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Abstract. Dengue hemorrhagic fever (DHF) is a disease caused by dengue virus and transmitted through the bite of Aedesaegypti. The way to decrease this disease is by breaking the spread chain of A. aegypti as a vector of DHF using insecticides. Insecticides commonly used are organophosphates that can cause death in non-target organisms. Since 1985, WHO recommended to look for bioinsecticides that does not cause negative impact on non-target organisms. One of bioinsecticide is betel leaf and srikaya seed. Testing the toxicity of this bioinsecticide to non-target organisms used to monitor the environment seen through observation of fish health conditions. Non-target organisms used are Cyprinuscarpio. Testing of this bioinsecticide with concentrations of 0.25 g/10 L, 0.5 g/10 L, 0.75 g/10 L and 1 g/10 L were performed on goldfish for 96 hours. Observations made on fish mortality, morphological and histopathological changes of intestinum, liver and kidney organs. The result showed that LC50 granule mixture of betel leaf and srikaya seeds extract was 1.14 g/10 L, classified as low toxicity. Granules with a concentration of 1 g/10 L can kill 95% A. aegypti larvae within 105 min and did not do cause any damage to goldfish within 48 hours.

1. Introduction

Insecticide is one type of pesticide that can kill insects pests [21]. Insecticide can be used to overcome the increase of Dengue Hemorrhagic Fever (DHF) incidence by breaking the spreading chain of *Aedesaegypti* mosquito as a vector of dengue [20]. DHF is a dangerous viral disease because of its high morbidity and mortality. It is caused by dengue virus that enter the human blood circulation through the bite of *A. Aegypti*mosquito. The incidence of dengue fever in East Java tends to increase every year. Jember is a district in East Java that has a high rate of DHF incidence with 923 cases in 2015 and 148 cases in early January 2016 [4].

The commonly used insecticides are temepos (organophos-phates) that can cause death in non-target organisms [9],[15]. WHO since 1985, recommended to investigate natural insecticides that do not cause negative impacts on non-target organisms. This natural insecticide is called bioinsecticide [7]. The requirement of a natural substance to be used as a bioinsecticide is to have toxicity to the target organism and toxicity to non-target organisms is low or absent [22]. One of bioinsecticides to kill *A. aegypti* mosquito larvae is betel leaf and srikaya seeds. The research has been done to produce granule of toxic compound mixture of betel leaf and srikaya seed extract to *A. aegypti*mosquito larva and proven to kill 95% larvae of *A. aegypti* mosquito with 1 g/10 L water dosage in 105 minutes [8].

Granula mixture of betel leaf and srikaya seeds extract has been submitted to obtain HKI and has arrived at the publication stage with publication number 2015/01902. The next stage will be carried

out a physical examination and safety test for the environment, including non-target organisms. So, in this study the granule toxicity test was conducted on non-target organisms. Toxicity testing of non-target organisms is used as a biomarker to monitor the aquatic environment through observations of the health conditions of fish [17]. The fish used in this study are goldfish (*Cyprinuscarpio*). Goldfish is used because it is a type of freshwater fish that is sensitive to changes in the aquatic environment. Research on the testing of insecticidal toxicity in goldfish has been done previously using bioinsecticide of babandotan leaf extract (*Ageratum conyzoides*) [13]. Examination carried out on the toxicity test of non-target organisms is an examination of morphology and histopathology which includes, the digestive tract (intestine), liver, gills and the kidney of the goldfish. These organs function important in metabolism, so that they can be used as an initial diagnosis of health problems in fish [2]. It is expected that the granules of betel leaf and srikaya seed extract are not toxic to goldfish.

2. Method

2.1. Research Time and Area

This research was conducted from June to August 2017, at Kalisat Fish Seed Center, Jember, Parasitology Laboratory, Biology Education, University of Jember, and Biomedical Laboratory, Faculty of Dentistry, University of Jember.

2.2. Research Instruments and Materials

The tools used in this research were aquarium, aerator, surgical instruments, and microscope. The materials used in this study were goldfish age 3-4 months, fish pellets, live media fish, bioinsektisida granules blend of betel leaves and srikaya seeds (BioAe) products obtained from FKIP Biology University of Jember.

