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Acute Toxicity of Cinnamaldehyde in Profile Hematology and Gill Histology of Zebrafish

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ABSTRACT

In this research, acute toxicity (LC_{50}) of cinnamaldehyde and its effect on hematology profile, as well as gill histology, in zebrafish were conducted. Briefly, 4-5 cm zebrafish were exposed to the following cinnamaldehyde concentrations: 0; 1.8; 2.4; 3.2; 4.2; 6.5; or 8.7 ppm for 96 hours. The results showed that cinnamaldehyde affected the hematology of zebrafish by increasing significantly the micronuclei and blood glucose at a concentration of 8,7 ppm (21 cells/mm^3 and 193 mg/dL, respectively). Interestingly, the total leukocyte and the phagocytosis increased significantly at the concentration of 4.2 ppm of cinnamaldehyde.. Furthermore, cinnamaldehyde promotes edema, and hyperplasia in the gill structure tissue was observed at the concentrations 4.2, 6.5, and 8.7 ppm with total damage around 1%, 11%, and 45%, respectively. Cinnamaldehyde has acute toxicity LC_{50} at concentration 7.2 ppm and up to that certain concentration promotes tissue health in the aquatic organism.

INTRODUCTION

Cinnamaldehyde (3-phenyl-2-propenal), a major component of the essential oil of cinnamon bark isolated from *Cinnamomum* trees, possesses multiple biological activities. Emerging studies have been performed over the past decades to evaluate its beneficial role in medicine and its complications. Cinnamaldehyde constitutes 98% of the essential oil of cinnamon bark. It is commonly used to treat various disorders and is known to exert antifungal **(Wang *et al.*, 2005)**, antioxidant **(Mathew & Abraham., 2006)**, nematicidal **(Kong *et al.*, 2007)**, insecticidal **(Cheng *et al.*, 2009)**, anticancer **(Koppikar *et al.*, 2010)** anti-inflammatory **(Tung *et al.*, 2010)**, antidiabetic **(Lu *et al.*, 2011)** and antibacterial **(Melo *et al.*, 2015)** effects. Cinnamaldehyde needs to be analyzed for its toxicity before

being used further. In general, the results of the toxicity test can provide information about the toxicity caused by cinnamaldehyde and help identifying possible side effects.

High and non-nutritional consumption of cinnamaldehyde may induce toxicity effects. The acute toxicity of cinnamaldehyde is low, with oral median lethal dose (LD_{50}) values ranging from a low of 0.6 g/kg body weight (BW)to a high of 3.4 g/kg BW in different species **(Gowder, 2014)**. Administered 20 times of effective dose (20 mg/kg) of this compound did not cause abnormal behavioral signs and disturbed serum chemistry values throughout the study **(Anand *et al.*, 2010)**. Several studies have been performed to detect the potential long term of toxicity of cinnamaldehyde. The results of the three-month studies showed that exposure to cinnamaldehyde increased the incidence of squamous epithelial hyperplasia of the fore-stomach. In addition, mice exposed to cinnamaldehyde also exhibited increased incidence of olfactory epithelial degeneration of the nasal cavity **(Hooth *et al.*, 2004)**. Thus, cautions must be excised when cinnamaldehyde is taken as a preventive measure. However, despite broad spectrum of acute toxicity (LC_{50}), clinical evidences are still lack to support the effect of cinnamaldehyde in the aquatic environment.

