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Research article

Larvicidal Activity of Red Betel (*Piper Crocatum*, L) Leaf Chloroform Extract Granule against *Aedes Aegypti* Larvae

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Abstract

Dengue fever is a disease caused by the bite of the Aedes aegypti mosquito. The development of natural larvicides needs to be done to reduce the risk of resistance and ensure environmental safety due to the use of chemical larvicides. Plants that have the potential as larvicides include red betel leaf, which contains alkaloids, flavonoids, tannins, and saponins. This study aims to determine the larvicidal activity of red betel leaf chloroform extract granules with LC_{50} and LC_{90} parameters against Aedes aegypti larvae. This study used a post-test control group design, where the chloroform maceration method was used in its extraction. The extract obtained was subjected to qualitative phytochemical identification and formulated into granules and tested for physical properties, namely: water content, flowability, and dispersion time and larvicidal activity test using a post-test control group design where the test group was divided into six groups, namely positive control (Abate®), negative control (placebo), treatment with extract concentrations of 0.18%; 0.24% and 0.48%. The results of the phytochemical test showed that the extract contained alkaloids, flavonoids, tannins, and saponins, while the granule test showed a water content of 3.02%, a flow rate of 2.07 g/second, and a dispersion time of 2.31 minutes. The granule concentration of 0.48% had a larvicidal activity of 98.67%, significantly different from placebo ($p<0.05$) and not significantly different from Abate ($p>0.05$). In conclusion, the chloroform extract granules of red betel leaves have larvicidal activity with an LC_{50} of 0.276% and an LC_{90} of 0.381% against Aedes aegypti larvae.

Keywords: *Aedes agypti*, extract, granule, larvacide, red betel

Introduction

Dengue fever (DF) is an epidemic disease with the highest frequency of occurrence in tropical countries, one of which is Indonesia. DF is a disease caused by the dengue virus, which is transmitted through the bite of the *Aedes aegypti* mosquito. In Indonesia, DF occurs in almost all regions or provinces so that some of Indonesia's areas are endemic areas [1], [2]. Prevention efforts have been carried out by the government and the community, one of which is the Jumantik program, where the community actively controls the number of mosquito larvae in their area as an effort to prevent dengue fever outbreaks. Through this Jumantik program, the government distributes larvicide to the community to be sprinkled in water reservoirs or ponds as a habitat for *Aedes aegypti* mosquito larvae, thereby preventing the growth of mosquito larvae.

However, these prevention efforts have not been effective, where data shows the number of DF sufferers is increasing, recorded in 2014 with 100,347 cases and IR 39.80 per 100,000 population, increasing in 2015 the number of DF sufferers reported was 129,650 cases and Incident Rate (IR) 50.75 per 100,000 population, in 2016 there were 202,314 sufferers, the number of deaths was 1,593 people and IR reached 78.85 per 100,000 population [2], [3]. The use of chemical larvicides such as temephos has a negative impact, namely the resistance of larvae to the compound so that it is necessary to increase the dose of administration which can increase the adverse impact on the environment and humans, where the temephos mechanism can inhibit the cholinesterase enzyme and stimulate nerves causing dizziness and nausea, at high concentrations can cause paralysis and death [4], [5]. For this reason, it is necessary to

develop a natural larvicide formula from plants that is safe and effective, one of which is the development of a red betel leaf extract granule formula as a natural larvicide.

Active compounds of natural ingredients which can be useful as larvicides include essential oils, alkaloids, tannins, saponins, flavonoids, and polyphenols. These compounds are toxic, if contact with larvae can result in larval death [1]–[3]. These compounds can also be found in red betel leaves (*Piper crocatum*) including essential oils that contain eugenol, alkaloids, flavonoids, tannins, saponins, and polyphenols so that the red betel leaf has the potential to be a biolarvicide [4], [5]. The development of a red betel leaf extract granule formula will increase the larvicidal activity capability, where the granule preparation is easier in determining the dosage and use so that it is effective in killing larvae.

Materials and Methods

Materials

The materials used in this study were red betel leaf (*Piper crocatum, L*), chloroform, potassium iodide, 2N HCL, Mayer reagent, Dragendorf reagent, NaOH 20%, $FeCl_3$ 4.5%, silica gel GF_{254}, ethyl acetate, methanol, distilled water, glacial acetic acid, butanol, lactose, polyvinyl pyrrolidone (PVP) and third instar *Aedes aegypti* larvae. The equipment used in the study include glassware, ovens, Flowmeter, *Halogen Moisture Analyze*r, siever with number of mesh screens 14 and 16, analytic scales.

