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The Editor-in-Chief: Pakistan Journal of Biological Sciences

Dear Sir,

Attached, please find our manuscript entitled:

Susceptibility of Aedes albopictus larvae, the competence vector for arboviruses to the larvicidal activity of three types of Derris elliptica extract

which we would like to submit to the scientific journal that you run as an original article.

Information on the exploration and evaluation of larvicidal activity of various plant extracts continues to grow, including research findings on *Derris elliptica* extracts. We explored the local species of this plant in an effort to obtain the bioactive compound for larvicide formulation, as an alternative effort to solve the problem of Dengue vector resistance to temephos. We would like to share our valued data that might be important in providing scientific information to develop the supporting material for the Dengue vector control in Indonesia.

We do believe that the manuscript would fill the data unavailability and also very much relevant to your reader.

I am looking forward to hearing your favorable reply

Sincerely yours,
Sayono Sayono
On behalf of the authors

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Susceptibility of *Aedes albopictus* larvae, the competence vector for arboviruses to the larvicidal activity of three types of *Derris elliptica* extract

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Susceptibility of *Aedes albopictus* larvae, the competence vector for arboviruses to the larvicidal activity of three types of *Derris elliptica* extract

Abstract.

Background and Objective: The methanol, ethyl acetate, and n-hexane extracts of *D. elliptica* root have high larvicidal activity against *Ae. aegypti* larvae, the primary vector of Dengue but have not been understood their potential against *Ae. albopictus* larvae, the secondary vector of Dengue that also transmits Chikungunya and Zika viruses. This invitro study aims to understand the larvicidal activity of the three extract types of *D. elliptica* root against *Ae. albopictus* larvae. **Materials and Methods:** The tuba root extract types were obtained from the sequential extraction process with three steps of liquid – liquid partition as described in the previous report. Six concentrations were occupied in this experiment ranging of 0.5, 1.0, 2.0, 4.0, 10.0, and 15.0 mg L^{-1} Each concentration was five time replicated and placed in a 250 mL plastic cups. As many as twenty of third instar larvae of *Ae. albopictus* were subjected in each treatment cup, and larval mortality was observed after 24 and 48 hours of exposure. **Results:** Larval mortality rates based on concentration ranged of 13.75-97.00 and 43,75-100%, 14.00-44.00 and 34.00-90.00%, and 12.00-47.00 and 28.00-88.00%, with the LC_{50} after 24 and 48 hours of exposure were 2.925 and 0.414, 16.184 and 2.900, and 15.789 and 4.380 mg L^{-1}, respectively for methanol, ethyl acetate, and n-hexane extracts. **Conclusion:** The methanol, ethyl acetate, and n-hexane extract of tuba root have high larvicidal activity against *Ae. albopictus* larvae. Further study on prototype formulation of larvicide and elucidation the specific phytochemical compounds of the extracts were necessary conducted.

Keywords: *Aedes albopictus*, arboviruses vector, *Derris elliptica* root extract, larvicidal activity, Dengue vector

INTRODUCTION

The competence of *Ae. albopictus* mosquito transmitted arboviruses such as Dengue[1,2], Chikungunya[3], and Zika[4] has been reported in several countries, and its ability in transmitting the other arboviral has been indicated[5]. The vectorial competence has triggered a community attention in the arboviral impacted areas for implementing the control measures[1]. Unfortunately, the preferred habitat for this species is places with lush trees and far from human settlements such as cemeteries and beaches[6]. Globally, the area affected by Dengue has expanded to 129 countries, mainly (70%) in Asia, and the number of new cases has increased more than eightfold in two decades[7]. This has sparked community efforts to control its vectors, including *Ae. albopictus*.

The use of chemical methods in dengue vector control for decades has resulted in the emergence of Aedes mosquito strains that are resistant to several insecticide formulations, including the Temephos larvicide in Southeast Asia[8]. Several studies also proved that *Ae. albopictus* was resistant to the larvicide Temephos in Mexico[9], Pakistan[10], India, Malaysia, Sri Lanka, China, and Central Africa[11]. This phenomenon also occurs in Indonesia, including in Surabaya[12] and Samarinda, East Kalimantan[13]. This resistance issue can hinder the success of arboviruses infection prevention efforts in affected areas. This is also exacerbated by other factors such as population mobility and high connectivity between rural and urban areas which can provide greater opportunities for arbovirus exposure by *Ae. albopictus* mosquitoes[14]. The emergence of Temephos resistant strain of *Ae. albopictus* can hinder efforts to control infectious diseases. This situation triggers researchers to develop alternative larvicides that are effective and environmentally friendly by exploring new active compounds[15], including chemical compounds from natural ingredients.