The zebrafish as a test animal in a study is increasingly being used, especially for acute toxicity tests. Zebrafish are often used in acute toxicity tests to study the relationship between exposure to toxic compounds on vertebrates **(Bambino & Chu, 2017)**. As vertebrates, 84 percent of the genes known to be associated with human disease are linked to zebrafish genes. In addition, zebrafish has a fairly high DNA similarity with humans and the similarity of toxic effects on humans and is sensitive to environmental changes so that it can be used as a pollutant bioindicator in toxicity research **(Bolis *et al.*, 2001; Silva *et al.*, 2008)**. This study aimed to analyse the toxic effect of cinnamaldehyde on aquatic organisms to ensure the safety of the resulting product on aquatic environment and humans. In this study, the toxicity of cinnamaldehyde on zebrafish test animals was carried out through LC_{50} for 96 hours. There are two stages in the research to be carried out, including the preliminary test to determine the critical concentration limit; namely, the concentration that can cause the largest death to approach 50% and the smallest death to approach 50%. After knowing the critical limit, the acute concentration was determined based on the logarithm series of concentrations modified in **Rossiana (2006)**. Next, the effect of cinnamaldehyde toxicity on zebrafish was observed through the occurring changes in zebrafish morphology. In this study, confirmation tests were carried out by analyzing hematological profiles (including red blood cells, white blood cells, micronuclei, blood sugar, phagosity) and histological alteration in gill tissue. Thus, the results obtained would provide information on the acute toxicity test of cinnamaldehyde and its effect on living things.

MATERIALS AND METHODS

1. Fish preparation

The 5-6cm of Zebrafish (*Danio rerio*) was maintained in well-aerated aquarium conditions in freshwater at 27°C. *Danio rerio* was maintained for one week for acclimatization before experiment. The fish were fed twice per day with commercial feed.

2. Acute toxicity test (LC_{50}-96h)

Ten of zebrafish were placed in fiberglass tank clean and pathogen-free facilities in the laboratory of freshwater aquaculture, Faculty of Fisheries and Marine Science, University of Brawijaya. First, a preliminary test and an acute toxicity (LC_{50}-96h) were performed as described in **Rossiana (2006)** with modification. First, cinnamaldehyde was prepared by diluting 100µl of cinnamaldehyde to 1ml of DMSO. Moreover, to determine the critical concentration limit, the fish specimens were exposed to cinnamaldehyde extract at the following concentrations (0.0, 0.01, 0.1, 1.0, 10, 100, or 1000ppm) for 96h, respectively. Test medium was not renewed during the experiment, and no food was provided to the animals. After 96 days, bloods were collected for hematology analysis, and gills were collected for histology analysis. Each experiment was repeated three times, respectively.

3. Hematology analysis

The measurement of the erythrocyte and leukocyte were performed as described in **Nemzek *et al*. (2001)**. Briefly, 0.5ml blood sample was collected from caudal vein using a capillary thoma pipette. Further, hayem solution (Merck, USA) was added for fixation erythrocyte and turk solution (Merck, USA) was used for the fixation of leukocyte. Immediately, the sample was homogenized by a gentle mixing. The erythrocyte and leukocyte were measured under microscope using the counting chamber and calculated with total cell erythrocytes multiplied by 10^4 cells per mm^3, and total cell leukocyte was multiplied by 50 cell per mm^3.

4. Micronuclei analysis

Micronuclei were obtained following the method of **Hussain *et al*. (2018)**; the observation of micronuclei can be done by observing red blood cells. Blood was taken and smeared on the preparations and allowed to stand for 5 minutes, stained using 10% Giemsa, and then allowed to stand for 30 minutes. The prepared preparations were then observed under a binocular microscope with a magnification of 1200×. Then, the observed erythrocytes and micronuclei were counted using the following formula:.

$$\text{Micronuclei} = \frac{\text{Total of micronuclei}}{\text{Total of cells}} \times 1000$$

5. Blood glucose level

The blood glucose level was measured using the Easy Touch GCU Model ET-301 following its protocol. Briefly, the blood glucose measurement code number was input to the Easy Touch GCU Model ET-301 tool. Then, the blood glucose strip was inserted following the sample attached to the strip. The result was obtained by waiting for 10 seconds until the blood glucose level results appeared.

