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Acetylcholinesterase Levels of *Aedes aegypti* Larvae after Exposure to The *Pandanus amaryllifolius* Leaf Extracts

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Abstract

Background: Dengue fever is a disease transmitted by *Ae. aegypti*. Controlling mosquitoes can use temephos. Temephos larvicides act primarily on the enzyme acetylcholinesterase (AChE) by inhibiting this enzyme. Temephos has drawbacks due to its high risk of resistance, so larvicides from natural ingredients such as pandan leaf extract (*P. amaryllifolius*) can be an alternative. The mechanism of action of *P. amaryllifolius* larvicide is still unclear.

Objective: This study aims to ascertain how LC_{85} pandan leaf methanol extract affects AChE levels in *Ae. aegypti*.

Methods: This is an actual experiment design with only a post-test control group research design. Tests were conducted by treating mosquito larvae with *P. amaryllifolius* LC_{85} extract, aquades, and temephos for 24 hours and measuring AChE levels with an ELISA Reader.

Results: The results showed that the AChE enzyme levels of *P. amaryllifolius* LC_{85} extract had an average AChE enzyme level of 147.19 $\pm$ 70.87 units/l. The AChE enzyme levels of larvae exposed to *P. amaryllifolius* LC_{85}, aquades, and temephos significantly differed ($p < 0.05$).

Conclusion: *P. amaryllifolius* has potential as a larvicidal, with a mechanism of action as a neurotoxin.

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INTRODUCTION

Dengue fever is a disease transmitted by *Ae. aegypti* and caused by the dengue virus of the genus Flavivirus. This disease can infect all age groups and is found throughout the year[1]. In 2019, 138,127 DHF cases were discovered, an increase from the previous year's total of 65,602 cases. Mortality due to DHF in 2019 is known to have increased from 2018, namely from 467 cases to 919 deaths[2]. Mosquito control can be done in several ways: environmental, biological, and chemical. Chemical control methods include the use of temephos larvicides and fogging. Insecticides are substances used to prevent, damage, repel, or reduce insects, for example, from the organophosphate group. This class of insecticides works primarily on the acetylcholinesterase (AChE) enzyme by inhibiting it[3]. Although effective in eradicating mosquitoes, insecticides have the disadvantage that they can cause resistance[4]. Temephos resistance has been found in Indonesia, namely in Demak, Banten, and Banjarnegara[5]. One way to deal with temephos resistance is to replace temephos with larvicides made from natural ingredients. Larvicides made from natural ingredients have been proven to be an alternative to reducing mosquito populations so that the number of diseases caused by mosquitoes can be reduced.

AChE enzymes can be inhibited by several plant compounds, one of which is alkaloids. Alkaloids can be found in various types of plants, such as the Pandanus. In the phytochemical tests, several active compounds were found in pandan leaves, such as alkaloids, steroids or triterpenoids, flavonoids, saponins, and hydroquinone phenol[27]. One of the natural ingredients that can be used as a substitute for temephos and as a natural larvicide is *Pandanus amaryllifolius,* which contains botanical larvicides[7]. *P. amaryllifolius* contains many active compounds, such as polyphenols (9.7%), flavonoids (17.18%), saponins (16.4%), and alkaloids (16.6%). Alkaloid compounds work as digestive system poisons and inhibit the AChE enzyme of mosquito larvae. This substance binds irreversibly to the AChE portion and inhibits the enzyme, causing acetylcholine (ACh) to accumulate in the mosquito's synaptic cleft[8]. The potential of these alkaloids is predicted to be realized in this study.

