Bioactivity of Ethanol Extract of *Muntingia calabura* L. Leaves on Mortality and Its Effect on Changes in Morphology and Midgut Tissue of Aedes aegypti Larvae

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Abstract. The *Aedes aegypti* mosquito is a vector for **Dengue Hemorrhagic Fever** (DHF) spreading from one to another through its bite. Many efforts have been made to control mosquito vectors of DHP, both chemically and naturally. One natural control is the use of *Muntingia calabura* (kersen) leaf extract, as a larval poison. *M. calabura* is considered to be potentially toxic to larvae since it contains secondary metabolites, presumably toxic to them. The aim of this study was to determine secondary metabolites contained in the *M. calabura* ethanolic extract followed by determining their bioactivity on mortality and their effect on morphology changes and midgut tissue of *A. aegypti* larvae. The ethanolic extract on *M. calabura* leaf contained saponins, tannins, flavonoids, steroids and alkaloids. Bioactivity of the extract indicated by the death of *Ae.aegypti* larvae, the highest was at a concentration of 1% (100% death) and the lowest was at a concentration of 0.25% (53.75% death). Shortening, shrunken, and darker color of the larvae body was indicated after administration of the extract. Histopathological observations on the midgut tissue found some damage in the peritrophic membrane, epithelial cells, basement membrane, as well as damage of the structure of the midgut cells.

Keywords: *Aedes aegypti*, *Muntingia calabura*, Bioactivity, Mortality.

1 Introduction

Mosquito-borne diseases such as dengue fever (DHF), *chikungunya*, and malaria continue to increase and pose a threat to public health, especially in tropical areas. In recent years, there has been an increase in the mosquito population and the number of cases that are thought to be related to climate change and deforestation [3].

Various efforts have been made to prevent and control mosquito populations, either in the form of repellents or directly as controllers in breeding areas. So far, control efforts still use synthetic chemicals that are not environmentally friendly, which affect the ecosystem and also cause resistance from target organisms. Therefore, larval control in this study is carried out using natural materials that are safe for the environment [15], one of which is the *Muntingia calabura plant (kersen*).

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Muntingia calabura is known as a protective plant because of its shady leaves, growing and fruiting throughout the year, in addition, the leaves of the *M. calabura* plant are commonly used as alternative medicine for various diseases such as: gout, antiseptic, antioxidant, antimicrobial, anti-inflammatory, antidiabetic, and antitumor [12].

According to Hidriya, et al. [6], *Muntingia calabura* leaves contain secondary metabolite compounds that have the potential to be larvicidal. This is because *M. calabura* leaves contain secondary metabolites such as: tannins, saponins, and flavonoids. These secondary metabolites contained in plant foods can act as toxins that can inhibit the activity of digestive enzymes in insects by reducing appetite, inhibiting growth, and damaging cell membranes resulting in death due to inability to breathe [15].

2 Material and Methods

2.1 Preparation of *Aedes aegypti* Larvae

The *Aedes aegypti* eggs were hatched in 30x15 cm trays filled with water for 1-2 days. Mashed fish pellets was used to feed the larvae and they were reared for 3-5 days reaching the first to third instar phases. From which, they ready to be used after reaching the third instar phase.

2.2 *Muntingia calabura* leaf extraction

M. calabura leaf was washed and mashed to become powder (as simplicia). In dark bottle, 500 g of simplicia then was macerated with 96% ethanol (ratio of 1:10) and left for 24 hours. Filter paper then was used to filter the maceration extract, followed by separation of the ethanol solvent using a rotary evaporator at 40 °C to obtain *M. calabura* leaf extract [4].

2.3 Qualitatively Determining of Phytochemical of *Muntingia calabura* leave

In order to determine the qualitative content of secondary metabolites in the ethanol extract of *M. calabura* leaf, phytochemical test was run [5], [11].

1. Determination of Saponin: 0.5 mL of sample was added with 5 mL of distilled water, then was shaken for 30 seconds. Foam was presence indicating positive result.
2. Determination of Tannin: 1 mL sample was added with 3 drops of 10% FeCl3 solution. A positive content of tannin could be obtained from bluishblack color sample alteration.
3. Determination of Flavonoid: 0.5 mL sample was added with 0.5 g Mg powder and 5 mL concentrated HCl. A positive content of flavonoid could be obtained by red/yellow color alteration and the presence of foam of the sample.
4. Determination of Alkaloid: 0.5 mL of sample was added with 5 drops of chloroform liquid and 5 drops of Mayer's reagent (consisted of 1 g of Kl dissolved

in 20 mL of distilled water then added with 0.271 g of $HgCl_2$). A positive content if alkaloid could be obtained by brownishwhite color alteration of the sample.
5. Determination of Steroid: 0.5 mL sample was added with 0.5 mL glacial acetic acid and 0.5 mL H_2SO_4. A positive content of steroid could be obtained blue or plurple color alteration of the sample.
6. Determination of Terpenoid: 0.5 mL sample was added 0.5 mL glacial acetic acid and 0.5 mL H_2SO_4. A positive content of terpenoid could be obtained by red or yellow color alteration of the sample.