Methods

Preparation of extract red piper betel leaf

The harvested red betel leaves were sorted, washed, then dried at 50oC for 24 hours. The dried leaves were reduced in size using a blender and sieved with a 40 mesh sieve. Extraction of 700 g of red betel leaf powder using the maceration method with chloroform solvent for 24 hours, then the extract was filtered and the chloroform was evaporated until a thick mass extract was obtained. Phytochemical screening for identification of chemical content and formulation of granule preparations and the extract yield is calculated.

Identification test for chloroform-free content in extracts

The weighed extract as much as 10 mg was then added in 10 ml of water, then added 0.1 ml of potassium iodide LP, shaken for 2 minutes, the liquid was allowed to separate, if positive the lower layer was purple [6].

Qualitative identification of phytochemical content in extracts

The identified phytochemical compounds are compounds that are suspected to have the ability as larvicides. The phytochemical compounds in the extract are as follows:

Identification of alkaloid compound

A porcelain cup filled with 2 ml test solution then evaporated to obtain residue. The residue was dissolved in 2 ml HCl as much as 5 ml. The solution is filtered after cold. The solution is divided into 3 test tubes. The first tube was used as a control, the second tube was added 3 drops of *Dragendorf* reagent through the tube wall, there was an orange precipitate showing positive results containing alkaloids. The last tube was added 3 drops of *Mayer* reagent through the tube wall, there was a yellow precipitate showing a positive result of the presence of alkaloids [3], [6], [7].

Identification of flavonoid compound

A total of 1 ml of each test solution was put into 2 test tubes. The first tube was used as a control and the second tube was added with a few drops of NaOH 20%, a positive result containing flavonoids if it formed yellow. Other colors that can arise include red to brown [7], [8].

Identification of tannin compound

A total of 2 ml test solution was put into 2 test tubes, tube 1 as control and tube 2 added a few drops of $FeCl_3$ 4.5%, positive results were indicated by the formation of dark green or blue [3], [8].

Identification of saponin compound

A total of 4 ml of test solution was added with 5 ml of aquadest, shake, see the presence of stable foam. A little extract is added with 5 ml of water, shake in a test tube, form a stable foam (foam as high as 1 cm and stable for 30 minutes). The test tube as a control was inserted 4 ml of the test solution [8].

Identification of phytochemical content in extracts using Thin Layer Chromatography (TLC)

The test solution was prepared by dissolving 500 mg of extract in 100 ml of chloroform.

Alkaloids contain test

The test solution was sprayed in the stationary phase of silica gel F_{254} eluted with the mobile phase of ethyl acetate: methanol: water (9: 2: 2). Dragendorf spray reagent is used to reveal stains. The formation of orange shows positive results in the presence of alkaloid content in red betel leaf chloroform extract [3], [9].

Flavonoids contain test

Test solutions and quercetin standards were sprayed on silica gel F_{254} plate eluted with the mobile phase of glacial acetic acid: butanol: water (1: 4: 5). Ammonia vapor is used to reveal stains. The formation of brown yellow stains after ammonia steaming shows positive results in the presence of flavonoids in visible light observations [3], [7].

Tannins compounding test

Test solutions and tannin standards were sprayed on a silica gel F_{254} plate eluted with a mobile phase of methanol: water (6: 4). $FeCl_3$ 4.5% reagent was used to reveal stains. The formation of black stains shows positive results of tannins in red betel leaf chloroform extract [10].

Saponin contain test

The test solution and standard saponins were treated with silica gel F_{254} plate eluted with the mobile phase of ethyl acetate: methanol: water (10 : 1.35 : 1). If the stain is not visible, Liebermann Burchard spray reagent is used [10].

Formulation of granule red betel leaf extract

In this study, the extract was made into granule preparations of three formulas with concentration variations of 0.18%; 0.24%; and 0.48%. The method of making granules used wet granulation, where PVP was mixed into the red betel leaf extract, then lactose was added up to 100 g. The mixture mass was sieved using a mesh sieve number 14, then the granules were dried in an oven at a temperature of 50°C for approximately 1 hour until dry granules were formed, then the granules were sieved with a mesh sieve number 16. The granule formula can be seen in Table 1.