Previous research tested 150 *Ae. aegypti* after 24 hours of exposure to *P. amaryllifolius* extract. Larval mortality was obtained at each extract concentration, with the average percentage of *Ae. aegypti* is the highest at a concentration of 50%, with a value of 80%. The lowest larval mortality rate was at a concentration of 10% with a value of 30%[9]. AChE enzyme activity in *L. acetivum*, which contains mainly alkaloids, has AChE activity at an LC_{50} dose of 0.20 mg/mL. This shows that the alkaloids have an inhibitory effect on the AChE enzyme in insects[10]. Lethal concentration (LC) is the ability of the extract to kill mosquito larvae. LC_{85} is the extract concentration needed to kill 85% of mosquito larvae[11]. The purpose of LC_{85} is to maintain the balance of the food chain in the ecosystem. This study used the methanol extract of *P. amaryllifolius* LC_{85}. This study aimed to determine the effect of a methanol extract of *P. amaryllifolius* LC_{85} on levels of the AChE enzyme in *Ae. aegypti.*

METHODS

This study employs a pure experimental (true-experimental) design with a post-test-only control group research. This research has been declared ethically feasible with letter number No. 008/EC/KEPK- FKUC/VII/2022. The materials used in this study were *P. amaryllifolius*, methanol, coarse filter paper, tween 20, aquades and QuantiChromTM Acetylcholine esterase Assay Kit, which contains an assay buffer, reagent and calibrator, gloves, masks, temephos, aquades, DMSO, Tris buffer pH 7.8, acetylcholine iodide solution (ATCI), DTNB solution (5,5 -dithiobis-2-nitrobenzoic acid, Ellman reagent), and three tips [blue tip, yellow tip, white tip]. The tools used are glass jars, funnels, 1000 ml Erlenmeyer, 500 ml beaker, rotary evaporator, spatula, analytical balance, plastic cup, gauze, thermometer, litmus paper, psychrometer, handcounter, microplate 96 wells, ELISA reader, container transparent sample (cuvette), and micropipette.

The P. amaryllifolius species was determined at the Faculty of Pharmacy, Widya Mandala University, Surabaya, to ensure that the species was P. amaryllifolius officially. The *P. amaryllifolius* was collected from Made, Sambikerep District, Surabaya City, East Java (7°16' 33.7" S 112°38' 42.6" E). The leaves are cut and dried for a month to make the extract. After drying, the leaves are crushed into powder (simplicia) and soaked in methanol (maceration) for 2 weeks. The maceration results were filtered using a funnel and filter paper and evaporated using a rotary evaporator to obtain the extract.

Using several doses, preliminary tests were carried out first to ensure the correct extract concentration was LC85. The dose to be used in this experiment is 35.000 ppm. After obtaining the right concentration, the actual test is carried out. The mother liquor is prepared using the following formula:

$$A\ ppm = \frac{A\ mg\ extract}{1000\ ml\ aquades}$$

After that, the main solution of the methanol extract of *P. amaryllifolius* was diluted into several concentrations using the dilution formula:

$$V1 \times N1 = V2 \times N2$$

Information

V1 = volume of mother liquor
N1 = concentration of mother liquor
V2 = desired volume
N2 = desired concentration

The larvicidal test was conducted at the Entomology Laboratory of the Institute of Tropical Diseases, Airlangga University, Surabaya. The mosquito larvae of *Ae. aegypti,* as many as 20 larvae were put into a clear glass, and each would be given a different treatment. Referring to WHO, the larvae used will be taken randomly from the tray, with each container containing 20 larvae. Container 1 will be given aquades (a negative control), container 2 will be given temephos (a positive control), and container three will be given *P. amaryllifolius* LC_{85} extract. The treatment was left for 24 hours and after that. The enzymes were measured in the larvae.

