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Semarang, 7 November 2021

The Editor-in-Chief: Biodiversitas
Dear Sir,

Attached, please find our manuscript entitled:

Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of *Derris elliptica* root against the Temephos-susceptible strain of Aedes aegypti larvae

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 work 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, especially about chemical compound isolated from specific plant, Derris elliptica.

I am looking forward to hearing your favorable reply

Sincerely yours,
Sayono Sayono
On behalf of the authors

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Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of Derris elliptica root against the Temephos-susceptible strain of Aedes aegypti larvae

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Abstract. Aedes aegypti is the main vector of Dengue, Chikungunya, and Zika diseases. Temephos resistance of this species has hampered vector control efforts worldwide. Studies proved that D. elliptica extracts were effective in controlling Aedes larvae, so the active compound needed to be isolated. This study evaluated the larvicidal activity of the chemical compounds isolated from n-hexane fractions of Tuba roots against the temephos-susceptible Ae. aegypti larvae. Six isolates were obtained from three of the seven n-hexane fractions. Three levels of concentration of each isolate were formulated for a preliminary bioassay test, and resulted in the two most active compounds, isolates 3 and 6. Results of the final bioassay test indicated that isolate 3 was more active than isolate 6 with LC_{50} and LC_{90} after 24 hours of exposure were 1.607 and 7.399 ppm, and after 48 hours of exposure were 0.926 and 3.206 ppm respectively. Isolate 6 also had high larvicidal activity after 48 hours of exposure, with LC_{50} and LC_{90} were 1.056 and 4.647 ppm. Further studies need to be carried out to determine the chemical structures and toxicity mechanisms of the bioactive compounds.
Keywords: Aedes aegypti larvae, chemical isolates, Derris elliptica, larvicidal activity, n-hexane fraction, Temephos-susceptible strain

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Abstract. *Aedes aegypti* is the main vector of Dengue, Chikungunya, and Zika diseases. Temephos resistance of this species has hampered vector control efforts worldwide. Studies proved that *D. elliptica* extracts were effective in controlling Aedes larvae, so the active compound needed to be isolated. This study evaluated the larvicidal activity of the chemical compounds isolated from n-hexane fractions of Tuba roots against the temephos-susceptible *Ae. aegypti* larvae. Six isolates were obtained from three of the seven n-hexane fractions. Three levels of concentration of each isolate were formulated for a preliminary bioassay test, and resulted in the two most active compounds, isolates 3 and 6. Results of the final bioassay test indicated that isolate 3 was more active than isolate 6 with LC_{50} and LC_{90} after 24 hours of exposure were 1.607 and 7.399 ppm, and after 48 hours of exposure were 0.926 and 3.206 ppm respectively. Isolate 6 also had high larvicidal activity after 48 hours of exposure, with LC_{50} and LC_{90} were 1.056 and 4.647 ppm. Further studies need to be carried out to determine the chemical structures and toxicity mechanisms of the bioactive compounds.

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INTRODUCTION

Ae. aegypti is the principle vector of human viral diseases including Dengue, Chikungunya, Yellow fever, and Zika (Powell et al 2018). Since these arboviral diseases have become a threat and a global public health problem (Marchi et al 2018, Girad et al 2020), community attention to

the species increased according to the escalation and expansion of the disease occurrence from Africa to other regions worldwide (Weetman et al 2018). In Dengue endemic areas, efforts to control this mosquito species have become a priority since there are no antiviral drugs and vaccines are still being developed (Arredondo-García et al 2018, Plennevauz et al 2018). In this case, people in endemic areas prefer to use insecticides to control these arboviral disease vectors where the organophosphate group is the most dominant (WHO 2009, Manjarres-Suarez and Alivero-Verbel 2013). The high intensity of community use with uncontrolled doses has led to resistance of Aedes aegypti to various classes of insecticides. This condition has spread in many countries, especially in America, Asia, and Africa (Manjarres-Suarez and Olivero-Verbel 2013).

Temephos is an active insecticide compound in the organophosphate group that is most widely used in the control of *Ae. aegypti* larvae in endemic areas of arboviruses worldwide (WHO 2009) for seven decades (Manjarres-Suarez and Alivero-Verbel 2013), although it does not always reduce the density of the Dengue vector population. This condition is due to inconsistencies in use (George et al 2015, Arosteguí et al 2017), low coverage of exposed water containers, especially in rural areas (Legorreta-Soberanis et al 2017). On the other hand, long-term use of temephos with operational deficiency has led to the emergence of resistant-strains of *Aedes aegypti* to this active ingredient (Cediak et al 2016) and has become a serious problem in controlling this arbovirus vector. To solve this problem the researchers conducted an exploration to find active compounds from natural materials that are biodegradable, non-persistent, and not bio-accumulative in the environment (Arnason et al 2012).

Phytochemical screening and larvicidal activity evaluation have been carried out on various plant species, including *D. elliptica* with varying results (Komalamisra et al 2005). The wild plant in the agricultural farm which is commonly found in South to Southeast Asia has been traditionally

used bay community for a long time as a fish poison and plant pest insecticide (Starr et al 2003, Sirrichamorn et al 2012). Studies on the larvicidal activity of various phytochemical compounds in *D. elliptica* extract against *Ae. aegypti* larvae have been reported from several countries with varying methods and results. Studies in Thailand showed that the effective doses (LC_{50} and LC_{90}) of the ethanol extract of *D. elliptica* against *Ae. aegypti* larvae were 20.49 and 47.49 ppm (Komalamisra et al 2005), whereas a study in India reported the lower effective doses of petroleum ether extract (0.616 and 1.44 ppm) and methyl chloride (4.21 and 12.40 ppm) (Dohuita et al 2015). Study with specific extraction methods shows that the solvent combinations of methyl chloride: methanol 1:1 produces an effective dose (LC_{50}) of 24 ppm) (Zubairi et al 2015). The bioassay test of four *D. elliptica* extract fractions with different polarity, namely water, methanol, ethyl acetate, and n-hexane on *Ae. aegypti* larvae showed different larvicidal activity, and n-hexane extract was the most active extract with LC_{50} of 4,088 ppm (Sayono et al 2020), and isolation of specific compounds from this fraction is recommended. This study aims to evaluate the larvicidal activity of chemical compounds isolated from the n-hexane extract of *D. elliptical* root against susceptible-Temephos *Ae. aegypti* larvae.

MATERIAL AND METHODS

Extraction, fractionation, and isolation of *D. elliptica* roots.

The n-hexane fraction of Derris elliptica was obtained from the sequential extraction process of these plant roots (Sayono et al 2020). Then, this fraction was separated by using liquid-vacuum chromatography with n-hexane: ethyl acetate: methanol 10% gradient eluents, and resulted in seven grouped-fractions, namely n-hexane fraction 1 (FH1) to FH7. As much as 200 mg of FH2

was separated by using column-gravitation chromatography with n-hexane: ethyl acetate eluent (9:1) resulting in five subfractions, namely FH2A to FH2E. FH2B subfraction was purified by using column-gravitation chromatography with the same eluent resulting in as much as 30 mg of isolate 1. The separation process of FH4 (420 mg) used column-gravitation chromatography with n-hexane: ethyl acetate eluent (8:2) resulting in six subfractions of FH4A to FH4F. As much as 40 mg of FH4A subfraction was recrystallized with methanol to obtain isolate 2. FH4C subfraction (120 mg) was purified by using column-gravitation chromatography with eluent solvent of n-hexane: ethyl acetate (8:2) resulting in as much as 15 mg of isolate 3. Isolate 4 was purified from FH4E subfraction using column-gravitation chromatography with eluent solvent n-hexane: ethyl acetate (7:3). FH5 fraction was separated by using column-gravitation chromatography with eluent solvent n-hexane gradually from 7:3 to 0:10 and resulted in four subfractions, namely FH5A to FH5D. Isocratic purification of FH5B subfraction used column-gravitation chromatography with n-hexane: ethyl acetate eluent solvent (7:3) resulting in isolate 5 and 6.

Collecting and rearing the *Ae. aegypti* larvae

Larval surveys were conducted from January to March 2020 in Sambiroto village, Semarang municipality, Central Java Province, Indonesia. Morphological identification of mosquito species was carried out in the Laboratory of Epidemiology and Tropical Diseases of Public Health Faculty, Universitas Muhammadiyah Semarang based on the Walter Reed guideline (WRBU 2020). *Ae. aegypti* mosquitoes were reared through the fourth generation to obtain sufficient numbers with uniform age. The 3rd instar larvae were subjected to a bioassay test to evaluate the larvicidal activity of secondary metabolites of Derris elliptica root after their susceptibility status to Temephos were determined (WHO 2016).

Larvicidal bioassay test

Initial bioassay tests of this study were performed by using the previous concentration range of n-hexane extract of *D. elliptica* (Sayono et al 2020) with slight modification. Based on the Lethal Concentration 50% and 90% (LC_{50} and LC_{90}) of the study, the new concentration range of 1, 4, and 7 mg L^{-1} was set and applied to the isolates. Preliminary bioassay test results are used to determine the new lower concentration ranges at the next testing steps until the lowest effective concentrations (LC_{50} and LC_{90}) are obtained.

Data analysis

Mortality rate, Probit analysis to determine the LC_{50} and LC_{90} was performed statistically by using SPSS 16.0 version.

Ethical consideration.

The protocol of this study was reviewed by the Ethic Committee of Health Research of Public Health Faculty of Universitas Muhammadiyah Semarang with registration number 231/KEPK-FKM/UNIMUS/2019.

RESULTS AND DISCUSSION

This study is a part of the exploration of phytochemicals with larvicidal potential and focuses on the *D. elliptica* or tuba root. This plant is interesting for further investigation because of several aspects: (i) its toxicity has been used by traditional communities as fish poison and insecticide for

plant pests (Starr et al 2003); (ii) the potential for larvacide varies widely based on geography and the screening method applied (Komalamisra et al 2005, Dohutia et al 2015, Zubairi et al 2015, Sayono et al 2020), and (iii) agricultural weeds that grow abundantly. *D. elliptica* is a vine both horizontally on the ground and wrapped around and covering towering trees commonly found in South to Southeast Asia, and even spread to Africa and America. These plants are invasive to moderate to high levels and grow rapidly in tropical climates (CABI 2020). Utilization of this plant has a positive and strategic impact from a health and environmental aspects, especially the promising potential of larvicides, as well as eradicating weeds.

This study applied a tiered or serial, non-parallel screening method guided bioassay test to evaluate the larvicidal potential of outcomes at each stage. This is intended to evaluate the larvicidal potential of each type of extract produced. The screening process starts from the extraction of the polar tuba root compound with methanol solvent, and then the polar methanol extract is partitioned with non-polar n-hexane to bind the non-polar compounds, leaving other parts in the water solution. The other part was partitioned with ethyl acetate to bind the semi-polar compound, leaving an aqueous extract. The bioassay test results of each type of extract showed the potential sequence of n-hexane, methanol, and ethyl acetate larvicides, while water extracts did not show larvicidal potential (Sayono et al 2020).

The focus of this research is to isolate pure chemical compounds (secondary metabolites) from n-hexane extract which are non-polar and have the highest larvicidal potential. Results of fractionation and isolation of chemical compounds (Fig 1) of n-hexane extract of *D. elliptica* roots are showed in Table 1. Initial bioassay test showed that the six isolates have different larvicidal potential (Table 2). There are four groups of chemical compounds resulted from six isolates. Isolates 1, 2, and 4 originate from the different groups namely Beta-sitosterol, Sitosterol, and

Triterpenoid, while isolates 3, 5, and 6 origins from the one group of chemical compounds, indicating the flavonoids. Visual characteristics showed that isolates 1, 2, and 4 are transparent (colorless) crystals while isolates 3, 5, and 6 are yellow crystals (Fig 2). Based on the concentration range of 1, 4, and 7 ppm for 24 hours, isolate 1 and 2 did not indicate the larvicidal activity. There is no dead *Ae. aegypti* larvae were found after exposure to the compounds. The six isolates of chemical compounds were produced from a combination of chromatography and purification methods (Ingle et al 2017), which are visually differentiated into four secondary metabolite groups, and only the flavonoid group that has high larvicidal potential. These findings indicate that the larvicide potency of n-hexane extract is influenced by the flavonoid content. This is shown by the difference in the larvicidal effect of isolates 1, 2, and 4 (non-flavonoids) with isolates 3 and 6 (flavonoid group) which have three times more potency than in the form of extracts (Sayono et al 2020, Zubairi et al 2015, and Komalamisra et al 2005), although slightly lower than petroleum ether extract and equivalent to methyl chloride extract (Dohutia et al 2015). The highest larvicidal activity was found in isolate 3 with a mortality rate of 45–92.5%, followed by isolate 6 (20-60%) and isolates 4 and 5 (< 10%). The mortality rate of isolate 4, 5, and 6 increased after 48 hours of exposure namely 22.5–57.5%, 45–60.0%, and 45–92.5% respectively. Based on the results, isolates 3 and 6 were used to determine the effective larvicidal activity by the next step of the bioassay test with the lower concentration range of 0.5, 1, 2, 4, and 6 ppm.