2.3. Research Procedure

2.3.1. Testing of Mixed Granules Betel Leaf and Srikaya Seeds Extracts against Non-Target Organisms. The granular concentrations were determined based on previous studies. The test phase in goldfish was as follows:

a. 25 aquariums each filled with 10 L of well water as medium and aerated on each test medium, idle overnight.
b. The 3-4 month old goldfish with an average weight of 20-25 grams [3] put into the aquarium each of 5 tails.
c. The mixed granules were fed into a fish-filled aquarium with concentrations of 0.25 g/10 L, 0.5 g/10 L, 0.75 g/10 L and 1 g/10 L. Control negative without granulation.
d. Testing was done for 96 hours and observation every 24 hours. At the end of the observation surgery on both live and dead fish to know the changes of fish organs.

The level of toxicity of the granule was determined by criteria based on EPA (Environmental Protection Agency):

Table 1: Criteria of LC50 toxicity value-96 hourin aquatic environment

No.	Category	Unit
1.	Low toxic	>100 mg/ L
2.	Medium	10-100 mg/L
3.	High toxic	1-10 mg/L
4.	Very toxic	< 1 mg/L

Source: EPA, 1999 [13].

2.3.2. Morphological Observation. The morphology observed, namely: color change, granular accumulation, black spot and size changes in the intestinum, liver and kidey of goldfish.

2.3.3. Histopathological Observation. Histopathological observation was performed by making histopathologic preparations [12]. Observations of preparations using a microscope were performed to see tissue damage in the intestinum, liver and kidey of goldfish.

2.4. Data Analysis

Data on the number of goldfish mortality obtained, analyzed by ANOVA one way 99% confidence level, followed by DMRT analysis. Test results may be accepted if 90% of test animals at control at the end of the observation are alive. Determination of LC50 value was analyzed by probit regression analysis.

3. Research Result

3.1. Number of Deaths of Fish and LC50 Value

The number of fish deaths is calculated for 96 hours with observation every 24 hours. The results of the calculation of the number of goldfish deaths can be seen in Table 2. The results of granular probit analysis showed LC50 value of 113.7 mg/L, classified as low toxicity due to LC50> 100 mg / L [13].

Table 2: Average number of fish mortality

Treatment	Number of fish mortality				
	24 H	48 H	72 H	96 H	± SD
K-	0	0	0	0	0,00 ± 0,00[a]
K1	0	0	0	0	0,00 ± 0,00[a]
K2	0	0	0	2	0,40 ± 0,05[a]
K3	0	0	3	5	1,60 ± 0,55[b]
K4	0	0	4	5	1,80 ± 0,45[b]

Description: K-) Control, K1) Concentration 0.25 g/10 L, K2) 0.5 g/10 L, K3) 0.75 g/10 L, K4) 1 g/ 10 L. Number followed by different letters show a significant different at α <0,01.

Table 3: Morphology and histophatology of goldfish's

Organ	Treatment	Parameter					
		Morphology				Histopathology	
		D	AG	BS	S	N	H
Intestine	K-	-	-	-	3,53[a]	-	-
	K1	+	+	-	3,53[a]	-	-
	K2	+	+	-	3,53[a]	-	-
	K3	+	+	+	3,57[a]	+	-
	K4	+	+	+	3,59[a]	+	-
Liver	K-	-	-	-	1,29[a]	-	-
	K1	-	-	-	1,30[a]	-	-
	K2	-	-	-	1,31[a]	-	-
	K3	-	-	+	1,32[a]	+	-
	K4	-	-	+	1,32[a]	++	-
Kidney	K-	-	-	-	1,99[a]	-	-
	K1	+	-	-	2,02[a]	-	-

Organ	Treatment	Parameter					
		Morphology				Histopathology	
		D	AG	BS	S	N	H
	K2	+	-	-	2,05[a]	-	-
	K3	+	-	+	2,04[a]	+	+
	K4	+	-	+	2,06[a]	+	+

Description: (D) Discoloration, (AG) Accumulation Granule, (BS) Black Spot, (S) Size, (N) Necrosis, (H) Hemorrhagi. (K-) Control, (K1) Concentration 0.25 g/10 L, (K2) 0.5 g/10 L, (K3) 0.75 g/10 L dan (K4) 1 g/10 L. (-) no difference compared to control (+) there are difference compared to control. Number followed by different letters show a significant different at $\alpha < 0{,}01$.