2.4 Mortality Test of *Muntingia calabura* against *Aedes aegypti* Larvae

Extract *M. calabura leaf* as much as 200 ml at different concentrations namely, 0.25%, 0.50%, 0.75%, and 1% and 0% (as control solution). Twenty five 25 instar III of *Ae. aegypti* larvae were put into separated plastic cups and soaked for 24 hours. The mortality of larvae was obtained at series of times (1/2, 1, 24 hours) [13]. Dead larvae could be seen unresponsive and sinking into the bottom of the plastic cup.

2.5 Tissue Processing of *Aedes aegypti* Larvae

10% formaldehyde solution was used to fix the larvae and they were soaked for 24 hours. Then, graded ethanol (70%, 80%, 96%, 100%) was used for dehydration process for 24 hours and and xylol was used to clear the alcohol. Infiltration then was apply to the larvae by using liquid paraffin for 30 minutes and followed by putting them in paraffin blocks. Paraffin blocks were cut crosswise using a microtome with 4-6 μm thickness. Staining was performed to the cutting paraffin tape with Hematoxylin - Eosin. The damage of the polytropic membrane, epithelial cells, and basement membrane was observed [9].

3 RESULTS AND DISCUSSION

Phytochemical test of *Muntingia calabura* leaf extract could be seen as follow (Table 1).

Table 1. Phytocompounds in ethanol extract of *Muntingia calabura* leaf.

Types of Phytochemicals	Observation Result	Test Results*
Saponin	Solution forms a foam	+
Tanin	Solution turned into blue until black	+
Flavonoid	Yellow color and foam	+
Alkaloid	Two layers forms, transparent and brown color	+
Steroid	Blue color	+
Terpenoid	No color alteration occurs	-

Note: * indicate the existing of chemical substance

In Table 1, it could be seen that the phytochemical content of *Muntingia calabura* leaf extract contained of saponins, tannins, flavonoids, alkaloids, steroids based on Harborne [5] and Robinson [11] test procedures. While, terpenoid could not be indicated in etanolic extract of *Muntingia calabura* leaf.

The bioactivity potential of *Muntingia calabura* leaf extract on the larvae *of Ae aegypt* could be seen in Table 2.

Table 2. Bioactivity of *Muntingia calabura* Leaf Extract on Mortality of *Aedes aegypti*.

Extract Concentration	N	Larvae Mortality (Mean ±St. Dev)
0% (Control)	4	0±0,000 (a)
0,25%	4	10,75±0,95743 (b)
0,50%	4	12,75±0,95743 9 (b)
0,75%	4	17,75±1,70783 (c)
1%	4	20,00±0,000 (c)

Note: Numbers followed by different letters indicate different larval mortality at different

From the results of further post-hoc tests of the bioactivity of *Muntingia calabura* leaf extract against the death of *Ae. aegypti* larvae, the highest mortality percentage was at a concentration of 1% at 100% (20 individuals), and the lowest mortality was at a concentration of 0.25% at 53.75% (11 individuals) (Table 2). These results indicated that the bioactivity of *M. calabura* leaf extract had the potential to be a larvicide *for Ae.aegypti* mosquitoes, this is due to the content of secondary metabolites found in *M. calabura* extract. The results of the phytochemical test of *M. calabura* extract contained several compounds whose effects causing damage to the death of larvae. These compounds presumably had properties of prevent nutrient absorption, have antifeedant, and cause failure of the molting process. Saponins are toxic to living organisms, especially at high concentrations. Another effect of saponins is that they act as antifeedants, which can cause a decrease in appetite in insects with a bitter taste [8], [10]. Tannins are believed to prevent the absorption of nutrients in the digestive processes, in addition, they can also bind to the proteins in the larval skin making the molting

process fails which eventually the larvae die before becoming pupae. Tong et al. [14], alkaloid compounds when conjugated with acids forms salt that dissolve in water, this salt has a very strong bitter taste causing the larvae may not like it [2].

Flavonoids act as enzyme inhibitors that cause death in insects, in addition, flavonoids can also cause molting failure in insects since they can inhibit the synthesis of steroid hormones (ecdysteroids) [1], [7]. These effects of this compound then cause the death in mosquito larvae in doses manner, the increase the concentration of the extract given the more the number of death of the larvae.

3.1 Morphological change of larvae after exposure to *Muntingia calabura* leaf extract

The morphological change of the larvae after exposure to the ethanolic *Muntingia calabura* leaf extract could be seen in Fig 1 as follow.