The final result of bioassay test showed that the larvicidal activity of isolate 3 better than isolate 6 (Table 3 and Table 4). Exposure to isolate 3 for 24 hours has caused mortality rates for *Ae. aegypti* larvae of 17.5-90% with LC_{50} and LC_{90} were 1.607 (1.250–2.025) and 7.399 (5.147–13.284) ppm, while exposure to isolate 6 caused mortality rates of 5–75% with LC_{50} and LC_{90} were 2.509 (2.098–3.048) and 13.894 (9.602–24.084) ppm respectively. However, both of isolate

3 and 6 had good larvicidal activity after 48 hours of exposure where the mortality rate of isolate 3 ranged from 27.5-97.5% with LC50 and LC90 were 0.926 (0.714-1.143) and 3.206 (2.459-4.782) ppm, and mortality rate of isolate 6 ranged from 25-100% with LC_{50} and LC_{90} were 1.056 (0.868-1249) and 4,647 (3,661-6,459) ppm, respectively. These findings indicated that the larvicidal activity of isolate 6 was slightly lower and slower than isolate 3. There were two solvents produced extracts and isolates that have high larvicidal potential, namely petroleum ether, n-hexane, and methyl chloride. Flavonoids include more than 4,000 specific compounds which are grouped into flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanidins (Paula-Ribeiro-Povinelli et al 2019). The main target site for flavonoid compounds is Acetylcholinesterase where the compound works to inhibit the activity of this enzyme (Perumalsamy et al 2015). These compounds also disrupt the endocrine and hormonal systems (Ge et al 2015) and reducing the esterase and monooxygenase enzymes (Visetson et al 2001).

CONCLUSION

This study obtained six secondary metabolites isolating from n-hexane fraction of Derris elliptica root, namely isolate 1 to 6. Two of the six isolates (number 3 and 6) have high larvicidal activity against the Temephos-susceptible *Aedes aegypti* larvae. Elucidation of a chemical structure and toxication mechanisms of the compounds are necessary conducted to prepare the technical grade of larvicide for this finding.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

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Figure 1.

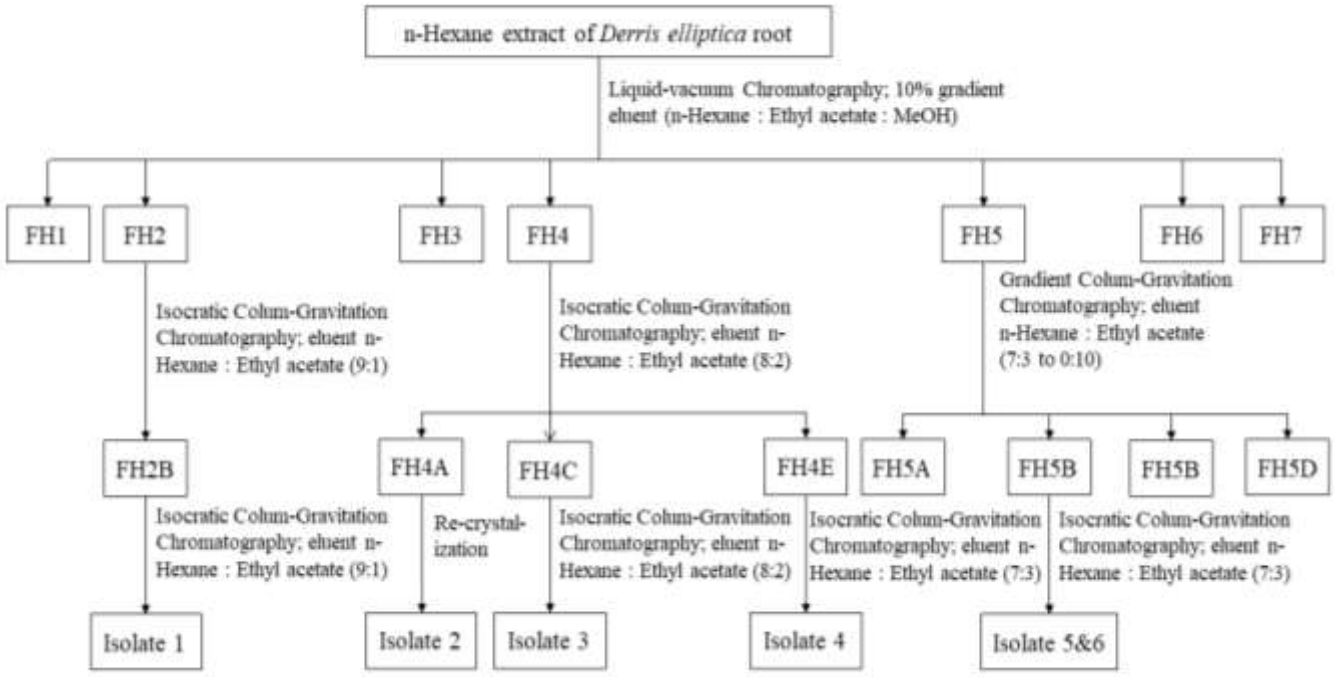

Fig 1. Fractionation and isolation of phytochemical compound from n-hexane extract of *Derris elliptica* roots. Seven fractionates and six isolates were obtained from the n-hexane fraction of *Derris elliptica*. FH1 – FH7: n-hexane fraction number 1 to 7. FH2B: n-hexane fraction number 2 subfraction B, etc.

Figure 2

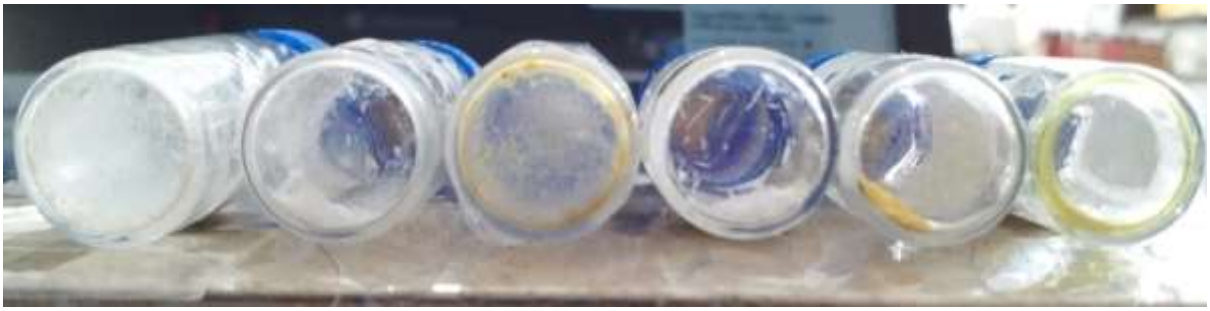

Fig 2. Visual characteristics of the six chemical compound isolates of n-hexane fraction of *Derris elliptica* roots. Isolates number 1, 2, and 4 are colorless crystals while isolates number 3, 5, and 6 are yellowish crystals.

Table 1.

Table 1. Group of secondary metabolites of chemical compound isolated from n-hexane fraction of *Derris elliptica* roots

Isolate number	Secondary metabolite group	Visual characteristics
I	Beta-sitosterol	Colorless crystal
II	Sitosterol	Colorless crystal
III	Flavonoid	Yellow crystal
IV	Triterpenoid	Colorless crystal
V	Flavonoid	Yellow crystal
VI	Flavonoid	Yellow crystal

Table 2

Table 2. Results of initial bioassay test of isolate number I, II, dan III of *Derris elliptica* against *Aedes aegypti* larvae

Isolates	Dosages (ppm)	Larval mortality rate (%)	
		24 hrs	48 hrs
I	1	0	-
	4	0	-
	7	0	-
II	1	0	-
	4	0	-
	7	0	-
III	1	45.0	-
	4	70.5	-
	7	92.5	-
IV	1	5.0	22.5
	4	5.0	47.5
	7	7.5	57.5
V	1	5.0	45.0
	4	7.5	60.0
	7	10.0	60.0
VI	1	20.0	57.5
	4	30.0	80.0
	7	65.0	92.5

Table 3

Table 3. Results of larvicidal activity determination of isolates III and VI of *Derris elliptica* against *Aedes aegypti* larvae

Isolates	Dosages (ppm)	Larval mortality rate (%)	
		24 hrs	48 hrs
III	0,5	17,5	27.5
	1	30,0	45.0
	2	65,0	77.5
	4	72,5	92.5
	6	90,0	100.0
VI	0,5	5,00	25,0
	1	26,3	51,3
	2	41,3	61,3
	4	46,3	71,3
	6	75,0	100,0

Table 4

Table 4. Results of Probit analysis showed the LC_{50} and LC_{90} of isolates III and VI of *Derris elliptica* against Aedes aegypti larvae

Isolates	Exposure time (hours)	Regression equation	Lethal Concentration (ppm)		Chi-Square	p-value
			LC_{50} (95% Confidence limits)	LC_{90} (95% Confidence limits)		
III	24	Y = -0.398+1.932X	1.607 (1.250 – 2.025)	7.399 (5.147 – 13.284)	6.539	0.587
	48	Y= 0.079+2.377X	0.926(0.714 – 1.143)	3.206(1.459 – 4.782)	7.594	0.474
VI	24	Y = -0.689+1.724X	2.509 (2.098 – 3.048)	13.894 (9.602 – 24.084)	12.948	0.795
	48	Y = -0.047+1.992X	1.056 (0.868 – 1.249)	4.647 (3.661 – 6.459)	16.865	0.532

Gmail **Sayono Sayono <say.epid@gmail.com>**

[biodiv] Submission Acknowledgement

1 pesan

Ahmad Dwi Setyawan <smujo.id@gmail.com> 7 November 2021 pukul 12.09
Kepada: Sayono Sayono <say.epid@gmail.com>

Sayono Sayono:

Thank you for submitting the manuscript, "Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of Derris elliptica root against the Temephos-susceptible strain of Aedes aegypti larvae" to Biodiversitas Journal of Biological Diversity. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/9804
Username: sayono

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Ahmad Dwi Setyawan

Biodiversitas Journal of Biological Diversity

Gmail **Sayono Sayono <say.epid@gmail.com>**

[biodiv] Editor Decision

2 pesan

Smujo Editors <smujo.id@gmail.com> 8 Desember 2021 pukul 19.03
Kepada: Sayono Sayono <say.epid@gmail.com>, Herbal Medicine Research of Dentistry Faculty of Universitas Muhammadiyah Semarang <riezdrgms@gmail.com>, "Laboratory of Epidemiology and Tropical Diseases of Public Health Faculty of Universitas Muhammadiyah Semarang, Semarang, Indonesia" <didik.24272@gmail.com>

Sayono Sayono, Herbal Medicine Research of Dentistry Faculty of Universitas Muhammadiyah Semarang, Laboratory of Epidemiology and Tropical Diseases of Public Health Faculty of Universitas Muhammadiyah Semarang, Semarang, Indonesia:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of Derris elliptica root against the Temephos-susceptible strain of Aedes aegypti larvae".

Our decision is: Revisions Required

Reviewer A:

Dear Authors,

Please see comments in the attached file.

Thank you

Recommendation: Revisions Required

Biodiversitas Journal of Biological Diversity

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Sayono Sayono <say.epid@gmail.com> 20 Desember 2021 pukul 22.57
Kepada: Smujo Editors <smujo.id@gmail.com>

Dear Smujo Editors

We have revised the attached-manuscript, entitled: "Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of Derris elliptica root against the Temephos-susceptible strain of Aedes aegypti larvae" based on the reviewer comments. Attached two files:
- File 1: Revised manuscript without the reviewer comments
- File 2: Revised manuscript with the reviewer comments.

We wait for the good news.

Regards,

Sayono
Department of Epidemiology and Tropical Diseases
School of Public Health of Universitas Muhammadiyah Semarang
Jalan Kedung Mundu Raya 18, Semarang, 50273
Indonesia

[Kutipan teks disembunyikan]

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Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of *Derris elliptica* root against the Temephos-susceptible strain of *Aedes aegypti* larvae

Abstract. *Aedes aegypti* is the main vector of Dengue, Chikungunya, and Zika diseases. Temephos resistance of this species has hampered vector control efforts worldwide. Studies proved that *D. elliptica* extracts were effective in controlling Aedes larvae, so the active compound needed to be isolated. This study evaluated the larvicidal activity of the chemical compounds isolated from n-hexane fractions of Tuba roots against the temephos-susceptible *Ae. aegypti* larvae. Six isolates were obtained from three of the seven n-hexane fractions. Three levels of concentration of each isolate were formulated for a preliminary bioassay test, and resulted in the two most active compounds, isolates 3 and 6. Results of the final bioassay test indicated that isolate 3 was more active than isolate 6 with LC_{50} and LC_{90} after 24 hours of exposure were 1.607 and 7.399 ppm, and after 48 hours of exposure were 0.926 and 3.206 ppm respectively. Isolate 6 also had high larvicidal activity after 48 hours of exposure, with LC_{50} and LC_{90} were 1.056 and 4.647 ppm. Further studies need to be carried out to determine the chemical structures and toxicity mechanisms of the bioactive compounds.