3.2. Morphology and Histopathology of Intestinum

Based on observations of intestinum morphology, it is known that there is a change of color and granular accumulation in all the concentrations tested, black spots at consentration of 0.75 g/10 L and 1 g/10 L, there is no changes in organ size significantly in all tested concentrations. Based on the results of histopathologic observations of the intestinum, it is known that necrosis cells at concentration of 0.75 g/10 L and 1 g/10 L.

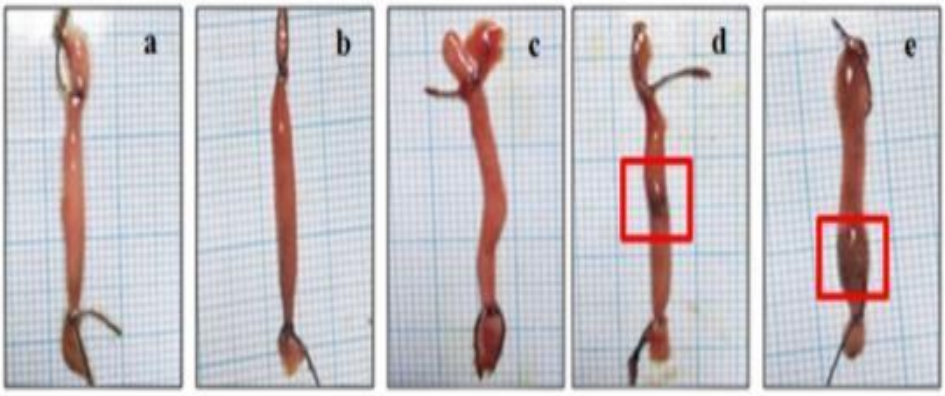

Figure 1. Morphology of goldfish's intestinum.
a) Control, b) Concentration 0.25 g/10 L, c) 0.5 g/10 L, d) 0.75 g/10 L, e) 1 g/10 L. Red square shows black spot.

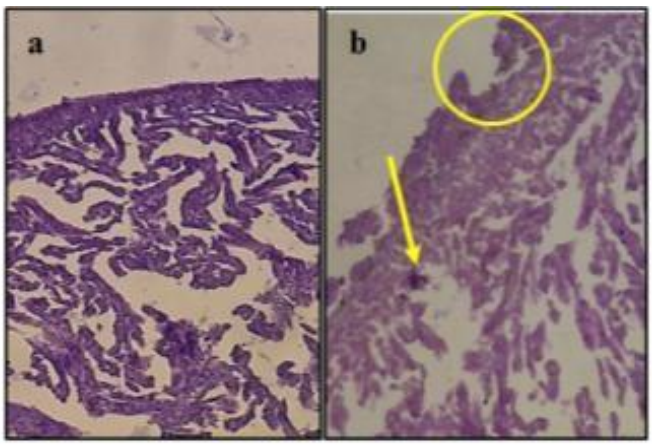

Figure 2. Histopathology of goldfish's intestinum.

a) Normal histology, b) Histology of the intestinum with cell necrosis. Yellow circle and arrow shows cell necrosis. Observed by microscope with 1000x magnification.

3.3. Morphology and Histopathology of Liver

Based on observations of liver morphology, it is known that there is no change in color, granular accumulation, and changes in organ size significantly in all tested concentrations. There is black spots at consentration of 0.75 g/10 L and 1 g/10 L. Based on the results of histopathologic observations of the liver, it is known that necrosis cells at concentration of 0.75 g/10 L and 1 g/10 L. Necrosis occurs in hepatocyte cells.