Studies on the larvicidal activity of various plant extracts have been carried out, especially for *Ae. aegypti* larvae. Previous studies resulted in ranking the effectiveness of plant extract larvicides, namely high, moderate, low, and ineffective based on the lethal concentration 50% (LC_{50}) values

<50, 50-100, 100-750, and higher than 750 mg L^{-1} [16]. *D. elliptica* is one of the local plants that has high larvicidal potential against *Ae. aegypti* larvae. Experiments with three types of plant extracts, namely methanol, ethyl acetate, and n-hexane, showed a low effective concentration (LC_{50}), of 14.066, 21.063, and 4.086 mg L^{-1}, respectively[17]. In particular, the results of the bioassay test for ethyl acetate of Derris elliptica extract also showed effective larvicidal activity even though it was exposed to larvae from *Ae. aegypti* mosquitoes that were resistant to Cypermethrin 0.05%, with an LC_{50} of 34.945 mg L^{-1} [18]. These results are interesting to apply to the secondary vector of Dengue, the *Ae. albopictus* mosquito. This in vitro study was aimed to determine the larvicidal activity of methanol, ethyl acetate, and n-hexane extracts of tubal roots against *Ae. albopictus* larvae.

MATERIAL AND METHODS

Study site, tuba root collection and processing. The origin, collection, and processing of tubal roots were described as in previous studies[17]. The extracts have been processed since April 2021, and stored in a refrigerator at 4-8^{0}C.

Mosquito collection and rearing. The Aedes albopictus mosquito was obtained from larval surveys around the Muhammadiyah University Semarang campus, especially in breeding places far from human habitation, cemeteries and gardens with lush trees[6]. Mosquito larvae from the survey were reared into adult mosquitoes and subjected to morphological species identification. During rearing, the larvae are fed with dog food. Breeding is continued until the second generation of eggs is obtained. During breeding, mosquitoes were fed with a solution of 10% sugar and guinea pig blood, and the environmental conditions were maintained at a temperature of 28±2^{0}C and a humidity of 75±10%. The eggs of the second offspring were bred into third instar larvae and subjected to experiments, as many as twenty larvae per treatment.

Experiments. The bioassay test was carried out in several stages. Preliminary tests were carried out with concentration ranges of 4, 25, and 40 mg L^{-1} based on the previous study's LC_{50} and LC_{90}[17] and obtained larval mortality of 27, 87, and 98 percent, respectively. Based on these results, a bioassay test was determined with a lower concentration range of 2, 4, 10, and 20 mg L^{-1} and resulted in a larval mortality range of 24 – 97%. Lower concentration ranges were achieved in the third stage of the bioassay test, namely 0.5, 1.0, 2.0, 4.0, 10.0, and 15.0 mg L^{-1}. Each concentration level was carried out in five replications. Experiments were compared with two control groups, namely Temephos 0.02 mg L^{-1} as a positive control as well as a standard concentration, while the negative control was distilled water. The research subjects were third instar Aedes albopictus larvae with active movement conditions. Twenty larvae were subject to each treatment, and larval mortality rate was observed at 24th and 48th hours post-exposure.

Data analysis. Larval mortality data were analysed descriptively in the form of tables and graphs, and analytically with Probit and Two Way Anova tests to determine the effective concentration and significance of mortality based on the type and concentration of the extract. Data analysis using SPSS and excel software.

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

RESULTS

Overall, the tuba root extracts showed high larvicidal activity against *Ae. albopictus* larvae. After 48 hours of exposure, the results showed that the mortality rates based on the lowest to highest concentrations ranged from 43.75-100%, 34-90%, and 28-88%, respectively, for methanol, ethyl acetate, and n-hexane extracts (**Table 1**). Based on observation time of 24 hours after exposure, only methanol extract showed high larvicidal potential with a mortality rate of 13.75-97.00%. However, the three types of extracts showed a high trend of increasing larvicidal activity based on observations 48 hours after exposure (**Fig. 1**). The results of statistical analysis showed significant differences in larval mortality based on extract type, concentration, and interaction of extract type and concentration (**Table 2**). Based on the types of extracts, there were significant differences in larval mortality with the order of larvicidal activity from highest to lowest were methanol, ethyl acetate, and n-hexane extracts (**Table 3**). The final results of the bioassay test showed that the effective concentrations (LC_{50} and LC_{90}) of each type of extract were 2.925 and 4.886, 16.184 and 34.899, and 15.789 and 32.022 mg L^{-1} at 24 hours observation, and then 0.414 and 3.938, 2.900 and 13.473, and 4.380 and 14.767 mg L^{-1} at 48 hours observation, respectively for methanol, ethyl acetate, and n-hexane extracts (**Table 4**). The dead larvae were not found in the negative control and 100% mortality was found in the positive control groups. This finding indicated that the mortality of larvae in the treatment group was caused by the larvicidal activity of tuba root extracts.

DISCUSSION

In general, the results showed that the three types of tuba root extract indicated a high larvicidal activity, according to the classification of larvicidal effectiveness of plant extracts that had been previously reported[16]. Larval mortality increased with increasing concentration and exposure time. The highest potency was shown by the methanol extract, both after 24 and 48 hours of exposure, equivalent to the combined extract of petroleum-ether and methanol-chloroform[19]. From the aspect of cost, time, and resources, this study is more efficient because it applies a sequential extraction which is carried out in a series of processes and with cheaper solvents, although both sequential and direct extraction have advantages and disadvantages[20].