Keywords: *Aedes aegypti* larvae, chemical isolates, *Derris elliptica*, larvicidal activity, n-hexane fraction, Temephos-susceptible strain

INTRODUCTION

Ae. aegypti is the principle vector of human viral diseases including Dengue, Chikungunya, Yellow fever, and Zika (Powell et al 2018). Since these arboviral diseases have become a threat and a global public health problem (Marchi et al 2018, Girad et al 2020), community attention to the species increased according to the escalation and expansion of the disease occurrence from Africa to other regions worldwide (Weetman et al 2018). In Dengue endemic areas, efforts to control this mosquito species have become a priority since there are no antiviral drugs and vaccines are still being developed (Arredondo-García et al 2018, Plennevauz et al 2018). In this case, people in endemic areas prefer to use insecticides to control these arboviral disease vectors where the organophosphate group is the most dominant (WHO 2009, Manjarres-Suarez and Alivero-Verbel 2013). The high intensity of community use with uncontrolled doses has led to resistance of Aedes aegypti to various classes of insecticides. This condition has spread in many countries, especially in America, Asia, and Africa (Manjarres-Suarez and Olivero-Verbel 2013).

Temephos is an active insecticide compound in the organophosphate group that is most widely used in the control of *Ae. aegypti* larvae in endemic areas of arboviruses worldwide (WHO 2009) for seven decades (Manjarres-Suarez and Alivero-Verbel 2013), although it does not always reduce the density of the Dengue vector population. This condition is due to inconsistencies in use (George et al 2015, Arosteguí et al 2017), low coverage of exposed water containers, especially in rural areas (Legorreta-Soberanis et al 2017). On the other hand, long-term use of temephos with operational deficiency has led to the emergence of resistant-strains of *Aedes aegypti* to this active ingredient (Cediak et al 2016) and has become a serious problem in controlling this arbovirus vector. To solve this problem the researchers conducted an exploration to find active compounds from natural materials that are biodegradable, non-persistent, and not bio-accumulative in the environment (Arnason et al 2012).

Phytochemical screening and larvicidal activity evaluation have been carried out on various plant species, including *D. elliptica* with varying results (Komalamisra et al 2005). The wild plant in the agricultural farm which is commonly found in South to Southeast Asia has been traditionally used bay community for a long time as a fish poison and plant pest insecticide (Starr et al 2003, Sirrichamorn et al 2012). Studies on the larvicidal activity of various phytochemical compounds in *D. elliptica* extract against *Ae. aegypti* larvae have been reported from several

countries with varying methods and results. Studies in Thailand showed that the effective doses (LC_{50} and LC_{90}) of the ethanol extract of *D. elliptica* against *Ae. aegypti* larvae were 20.49 and 47.49 ppm (Komalamisra et al 2005), whereas a study in India reported the lower effective doses of petroleum ether extract (0.616 and 1.44 ppm) and methyl chloride (4.21 and 12.40 ppm) (Dohuita et al 2015). Study with specific extraction methods shows that the solvent combinations of methyl chloride: methanol 1:1 produces an effective dose (LC_{50}) of 24 ppm) (Zubairi et al 2015). The bioassay test of four *D. elliptica* extract fractions with different polarity, namely water, methanol, ethyl acetate, and n-hexane on *Ae. aegypti* larvae showed different larvicidal activity, and n-hexane extract was the most active extract with LC_{50} of 4,088 ppm (Sayono et al 2020), and isolation of specific compounds from this fraction is recommended. This study aims to evaluate the larvicidal activity of chemical compounds isolated from the n-hexane extract of *D. elliptical* root against susceptible-Temephos *Ae. aegypti* larvae.

MATERIAL AND METHODS

Extraction, fractionation, and isolation of *D. elliptica* roots.

The n-hexane fraction of ~~*Derris D.*~~ *elliptica* was obtained from the sequential extraction process of these plant roots (Sayono et al 2020). Then, this fraction was separated by using liquid-vacuum chromatography with n-hexane: ethyl acetate: methanol 10% gradient eluents, and resulted in seven grouped-fractions, namely n-hexane fraction 1 (FH1) to FH7. As much as 200 mg of FH2 was separated by using column-gravitation chromatography with n-hexane: ethyl acetate eluent (9:1) resulting in five subfractions, namely FH2A to FH2E. FH2B subfraction was purified by using column-gravitation chromatography with the same eluent resulting in as much as 30 mg of isolate 1. The separation process of FH4 (420 mg) used column-gravitation chromatography with n-hexane: ethyl acetate eluent (8:2) resulting in six subfractions of FH4A to FH4F. As much as 40 mg of FH4A subfraction was recrystallized with methanol to obtain isolate 2. FH4C subfraction (120 mg) was purified by using column-gravitation chromatography with eluent solvent of n-hexane: ethyl acetate (8:2) resulting in as much as 15 mg of isolate 3. Isolate 4 was purified from FH4E subfraction using column-gravitation chromatography with eluent solvent n-hexane: ethyl acetate (7:3). FH5 fraction was separated by using column-gravitation chromatography with eluent solvent n-hexane gradually from 7:3 to 0:10 and resulted in four subfractions, namely FH5A to FH5D. Isocratic purification of FH5B subfraction used column-gravitation chromatography with n-hexane: ethyl acetate eluent solvent (7:3) resulting in isolate 5 and 6. The secondary metabolites were isolated from the n-hexane extract and identified by IR and NMR, and the relevant literature was consulted.

Collecting and rearing the *Ae. aegypti* larvae

Larval surveys were conducted from January to March 2020 in Sambiroto village, Semarang municipality, Central Java Province, Indonesia. Morphological identification of mosquito species was carried out in the Laboratory of Epidemiology and Tropical Diseases of Public Health Faculty, Universitas Muhammadiyah Semarang based on the Walter Reed guideline (WRBU 2020). *Ae. aegypti* mosquitoes were reared through the fourth generation to obtain sufficient numbers with uniform age. The 3rd instar larvae were subjected to a bioassay test to evaluate the larvicidal activity of secondary metabolites of *D. elliptica* root after their susceptibility status to Temephos were determined (WHO 2016).

Larvicidal bioassay test

Initial bioassay tests of this study were performed by using the previous concentration range of n-hexane extract of *D. elliptica* (Sayono et al 2020) with slight modification. Based on the Lethal Concentration 50% and 90% (LC_{50} and LC_{90}) of the study, the new concentration range of 1, 4, and 7 mg L^{-1} was set and applied to the isolates (Table 2). Five experiment replicates were involved in each concentration level, and each replicate contains twenty third instar larvae of *Ae. aegypti*. Preliminary bioassay test results are used to determine the new lower concentration ranges at the next testing steps until the lowest effective concentrations (LC_{50} and LC_{90}) are obtained. The final concentration ranges were 0.5, 1, 2, 4, and 6 ppm. The larvicidal bioassay test experiment was accompanied by a positive control (Temephos 0.02 ppm) and a negative control (aquadest).

Data analysis

The mortality rate was performed descriptively with descriptive statistical techniques, while the effective concentration (the LC_{50} and LC_{90}) was determined using the Probit technique. Data analysis was performed by using SPSS 16.0 version.

Ethical consideration.

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The protocol of this study was reviewed by the Ethic Committee of Health Research of Public Health Faculty of Universitas Muhammadiyah Semarang with registration number 231/KEPK-FKM/UNIMUS/2019.

RESULTS AND DISCUSSION

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Performance of n-hexane extract, pure compound isolation, and initial bioassay

This study is a part of the exploration of phytochemicals with larvicidal potential and focuses on the *D. elliptica* or tuba root. This plant is interesting for further investigation because of several aspects: (i) its toxicity has been used by traditional communities as fish poison and insecticide for plant pests (Starr et al 2003); (ii) the potential for larvacide varies widely based on geography and the screening method applied (Komalamisra et al 2005, Dohutia et al 2015, Zubairi et al 2015, Sayono et al 2020), and (iii) agricultural weeds that grow abundantly. *D. elliptica* is a vine both horizontally on the ground and wrapped around and covering towering trees commonly found in South to Southeast Asia, and even spread to Africa and America. These plants are invasive to moderate to high levels and grow rapidly in tropical climates (CABI 2020). Utilization of this plant has a positive and strategic impact from a health and environmental aspects, especially the promising potential of larvicides, as well as eradicating weeds.

This study applied a tiered or serial, non-parallel screening method guided bioassay test to evaluate the larvicidal potential of outcomes at each stage. This is intended to evaluate the larvicidal potential of each type of extract produced. The screening process starts from the extraction of the polar tuba root compound with methanol solvent, and then the polar methanol extract is partitioned with non-polar n-hexane to bind the non-polar compounds, leaving other parts in the water solution. The other part was partitioned with ethyl acetate to bind the semi-polar compound, leaving an aqueous extract. The bioassay test results of each type of extract showed the potential sequence of n-hexane, methanol, and ethyl acetate larvicides, while water extracts did not show larvicidal potential (Sayono et al 2020).

The focus of this research is to determine the highest larvicidal potential of the chemical compound isolated from the non-polar, n-hexane extract. Results of fractionation and isolation of chemical compounds (Fig 1) of n-hexane extract of *D. elliptica* roots are showed in Table 1. Initial bioassay test showed that the six isolates have different larvicidal potential (Table 2). There are four groups of chemical compounds resulted from six isolates. Isolates 1, 2, and 4 originate from the different groups namely Beta-sitosterol, Sitosterol, and Triterpenoid, while isolates 3, 5, and 6 origins from the one group of chemical compounds, indicating the flavonoids. Visual characteristics showed that isolates 1, 2, and 4 are transparent (colorless) crystals while isolates 3, 5, and 6 are yellow crystals (Fig 2). Based on the concentration range of 1, 4, and 7 ppm for 24 hours, isolate 1 and 2 did not indicate the larvicidal activity. There is no dead *Ae. aegypti* larvae were found after exposure to the compounds. The six isolates of chemical compounds were produced from a combination of chromatography and purification methods (Ingle et al 2017), which are visually differentiated into four secondary metabolite groups, and only the flavonoid group that has high larvicidal potential. These findings indicate that the larvicide potency of n-hexane extract is influenced by the flavonoid content. This is shown by the difference in the larvicidal effect of isolates 1, 2, and 4 (non-flavonoids) with isolates 3 and 6 (flavonoid group) which have three times more potency than in the form of extracts (Sayono et al 2020, Zubairi et al 2015, and Komalamisra et al 2005), although slightly lower than petroleum ether extract and equivalent to methyl chloride extract (Dohutia et al 2015). The highest larvicidal activity was found in isolate 3 with a mortality rate of 45–92.5%, followed by isolate 6 (20-60%) and isolates 4 and 5 (< 10%). The mortality rate of isolate 4, 5, and 6 increased after 48 hours of exposure namely 22.5–57.5%, 45–60.0%, and 45–92.5% respectively. Based on the results, isolates 3 and 6 were used to determine the effective larvicidal activity by the next step of the bioassay test with the lower concentration range of 0.5, 1, 2, 4, and 6 ppm.

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Final bioassay test and the effective concentration

The final result of bioassay test showed that the larvicidal activity of isolate 3 better than isolate 6 (Table 3). Exposure to isolate 3 for 24 hours has caused mortality rates for *Ae. aegypti* larvae of 17.5-90% with LC_{50} and LC_{90} were 1.607 (1.250–2.025) and 7.399 (5.147–13.284) ppm, while exposure to isolate 6 caused mortality rates of 5–75% with LC_{50} and LC_{90} were 2.509 (2.098–3.048) and 13.894 (9.602–24.084) ppm respectively. However, both of isolate 3 and 6 had good larvicidal activity after 48 hours of exposure where the mortality rate of isolate 3 ranged from 27.5-97.5% with LC50 and LC90 were 0.926 (0.714-1.143) and 3.206 (2.459-4.782) ppm, and mortality rate of isolate 6 ranged from 25-100% with LC_{50} and LC_{90} were 1.056 (0.868-1249) and 4,647 (3,661-6,459) ppm, respectively (Table 4). These findings indicated that the larvicidal activity of isolate 6 was slightly lower and slower than isolate 3. There were three solvents produced extracts and isolates that have high larvicidal potential, namely petroleum ether, n-hexane, and methyl chloride. Flavonoids include more than 4,000 specific compounds which are

grouped into flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanidins (Paula-Ribeiro-Povinelli et al 2019). The main target site for flavonoid compounds is Acetylcholinesterase where the compound works to inhibit the activity of this enzyme (Perumalsamy et al 2015). These compounds also disrupt the endocrine and hormonal systems (Ge et al 2015) and reducing the esterase and monooxygenase enzymes (Visetson et al 2001).

CONCLUSION

This study obtained six secondary metabolites isolating from n-hexane fraction of *D. elliptica* root, namely isolate 1 to 6. Two of the six isolates (number 3 and 6) have high larvicidal activity against the Temephos-susceptible *Aedes aegypti* larvae. Elucidation of a chemical structure and toxication mechanisms of the compounds are necessary conducted to prepare the technical grade of larvicide for this finding.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGMENT

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Figure 1.