An AChE enzyme examination was carried out at the Laboratory of Professor Nidom Foundation in Surabaya. Five dead mosquito larvae were taken randomly from each treatment, and the AChE enzyme levels were measured using the QuantiChrom™ Acetylcholine Esterase Assay Kit. The mosquito larvae were crushed individually and made into a homogenate,

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which was dissolved in 0.5 ml of 0.1 M phosphate-buffered saline (PBS) solution with a pH of 7.5[3]. The homogenate was then centrifuged at 14,000 rpm for 5 minutes, and the supernatant was obtained, which was transferred to the microplate using a micropipette. The reagent was prepared by adding 200 µl of assay buffer to 2 mg of reagent and then vortexing it until it dissolved. In each well with the supernatant on the microplate, 190µl of the reagent mixture was added. The microplate is inserted into the ELISA reader, and the optical density (OD), or what can be called the adsorption, is read at a wavelength of 405 nm in the first 5 minutes. The percentage of AChE inhibition can be calculated based on the adsorption value using the following formula[12]:

$$\% \text{ Inhibition} = \left(1 - \frac{A_t}{A_0}\right) \times 100$$

Information:
A0: Absorbance control
At: The adsorption of the compound tested.

AChE enzyme levels are expressed as Mean ± Standard Deviation (SD) and visualized in graphical form. The data that has been obtained will be tested for normality first. If p> 0.05, then the data will normally be distributed. Then, the data is tested for homogeneity (if sig. > 0.05, then the data is homogeneous). The one-way ANOVA test will be continued if normal distribution data is obtained. If data with an abnormal distribution is obtained, the Kruskal Wallis test will be carried out. After that, the Post hoc test is repeated, with the Tukey/Duncan test used if the data is homogeneous and the Mann-Whitney test used if it is not.

RESULTS

In the preliminary test, it was found that the concentration of the extract needed to kill 85% of mosquito larvae was 35.000 ppm. For complete preliminary test results, see Table 1 and the biolarvicidal test (the actual test), the mortality of *Ae. aegypti* is as follows in Table 2.

Table 1. Preliminary test result

Concentrations	Replication	Mortality
1.000 ppm	1	8
	2	6
5.000 ppm	1	12
	2	12
8.000 ppm	1	15
	2	18
12.000 ppm	1	15
	2	19
15.000 ppm	1	17
	2	16
35.000 ppm	1	18
	2	17

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This research will analyze the effects of the methanol solution of *P. amaryllifolius* LC_{85} on AChE levels in *Ae—aegypti* instar III. The first step is to describe the results of AChE enzyme levels (units/l) using descriptive statistics. Table 3 shows the characteristic results of AChE enzyme levels in each treatment.

The Kruskall-Wallis statistical test determines significant differences in AChE enzyme levels in different solutions, as shown in Table 4. At a significant level of 5%, if the p-value <0.05, there is a significant difference between the extract solutions; if the p-value is >0.05, there is no significant difference between the solutions. The following table shows the results of the Kruskall-Wallis test.

The results of the statistical tests showed significant differences in the levels of the AChE enzyme in each group of pandan LC_{85}, aquades, and temephos solutions. The post hoc test with the Mann-Whitney test was used to determine which solution had the highest levels of the AChE enzyme and had a significantly different value from the other solutions, as shown in Table 5. Post-hoc test results (Mann Whitney) showed that the AChE enzyme levels of larvae exposed to P. amaryllifolius LC_{85} equate, and temephos had a significant difference (p <0.05). Based on Table 5, the median value of P. amaryllifolius LC_{85} was 129.87 units/l, 311.69 units/l, and temephos 277.06 units/l. This indicated that treatment with *P. amaryllifolius* LC_{85} had the lowest levels of the enzyme AChE and was significantly different from the aquadest and temephos groups. At the same time, the group with the highest enzyme levels was aquades.

The following Figure 1 illustrates the average AChE enzyme levels in each treatment.