6. Phagocytosis

Phagocytic activity was performed as described in **Wulansari (2009)**. Briefly, 50µl of blood sample was put into a sterile eppendorf tube, and 50 µl of baker's yeast cell suspension was added. Then, the sample was homogenized and incubated at room temperature for 20 minutes. Furthermore, 5ul of the mixture of blood and baker's yeast was observed using a glass slide with a size of 1 - 1.2mm with Giemsa staining. The staining process for the smear preparation with Giemsa was carried out to the following

procedure: Phagocytosis activity was expressed by the number of phagocytic cells/100 observed phagocytic cells multiplied by 100%.

7. Histology

Histopathological examination was performed as described in **Elsayed *et al.* (2006)**; the gill tissues of Zebrafish in each treatment were collected after acute toxicity test and immediately fixed in neutral buffered formalin 10% for 1.0 week; dehydration was done using ascending grades of ethanol (70, 80, 90, and 100% for 1 hour each). The specimens were then cleared in 2 changes of xylene. After blocking using soft paraffin, serial sections of 4µm thickness were done. The sections were stained using hematoxylin-eosin stain and the calculation of the amount of damage was evaluated following the succeeding formula:

$$\text{Histology damage (\%)} = \frac{\text{The total (square) damage of tissue}}{\text{The total (square) of tissue}}$$

The percentage of tissue damage was calculated based on the methods used as the amount of damaged tissue divided by the amount of tissue analyzed in percentage. The formula was obtained from tissue observations using one field of view, using the OlyVIA 2.9.1 application (Olympus, USA) instead of a microscope. In one field of view, it was divided into several squares, and each damaged square was counted and then recorded as the number of damaged tissue. The square representing the damaged tissue is recorded as the number of damaged tissue analyzed.

8. Data analysis

Data are expressed as mean ± SD. Statistical significance of pairwise differences among three or more groups were determined using one-way analysis of variance (ANOVA) followed by LSD test. $P<0.05$ was considered statistically significant. Analysis was performed using SPSS for windows (SPSS Inc., Version 20.0, Chicago, USA). Graph was performed using GraphPad Prism 7 (GraphPad Software, Inc. USA).

RESULTS

1. Acute toxicity of cinnamaldehyde

Cinnamaldehyde revealed the differences in lethal and toxicity in zebrafish at each concentration (Table 1). This study observed the behavior of swimming, operculum opening, and the color of zebrafish. Cinnamaldehyde showed no toxicity at a dose of 1.8ppm, 2.4ppm and 3.2ppm. At this concentration, it was found that the mortality of zebrafish was low. The mortality was thought to be unaffected by cinnamaldehyde toxicity due to the normal color of zebrafish at this concentration, the operculum opening, and the swimming pattern behavior. On the other hand, the toxicity of cinnamaldehyde was shown at concentrations of 6.5ppm and 8.7ppm, associated with an increase in the mortality of about 10 and 23 fish, respectively. In this concentration range, several health problems occurred, such as faded colors, unstable operculum opening, and reversed swimming pattern behavior with the ventral facing upwards. At a concentration of 8.7ppm, a fairly rapid death was observed after the exposure to cinnamaldehyde. This

indicates that concentrations above 8.7ppm, cinnamaldehyde has a negative impact and is toxic to organisms. Additionally, a probit test analysis was performed to determine the acute toxicity of LC_{50}. The results of the 96-hour LC_{50} probit test are presented in Fig. (1).

In the LC_{50} analysis, the probit test was carried out using the IBM SPSS Statistic 20 statistical program. The results showed that the lethal concentration causing death with toxicity up to 50% was obtained at a concentration of 7.2ppm with an R^2 of 0.905.

Table 1. Results of LC50 cinnamaldehyde acute toxicity test for 96 hours

Concentration (ppm)	Replication	∑ Fish	Mortality	∑ Mortality
control	1	10	0	0
	2	10	0	
	3	10	0	
1.8	1	10	0	1
	2	10	1	
	3	10	0	
2.4	1	10	0	1
	2	10	0	
	3	10	1	
3.2	1	10	1	3
	2	10	1	
	3	10	1	
4.2	1	10	1	4
	2	10	2	
	3	10	1	
6.5	1	10	3	10
	2	10	3	
	3	10	4	
8.7	1	10	7	23
	2	10	8	
	3	10	8	