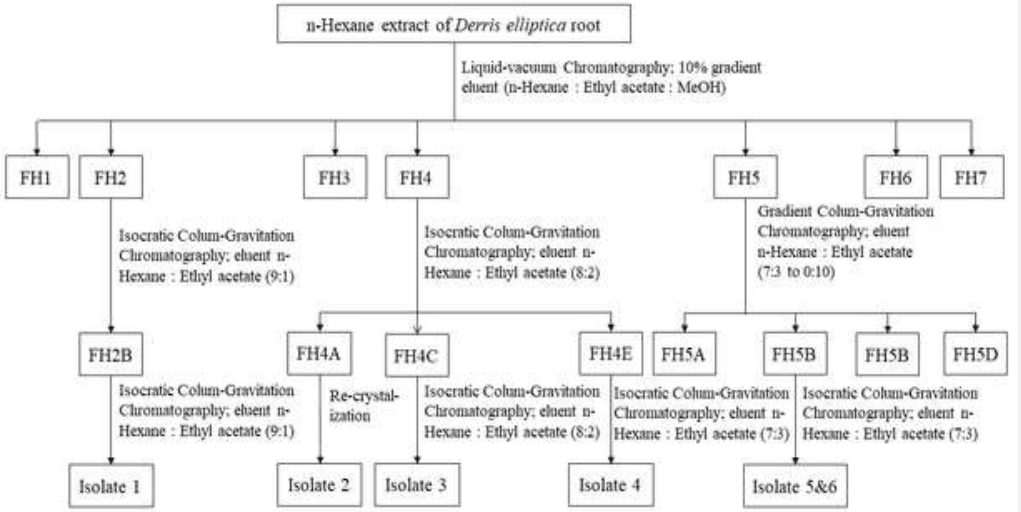

Fig 1. Fractionation and isolation of phytochemical compound from n-hexane extract of *D. elliptica* roots. Seven fractionates and six isolates were obtained from the n-hexane fraction of *D. elliptica.* FH1 – FH7: n-hexane fraction number 1 to 7. FH2B: n-hexane fraction number 2 subfraction B, etc.

Figure 2

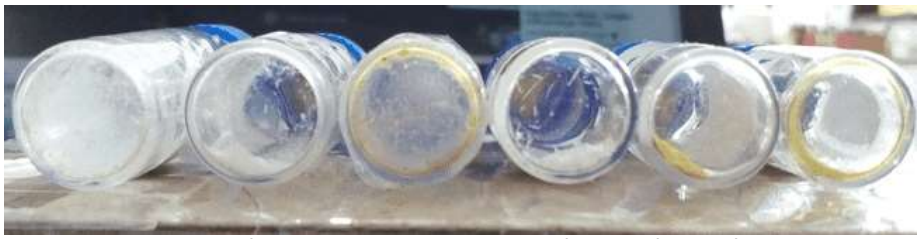

Fig 2. Visual characteristics of the six chemical compound isolates of n-hexane fraction of *D. elliptica* roots. Isolates number 1, 2, and 4 are colorless crystals while isolates number 3, 5, and 6 are yellowish crystals.

Table 1.

Table 1. Group of secondary metabolites isolated from n-hexane fraction of *D. elliptica* roots and identified by IR and NMR

Isolate number	Secondary metabolite group	Visual characteristics
I	Beta-sitosterol	Colorless crystal
II	Sitosterol	Colorless crystal
III	Flavonoid	Yellow crystal
IV	Triterpenoid	Colorless crystal
V	Flavonoid	Yellow crystal
VI	Flavonoid	Yellow crystal

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It can not be trusted only by visual characteristics.
This should be analysed by MS, IR and or NMR

Commented [su9]: If it is identified by IR/NMR, please also included the summary of IRs including wavelength and wavenumber

Table 2

Table 2. Results of initial bioassay test of the pure compound isolated from n-hexane fraction of *D. elliptica* extract against *Ae. aegypti* larvae

Isolates	Dosages (ppm)	Larval mortality rate (%)	
		24 hrs	48 hrs
I	1	0	-
	4	0	-
	7	0	-
II	1	0	-
	4	0	-
	7	0	-
III	1	45.0	-
	4	70.5	-
	7	92.5	-
IV	1	5.0	22.5
	4	5.0	47.5
	7	7.5	57.5
V	1	5.0	45.0
	4	7.5	60.0
	7	10.0	60.0
VI	1	20.0	57.5
	4	30.0	80.0
	7	65.0	92.5
Positive control (Temephos)	0.02	100	100
Negative control (Aquadest)	-	0	0

Commented [su10]: These include isolate 1 to 6 not only 1-3

Table 3

Table 3. Results of larvicidal activity determination of pure compound isolates number III and VI of *D. elliptica* against *Ae. aegypti* larvae using the final concentration ranges

Isolates	Dosages (ppm)	Larval mortality rate (%)	
		24 hrs	48 hrs
III	0,5	17,5	27.5
	1	30,0	45.0
	2	65,0	77.5
	4	72,5	92.5
	6	90,0	100.0
VI	0,5	5,00	25,0
	1	26,3	51,3
	2	41,3	61,3
	4	46,3	71,3
	6	75,0	100,0
Positive control (Temephos)	0.02	100	100
Negative control (Aquadest)	-	0	0

Table 4

Table 4. Results of Probit analysis showed the LC_{50} and LC_{90} of isolates III and VI of *D. elliptica* against *Ae. aegypti* larvae based on final concentration ranges

Isolates	Exposure time (hours)	Regression equation	Lethal Concentration (ppm)	
			LC_{50} (95% Confidence limits)	LC_{90} (95% Confidence limits)
III	24	Y = -0.398+1.932X	1.607 (1.250 – 2.025)	7.399 (5.147 – 13.284)
	48	Y= 0.079+2.377X	0.926(0.714 – 1.143)	3.206(1.459 – 4.782)
VI	24	Y = -0.689+1.724X	2.509 (2.098 – 3.048)	13.894 (9.602 – 24.084)
	48	Y = -0.047+1.992X	1.056 (0.868 – 1.249)	4.647 (3.661 – 6.459)

Commented [su11]: These based on which dosage concentration?

Is there any data larvicidal activity using the pure compounds of isolates III and VI?

Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of *Derris elliptica* root against the Temephos-susceptible strain of *Aedes aegypti* larvae

Abstract. *Aedes aegypti* is the main vector of Dengue, Chikungunya, and Zika diseases. Temephos resistance of this species has hampered vector control efforts worldwide. Studies proved that *D. elliptica* extracts were effective in controlling Aedes larvae, so the active compound needed to be isolated. This study evaluated the larvicidal activity of the chemical compounds isolated from n-hexane fractions of Tuba roots against the temephos-susceptible *Ae. aegypti* larvae. Six isolates were obtained from three of the seven n-hexane fractions. Three levels of concentration of each isolate were formulated for a preliminary bioassay test, and resulted in the two most active compounds, isolates 3 and 6. Results of the final bioassay test indicated that isolate 3 was more active than isolate 6 with LC_{50} and LC_{90} after 24 hours of exposure were 1.607 and 7.399 ppm, and after 48 hours of exposure were 0.926 and 3.206 ppm respectively. Isolate 6 also had high larvicidal activity after 48 hours of exposure, with LC_{50} and LC_{90} were 1.056 and 4.647 ppm. Further studies need to be carried out to determine the chemical structures and toxicity mechanisms of the bioactive compounds.

Keywords: *Aedes aegypti* larvae, chemical isolates, *Derris elliptica*, larvicidal activity, n-hexane fraction, Temephos-susceptible strain

INTRODUCTION

Ae. aegypti is the principle vector of human viral diseases including Dengue, Chikungunya, Yellow fever, and Zika (Powell et al 2018). Since these arboviral diseases have become a threat and a global public health problem (Marchi et al 2018, Girad et al 2020), community attention to the species increased according to the escalation and expansion of the disease occurrence from Africa to other regions worldwide (Weetman et al 2018). In Dengue endemic areas, efforts to control this mosquito species have become a priority since there are no antiviral drugs and vaccines are still being developed (Arredondo-García et al 2018, Plennevauz et al 2018). In this case, people in endemic areas prefer to use insecticides to control these arboviral disease vectors where the organophosphate group is the most dominant (WHO 2009, Manjarres-Suarez and Alivero-Verbel 2013). The high intensity of community use with uncontrolled doses has led to resistance of Aedes aegypti to various classes of insecticides. This condition has spread in many countries, especially in America, Asia, and Africa (Manjarres-Suarez and Olivero-Verbel 2013).

Temephos is an active insecticide compound in the organophosphate group that is most widely used in the control of *Ae. aegypti* larvae in endemic areas of arboviruses worldwide (WHO 2009) for seven decades (Manjarres-Suarez and Alivero-Verbel 2013), although it does not always reduce the density of the Dengue vector population. This condition is due to inconsistencies in use (George et al 2015, Arosteguí et al 2017), low coverage of exposed water containers, especially in rural areas (Legorreta-Soberanis et al 2017). On the other hand, long-term use of temephos with operational deficiency has led to the emergence of resistant-strains of *Aedes aegypti* to this active ingredient (Cediak et al 2016) and has become a serious problem in controlling this arbovirus vector. To solve this problem the researchers conducted an exploration to find active compounds from natural materials that are biodegradable, non-persistent, and not bio-accumulative in the environment (Arnason et al 2012).

Phytochemical screening and larvicidal activity evaluation have been carried out on various plant species, including *D. elliptica* with varying results (Komalamisra et al 2005). The wild plant in the agricultural farm which is commonly found in South to Southeast Asia has been traditionally used bay community for a long time as a fish poison and plant pest insecticide (Starr et al 2003, Sirrichamorn et al 2012). Studies on the larvicidal activity of various phytochemical compounds in *D. elliptica* extract against *Ae. aegypti* larvae have been reported from several

countries with varying methods and results. Studies in Thailand showed that the effective doses (LC_{50} and LC_{90}) of the ethanol extract of *D. elliptica* against *Ae. aegypti* larvae were 20.49 and 47.49 ppm (Komalamisra et al 2005), whereas a study in India reported the lower effective doses of petroleum ether extract (0.616 and 1.44 ppm) and methyl chloride (4.21 and 12.40 ppm) (Dohuita et al 2015). Study with specific extraction methods shows that the solvent combinations of methyl chloride: methanol 1:1 produces an effective dose (LC_{50}) of 24 ppm) (Zubairi et al 2015). The bioassay test of four *D. elliptica* extract fractions with different polarity, namely water, methanol, ethyl acetate, and n-hexane on *Ae. aegypti* larvae showed different larvicidal activity, and n-hexane extract was the most active extract with LC_{50} of 4,088 ppm (Sayono et al 2020), and isolation of specific compounds from this fraction is recommended. This study aims to evaluate the larvicidal activity of chemical compounds isolated from the n-hexane extract of *D. elliptical* root against susceptible-Temephos *Ae. aegypti* larvae.

MATERIAL AND METHODS

Extraction, fractionation, and isolation of *D. elliptica* roots.

The n-hexane fraction of *D. elliptica* was obtained from the sequential extraction process of these plant roots (Sayono et al 2020). Then, this fraction was separated by using liquid-vacuum chromatography with n-hexane: ethyl acetate: methanol 10% gradient eluents, and resulted in seven grouped-fractions, namely n-hexane fraction 1 (FH1) to FH7. As much as 200 mg of FH2 was separated by using column-gravitation chromatography with n-hexane: ethyl acetate eluent (9:1) resulting in five subfractions, namely FH2A to FH2E. FH2B subfraction was purified by using column-gravitation chromatography with the same eluent resulting in as much as 30 mg of isolate 1. The separation process of FH4 (420 mg) used column-gravitation chromatography with n-hexane: ethyl acetate eluent (8:2) resulting in six subfractions of FH4A to FH4F. As much as 40 mg of FH4A subfraction was recrystallized with methanol to obtain isolate 2. FH4C subfraction (120 mg) was purified by using column-gravitation chromatography with eluent solvent of n-hexane: ethyl acetate (8:2) resulting in as much as 15 mg of isolate 3. Isolate 4 was purified from FH4E subfraction using column-gravitation chromatography with eluent solvent n-hexane: ethyl acetate (7:3). FH5 fraction was separated by using column-gravitation chromatography with eluent solvent n-hexane gradually from 7:3 to 0:10 and resulted in four subfractions, namely FH5A to FH5D. Isocratic purification of FH5B subfraction used column-gravitation chromatography with n-hexane: ethyl acetate eluent solvent (7:3) resulting in isolate 5 and 6. The secondary metabolites were isolated from the n-hexane extract and identified by IR and NMR, and the relevant literature was consulted.

Collecting and rearing the *Ae. aegypti* larvae

Larval surveys were conducted from January to March 2020 in Sambiroto village, Semarang municipality, Central Java Province, Indonesia. Morphological identification of mosquito species was carried out in the Laboratory of Epidemiology and Tropical Diseases of Public Health Faculty, Universitas Muhammadiyah Semarang based on the Walter Reed guideline (WRBU 2020). *Ae. aegypti* mosquitoes were reared through the fourth generation to obtain sufficient numbers with uniform age. The 3rd instar larvae were subjected to a bioassay test to evaluate the larvicidal activity of secondary metabolites of *D. elliptica* root after their susceptibility status to Temephos were determined (WHO 2016).

Larvicidal bioassay test

Initial bioassay tests of this study were performed by using the previous concentration range of n-hexane extract of *D. elliptica* (Sayono et al 2020) with slight modification. Based on the Lethal Concentration 50% and 90% (LC_{50} and LC_{90}) of the study, the new concentration range of 1, 4, and 7 mg L^{-1} was set and applied to the isolates (Table 2). Five experiment replicates were involved in each concentration level, and each replicate contains twenty third instar larvae of *Ae. aegypti*. Preliminary bioassay test results are used to determine the new lower concentration ranges at the next testing steps until the lowest effective concentrations (LC_{50} and LC_{90}) are obtained. The final concentration ranges were 0.5, 1, 2, 4, and 6 ppm. The larvicidal bioassay test experiment was accompanied by a positive control (Temephos 0.02 ppm) and a negative control (aquadest).