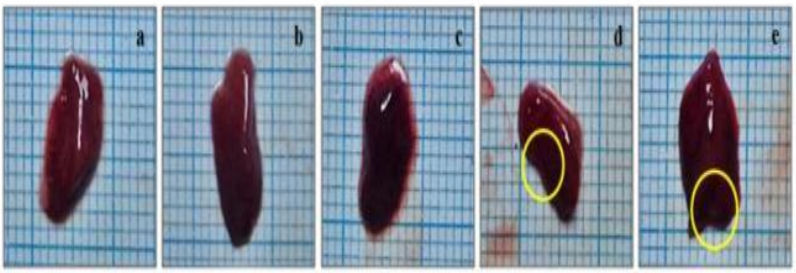

Figure 3. Morphology of goldfish's liver.
a) Control, b) Concentration 0.25 g/10 L, c) 0.5 g/10 L, d) 0.75 g/10 L, e) 1 g/10 L. Yellow circle shows black spot.

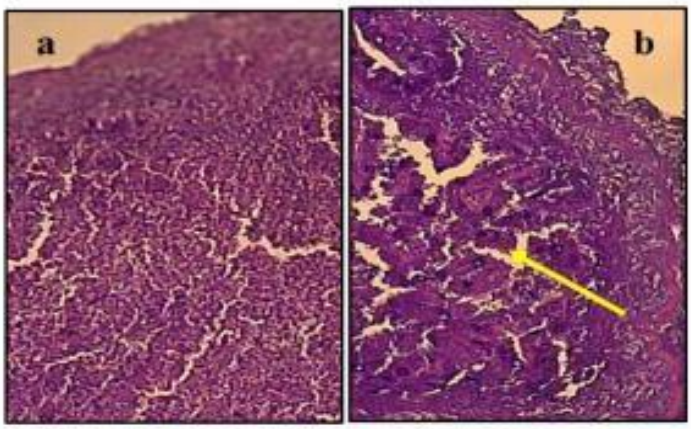

Figure 4. Histopathology of goldfish's liver.
a) Normal hepatic histology, b) Histology of the liver with cell necrosis.Yellow arrow shows cell necrosis. Observed by microscope with 1000x magnification.

3.4. Morphology and Histopathology of Kidney

Based on the results of kidney morphology observations, it is known that there is a change of color in all the concentrations tested and there is black spots at consentration of 0.75 g/10 L and 1 g/10 L. Based on gill histopathology observation, it is known that necrosis cells and hemorrhagi at concentration of 0.75 g/10 L and 1 g/10 L.

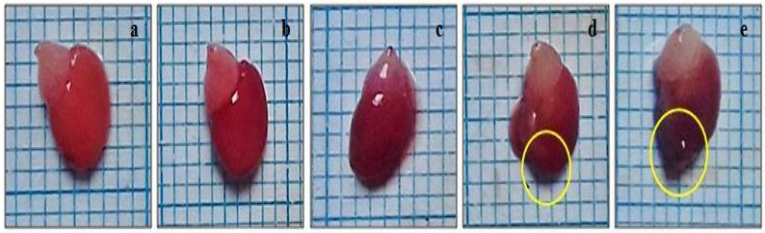

Figure 5. Morphology of goldfish's kidney. a) Control, b) Concentration 0.25 g/10 L, c) 0.5 g/10 L, d) 0.75 g/10 L, e) 1 g/10 L. Yellow circle shows black spot.

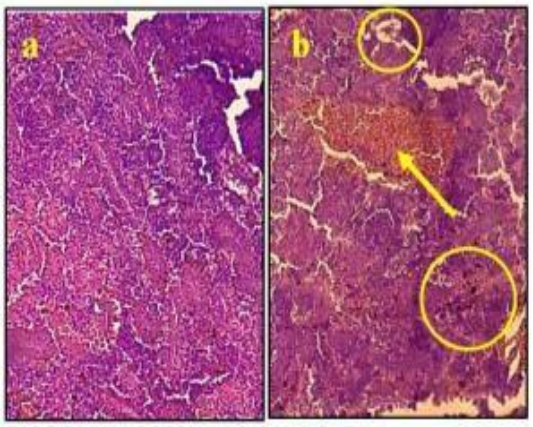

Figure 6. Histopathology of goldfish's Kidney.
a) Normal kidney histology, b) Kidney histology with cell necrosis and hemorrhage. Yellow circle shows cell necrosis and yellow arrow shows hemorrhage. Observed by microscope with 1000x magnification.