Table 2. The number of mortality of *Ae. aegypti*

Treatment type	Replication	Mortality of *Ae. aegypti* larvae	
		Average (amount)	Average (%)
P. amaryllifolius	1	17	85%
	2	16	80%
	3	18	90%
Aquades	1	0	0%
	2	0	0%
	3	0	0%
Temephos	1	20	100%
	2	20	100%
	3	20	100%

Table 3. Characteristics AChE enzyme levels (unit/l)

Groups	Min	Max	Median	(Mean ± SD)
P. amaryllifolius	86,58	225,11	129,87	147,19 ± 70,87
Aquades	294,37	363,64	311,69	323,23 ± 36,05
Temephos	277,06	285,71	277,06	279,94 ± 4,99

Table 4. Kruskall Wallis test results AChE enzyme levels (unit/l)

Groups	Median	(Mean ± SD)	*p*	Description
Pandan LC_{85}	129,87	147,19 ± 70,87	0,027	Significantly different
Aquades	311,69	323,23 ± 36,05		
Temephos	277,06	279,94 ± 4,99		

Description: (*) = Significantly different at a significant level of 5% ($p < 0,05$)

Table 5. Post-hoc test results (Mann Whitney) AChE enzyme levels (unit/l)

Groups	Pandan LC_{85}	Aquades	Temephos
P. amaryllifolius LC_{85}			
Aquades	0,050*		
Temephos	0,046*	0,046*	

Description: Sign (*) = Significantly different at a significant level of 5% ($p < 0,05$)

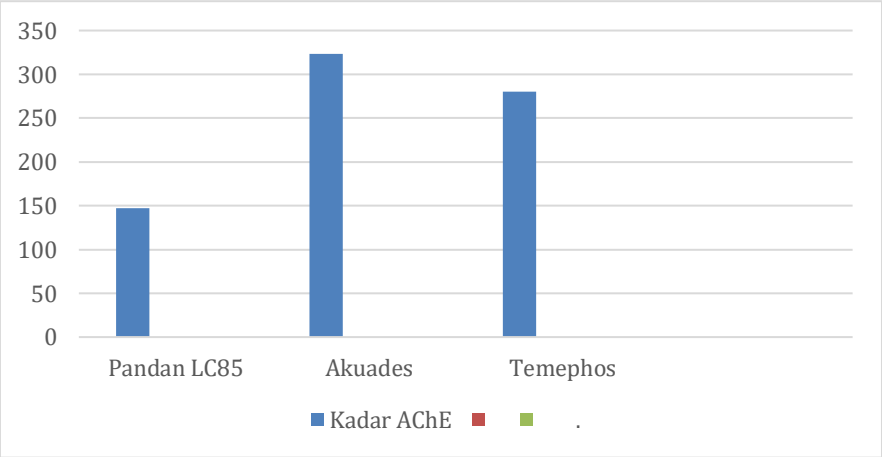

Figure 1. Diagram of average AChE enzyme levels in each group

DISCUSSION

P. amaryllifolius influences the mortality of *Ae. aegypti* at a concentration of 35,000 ppm, which is 85%. This ability is similar to research on the effect of *P. amaryllifolius* at a concentration of 200 ppm, which will kill an average of 25 (100%) *Aedes sp.* larvae and is proven to be used as a larvicidal mosquito, *Ae. aegypti*. The use of solvents in extraction will affect the quality of the extract and the compound content in plant extracts because ethanol will function as a solvent and pull out the active compounds in the extract[13,14,15]. This is by research that examines the differences in the content of extracts with ethanol solvents and without solvents (water). This study showed that extracts with ethanol solvents contained alkaloids, flavonoids, saponins, tannins, and phenols, while extracts without solvents (water) contained flavonoids, tannins, and phenols[16].

P. amaryllifolius toxicity was also found in *Musca domestica*, with the lowest mortality of one fly (5%) and the highest mortality of 13 flies (15%). This study showed that *P. amaryllifolius* extract was toxic to insects[17]. In addition, the toxicity ability of *P. amaryllifolius* to *Anopheles* sp. at a concentration of 1,000 ppm, an average death rate of 19 larvae (94%) and at a concentration of 400 ppm, an average death rate of 14 larvae (60%)[18].