Table 1. Formulas of granules red betle leaves extract.

Material	F1	F2	F3	F4
Red betle leaves extract(g)	-	0,18	0,24	0,48
Poly vinyl pyrrolidonen (g)	2	2	2	2
Distillation water (g)	qs	q.s	q.s	q.s
Lactosa (g)	Ad 100	ad 100	ad 100	ad 100

Physical properties test of granule red betel leaf extract

The physical properties test of red betel leaf extract granule preparation aims to determine the effect of extract concentration on humidity, flow properties and dispersion time. Furthermore, the results of the physical properties test are used to determine the best formula based on its physical properties characteristics.

Moisture content of granule red betel leafs extract

Moist content test is done by using a minimum moisture balance tool to insert 500 mg of granule then wait for the process to finish until the lights on the appliance die. Evaluate the moisture content to prevent granules from becoming moist which can accelerate the growth of microbes and fungi. A good granule has a moisture content of around 2-5% [11].

Flowability of granule red betel leaf extract

Twenty-five grams of granules are included in the flowmeter test equipment which has been covered in the bottom. When the bottom cover is pulled, the stopwatch is turned on then the time is recorded. Good flow velocity requirements are less than 10 g/sec or have easy flowing properties [11]–[13].

Dispersion time of granule red betel leaf extract

Approximately 400 mg of granules is weighed, poured into a glass, and then added to about one liter of water, then stirred, and the time is recorded until the granule dissolves completely. The time requirements for granules to dissolve, which is less than 5 minutes [14]–[16]

Activity test of granules of red betel leaf extract

Larvicidal activity test using a container filled with 100 ml of water and 25 third instar *Aedes aegypti* larvae. Temperature and humidity are set between 25-30°C and 60-80%, and water pH between 4-10. The treatment group consists of 3 concentration groups of 0.18%, 0.24%; 0.48%, and positive control using temephos (Abate®), and negative control (placebo) with 3 replications. Observation of the activity test was carried out for 24 hours by calculating the number of larval deaths.

Data analysis

The research data of physical properties test and larvicide granule activity were analyzed using SPSS software version 24. The data were normally distributed and homogeneous, then the data analysis used One-Way Anova. And if the data was not normally distributed and not homogeneous, then the data analysis used Kruskal Wallis and Mann-Whitney. Furthermore, the data determined the LC_{50} and LC_{90} values using probit analysis.

Results and Discussion

Qualitative identification of phytochemical content in extracts

Based on the results of the phytochemical identification test of the chloroform extract of red betel leaves, the phytochemical compound is shown in Table 2.

Table 2. Data of identification test of phytochemical compound in extract

Class of compounds	Parameter test	Results
Alkaloids	There is a brick yellow precipitate	Positive chemical content
Flavonoids	Formed brownish yellow color	Positive chemical content
Tannins	Formed in blue	Positive chemical content
Saponins	Formed foam	Positive chemical content

Phytochemical compound identification tests by Thin Layer Chromatography (TLC)

Phytochemical identification test results in extracts using thin layer chromatography were obtained as shown in table 3.

Table 3. Data of identification of phytochemical compound using TLC method

Class of compounds	Rf	After elution	After spray	Result
Alkaloids	0,78	Blue	Black	Positive chemical content
Flavonoids	0,79	Black	Black	Positive chemical content
Tanins	0,88	Black	Black	Positive chemical content
Saponins	0,84	Yellow	Yellow	Positive chemical content

Formulation of granules red betle leaf extract

The evaporated red betel leaf extract was then made into granule preparations with 3 extract concentrations, namely formula 1 (Placebo); formula 2 (0.18%); formula 3 (0.24%) and formula 4 (0.48%). Granulation of red comb leaf extract can be seen in Figure 1.