Data analysis

The mortality rate was performed descriptively with descriptive statistical techniques, while the effective concentration (the LC_{50} and LC_{90}) was determined using the Probit technique. Data analysis was performed by using SPSS 16.0 version.

Ethical consideration.

The protocol of this study was reviewed by the Ethic Committee of Health Research of Public Health Faculty of Universitas Muhammadiyah Semarang with registration number 231/KEPK-FKM/UNIMUS/2019.

RESULTS AND DISCUSSION

Performance of n-hexane extract, pure compound isolation, and initial bioassay

This study is a part of the exploration of phytochemicals with larvicidal potential and focuses on the *D. elliptica* or tuba root. This plant is interesting for further investigation because of several aspects: (i) its toxicity has been used by traditional communities as fish poison and insecticide for plant pests (Starr et al 2003); (ii) the potential for larvacide varies widely based on geography and the screening method applied (Komalamisra et al 2005, Dohutia et al 2015, Zubairi et al 2015, Sayono et al 2020), and (iii) agricultural weeds that grow abundantly. *D. elliptica* is a vine both horizontally on the ground and wrapped around and covering towering trees commonly found in South to Southeast Asia, and even spread to Africa and America. These plants are invasive to moderate to high levels and grow rapidly in tropical climates (CABI 2020). Utilization of this plant has a positive and strategic impact from a health and environmental aspects, especially the promising potential of larvicides, as well as eradicating weeds.

This study applied a tiered or serial, non-parallel screening method guided bioassay test to evaluate the larvicidal potential of outcomes at each stage. This is intended to evaluate the larvicidal potential of each type of extract produced. The screening process starts from the extraction of the polar tuba root compound with methanol solvent, and then the polar methanol extract is partitioned with non-polar n-hexane to bind the non-polar compounds, leaving other parts in the water solution. The other part was partitioned with ethyl acetate to bind the semi-polar compound, leaving an aqueous extract. The bioassay test results of each type of extract showed the potential sequence of n-hexane, methanol, and ethyl acetate larvicides, while water extracts did not show larvicidal potential (Sayono et al 2020).

The focus of this research is to determine the highest larvicidal potential of the chemical compound isolated from the non-polar, n-hexane extract. Results of fractionation and isolation of chemical compounds (Fig 1) of n-hexane extract of *D. elliptica* roots are showed in Table 1. Initial bioassay test showed that the six isolates have different larvicidal potential (Table 2). There are four groups of chemical compounds resulted from six isolates. Isolates 1, 2, and 4 originate from the different groups namely Beta-sitosterol, Sitosterol, and Triterpenoid, while isolates 3, 5, and 6 origins from the one group of chemical compounds, indicating the flavonoids. Visual characteristics showed that isolates 1, 2, and 4 are transparent (colorless) crystals while isolates 3, 5, and 6 are yellow crystals (Fig 2). Based on the concentration range of 1, 4, and 7 ppm for 24 hours, isolate 1 and 2 did not indicate the larvicidal activity. There is no dead *Ae. aegypti* larvae were found after exposure to the compounds. The six isolates of chemical compounds were produced from a combination of chromatography and purification methods (Ingle et al 2017), which are visually differentiated into four secondary metabolite groups, and only the flavonoid group that has high larvicidal potential. These findings indicate that the larvicide potency of n-hexane extract is influenced by the flavonoid content. This is shown by the difference in the larvicidal effect of isolates 1, 2, and 4 (non-flavonoids) with isolates 3 and 6 (flavonoid group) which have three times more potency than in the form of extracts (Sayono et al 2020, Zubairi et al 2015, and Komalamisra et al 2005), although slightly lower than petroleum ether extract and equivalent to methyl chloride extract (Dohutia et al 2015). The highest larvicidal activity was found in isolate 3 with a mortality rate of 45–92.5%, followed by isolate 6 (20-60%) and isolates 4 and 5 (< 10%). The mortality rate of isolate 4, 5, and 6 increased after 48 hours of exposure namely 22.5–57.5%, 45–60.0%, and 45–92.5% respectively. Based on the results, isolates 3 and 6 were used to determine the effective larvicidal activity by the next step of the bioassay test with the lower concentration range of 0.5, 1, 2, 4, and 6 ppm.

Final bioassay test and the effective concentration

The final result of bioassay test showed that the larvicidal activity of isolate 3 better than isolate 6 (Table 3). Exposure to isolate 3 for 24 hours has caused mortality rates for *Ae. aegypti* larvae of 17.5-90% with LC_{50} and LC_{90} were 1.607 (1.250–2.025) and 7.399 (5.147–13.284) ppm, while exposure to isolate 6 caused mortality rates of 5–75% with LC_{50} and LC_{90} were 2.509 (2.098–3.048) and 13.894 (9.602–24.084) ppm respectively. However, both of isolate 3 and 6 had good larvicidal activity after 48 hours of exposure where the mortality rate of isolate 3 ranged from 27.5-97.5% with LC50 and LC90 were 0.926 (0.714-1.143) and 3.206 (2.459-4.782) ppm, and mortality rate of isolate 6 ranged from 25-100% with LC_{50} and LC_{90} were 1.056 (0.868-1249) and 4,647 (3,661-6,459) ppm, respectively (Table 4). These findings indicated that the larvicidal activity of isolate 6 was slightly lower and slower than isolate 3. There were three solvents produced extracts and isolates that have high larvicidal potential, namely petroleum ether, n-hexane, and methyl chloride. Flavonoids include more than 4,000 specific compounds which are

grouped into flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanidins (Paula-Ribeiro-Povinelli et al 2019). The main target site for flavonoid compounds is Acetylcholinesterase where the compound works to inhibit the activity of this enzyme (Perumalsamy et al 2015). These compounds also disrupt the endocrine and hormonal systems (Ge et al 2015) and reducing the esterase and monooxygenase enzymes (Visetson et al 2001).

CONCLUSION

This study obtained six secondary metabolites isolating from n-hexane fraction of *D. elliptica* root, namely isolate 1 to 6. Two of the six isolates (number 3 and 6) have high larvicidal activity against the Temephos-susceptible *Aedes aegypti* larvae. Elucidation of a chemical structure and toxication mechanisms of the compounds are necessary conducted to prepare the technical grade of larvicide for this finding.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGMENT

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Figure 1.

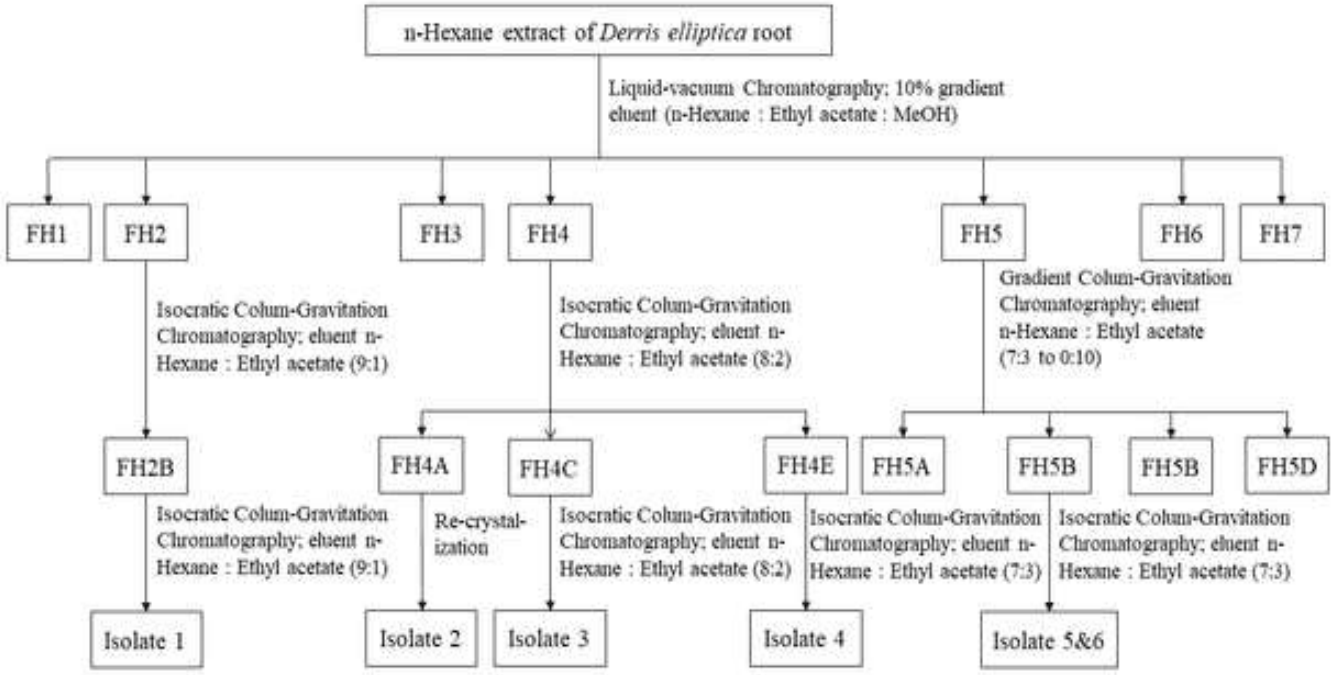

Fig 1. Fractionation and isolation of phytochemical compound from n-hexane extract of *D. elliptica* roots. Seven fractionates and six isolates were obtained from the n-hexane fraction of *D. elliptica*. FH1 – FH7: n-hexane fraction number 1 to 7. FH2B: n-hexane fraction number 2 subfraction B, etc.

Figure 2

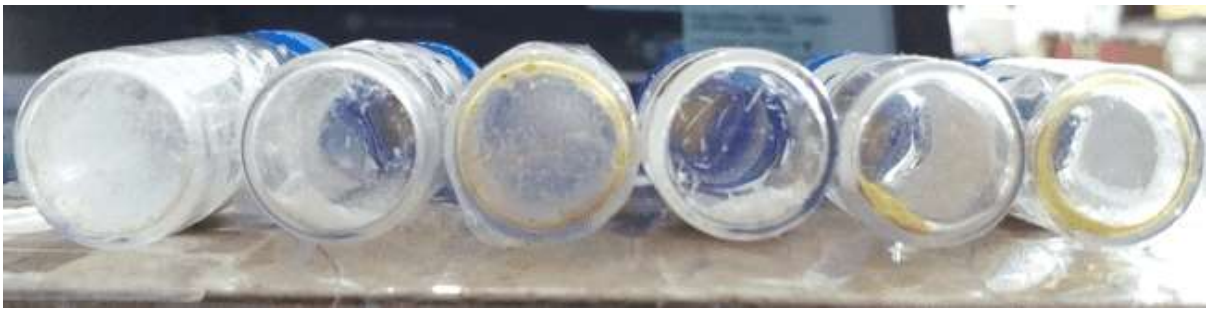

Fig 2. Visual characteristics of the six chemical compound isolates of n-hexane fraction of *D. elliptica* roots. Isolates number 1, 2, and 4 are colorless crystals while isolates number 3, 5, and 6 are yellowish crystals.

Table 1.

Table 1. Group of secondary metabolites isolated from n-hexane fraction of *D. elliptica* roots and identified by IR and NMR

Isolate number	Secondary metabolite group	Visual characteristics
I	Beta-sitosterol	Colorless crystal
II	Sitosterol	Colorless crystal
III	Flavonoid	Yellow crystal
IV	Triterpenoid	Colorless crystal
V	Flavonoid	Yellow crystal
VI	Flavonoid	Yellow crystal

Table 2

Table 2. Results of initial bioassay test of the pure compound isolated from n-hexane fraction of *D. elliptica* extract against *Ae. aegypti* larvae

Isolates	Dosages (ppm)	Larval mortality rate (%)	
		24 hrs	48 hrs
I	1	0	-
	4	0	-
	7	0	-
II	1	0	-
	4	0	-
	7	0	-
III	1	45.0	-
	4	70.5	-
	7	92.5	-
IV	1	5.0	22.5
	4	5.0	47.5
	7	7.5	57.5
V	1	5.0	45.0
	4	7.5	60.0
	7	10.0	60.0
VI	1	20.0	57.5
	4	30.0	80.0
	7	65.0	92.5
Positive control (Temephos)	0.02	100	100
Negative control (Aquadest)	-	0	0

Table 3

Table 3. Results of larvicidal activity determination of pure compound isolates number III and VI of *D. elliptica* against *Ae. aegypti* larvae using the final concentration ranges

Isolates	Dosages (ppm)	Larval mortality rate (%)	
		24 hrs	48 hrs
III	0,5	17,5	27.5
	1	30,0	45.0
	2	65,0	77.5
	4	72,5	92.5
	6	90,0	100.0
VI	0,5	5,00	25,0
	1	26,3	51,3
	2	41,3	61,3
	4	46,3	71,3
	6	75,0	100,0
Positive control (Temephos)	0.02	100	100
Negative control (Aquadest)	-	0	0

298 Table 4
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300
301 Table 4. Results of Probit analysis showed the LC_{50} and LC_{90} of isolates III and VI of *D. elliptica* against *Ae. aegypti*
302 larvae based on final concentration ranges

Isolates	Exposure time (hours)	Regression equation	Lethal Concentration (ppm)	
			LC_{50} (95% Confidence limits)	LC_{90} (95% Confidence limits)
III	24	Y = -0.398+1.932X	1.607 (1.250 – 2.025)	7.399 (5.147 – 13.284)
	48	Y= 0.079+2.377X	0.926(0.714 – 1.143)	3.206(1.459 – 4.782)
VI	24	Y = -0.689+1.724X	2.509 (2.098 – 3.048)	13.894 (9.602 – 24.084)
	48	Y = -0.047+1.992X	1.056 (0.868 – 1.249)	4.647 (3.661 – 6.459)

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[biodiv] Editor Decision

1 pesan

Smujo Editors <smujo.id@gmail.com> 28 Desember 2021 pukul 22.56
Kepada: Sayono Sayono <say.epid@gmail.com>, Herbal Medicine Research of Dentistry Faculty of Universitas Muhammadiyah Semarang <riezdrgms@gmail.com>, "Laboratory of Epidemiology and Tropical Diseases of Public Health Faculty of Universitas Muhammadiyah Semarang, Semarang, Indonesia" <didik.24272@gmail.com>

Sayono Sayono, Herbal Medicine Research of Dentistry Faculty of Universitas Muhammadiyah Semarang, Laboratory of Epidemiology and Tropical Diseases of Public Health Faculty of Universitas Muhammadiyah Semarang, Semarang, Indonesia:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of Derris elliptica root against the Temephos-susceptible strain of Aedes aegypti larvae".