Apart from *P. amaryllifolius*, several other types of Pandanus contain chemical compounds similar to *P. amaryllifolius*. Previous studies found the content of alkaloids, steroids, phenols, tannins, terpenes, flavonoids, saponins, and glycosides in *P. tectorius* extract[19]. The content of secondary metabolites in red fruit (*Pandanus commodious Lam.) was determined by extraction using n-hexane, methanol, ethyl acetate, and water solvent. However,* different solvents were obtained; all samples contained flavonoids, terpenoids, and alkaloids, except for samples with n-hexane solvent[20]. The *Pandanus* genus, such as *P. amaryllifolius*, *P. dubius*, and *P. utility,* are sources of secondary metabolites, namely steroids, terpenoids, flavonoids, lignans, benzenoids, and alkloids[21].

AChE is a hydrolytic enzyme that functions in cholinergic nerve transmission by hydrolyzing acetylcholine (ACh) into acetate and choline. Organophosphate larvicides will act on the AChE enzyme, which has a mechanism of action to permanently bind the amino acid serine. AChE, which is inhibited, will increase muscle impulses so that they will contract continuously, muscle spasms occur, and end in insect death[22]. Temephos will enter the larvae through the exoskeleton through the tarsus, and its mechanism of action involves inhibiting the AChE enzyme so that ACh buildup will occur in the nerve endings, which will cause hyperexcitation, seizures, muscle paralysis, and death[23].

P. amaryllifolius contains polyphenolic compounds, flavonoids, saponins, essential oils, tannins, and alkaloids[24]. One of these compounds, namely alkaloids, is a neurotoxin that works by inhibiting the AChE enzyme so that it cannot hydrolyze ACh into acetate and choline. This inhibition causes ACh to accumulate in the nerve endings, increasing the work of the larval body in constantly sending commands to the larval muscles, causing the muscles to contract continuously and be challenging to control[25].

The AChE enzyme has an essential function at nerve synapses. AChE levels of *Ae. aegypti* and *Ae. albopictus* after exposure to *Bryopsis pennata, Padina australis*, and *Sargassum binderi* at LC_{50} was between 1-10 mg/ml. AChE levels of larvae after exposure to *Sargassum angustifolium* LC_{50} were 5.4 mg/ml, *Sargassum boveanum* LC_{50} were 1.0 mg/ml, *Sargassum oligocystum* LC_{50} were 2.5 mg/ml and *Sargassum Sp.* from India LC_{50} of 1.0 mg/ml. AChE levels after exposure to *P. australis* LC_{50} showed better results, namely 6.3 mg/ml[26]. AChE activity in mosquito larvae exposed to temephos was 70% and 60%. This indicates that inhibition of AChE will cause AChE activity to decrease and cause the death of the larvae[27]. Reduced levels of the enzyme AChE appear to inhibit the enzyme's synthesis. This requires further research at the molecular and protein (proteomic) levels. In this study, the AChE levels of mosquito larvae exposed to *P. amaryllifolius* LC_{85} extract had an average AChE enzyme level of 147.19 + 70.87 units/l with the lowest level being 86.58 units/l and the highest level being 225.11 units/l.

The mechanism of action of the alkaloids is that when they enter the body of the larvae through the mouth and skin, the alkaloids inhibit the AChE enzyme, disrupt endocrine functions by interfering with the molting function, and are toxic to nerves. AChE enzyme inhibition will lead to the accumulation of ACh at the synapses and trigger constant excitation of the larvae, ataxia, and a lack of muscular coordination until it ends with the death of the larvae[28,29].

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CONCLUSION

In this experiment, the conclusion is obtained that the methanol extract of P. amaryllifolius LC_{85} affects the levels of the acetylcholinesterase enzyme. AChE enzyme levels of *P. amaryllifolius* LC_{85} extract had an average AChE enzyme level of 147.19 + 70.87 units/l, with the lowest level being 86.58 units/l and the highest level being 225.11 units/l. There were significant differences in the levels of the AChE enzyme in each group of pandanus LC_{85} solution, aquades, and temephos.

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