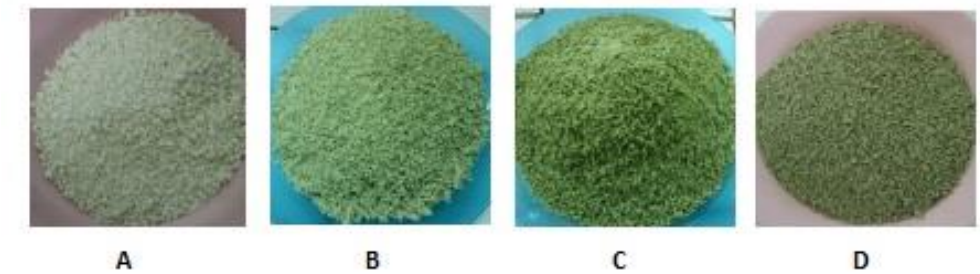

Figure 1. Red betel leaf extract granules (A). F1: placebo, (B). F2: granules with 0.18% extract, (C). F3: granules with 0.24% extract and (D). F4: granules with 0.48% extract

Based on the data in Figure 1, it shows that with the increase in the concentration of red betel leaf extract, the color of the granules becomes more concentrated. This is related to the chlorophyll content or green leaf dye contained in the extract. Based on the results of the study, it can be concluded that the red betel leaf extract obtained using the maceration technique with methanol solvent has a density of 0.69 gr/mL with a boiling point of 43°C. The phytochemical content in the extract found was alkaloids, flavonoids, saponins and tannins. While the results of

qualitative tests, extraction methods and ethanol solvent concentrations of 50%, 70% and 90% used gave positive results for secondary metabolite compounds in the form of flavonoids, polyphenols, saponins, and tannins, alkaloids, terpenoids (triterpenoids). and showed negative results for steroid compounds. Ethanol solvents of 50%, 70% and 90% gave negative results for steroid compounds, this is suspected because of the possibility of the absence of these compounds in red betel [3], [17]

Moisture content test of granule red betel leaf extract

The moisture content test data for red betel leaf extract granules can be seen in Figure 2.

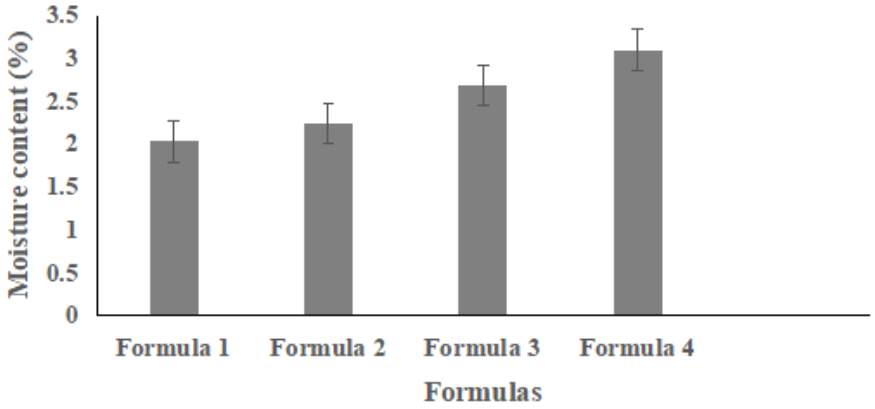

Figure 2. Moisture content of red betel extract granules

The data from the water content test of red betel leaf extract granules showed that the water content of formula granules 1, 2, 3, and 4 showed a water content between 1-2.3%, where the water content requirement in the granules meets the requirements if the water content is 2-4% [12], [13]. If the water content in the granules is high, it will be easy for mold to grow, so that the quality of the granules will decrease. Meanwhile, if the water content in the granules is too low, it will easily change shape into powder because of its fragile nature, thus affecting the primary packaging process. The results of the statistical test of the water content in the granules of each formula have a P-value <0.05, This shows that there is a significant difference in the water content of each formula. The data in Figure 2 shows that adding the concentration of red betel leaf extract will increase the water content in the granules, but still meets the requirements for good granules.

Flow-ability test of granule red betel leaf extract

The flow-ability test data of red betel leaf extract granules are shown in Figure 3.

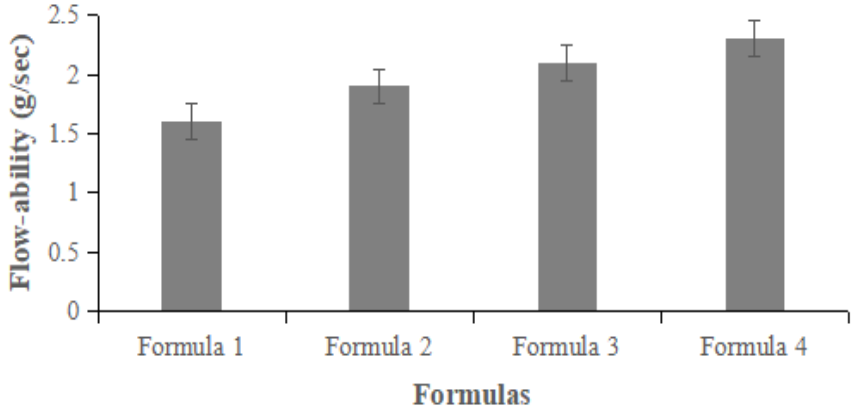

Figure 3. Data flow-ability tester of red betel leaf extract granules.