The effective concentration in this study was lower than the exposure of the same extract to *Ae. aegypti* larvae in the previous study[17]. This indicates that the larvicidal activity or susceptibility of

Ae. albopictus larvae is higher. Aedes albopictus larvae were more susceptible to the larvicidal activity of tuba root extract. This vulnerability can be attributed to the habitat preferences and flight ability of this species. The population of Aedes albopictus has a different habitat preference from Aedes aegypti, although co-occurrence often occurs[21]. The Aedes albopictus mosquito occupies habitats far from human settlements[6] so it has a low chance of exposure to insecticides from dengue vector control programs, including the larvicide Temephos. The fact shows that there are fewer reports of monitoring *Ae. albopictus* resistance to insecticides from arboviruses control programs than *Ae. Aegypti*[22], including in Indonesia[12,13] which is only reported from a limited number of locations. This low history of exposure to insecticides causes the development of resistance mechanisms, both knockdown and lower metabolic rates[8]. Several studies have shown that this species is more dominant in rural and suburban areas than in urban areas[23], but this species is also dominant in urban and suburban environments in low temperature areas[24], or in settlements where small breeding sites are found outdoors[21]. This low susceptibility is also supported by other factors, namely the flight distance of *Ae. albopictus* which reaches more than 200 m which allows it to avoid exposure to adulticides[25].

This study indicated that the methanol extract had a higher and faster larvicidal potential than the ethyl acetate and n-hexane extract types. Methanol is a solvent that can produce high extract products and phytochemical constituents, namely phenolics, alkaloids, flavonoids, and terpenoids [26]. Flavonoids are secondary metabolites that are widely found in tubal plants[27-29]. Flavonoids work by inhibiting the enzyme acetylcholinesterase by prolonging the effect of acetylcholine which increases nerve impulses at synapses[30], causing the larvae to spasm and die. Rotenone is one of the dominant flavonoids in *D. elliptica*. The activity of these compounds affects electron transport or oxidative phosphorylation which inhibits cellular oxygen uptake so that energy production drops drastically. This situation triggers anaerobic cellular metabolism leading to increased lactic acid production and tissue acidosis and anoxia, and death from heart and nervous system failure[31].

The larvicidal activity of the n-hexane extract in this study was lower than previous findings[17], with the order of highest to lowest larvicidal potency being n-hexane, methanol, and ethyl acetate. In this study, the larvicidal activity of n-hexane extract was the lowest after methanol and ethyl acetate. This condition is thought to be due to the degradation of phytochemical compounds, especially polyphenols, as reported by a study which proved that storage of fresh extracts for a period of three to six months caused the degradation of these compounds, except for methanol extracts which tended to be more stable[32]. The three types of tuba root extract used in this study were extracted six months ago and stored in a refrigerator at 4-8^{0}C so that this degradation factor is suspected to occur. Nevertheless, the larvicidal activity of this tubal root extract shows promising potential to be developed to the level of larvicidal prototype formulation, and further exploration of the specific phytochemical compounds that play a role is also carried out.

Conclusion

Three types of Tuba root extract, namely methanol, ethyl acetate, and n-hexane, respectively have high larvicidal potential against the *Ae. albopictus* larvae. Further studies on technical grade of larvicidal prototype and elucidation of specific chemical compounds are necessary done.

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SIGNIFICANCE STATEMENT

This study found the high larvicidal activity in three 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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Table 1. Larval mortality of *Aedes albopictus* based on the types and concentrations Tuba root extract

Extract type	Concentration (ppm)	Post exposure larval mortality (%)					
		24 h			48 h		
		Min	Max	Mean	Min	Max	Mean
Methanol							
	0.1	10.0	25.0	13.75	40.0	50.0	43.75
	0.5	15.0	25.0	16.25	65.0	70.0	66.25
	1.0	25.0	25.0	22.00	80.0	90.0	83.00
	2.0	25.0	30.0	24.00	85.0	90.0	86.00
	4.0	25.0	30.0	26.00	90.0	95.0	91.00
	10.0	40.2	86.0	83.00	85.0	100.0	98.00
	15.0	96.0	100.0	97.00	100.0	100.0	100.00
Ethyl acetate							
	0.5	10.0	20.0	14.00	25.0	40.0	34.00
	1.0	15.0	20.0	15.00	30.0	55.0	40.00
	2.0	15.0	25.0	17.00	35.0	70.0	49.00
	4.0	15.0	30.0	21.00	55.0	85.0	66.00
	10.0	30.0	45.0	37.00	60.0	95.0	81.00
	15.0	35.0	50.0	44.00	85.0	95.0	90.00
n-Hexane							
	0.5	10.0	15.0	12.00	25.0	35.0	28.00
	1.0	10.0	15.0	13.00	25.0	35.0	30.00
	2.0	15.0	15.0	15.00	35.0	60.0	47.00
	4.0	15.0	20.0	17.00	45.0	70.0	55.00
	10.0	25.0	50.0	33.00	50.0	90.0	78.00
	15.0	40.0	60.0	47.00	80.0	95.40	88.00
Temephos*	0.02	100	100	100	-	-	-
Aquadest#	0	0	0	0	0	0	0