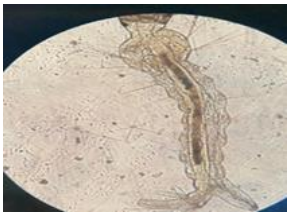
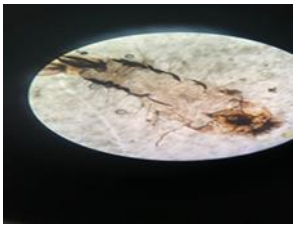
a		Picture (a) is the condition of larvae that are not exposed to the *M. calabura* extract, no damage to the outside of the body and shows active movement**.**
b		Picture (b) is the condition of the mosquito larva's body after exposure to the *M. calabura* extract, it is shorter, with some black parts shrinking.

Fig. 1. Morphology of larvae after exposure to the extract.

Compare to the control, the morphological changes occur in mosquito larvae, the body becomes shorter, shrunken and some parts become black due to the effects of *M. calabura* leaf extract. It has already mentioned before, that phytochemical contains of *M. calabura* leaf extract may cause this morphological changes. The extract of *M. calabura* contains tannin that affects the larvae morphologically and anatomically.

3.2 Histopathological observations of *Muntingia calabura* leaf extract on midgut damage of *Ae. aegypti* larvae at various concentrations

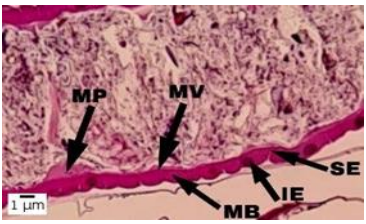
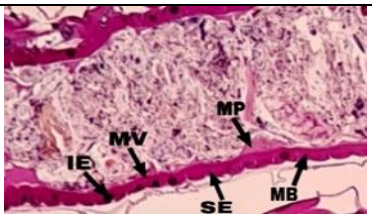
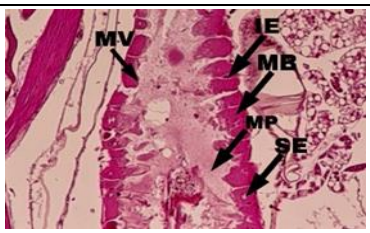
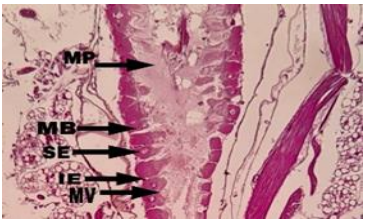
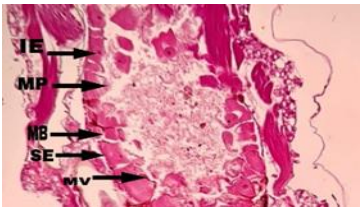
Histophatological observation was made on the midgut of the larvae of *Ae aegypti*. It could be seen in Fig 2.

The results of histopathological observations of the larval midgut with H&E staining after 24 hours exposure to the extract showed that damage began to be seen at a concentration of 0.25% upto a concentration of 1%. Damage was seen from mild to severe as the concentration increased, this was due to the bioactivity of secondary metabolites found in *M. calabura* leaf extract, such as saponins, tannins, alkaloids, flavonoids, steroids that all might contribute to death and damage to the midgut of the larvae.

Saponins contained in *M. calabura* leaf extract can cause peritrophic permeability disruption. When contacted with microvilli and midgut epithelium, it caused a decrease in surface tension leading to damage of the peritrophic membrane [16]. Furthermore, this also was able to reduce the permeability of the larval cuticle membrane, resulting in hemolymph disruption, eventually causing lysis in the larvae [9].

According to Mugford and Osbourn [10], saponins are amphipathic compound and can penetrate the intestinal membrane through sterol bonds, subsequently forming pores and resulting in apoptosis.

In addition to, the toxicity of alkaloid compound was also able to cause degradation of cell membranes. It caused damage to the epithelial cells of the larval midgut, due to a reduction in cell surface tention and damage to the basement membrane [9].

	Concentration (0%): visible peritrophic membrane, basal membrane, intact epithelial cells, normal cell nuclei, thick microvilli.
	Concentration (0.25%): the peritrophic membrane begins to experience slight damage, while the basal membrane, epithelial cells, cell nuclei are still normal, the microvilli experience thinning.
	Concentration (0.50%): peritrophic membrane, damaged basal membrane, normal epithelial cells, thinning of microvilli
	Concentration (0.75%): peritrophic membrane is slightly impaired, some epithelial cells begin to separate from the basal membrane, the basal membrane is impaired, epithelial cell bulge occurs, and microvilli become thin.
	Concentration (1%): peritrophic membrane damaged, epithelial cells detached from the basal membrane, epithelial cells swollen, basal membrane damaged, microvilli thinned and irregular, midgut cells loose in the lumen, irregular cell structure.

Description: MP = Peritropic membrane; MV = Microvilli; SE = Epithelial cell; IE = Epithelial cell nucleus; MB = basement membrane

Fig. 2. Histopathology of the Midgut of *Ae.aegypti* larvae after exposure to *Muntingia calabura* leaf extract in various concentrations after 24 hours (100x).

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