The granule flow test data in Figure 3 shows that the granule flow has a good flow rate, where the average granule flow of formulas 1, 2, 3, and 4 has a granule flow of less than <10 seconds/gram [13], [18]. This shows that the addition of extract concentration does not have an extreme effect on changes in granule flow. This test aims to maintain the uniformity of granule weight during the packaging process. Uniformity of granule weight will affect the uniformity of the active substance content of each package so that it will affect its larvicidal activity. Based on the statistical test data, the value of each comparison formula between groups P value < 0.05, this shows a significant

difference between groups, meaning that the greater the concentration of extract in the formula shows an increase in its flow rate. Although the increase in extract concentration increases the water content in the granule, it does not affect the granule flow rate because the water content in the granule is still below the specified water content requirements.

Dispersion time test of granule red betel leaf extract

The dispersion time test data of red betel leaf extract granules aims to determine the length of dispersion time of granules in water so that this dispersion time will affect the release of the extract in its solvent. The dispersion time test results data are shown in Figure 4.

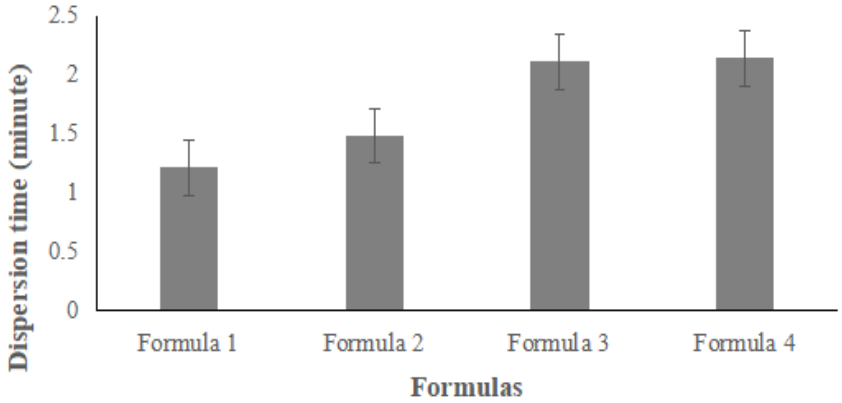

Figure 4. Data of the dispersion time of red betel leaf extract granules.

The dispersion time test data of the granules showed that all red betel leaf extract granule formulas had good dispersion times, where the dispersion time results showed that none of the dispersion times of each formula were more than 5 minutes. According to the provisions of the Indonesian pharmacopoeia, a good granule dispersion time is less than 5 minutes. This test aims to ensure that the granules are completely dissolved in aquadest. If the dispersion ability increases, it will affect the increase in larvicidal activity.

Activity larvacide test of granule red betel leaf extract

The data of the larvicidal activity test of red betel leaf extract granules are shown in Figure 5.

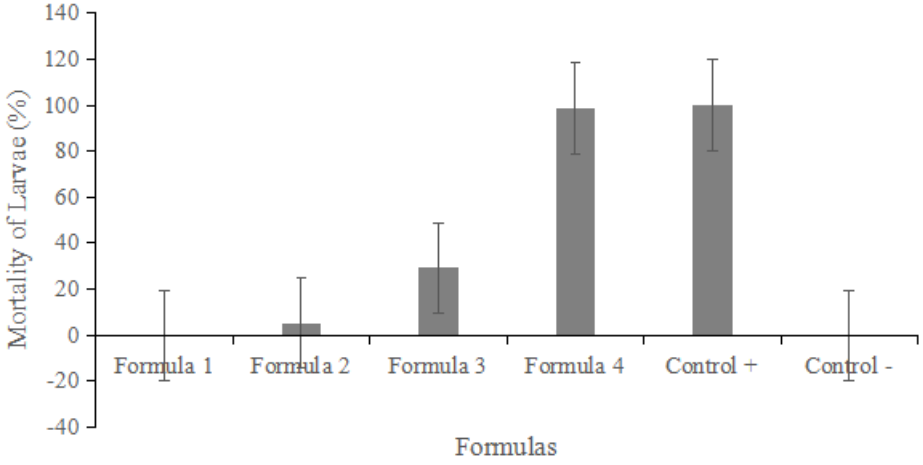

Figure 5. Data from the results of granule the red betel leaf extract larvicide test.