*positive control; #negative control

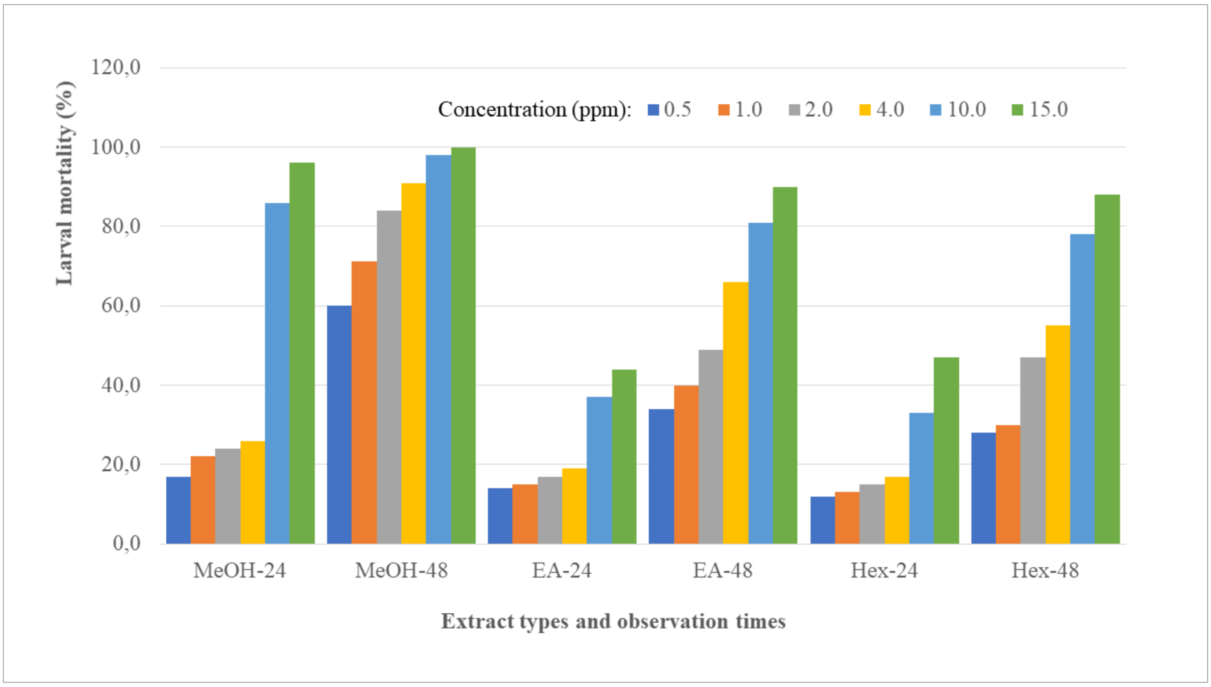

Figure 1. Mortality rate of *Aedes albopictus* larvae after 24 h and 48 h exposure to three extract type, namely methanol (MeOH), Ethyl acetate (EA), and n-hexane (Hex). MeOH extract type showed the rapid progress on mortality of research subject.

Table 2. Effect of extract type, concentration, and their interaction on larval mortality

Variables	**F**	**p**
Intercept	62.538	0.001
Extract types	37.662	0.000
Concentrations	31.564	0.000
Extract types and concentrations	2.360	0.018

Table 3. Multiple comparison of extract types on larval mortality

Extract types	Mean difference	p	95% Confidence Interval
Methanol – Ethyl acetate	23.83	0.000	19.10-28.57
Methanol – n-Hexane	30.00	0.000	25.26-34.74
Ethyl acetate – n-hexane	6.17	0.011	1.43-10.90

Table 4. The Lethal Concentration (LC_{50} and LC_{90}) of the methanol, ethyl acetate, and n-hexane extract types on mortality of *Aedes albopictus* larvae

Extract types	**Regression equation**	**Lethal Concentrationa (ppm)**		**Chi Square**	**p**
		LC_{50} (95% CI)	**LC_{90} (95% CI)**		
24 h exposure					
Methanol	Y = -1.915+0.655X	2.925 (2.641-3.200)	4.882 (4.487-5.423)	52.713	0.002
Ethyl acetate	Y = -1.108_0.068	16.184 (13.492-20.751)	34.899 (28.239-46.937)	12.013	0.999
n-hexane	Y = -1.246+0.079	15.789 (13.455-19.471)	32.022 (26.596-41.130)	10.400	0.999
48 h exposure					
Methanol	Y = -0.151+0.364X	0.414 (-11.872-1.863)	3.938 (2.291-40.334)	467.885	0.000
Ethyl acetate	Y = -0.351+0.121	2.900 (1.527-4.092)	13.473 (11.262-17.051)	49.079	0.011
n-hexane	Y = -0.540+0.123	4.380 (3.283-5.468)	14.767 (12.648-17.972)	40.834	0.071

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The article has been accepted for publication after revision. A Peer Review report is available online and you can access this report after log in to your account with User ID: say.epid@gmail.com.