Our decision is: Revisions Required

Reviewer A:

Dear Authors and editors,

Please see comments in the attached files and also please consider with other reviewer comments regarding this manuscript.

Thank you

Recommendation: Revisions Required

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Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of *Derris elliptica* root against the Temephos-susceptible strain of *Aedes aegypti* larvae

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Abstract. *Sayono S, Anwar R, Sumanto D. 2021. Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of* Derris elliptica *root against the Temephos-susceptible strain of* Aedes aegypti *larvae. Biodiversitas 23: xxxx. Aedes aegypti* is the main vector of Dengue, Chikungunya, and Zika diseases. Temephos resistance of this species has hampered vector control efforts worldwide. Studies proved that *D. elliptica* extracts were effective in controlling Aedes larvae, so the active compound needed to be isolated. This study evaluated the larvicidal activity of the chemical compounds isolated from n-hexane fractions of Tuba roots against the temephos-susceptible *Ae. aegypti* larvae. Six isolates were obtained from three of the seven n-hexane fractions. Three levels of concentration of each isolate were formulated for a preliminary bioassay test, and resulted in the two most active compounds, isolates 3 and 6. Results of the final bioassay test indicated that isolate 3 was more active than isolate 6 with LC_{50} and LC_{90} after 24 hours of exposure were 1.607 and 7.399 ppm, and after 48 hours of exposure were 0.926 and 3.206 ppm respectively. Isolate 6 also had high larvicidal activity after 48 hours of exposure, with LC_{50} and LC_{90} were 1.056 and 4.647 ppm. Further studies need to be carried out to determine the chemical structures and toxicity mechanisms of the bioactive compounds.

Keywords: *Aedes aegypti* larvae, chemical isolates, *Derris elliptica*, larvicidal activity, n-hexane fraction, Temephos-susceptible strain

INTRODUCTION

Ae. aegypti is the principle vector of human viral diseases including Dengue, Chikungunya, Yellow fever, and Zika (Powell et al 2018). Since these arboviral diseases have become a threat and a global public health problem (Marchi et al 2018, Girad et al 2020), community attention to the species increased according to the escalation and expansion of the disease occurrence from Africa to other regions worldwide (Weetman et al 2018). In Dengue endemic areas, efforts to control this mosquito species have become a priority since there are no antiviral drugs and vaccines are still being developed (Arredondo-García et al 2018, Plennevauz et al 2018). In this case, people in endemic areas prefer to use insecticides to control these arboviral disease vectors where the organophosphate group is the most dominant (WHO 2009, Manjarres-Suarez and Alivero-Verbel 2013). The high intensity of community use with uncontrolled doses has led to resistance of Aedes aegypti to various classes of insecticides. This condition has spread in many countries, especially in America, Asia, and Africa (Manjarres-Suarez and Olivero-Verbel 2013).

Temephos is an active insecticide compound in the organophosphate group that is most widely used in the control of *Ae. aegypti* larvae in endemic areas of arboviruses worldwide (WHO 2009) for seven decades (Manjarres-Suarez and Alivero-Verbel 2013), although it does not always reduce the density of the Dengue vector population. This condition is due to inconsistencies in use (George et al 2015, Arosteguí et al 2017), low coverage of exposed water containers, especially in rural areas (Legorreta-Soberanis et al 2017). On the other hand, long-term use of temephos with operational deficiency has led to the emergence of resistant-strains of *Aedes aegypti* to this active ingredient (Cediak et al 2016) and has become a serious problem in controlling this arbovirus vector. To solve this problem the researchers conducted an exploration to find active compounds from natural materials that are biodegradable, non-persistent, and not bio-accumulative in the environment (Arnason et al 2012).

Phytochemical screening and larvicidal activity evaluation have been carried out on various plant species, including *D. elliptica* with varying results (Komalamisra et al 2005). The wild plant in the agricultural farm which is commonly found in South to Southeast Asia has been traditionally used bay community for a long time as a fish poison and plant pest insecticide (Starr et al 2003, Sirrichamorn et al 2012). Studies on the larvicidal activity of various phytochemical compounds in *D. elliptica* extract against *Ae. aegypti* larvae have been reported from several countries with varying methods and results. Studies in Thailand showed that the effective doses (LC_{50} and LC_{90}) of the ethanol extract of *D. elliptica* against *Ae. aegypti* larvae were 20.49 and 47.49 ppm (Komalamisra et al 2005), whereas a study in India reported the lower effective doses of petroleum ether extract (0.616 and 1.44

ppm) and methyl chloride (4.21 and 12.40 ppm) (Dohuita et al 2015). Study with specific extraction methods shows that the solvent combinations of methyl chloride: methanol 1:1 produces an effective dose (LC_{50}) of 24 ppm) (Zubairi et al 2015). The bioassay test of four *D. elliptica* extract fractions with different polarity, namely water, methanol, ethyl acetate, and n-hexane on *Ae. aegypti* larvae showed different larvicidal activity, and n-hexane extract was the most active extract with LC_{50} of 4,088 ppm (Sayono et al 2020), and isolation of specific compounds from this fraction is recommended. This study aims to evaluate the larvicidal activity of chemical compounds isolated from the n-hexane extract of *D. elliptical* root against susceptible-Temephos *Ae. aegypti* larvae.

MATERIAL AND METHODS

The work sequent of this study consist of seven steps, namely extract fractionation and pure chemical compound isolation, screening larvicidal potency use the initial concentration, determination bioassay test, analyzing the effective concentration, and elucidating the chemical structure of pure isolate (Figure 1). *D. elliptica* extract used in this study is a product of previous experiment where n-hexane extract has the highest larvicidal potential among other types of extracts (Sayono et al 2020).

Fractionation and isolation of *D. elliptica* roots.

Fractions of *D. elliptica* were conducted by separated by using liquid-vacuum chromatography with n-hexane: ethyl acetate: methanol 10% gradient eluents, and resulted in seven grouped-fractions, namely n-hexane fraction 1 (FH1) to FH7. As much as 200 mg of FH2 was separated by using column-gravitation chromatography with n-hexane: ethyl acetate eluent (9:1) resulting in five subfractions, namely FH2A to FH2E. FH2B subfraction was purified by using column-gravitation chromatography with the same eluent resulting in as much as 30 mg of isolate 1. The separation process of FH4 (420 mg) used column-gravitation chromatography with n-hexane: ethyl acetate eluent (8:2) resulting in six subfractions of FH4A to FH4F. As much as 40 mg of FH4A subfraction was recrystallized with methanol to obtain isolate 2. FH4C subfraction (120 mg) was purified by using column-gravitation chromatography with eluent solvent of n-hexane: ethyl acetate (8:2) resulting in as much as 15 mg of isolate 3. Isolate 4 was purified from FH4E subfraction using column-gravitation chromatography with eluent solvent n-hexane: ethyl acetate (7:3). FH5 fraction was separated by using column-gravitation chromatography with eluent solvent n-hexane gradually from 7:3 to 0:10 and resulted in four subfractions, namely FH5A to FH5D. Isocratic purification of FH5B subfraction used column-gravitation chromatography with n-hexane: ethyl acetate eluent solvent (7:3) resulting in isolate 5 and 6.

Collecting and rearing the *Ae. aegypti* larva

Larval surveys were conducted from January to March 2020 in Sambiroto village, Semarang municipality, Central Java Province, Indonesia. Morphological identification of mosquito species was carried out in the Laboratory of Epidemiology and Tropical Diseases of Public Health Faculty, Universitas Muhammadiyah Semarang based on the Walter Reed guideline (WRBU 2020). *Ae. aegypti* mosquitoes were reared through the fourth generation to obtain sufficient numbers with uniform age. The 3rd instar larvae were subjected to a bioassay test to evaluate the larvicidal activity of secondary metabolites of *D. elliptica* root after their susceptibility status to Temephos were determined (WHO 2016).

Bioassay tests for determining the larvicidal activity of Tuba 1-6 isolates

Initial bioassay test of this study were performed by using the previous lethal concentration 50% of n-hexane extract of *D. elliptica* (Sayono et al 2020), namely 4.086 mg L^{-1}. Based on the LC50, a new concentration range of 1, 4, and 7 mg L^{-1} were set and occupied for the six isolates (Table 2). Five experiment replicates were involved in each concentration level, and each replicate contains twenty third instar larvae of *Ae. aegypti*. The results of the initial bioassay test were used to determine the new lower concentration ranges at the next testing steps until the lowest effective concentrations (LC_{50} and LC_{90}) are obtained. The final concentration ranges of tuba isolates were 0.5, 1, 2, 4, and 6 ppm. The larvicidal bioassay test experiment was accompanied by a positive control (Temephos 0.02 ppm) and a negative control (aquadest).

Elucidation of chemical compound

This step was carried out to determine the structure of chemical compounds from tuba root isolates which had high larvicidal activity. There were two isolates with the highest larvicidal activity, namely isolates III and VI, but only isolate VI had completed structural elucidation. The process of elucidating the chemical structure of isolate VI combines two methods, namely spectroscopy and Nuclear Magnetic Resonance (NMR). Spectroscopy uses ultraviolet (UV) light with a wavelength of 200-400 nm and infrared (IR). UV spectroscopy was used to identify double bonds and aromatic conjugates, while IR was used to identify functional groups. NMR of one dimension (^{13}C-NMR, ^{1}H-NMR dan DEPT 135^{0}) and two dimensions (*Heteronuclear Multiple Quantum Coherence* [HMQC], *Heteronuclear Multiple Bond Connectivity* [HMBC] dan ^{1}H-^{1}H COSY [*Correlation Spectroscopy*]) were used to understand the number, kind, and environment of carbon and proton. The spectrophotometer FTIR Perkin Elmer Spectrum One, JEOL JNM A-500 nuclear magnetic resonance (NMR) spectrometer, and TMS as internal standard and chemical shift (δ) in ppm units were used.

Data analysis

Larvicidal activity is indicated with the average of larval mortality of *Ae. aegypti* larvae. This variable was analyzed based on the five levels of isolates concentration, five replicates of each concentration level, and two different of observation times (24 and 48 hours) by using AN OVA test. The result was followed by Probit test to

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analyzed the effective concentration indicating by Lethal Concentration 50 (LC_{50}) and 90 (LC_{90}) percent. All data analysis was performed by using SPSS 16.0 version.

Ethical consideration.

The protocol of this study was reviewed by the Ethic Committee of Health Research of Public Health Faculty of Universitas Muhammadiyah Semarang with registration number 231/KEPK-FKM/UNIMUS/2019.

RESULTS AND DISCUSSION

***D. elliptica* root extract, fractionation, and pure compound isolation**

This study is a part of the exploration of phytochemicals with larvicidal potential and focuses on the *D. elliptica* or tuba root. This plant is interesting for further investigation because of several aspects: (i) its toxicity has been used by traditional communities as fish poison and insecticide for plant pests (Starr et al 2003); (ii) the potential for larvacide varies widely based on geography and the screening method applied (Komalamisra et al 2005, Dohutia et al 2015, Zubairi et al 2015, Sayono et al 2020), and (iii) agricultural weeds that grow abundantly. *D. elliptica* is a vine both horizontally on the ground and wrapped around and covering towering trees commonly found in South to Southeast Asia, and even spread to Africa and America. These plants are invasive to moderate to high levels and grow rapidly in tropical climates (CABI 2020). Utilization of this plant has a positive and strategic impact from a health and environmental aspects, especially the promising potential of larvicides, as well as eradicating weeds.

This study applied a tiered or serial, non-parallel screening method guided bioassay test to evaluate the larvicidal potential of outcomes at each stage. This is intended to evaluate the larvicidal potential of each type of extract produced. The screening process starts from the extraction of the polar tuba root compound with methanol solvent, and then the polar methanol extract is partitioned with non-polar n-hexane to bind the non-polar compounds, leaving other parts in the water solution. The other part was partitioned with ethyl acetate to bind the semi-polar compound, leaving an aqueous extract.