4. Discussion

4.1. Number of Deaths of Fish and LC50 Value

The granular mixture of betel leaf and srikaya seeds extract with a concentration of 1 g/10 L can kill 95% of *A. aegypti* mosquito larvae within 105 minutes [8]. Based on table 2 it can be seen that at 24 hours and 48 hours after administration of granule mixture, goldfish at all concentration of treatment did not experience death. This suggests that administration of 1 g/10 L granula for 48 hours to kill *A.aegypti* mosquito larvae has no effect on goldfish as non-target organism. Granules affect the goldfish at 72 hours and 96 hours after application. Goldfish died at concentrations of 0.75 g/10 L and 1 g/10 L. At concentrations of 0.25 g/10 L and 0.5 g/10 L goldfish still able to adapt and neutralize the effects of granules. Goldfish have a defense mechanism (immune system) against harmful foreign compounds from betel leaves and srikaya seeds. The ability of the immune system in the goldfish body increases after granular application because, besides containing potentially toxic compounds, betel leaves and srikaya seeds also contain antioxidant and immunostimulant flavonoid compounds. Flavonoids can enhance body immunity by stimulating the growth and growth of leukocytes as non-specific defense of fish [23].

At concentrations of 0.75 g/10 L and 1 g/10 L there is an inability to adapt the goldfish to granules, consequently goldfish is not able to neutralize the effects caused. Betel leaf contains saponins and tannins that have the potential to poison on [6]. Saponins can destroy red blood grains in cold-blooded animals including fish through hemolysis reactions [5], whereas tannins can cause protease enzyme inhibition. The seeds of srikaya contain potent acetogenin as stomach poison [19].

4.2. Morphology and Histopathology of Intestinum

The gastrointestinal tract has a great potential as the granular entry point into the fish body. Damage to the gastrointestinal tract will affect the activity and function of digestive enzymes that lead to impaired digestion and absorption of food [11]. Observation of morphology of goldfish'sintestinum after granulation for 96 hours showed that the color change of intestine become darker because of the accumulation of granules in fish intestine. Black spots on the intestinal surface suggest intestinal tissue damage, indicating the presence of necrosis in intestinal epithelial cells [18].

Histopathological observation of goldfish's intestinum after granule mixture for 96 hours showed that necrosis occurred. Necrosis occurs because saponins in betel leaves cause a disruption of physical

activity in the cells. Saponin type of aglikon contained in betel leaves will bind to the phospholipid on the cell wall, so that the phospholipid will change the structure and formed porus on the cell membrane. The difference in osmotic pressure inside the cell becomes larger than the osmotic pressure outside the cell, consequently the cell will lose much fluid [1].

Acetogenin in srikaya seeds can also cause necrosis. Acetylene has a furanon group that acts as a stomach poison. Acetogenin enters the intestinal cells with the help of the C1 channel receptor. The furanone groups in acetogenin will bind to ATPase vacuoles that serve both intra and extra cells, so that their activity is disrupted. Disturbance in the cell transport process causes the incoming nutrients in the cells to become blocked. The next condition will cause malnutrition in the intestinal cells. Acetylene also causes damage to intestinal epithelial cell wall through oxidation process and decrease of surface tension. The process causes cell damage resulting in cellular necrosis [16].

4.3. Morphology and Histopathology of Liver

The liver is an important organ that can detoxify toxins, bile production and homeostasis of body metabolism [10]. Liver is susceptible to damage caused by pollutants because the liver receives 89% of the blood supply from the portal vein that drains blood from the gastrointestinal system. Impaired liver damage causes the disruption of metabolism in the body.