The data of the larvicidal activity test results showed that the death of larvae in formula 1 (5.33±4.62%); formula 2 (29.33±4.62%); formula 3 (98.67±2.31%); positive control (100±0.00%); and negative control (98.67±2.31). Based on the data in Figure 6, it shows that the larvicidal activity of formula 4 with positive control is almost the same activity. The data of the statistical test results of larvicidal activity show that each formula shows a P-value <0.05, which indicates a significant difference in the larvicidal activity of red betel leaf chloroform extract granules.

After obtaining the percentage data of larval mortality, the data was then used to determine the LC50 and LC90 values, where the results of the probit test obtained an LC_{50} value of 0.276% and an LC_{90} of 0.381%. This shows that the larvicidal ability of the granular extract is classified as less toxic and is still far below the positive control (Abate) of 0.01%, but the granules can be effectively used as an alternative larvicidal in several areas that are resistant to Abate because their larvicidal activity is almost 100% [18]–[20].

Red betel leaf extract contains phytochemical compounds in the form of alkaloids, saponins, tannins and flavonoids, where alkaloids can cause cell death through the mechanism of stomach poisoning and inhibit the action of the acetylcholinesterase enzyme, causing digestive disorders, and inhibiting larval growth, causing death [21]–[23]. Tannin compounds have antibacterial activity by slowing the growth of fungal cells, and shrinking cell membranes, thereby limiting the development of cell membrane synthesis, distorting permeability, damage, and cell lysis, in addition tannins can bind to digestive system proteins that are essential for growth, resulting in disruption in the way protein is absorbed in the digestive system. Tannins target cell wall polypeptides and coagulate proteins. Tannins also reduce the activity of digestive enzymes, resulting in nutritional disorders and disrupted larval growth [24], [25]. While saponins can dilute lipids (lipophilic) and then reduce cell surface pressure with their ability to attract water (hydrophilic) and cause cell damage and in the study of tea leaf saponin activity showed that the insecticidal mechanism of saponins is related to the effect on insect detoxification enzymes, where saponins are able to reduce the activity of superoxide dismutase (SOD), catalase (CAT), acetylcholinesterase (AChE), and carboxyesterase (CES). In addition, the insecticidal activity of saponins through the mechanism of interaction with cholesterol causes interference with ecdysteroid synthesis, where these substances are protease inhibitors or cytotoxic to insects [26], [27].

In the extract of red betel leaves contains flavonoid compounds which have the potential to inhibit the digestive tract of insects. Flavonoids also cause the accumulation of acetylcholine, which will cause disruption in the impulse transmission system to the muscles, causing muscle spasms, paralysis and death. Acetylcholinesterase (AChE) is the main site of action of flavonoids, oleic acid, and palmitic acid [21], [25], [28], [29]. Research on the effect of chloroform extract of red betel leaf on the inhibition of mouse fibroblast growth (NIH3T3 cell line) showed that the higher the concentration of the extract, the lower the percentage of living cells, where the results of Flowcytometry of cell death were dominated by the necrosis pathway > 90%. Morphologically, with May-Grunwald-Giemsa staining, it showed that the percentage of apoptotic cells was highest at a concentration of 250 micrograms per milliliter, where concentrations of 125 and 250 micrograms per milliliter could induce cell death through the necrosis pathway [30].

Conclusion

Red betel extract granules have low larvicidal activity compared to temephos with an LC_{50} value of 0.276% and LC_{90} of 0.381%.

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Declarations

Author contribution	:	Azis Ikhsanudin and Haadiyatul Tri Hastuti contributed to the data collection of the study, Lolita Lolita contributed to the data analysis and preparation of the research report, and Saiful Nizam Tajjudin contributed to the writing of the published article.
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Conflict of interest	:	There are no conflicts of interest among the authors
Ethics Declaration	:	Number of ethical approval: 0110801006 issued by research ethics committee of Universitas Ahmad Dahlan, Yogyakarta, Indonesia
Additional information	:	No additional information is available for this paper.

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