There are seven fractions resulted from this study, and eight subfraction were identified from the stronger fraction, namely FH2, FH4, and FH5. Six isolates of pure chemical compound were resulted from these subfractions. Visual characteristics showed that isolates 1, 2, and 4 are transparent (colorless) crystals while isolates 3, 5, and 6 are yellow crystals. This study reached a new step in exploration of chemical compounds of *D. elliptica* and their potency.

Bioassay test

Initial bioassay test showed that there were two chemical compound isolates of *D. elliptica* root have a high larvicidal potency, namely isolate III and VI. Isolates IV and V showed a low larvicidal activity while isolates I and II did not result the larval mortality. It meant that only isolates III and VI contain an active ingredient. Based on the concentration range of 1, 4, and 7 ppm for 24 hours, isolate 1 and 2 did not indicate the larvicidal activity. There is no dead *Ae. aegypti* larvae were found after exposure to the compounds. The six isolates of chemical compounds were produced from a combination of chromatography and purification methods (Ingle et al 2017), which are visually differentiated into four secondary metabolite groups, and only the flavonoid group that has high larvicidal potential. These findings indicate that the larvicide potency of n-hexane extract is influenced by the flavonoid content. This is shown by the difference in the larvicidal effect of isolates 1, 2, and 4 (non-flavonoids) with isolates 3 and 6 (flavonoid group) which have three times more potency than in the form of extracts (Sayono et al 2020, Zubairi et al 2015, and Komalamisra et al 2005), although slightly lower than petroleum ether extract and equivalent to methyl chloride extract (Dohutia et al 2015). The highest larvicidal activity was found in isolate 3 with a mortality rate of 45-92.5%, followed by isolate 6 (20-60%) and isolates 4 and 5 (< 10%). The mortality rate of isolate 4, 5, and 6 increased after 48 hours of exposure namely 22.5-57.5%, 45-60.0%, and 45-92.5% respectively. Based on the results, isolates 3 and 6 were used to determine the effective larvicidal activity by the next step of the bioassay test with the lower concentration range of 0.5, 1, 2, 4, and 6 ppm.

Final bioassay test and the effective concentration

The final result of bioassay test showed that the larvicidal activity of isolate 3 better than isolate 6 (Table 3). Exposure to isolate 3 for 24 hours has caused mortality rates for *Ae. aegypti* larvae of 17.5-90% with LC_{50} and LC_{90} were 1.607 (1.250-2.025) and 7.399 (5.147-13.284) ppm, while exposure to isolate 6 caused mortality rates of 5-75% with LC_{50} and LC_{90} were 2.509 (2.098-3.048) and 13.894 (9.602-24.084) ppm respectively. However, both of isolate 3 and 6 had good larvicidal activity after 48 hours of exposure where the mortality rate of isolate 3 ranged from 27.5-97.5% with LC50 and LC90 were 0.926 (0.714-1.143) and 3.206 (2.459-4.782) ppm, and mortality rate of isolate 6 ranged from 25-100% with LC_{50} and LC_{90} were 1.056 (0.868-1249) and 4,647 (3,661-6,459) ppm, respectively (Table 4). These findings indicated that the larvicidal activity of isolate 6 was slightly lower and slower than isolate 3. There were three solvents produced extracts and isolates that have high larvicidal potential, namely petroleum ether, n-hexane, and methyl chloride. Flavonoids include more than 4,000 specific compounds which are grouped into flavonols, flavones, flavanones, flavanols, isoflavones, and anthocyanidins (Paula-Ribeiro-Povinelli et al 2019). The main target site for flavonoid compounds is Acetylcholinesterase where the compound works to inhibit the activity of this enzyme (Perumalsamy et al 2015). These compounds also disrupt the endocrine and hormonal systems (Ge et al 2015) and reducing the esterase and monooxygenase enzymes (Visetson et al 2001).

Chemical structure of isolate VI

UV spectroscopy in 200-400 nm wave length is used to identify the double-bond and aromatic conjugation (Supratman 2010). The results (Figure_3a) show the presence of a 315-350 nm peak which is a conjugated carbonyl (C=CC=O) with an electron transition of n→π* and a 245-265 nm peak with an electron transition of →π*, indicating the existence of a conjugated double bond (C=C-C=C) (Mabry et al 1975). Figure_3b showed the presence of typical absorptions such as free -OH groups at max 3,452 cm^{-1}, C-H stretching vibrations at max 2,938 cm^{-1}, chelated α,β-unsaturated carbonyl groups bonded to aromatics at max 1,674, 1,607, and 1,509 cm^{-1}, the stretching vibration of C-O at max 1,262 cm^{-1} and the methoxy group at max 1,088 cm^{-1} (Pavia et al 2008). Interpretation of IR spectrum of Tuba VI isolate showed in Table 4.

The spectrum of the 13C-NMR showed the presence of twenty-three carbon signals consisting of one carbonyl signal at δC 191.3 ppm, five oxygenated aromatic carbon signals at δC 168.2; 157.8; 151.2; 148.5; and 144.1 ppm, one oxygenated methylene carbon signal at δC 67.7 ppm, two methylene carbon signals at δC 31.3 and 112.9 ppm, two oxygenated methine signals at δC 88.2 and 76.2 ppm, one signal oxygenated quaternary carbon at δC 64.0 ppm, four methine signals at δC 130.3; 109.3; 105.5; and 101.2 ppm, two methoxy signals at δC 56.5 and 56.0 ppm, one methyl at δC 17.3 ppm and six quaternary carbon signals at δC 143.0; 113.4; 111.9; and 108.8 ppm. DEPT 135° analysis showed that the methoxy and methoxy carbons were the top peaks (positive) while the curtener carbon did not appear as the peaks (Figure 3c). The 1H-NMR spectrum showed a shift at δH 7.82 ppm (1H, d, J = 8.5 Hz); 6.54 (1H, s); 6.52 (1H, s); 6.48 (1H, s); 5.23 (1H, t, J = 9 Hz) and 4.59 (1H, m) indicated the presence of six methines. Then there is a shift in δH 4.93 & 5.06 (2H, s); 4.49 (2H, m, J = 12 Hz); and 2.93 & 3.29 (2H, q, J = 7.75 & 15.5 Hz) indicated the presence of three methylene, shifts of δH 3.81 (3H, s) and 3.72 (3H, s) indicated the presence of two methoxy and δH shift of 1.75 ppm (3H, s) indicated the presence of methyl (Figure_3d).

The HMQC spectrum showed the six proton correlations with carbon atoms one bond apart (bond to each other). The correlation between H-1 at δH 1.75 ppm (3H, s) and C-1 at δC 17.3 ppm indicates the presence of methyl carbon. Correlation of H-2 at δH 2.93 & 3.29 ppm (2H, q, J = 7.75 & 15.5 Hz) with C-2 at δC 31.3 ppm; H-5 at δH 4.49 ppm (2H, m, J = 12 Hz) with C-5 at δC 64.0 ppm and H-14 at H 4.93 & 5.06 ppm (2H, s) with C -14 at δC 112.9 ppm confirmed that C-2, C-5, and C-14 were methylene carbon. Correlation of H-3 at δH 3.81 ppm (3H, s) with C-3 at δC 56.0 ppm and H-4 at δH 3.72 ppm (3H, s) with C-4 at δC 56.5 ppm showed carbon methoxy and correlation of H-7 at δH 4.59 ppm (1H, m) with C-7 at δC 76.2 ppm; H-8 at δH 5.23 ppm (1H, t, J = 9 Hz) with C-8 at δC 88.2 ppm; H-9 at δH 6.48 ppm (1H, s) with C-9 at δC 101.2 ppm; H-10 at δH 6.52 ppm (1H, s) with C-10 at δC 105.5 ppm; H-12 at δH 6.54 ppm (1H, s) with C-12 at δC 109.3 ppm; and H-16 at δH 7.82 ppm (1H, d, J = 8.5 Hz) with C-16 at C 130.3 ppm indicating the presence of proton methine (Figure 4a). One-dimensional NMR spectroscopic analysis of the Tuba_ɪVI compound in CHCl3 solvent obtained the data in Table 5 and it is suspected that this compound is a group of rotenoid compounds with an OH group at the C-6 position (Figure_4b). the ^{1}H-^{1}H COSY analysis was used to determine the proton-to-proton correlation that was three bonds apart. This correlation is indicated by the presence of a cross peak. The COSY ^{1}H-1H spectrum showed a correlation between H-10 at δH 6.52 ppm (1H, s) and H-16 at δH 7.82 ppm (1H, d, J = 8.5 Hz), H -8 at δH 5.23 ppm (1H, t, J = 9 Hz) with H-2 at δH 2.93 & 3.29 ppm (2H, q, J = 7.75 & 15.5 Hz), and δH-7 at H 4.59 ppm (2H, m) with H-5 at δH 4.49 (2H, m, J = 12 Hz) so it can be concluded that C-10 at δC 105.5 ppm coexists with C-16 at δC 130.3 ppm, C-8 at δC 88.2 ppm coexists with C-2 at δC 31.3 ppm, and C-7 at δC 76.2 ppm coexists with C-5 at δC 64.0ppm with one bond distance (Figure 4c).

The HMBC spectrum was used to determine the proton-to-carbon correlation between two to three bonds, which is important to confirm that Tuba VI is a rotenoid compound (Figure 5a-g). The first correlation is H-7 which has a correlation of ^{1}J with C-7, ^{2}J with C-11 and C-6, ^{3}J with C-23, and ^{4}J with C-19. Furthermore, H-14 which has a correlation of ^{2}J with C-17 and ^{3}J with C-8. There is also a correlation between H-8 and C-17 as far as two bonds (^{2}J), C-14 and C-1 as far as three bonds (^{3}J). Then, H-9 correlated sequentially with C-19 and C-20 by two bonds (^{2}J), and C-18 and C-11 by three bonds (^{3}J). The protons of methine H-12 are correlated sequentially with C-18 by two bonds (^{2}J) C-6, C-19 and C-20 by three bonds (^{3}J) and C-13 by five bonds (^{5}J) while H-10 is correlated with C-13 by 3 bonds (^{3}J). The methylene protons C-5 correlated sequentially with C-7 by one bond (^{1}J), C-6 and C-23 by four bonds (^{4}J) while H-2 correlated sequentially with C-8 and C-21 by two bonds (^{2}J), C-22 and C-17 by three bonds (^{3}J) and C-14 by three bonds (^{3}J). The methoxy protons H-3 and H-4 are sequentially correlated with C-20 and C-18 by three bonds (3J). The methyl proton H-1 is sequentially correlated with C-17 by two bonds (^{2}J), C-8 and C-14 by three bonds (^{3}J). The correlation of Tuba VI compounds can be seen in Figure5h. Based on the analysis of one-dimensional NMR (^{13}C-NMR, ^{1}H-NMR and DEPT 135^{0}) and two-dimensional (HMQC, HMBC and ^{1}H-^{1}H COSY) compounds, Tuba VI was found in the form of 6a-hydroxy-8,9-dimethoxy-2-(prop-1-en-2-yl)-1,2,12,12a-tetrahydrochromeno[3,4-b]furo[2,3-h]chromen-6(6aH)-one or more commonly known as rotenolone.

In conclusion this study obtained six secondary metabolites isolating from n-hexane fraction of *D. elliptica* root, namely isolate 1 to 6. Two of the six isolates (Tuba III and VI) have high larvicidal activity against the Temephos-susceptible *Aedes aegypti* larvae. Elucidation of a chemical structure of isolate VI was finished and indicated a rotenolone compound. The chemical structure of isolate III, and toxication mechanisms of all active compounds are necessary conducted to prepare the technical grade of larvicide for this finding.

Commented [A6]: Figure 3a

Commented [A8]: Figure 4b

Commented [A7]: Figure 3b

Commented [A9]: Conclusion

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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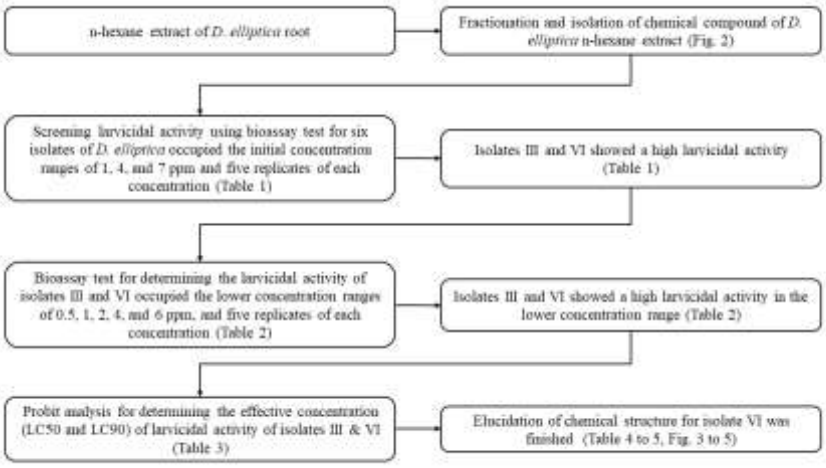

Figure 1. The consolidated report of trial. There were seven steps of the study started from fractionation and isolation of pure chemical compounds, screening bioassay use initial concentration, determination bioassay test for isolates III and VI, Probit analysis to determine the effective concentration, and elucidation of pure chemical structure of isolate VI.