Necrosis of goldfish liver after granulation occurs due to saponin in betel leaf causing disruption of physical activity in cells, consequently cells lose much fluid. Hepatocyte necrosis also occurs due to the presence of acetogenin. Acetogenin enters the cell with the help of the C1 channel receptor. The furanone group of acetogenin will bind to the ATPase vacuole that functions for intra and extra cell transport, thus disrupting the activity. Disturbance in the cell transport process causes malnutrition in cells [20]. In addition, acetogenin in srikaya seeds also causes cell wall damage through the oxidation process.

4.4. Morphology and Histopathology of Kidney

The kidneys are the vital organs in fish that have the primary function of keeping homeostasis, are involved in the cleansing of waste from the blood, and are responsible for reabsorption that helps maintain blood volume and body fluids and fluids [14]. Disorders of the kidneys can cause disruption of homeostasis, so it is necessary to observe the morphology and histopathology of the kidneys.

Morphological changes that occur in the kidney organs after 96 hours of granule are color changes and there are black spots. Color changes can occur due to inflammation of the kidneys. Inflammation is a local physiologic response to mild injury and is not a disease that causes death. Inflammation is an infection reaction due to the entry of toxicity (in this study granules mixture of betel leaf and srikaya seed extract) into the blood [2]. Color changes can also occur due to the reaction of hemolysis that occurs due to the influence of saponin compounds in betel leaves. The reaction of hemolysis is due to Hb escape from the stroma to the surrounding liquid so that the color of the gill becomes darker due to the large amount of Hb in the liquid [6]. This is in accordance with study which states that fish renal color mas will change from bright red to blackish after pesticide [18]. Black spots are present at concentrations of 0.75 g / 10 L and 1 g / 10 L. Black spots in the kidney show tissue damage in the form of necrosis in renal cells [18]. While on the parameters of granular accumulation, cavities and organ size changes there was no difference in all tested concentration when compared with control.

Histopathological changes that occur in the kidney organ after 96 hours of granular are the occurrence of hemorrhage and necrosis in the kidney organs of goldfish. Hemorrhage occurs because the blood vessels burst. Rupture of blood vessels due to the presence of saponins in betel leaves that are toxic to coldblooded animals [6]. Necrosis occurs due to the presence of saponins in betel leaves. Saponin type of aglikon contained in betel leaves will bind to the phospholipid on the cell wall, so that the phospholipid will change the structure and formed porus on the cell membrane. The osmotic pressure difference inside the cell becomes larger than the osmotic pressure outside the cell, consequently the cell will lose a lot of fluid [1]. Acetogenin in srikaya seed also causes necrosis through the mechanism of cell wall damage due to the oxidation process and the decrease of surface

tension [16]. Necrosis is characterized by pynosis (the core looks more dense and darker) and caricinical (breakup of the cell nucleus and chromatin breakdown).

5. Conclusion

The result showed that LC_{50} of granular mixture of betel leaf and srikaya seeds extract was 1.137 g/10 L, can be classified as low toxicity. Granular bioinsecticide at concentration of 1 g/10 L after 48 hours of treatment can kill 95% mosquito larvae in 105 minutes and did not cause mortality in goldfish. Bioinsecticides at concentration of 1 g/10 L can cause mortality in goldfish after 96 hours amount 36 % (below LC_{50}). Morphological and histopathological abnormalities occured after 96 hours of treatment.

Morphological observation showed that there were discoloration in goldfish's intestinum, kidney, black spot in goldfish's intestinum, liver and kidney, and granular accumulation in goldfish's intestinum after granular mixture of betel leaf and srikaya seeds extract treatment for 96 hours. Histopathological observation showed there were necrosis in goldfish's intestinum, liver and kidney after granular treatment at concentration of 0.75 g/10 L and 1 g/10 L for 96 hours, and there were hemorrhage in goldfish's kidney after granular treatment at concentration of 0.75 g/10 L dan 1 g/10 L for 96 hours.

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