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Susceptibility of *Aedes albopictus* larvae, the competence vector for arboviruses to the larvicidal activity of three types of *Derris elliptica* extract

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Running title: Susceptibiliy of *Aedes albopictus* larvae to the *Derris elliptica* extracts

Conflict of interest: All authors declare no conflict of interest

Author's Contribution: SS designed the study, collected natural materials, carried out experiments and bioassay tests, analyzed data, and wrote a manuscript. RA compiled the extraction method, carried out the extraction, analyzed the data on chemical compounds, and wrote some of the results and research methods. DS mosquito rearing, bioassay testing, data analysis, and co-writing the manuscript. EN perform the mosquito rearing and bioassay test; and FFA perform extraction and experiment of chemical compounds in the natural materials chemistry laboratory.

Abstract.

Background and Objective: The methanol, ethyl acetate, and n-hexane extracts of *D. elliptica* root have high larvicidal activity against *Ae. aegypti* larvae, the primary vector of Dengue but have not been understood their potential against *Ae. albopictus* larvae, the secondary vector of Dengue that also transmits Chikungunya and Zika viruses. This *in-vitro* study aims to understand the larvicidal activity of the three extract types of *D. elliptica* root against *Ae. albopictus* larvae. **Materials and Methods:** The tuba root extract types were obtained from the sequential extraction process with three steps of liquid—-liquid partition as described in the previous report. Six concentrations were occupied in this experiment ranging of 0.5, 1.0, 2.0, 4.0, 10.0, and 15.0 mg L^{-1} Each concentration was five times replicated and placed in ~~a~~ 250 mL plastic cups. As many as twenty of third instar

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larvae of *Ae. albopictus* were subjected in each treatment cup, and larval mortality was observed after 24 and 48 hours of exposure. **Results:** Larval mortality rates based on concentration range~~d~~ of 13.75-97.00 and 43,75-100%, 14.00-44.00 and 34.00-90.00%, and 12.00-47.00 and 28.00-88.00%, with the LC_{50} after 24 and 48 hours of exposure were 2.925 and 0.414, 16.184 and 2.900, and 15.789 and 4.380 mg L^{-1}, respectively for methanol, ethyl acetate, and n-hexane extracts. **Conclusion:** The methanol, ethyl acetate, and n-hexane extract of tuba root have high larvicidal activity against *Ae. albopictus* larvae. Further study on prototype formulation of larvicide and elucidation of the specific phytochemical compounds of the extracts were necessarily conducted.

Keywords: *Aedes albopictus*, arboviruses vector, *Derris elliptica*, methanol extract, ethyl acetate extraxt, n-hexane extraxt, larvicidal activity

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INTRODUCTION

The competence of *Ae. albopictus* ~~mosquito~~ mosquito-transmitted arboviruses such as Dengue[1,2], Chikungunya[3], and Zika[4] has been reported in several countries, and its ability in transmitting the other arboviral has been indicated[5]. The vectorial competence has triggered ~~a~~ community attention in the arboviral impacted areas for implementing the control measures[1]. Unfortunately, the preferred habitat for this species is ~~places~~ placed with lush trees and far from human settlements such as cemeteries and beaches[6]. Globally, the area affected by Dengue has expanded to 129 countries, mainly in Asia, and the number of new cases has increased more than eightfold in two decades[7]. This has sparked community efforts to control its vectors, including *Ae. albopictus*.

The use of chemical methods in dengue vector control for decades has resulted in the emergence of Aedes mosquito strains that are resistant to several insecticide formulations, including the Temephos larvicide in Southeast Asia[8]. Several studies also proved that *Ae. albopictus* was resistant to the larvicide Temephos in Brazil[9], Pakistan[10], India, Malaysia, Sri Lanka, China, and Central Africa[11]. This phenomenon also occurs in Indonesia, including in Surabaya[12] and Bengkulu[13] where *Ae albopictus* resistant to organophosphate insecticides (Temephos and Malathion). This resistance issue can hinder the success of arboviruses infection prevention efforts in affected areas. This is also exacerbated by other factors such as population mobility and high connectivity between rural and urban areas which can provide greater opportunities for arbovirus exposure by *Ae. albopictus* mosquitoes[14]. The emergence of Temephos resistant strain of *Ae. albopictus* can hinder efforts to control infectious diseases. This situation triggers researchers to develop alternative larvicides that are effective and environmentally friendly by exploring new active compounds[15], including chemical compounds from natural ingredients.