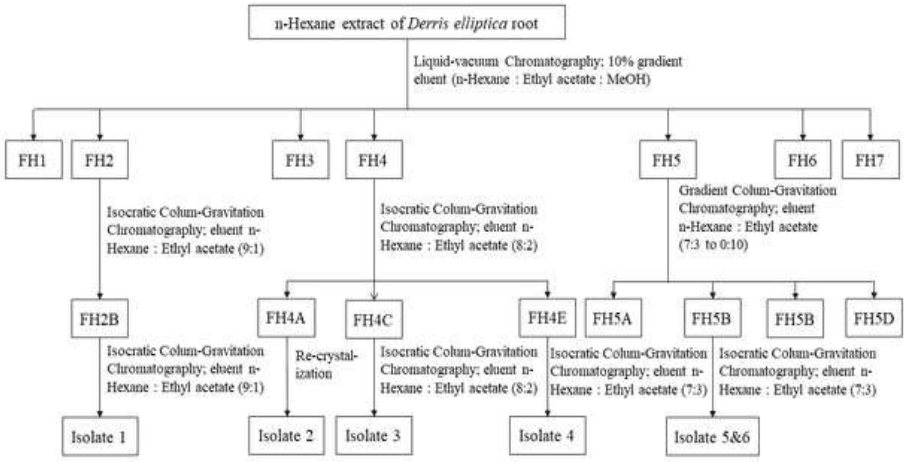

Figure 2. Fractionation and isolation of phytochemical compound from n-hexane extract of *D. elliptica* roots. Seven fractionates and six isolates were obtained from the n-hexane fraction of *D. elliptica*. FH1 – FH7: n-hexane fraction number 1 to 7. FH2B: n-hexane fraction number 2 subfraction B, etc.

Table 1. Results of initial bioassay test of the pure compound isolated from n-hexane fraction of *D. elliptica* extract against *Ae. aegypti* larvae

Isolates	Dosages (ppm)	Larval mortality rate (%)	
		24 hrs	48 hrs
I	1	0	-
	4	0	-
	7	0	-
II	1	0	-
	4	0	-
	7	0	-
III	1	45.0	-
	4	70.5	-
	7	92.5	-
IV	1	5.0	22.5
	4	5.0	47.5
	7	7.5	57.5
V	1	5.0	45.0
	4	7.5	60.0
	7	10.0	60.0
VI	1	20.0	57.5
	4	30.0	80.0
	7	65.0	92.5
Positive control (Temephos)	0.02	100	100
Negative control (Aquadest)	-	0	0

Table 2. Results of larvicidal activity determination of pure compound isolates number III and VI of *D. elliptica* against *Ae. aegypti* larvae using the final concentration ranges of 0.5, 1, 2, 4, and 6 mg L^{-1}

Isolates	Dosages (mg L^{-1})	Larval mortality rate (%)	
		24 hrs	48 hrs
III	0,5	17,5	27.5
	1	30,0	45.0
	2	65,0	77.5
	4	72,5	92.5
	6	90,0	100.0
VI	0,5	5,00	25,0
	1	26,3	51,3
	2	41,3	61,3
	4	46,3	71,3
	6	75,0	100,0
Positive control (Temephos)	0.02	100	100
Negative control (Aquadest)	-	0	0

Table 3. Results of Probit analysis showed the LC_{50} and LC_{90} of isolates III and VI of *D. elliptica* against *Ae. aegypti* larvae based on final concentration ranges of 0.5, 1, 2, 4, and 6 mg L^{-1}

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Isolates	Exposure time (hours)	Regression equation	Lethal Concentration (ppm) LC50 (95% Confidence limits)	LC90 (95% Confidence limits)	Chi-Square	p-value
III	24	Y = -0.398+1.932X	1.607 (1.250 – 2.025)	7.399 (5.147 – 13.284)	6.539	0.587
	48	Y= 0.079+2.377X	0.926(0.714 – 1.143)	3.206(1.459 – 4.782)	7.594	0.474
VI	24	Y = -0.689+1.724X	2.509 (2.098 – 3.048)	13.894 (9.602 – 24.084)	12.948	0.795
	48	Y = -0.047+1.992X	1.056 (0.868 – 1.249)	4.647 (3.661 – 6.459)	16.865	0.532

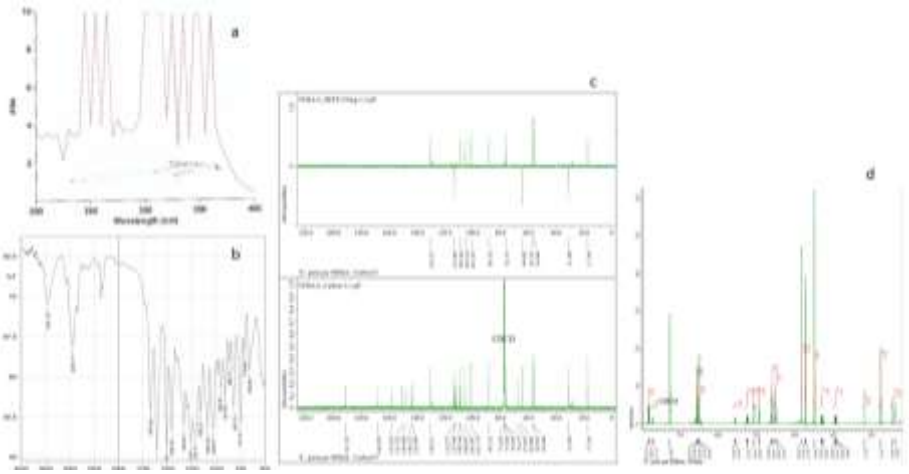

Figure 3. The spectrum results of spectroscopy and NMR: (a) UV and (b) IR spectroscopy; (c) ^{13}C-NMR and DEPT 135° (500MHz, $CDCl_3$); and (d) ^{1}H-NMR (500 MHz, $CHCl_3$) of Tuba-VI pure chemical compound isolate.

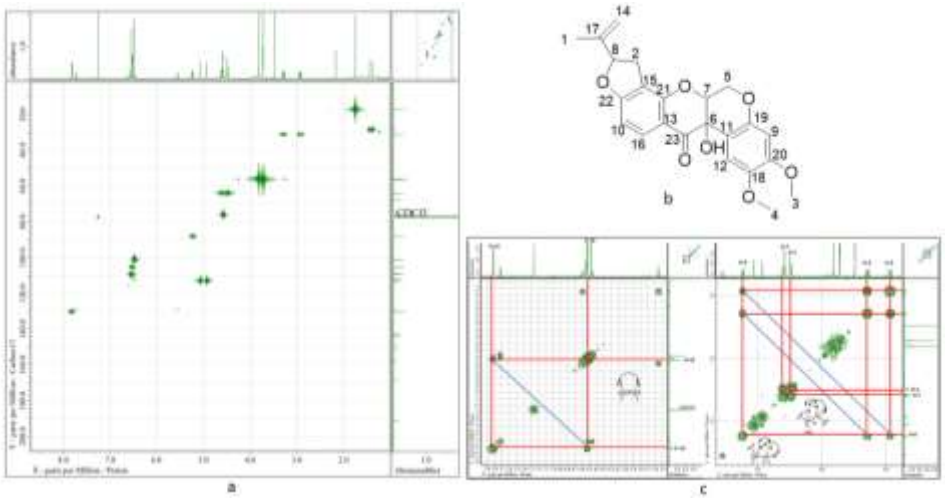

Figure 4. The HMCQ spectrum (a), prediction chemical structure (b), and ^{1}H-^{1}H COSY (c).

Table 4. Intrepretation of IR spectrum of TUBA-6 compound

υ_{max} / cm^{-1}	Band Shape	Intensity	Prediction
3452	Sharp	Low	Free OH
2938	Sharp	Medium	C-H stretch
1674	Sharp	High	C=O stretch
1607	Sharp	High	C=C stretch
1509	Sharp	High	C=C stretch
1088	Sharp	High	Oxygenized-Methyl

Table 5. Interpretation of NMR 1D dan HMQC (500 MHz, $CHCl_3$) Data

C position	δC (ppm)	δH (ppm), Mult, *J* (Hz)	Prediction
1	17.3	1,75 (3H, s)	-Cq-CH_3
2	31.3	2,93 & 3,29 (2H, q, *J* = 7,75 & 15,5 Hz)	-CH_2-CH-
3	56.0	3,81 (3H, s)	-O-CH_3
4	56.5	3,72 (3H, s)	-O-CH_3
5	64.0	4,49 (2H, m, *J* = 12 Hz)	-CH_2-CH-
6	67.7		
7	76.2	4,59 (1H, m)	-CH-CH_2-
8	88.2	5,23 (1H, t, *J* = 9 Hz)	-CH-CH_2
9	101.2	6,48 (1H, s)	-CH-
10	105.5	6,52 (1H, s)	-CH-
11	108.8		Cq
12	109.3	6,54 (1H, s)	-CH-Cq-
13	111.9		Cq
14	112.9	4,93 & 5,06 (2H, s)	-CH_2-
15	113.4		Cq
16	130.3	7,82 (1H, d, *J* = 8,5 Hz)	-CH-CH-
17	143.0		Cq
18	144.1		-O-Cq
19	148.5		-O-Cq
20	151.2		-O-Cq
21	157.8		-O-Cq
22	168.2		-O-Cq
23	191.3		C=O

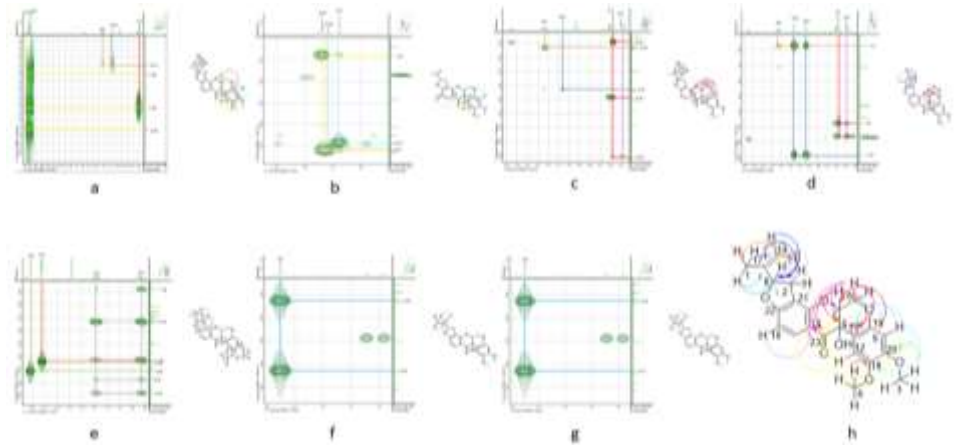

Figure 5. The HMBC spectrum (a-g) and correlation (h) of chemical compound of Tuba VI is indicate a rotenolone

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Sayono Sayono <say.epid@gmail.com>

[biodiv] Editor Decision

1 pesan

Anisa Septiasari <smujo.id@gmail.com> 28 Januari 2022 pukul 15.39
Kepada: SAYONO SAYONO <say.epid@gmail.com>, RISYANDI ANWAR <riezdrgms@gmail.com>, DIDIK SUMANTO <didik.24272@gmail.com>

SAYONO SAYONO, RISYANDI ANWAR, DIDIK SUMANTO:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of Derris elliptica root against the Temephos-susceptible strain of Aedes aegypti larvae".

Our decision is to: Accept Submission

_____

Biodiversitas Journal of Biological Diversity

Gmail **Sayono Sayono <say.epid@gmail.com>**

[biodiv] Editor Decision

1 pesan

Smujo Editors <smujo.id@gmail.com> 30 Januari 2022 pukul 21.14
Kepada: SAYONO SAYONO <say.epid@gmail.com>, RISYANDI ANWAR <riezdrgms@gmail.com>, DIDIK SUMANTO <didik.24272@gmail.com>

SAYONO SAYONO, RISYANDI ANWAR, DIDIK SUMANTO:

The editing of your submission, "Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of Derris elliptica root against the Temephos-susceptible strain of Aedes aegypti larvae," is complete. We are now sending it to production.

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/9804

Biodiversitas Journal of Biological Diversity

Gmail **Sayono Sayono <say.epid@gmail.com>**

[biodiv] New notification from Biodiversitas Journal of Biological Diversity

4 pesan

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Link: https://smujo.id/biodiv/authorDashboard/submission/9804

Ahmad Dwi Setyawan

Biodiversitas Journal of Biological Diversity

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[Kutipan teks disembunyikan]

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Kepada: Sayono Sayono <say.epid@gmail.com>

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There is new activity in the discussion titled "BILLING" regarding the submission "Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of Derris elliptica root against the Temephos-susceptible strain of Aedes aegypti larvae".
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Sayono Sayono <say.epid@gmail.com> 27 Januari 2022 pukul 12.21
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Dear Sir/Madam,

Dear Sir/Madam

Attached is the payment receipt for my article processing charge article on Biodiversity, entitled: "Larvicidal activity evaluation of the chemical compounds isolated from n-hexane extract of Derris elliptica root against the Temephos-susceptible strain of Aedes aegypti larvae". Thank you.

Regards,

Sayono Sayono
[Kutipan teks disembunyikan]

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Link: https://smujo.id/biodiv/authorDashboard/submission/9804

Ahmad Dwi Setyawan

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