Studies on the larvicidal activity of various plant extracts have been carried out, especially for *Ae. aegypti* larvae. Previous studies resulted in ranking the effectiveness of plant extract larvicides, namely high, moderate, low, and ineffective based on the lethal concentration 50% (LC_{50}) values <50, 50-100, 100-750, and higher than 750 mg L^{-1} [16]. *D. elliptica* is one of the local plants that has high larvicidal potential against *Ae. aegypti* larvae. Experiments with three types of plant extracts, namely methanol, ethyl acetate, and n-hexane, showed a low effective concentration (LC_{50}), of 14.066, 21.063, and 4.086 mg L^{-1}, respectively[17]. In particular, the results of the bioassay test for ethyl acetate of Derris elliptica extract also showed effective larvicidal activity even though it was exposed to larvae from *Ae. aegypti* mosquitoes that were resistant to Cypermethrin 0.05%, with an LC_{50} of 34.945 mg L^{-1} [18]. These results are interesting to apply to the secondary vector of Dengue, the *Ae. albopictus* mosquito. This *in vitro* study was aimed to determine the larvicidal activity of methanol, ethyl acetate, and n-hexane extracts of tubal roots against *Ae. albopictus* larvae.

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MATERIAL AND METHODS

Study site,: This study was carried out at two different laboratory, namely the Natural Chemical Laboratory of Sciences and Mathematics Faculty of Garut University, West Java Province for extraction process, and Laboratory of Epidemiology and Tropical Diseases of Universitas Muhammadiyah Semarang, Central Java, Indonesia for mosquito collection, rearing and bioassay experiments.

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(The study was carried out at Microbiology Department, Quality Control Lab, Egypt from January 2018 to March 2019).

Tuba root collection and processing. The origin, collection, and processing of tubal roots were described as in the previous studies[17]. The extracts have been processed since April to June 2021, delivered and stored in a refrigerator at $4-8^0C$ in Laboratory of Epidemiologi and Tropical Diseases of Universitas Muhammadiyah Semarang.

Mosquito collection and rearing. The Aedes albopictus mosquito was obtained from larval surveys around the Muhammadiyah University Semarang campus, especially in breeding places far from human habitation, cemeteries and gardens with lush trees[6]. Mosquito larvae from the survey were reared into adult mosquitoes and subjected to morphological species identification. During rearing, the larvae are fed with dog food. Breeding is continued until the second generation of eggs is obtained. During breeding, mosquitoes were fed with a solution of 10% sugar and guinea pig blood, and the environmental conditions were maintained at a temperature of 28 ± 2^0C and a humidity of

75±10%. The eggs of the second offspring were bred into third instar larvae and subjected to experiments, as many as twenty larvae per treatment.

Experiments. The bioassay test was carried out in several stages. Preliminary tests were carried out with concentration ranges of 4, 25, and 40 mg L^{-1} based on the previous study's LC_{50} and LC_{90}[17] and obtained larval mortality of 27, 87, and 98 percent, respectively. Based on these results, a bioassay test was determined with a lower concentration range of 2, 4, 10, and 20 mg L^{-1} and resulted in a larval mortality range of 24 – 97%. Lower concentration ranges were achieved in the third stage of the bioassay test, namely 0.5, 1.0, 2.0, 4.0, 10.0, and 15.0 mg L^{-1}. Each concentration level was carried out in five replications. Experiments were compared with two control groups, namely Temephos 0.02 mg L^{-1} as a positive control as well as a standard concentration, while the negative control was distilled water. The research subjects were third instar Aedes albopictus larvae with active movement conditions. Twenty larvae were subject to each treatment, and the larval mortality rate was observed at 24th and 48th hours post-exposure.

Data analysis. Larval mortality data were analysed descriptively in the form of tables and graphs, and analytically with Probit and Two Way, Anova tests to determine the effective concentration and significance of mortality based on the type and concentration of the extract. Data analysis using SPSS and ~~excel~~ Excel software.

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

RESULTS

Overall, the tuba root extracts showed high larvicidal activity against *Ae. albopictus* larvae. After 48 hours of exposure, the results showed that the mortality rates based on the lowest to highest concentrations ranged from 43.75-100%, 34-90%, and 28-88%, respectively, for methanol, ethyl acetate, and n-hexane extracts (**Table 1**).

Based on observation time of 24 hours after exposure, only methanol extract showed high larvicidal potential with a mortality rate of 13.75-97.00%. However, the three types of extracts showed a high trend of increasing larvicidal activity based on observations 48 hours after exposure (**Fig. 1**).

The results of statistical analysis showed significant differences in larval mortality based on extract type, concentration, and interaction of extract type and concentration (**Table 2**). Based on the types of extracts, there were significant differences in larval mortality with the order of larvicidal activity from highest to lowest were methanol, ethyl acetate, and n-hexane extracts (**Table 3**).

The final results of the bioassay test showed that the effective concentrations (LC_{50} and LC_{90}) of each type of extract were 2.925 and 4.882, 16.184 and 34.899, and 15.789 and 32.022 mg L^{-1} at 24 hours observation, and then 0.414 and 3.938, 2.900 and 13.473, and 4.380 and 14.767 mg L^{-1} at 48 hours observation, respectively for methanol, ethyl acetate, and n-hexane extracts (**Table 4**). The dead larvae were not found in the negative control and 100% mortality was found in the positive control groups. This finding indicated that the mortality of larvae in the treatment group was caused by the larvicidal activity of tuba root extracts.

DISCUSSION

In general, the results showed that the three types of tuba root extract indicated a high larvicidal activity, according to the classification of larvicidal effectiveness of plant extracts that had been previously reported[16]. Larval mortality increased with increasing concentration and exposure time. The highest potency was shown by the methanol extract, both after 24 and 48 hours of exposure, equivalent to the combined extract of petroleum-ether and methanol-chloroform[19]. From the aspect of cost, time, and resources, this study is more efficient because it applies a sequential extraction which is carried out in a series of processes and with cheaper solvents, although both sequential and direct extraction have advantages and disadvantages[20].

The effective concentration in this study was lower than the exposure of the same extract to *Ae. aegypti* larvae in the previous study[17]. This indicates that the larvicidal activity or susceptibility of *Ae. albopictus* larvae ~~is~~ are higher. Aedes albopictus larvae were more susceptible to the larvicidal activity of tuba root extract. This vulnerability can be attributed to the habitat preferences and flight ability of this species. The population of Aedes albopictus has a different habitat preference from Aedes aegypti, although co-occurrence often occurs[21]. The Aedes albopictus mosquito occupies habitats far from human settlements[6] so it has a low chance of exposure to insecticides from dengue vector control programs, including the larvicide Temephos. The fact shows that there are fewer reports of monitoring *Ae. albopictus* resistance to insecticides from arboviruses control programs than *Ae. Aegypti*[22], including in Indonesia[12,13] which is only reported from a limited number of locations. This low history of exposure to insecticides causes the development of resistance mechanisms, both knockdown and lower metabolic rates[8]. Several studies have shown that this species is more dominant in rural and suburban areas than in urban areas[23], but this species is also dominant in urban and suburban environments in ~~low~~ low-temperature areas[24], or in settlements where small breeding sites are found outdoors[21]. This low susceptibility is also supported by other factors, namely the flight distance of *Ae. albopictus* which reaches more than 200 m which allows it to avoid exposure to adulticides[25].

This study indicated that the methanol extract had a higher and faster larvicidal potential than the ethyl acetate and n-hexane extract types. Methanol is a solvent that can produce high extract products and phytochemical constituents, namely phenolics, alkaloids, flavonoids, and terpenoids [26]. Flavonoids are secondary metabolites that are widely found in tubal plants[27-29]. Flavonoids work by inhibiting the enzyme acetylcholinesterase by prolonging the effect of acetylcholine which increases nerve impulses at synapses[30], causing the larvae to spasm and die. Rotenone is one of the dominant flavonoids in *D. elliptica*. The activity of these compounds affects electron transport or oxidative phosphorylation which inhibits cellular oxygen uptake so that energy production drops drastically. This situation triggers anaerobic cellular metabolism leading to increased lactic acid production and tissue acidosis and anoxia, and death from heart and nervous system failure[31].

The larvicidal activity of the n-hexane extract in this study was lower than previous findings[17], with the order of highest to lowest larvicidal potency being n-hexane, methanol, and ethyl acetate. In this study, the larvicidal activity of n-hexane extract was the lowest after methanol and ethyl acetate. This condition is thought to be due to the degradation of phytochemical compounds, especially polyphenols, as reported by a study ~~which~~ that proved that storage of fresh extracts for a period of three to six months caused the degradation of these compounds, except for methanol extracts which tended to be more stable[32]. The three types of tuba root extract used in this study were extracted six months ago and stored in a refrigerator at 4-8^{0}C so that this degradation factor is suspected to occur. Nevertheless, the larvicidal activity of this tubal root extract shows promising potential to be developed to the level of larvicidal prototype formulation, and further exploration of the specific phytochemical compounds that play a role is also carried out.

Conclusion

Three types of Tuba root extract, namely methanol, ethyl acetate, and n-hexane, respectively have high larvicidal potential against the *Ae. albopictus* larvae. Further studies on the technical grade of larvicidal prototypes and elucidation of specific chemical compounds are necessary done.

ACKNOWLEDGEMENT

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SIGNIFICANCE STATEMENT

This study found the high larvicidal activity in three 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 arrivedarrive at the near times.

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Table 1. Larval mortality of *Aedes albopictus* based on the types and concentrations of Tuba root extract

Extract type	**Concentration (ppm)**	~~**Post**~~ **Post-exposure larval mortality (%)**					
		24 h			**48 h**		
		Min	**Max**	**Mean**	**Min**	**Max**	**Mean**
Methanol							
	0.1	10.0	25.0	13.75	40.0	50.0	43.75
	0.5	15.0	25.0	16.25	65.0	70.0	66.25
	1.0	25.0	25.0	22.00	80.0	90.0	83.00
	2.0	25.0	30.0	24.00	85.0	90.0	86.00
	4.0	25.0	30.0	26.00	90.0	95.0	91.00
	10.0	40.2	86.0	83.00	85.0	100.0	98.00
	15.0	96.0	100.0	97.00	100.0	100.0	100.00
Ethyl acetate							
	0.5	10.0	20.0	14.00	25.0	40.0	34.00
	1.0	15.0	20.0	15.00	30.0	55.0	40.00
	2.0	15.0	25.0	17.00	35.0	70.0	49.00
	4.0	15.0	30.0	21.00	55.0	85.0	66.00
	10.0	30.0	45.0	37.00	60.0	95.0	81.00
	15.0	35.0	50.0	44.00	85.0	95.0	90.00
n-Hexane							
	0.5	10.0	15.0	12.00	25.0	35.0	28.00
	1.0	10.0	15.0	13.00	25.0	35.0	30.00
	2.0	15.0	15.0	15.00	35.0	60.0	47.00
	4.0	15.0	20.0	17.00	45.0	70.0	55.00

	10.0	25.0	50.0	33.00	50.0	90.0	78.00
	15.0	40.0	60.0	47.00	80.0	95.40	88.00
Temephos*	0.02	100	100	100	-	-	-
Aquadest#	0	0	0	0	0	0	0

Footnote: *positive control; #negative control

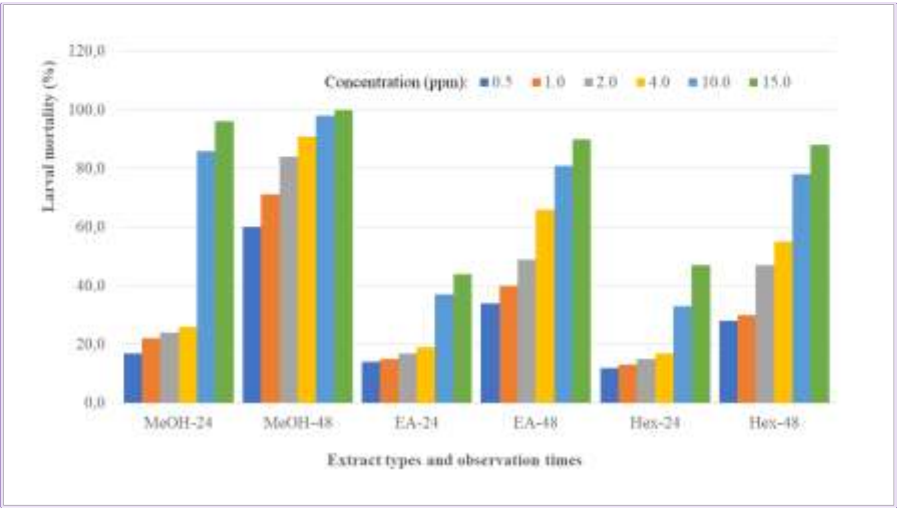

Figure 1. ~~Mortality~~ The mortality rate of *Aedes albopictus* larvae after 24 h and 48 h exposure to three extract types~~., namely methanol (MeOH), Ethyl acetate (EA), and n-hexane (Hex).~~

Footnote: three extract types, namely methanol (MeOH), Ethyl acetate (EA), and n-hexane (Hex).

MeOH extract type showed the rapid progress on mortality of research subject.

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Table 2. Effect of extract type, concentration, and their interaction on larval mortality

Variables	**F**	**p**
Intercept	62.538	0.001
Extract types	37.662	0.000
Concentrations	31.564	0.000
Extract types and concentrations	2.360	0.018

Table 3. Multiple comparison of extract types on larval mortality

Extract types	Mean difference	p	95% Confidence Interval
Methanol – Ethyl acetate	23.83	0.000	19.10-28.57
Methanol – n-Hexane	30.00	0.000	25.26-34.74
Ethyl acetate – n-hexane	6.17	0.011	1.43-10.90

Table 4. The Lethal Concentration (LC_{50} and LC_{90}) of the methanol, ethyl acetate, and n-hexane extract types on mortality of *Aedes albopictus* larvae

Extract types	Regression equation	Lethal Concentrationa (ppm)		Chi Chi-Square	p
		LC_{50} (95% CI)	LC_{90} (95% CI)		
24 h exposure					
Methanol	Y = -1.915+0.655X	2.925 (2.641-3.200)	4.882 (4.487-5.423)	52.713	0.002
Ethyl acetate	Y = -1.108_0.068	16.184 (13.492-20.751)	34.899 (28.239-46.937)	12.013	0.999
n-hexane	Y = -1.246+0.079	15.789 (13.455-19.471)	32.022 (26.596-41.130)	10.400	0.999
48 h exposure					
Methanol	Y = -0.151+0.364X	0.414 (-11.872-1.863)	3.938 (2.291-40.334)	467.885	0.000
Ethyl acetate	Y = -0.351+0.121	2.900 (1.527-4.092)	13.473 (11.262-17.051)	49.079	0.011
n-hexane	Y = -0.540+0.123	4.380 (3.283-5.468)	14.767 (12.648-17.972)	40.834	0.071

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