2. Profile hematology

To observe the effect of cinnamaldehyde on aquatic organisms, a hematological profile analysis was performed. In this study, further analysis of erythrocyte total, leukocyte total, phagocytosis and blood glucose were assessed. These results are shown in Fig. (2). Cinnamaldehyde induced an increase in several hematological profiles. Cinnamaldehyde at concentrations of 6.5ppm and 8.7ppm induced suppression of total erythrocytes about of 6.3×10^6 and 4.4×10^6 cells/$mm^3$with no significance, respectively. The toxicity of cinnamaldehyde at the concentrations of 6.5ppm and 8.7ppm induced physiological issues in the blood, resulting in a significant increase in micronuclei about 11 cells/mm^3 and 21 mm^3, respectively.

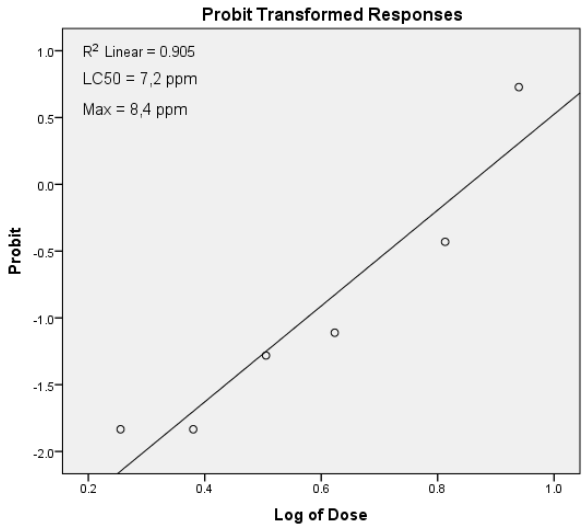

Fig. 1. Analysis of acute toxicity LC_{50} 96 H in probit analysis.

Furthermore, the maximum concentration of cinnamaldehyde toxicity, which is thought to be capable of causing death was obtained at 8.4 ppm. Based on the results of the probit test, it showed that at concentrations above a dose of 7.2ppm it can be suspected that it causes physiological disturbances in the organism so that the recommended dose for use is 7.2 ppm innamaldehyde because it does not have a good effect on mortality, morphological disturbances, and fish behavior. To determine the disturbance occurring in zebrafish caused by cinnamaldehyde toxicity, a hematological profile analysis was carried out, as well as an analysis of changes in the structure of the gill.

Cinnamaldehyde toxicity revealed a significant increase in zebrafish blood glucose at a concentration of 8.7ppm. Interestingly in this study it was observed that, cinamaldehyde at certain concentrations was able to significantly improve the immune system. At a concentration of 4.2 ppm, cinnamaldehyde increased significantly total leukocytes with about 8.8×10^5 cells/mm^3. To find out whether cinnamaldehyde is also able to increase the immune system, in this study a phagocytosis test was carried out. Interestingly, cinnamaldehyde was able to significantly increase phagocytic activity at a concentration of 4.2ppm with a value of 26%. On the other hand, the concentrations of 6.5ppm and 8.7ppm of cinnamaldehyde induced a decrease in the immune system with no significance. This is indicated by a decrease in erythrocyte, leukocyte and phagocytic activity, with values of 7.4×10^6 and 3.3×10^6 cells/mm^3; 3.3×10^5 and 1.9×10^5 cells/mm^3 and 3% and 1%, respectively. At concentrations above 6.5ppm, cinnamaldehyde can be concluded to have a toxic effect that is not good for aquatic organisms since, based on research results, it can cause suppression of the immune response.

3. Histology of gill zebrafish

Cinnamaldehyde has a toxic effect on the structure of the gill tissue so that there is an abnormality of respiratory activity through the opening speed of the operculum, hence an analysis was carried out detecting the changes in the structure of the tissue of the gills. The results of tissue structure morphology and total damage are shown in Fig. (3) and Table (2).

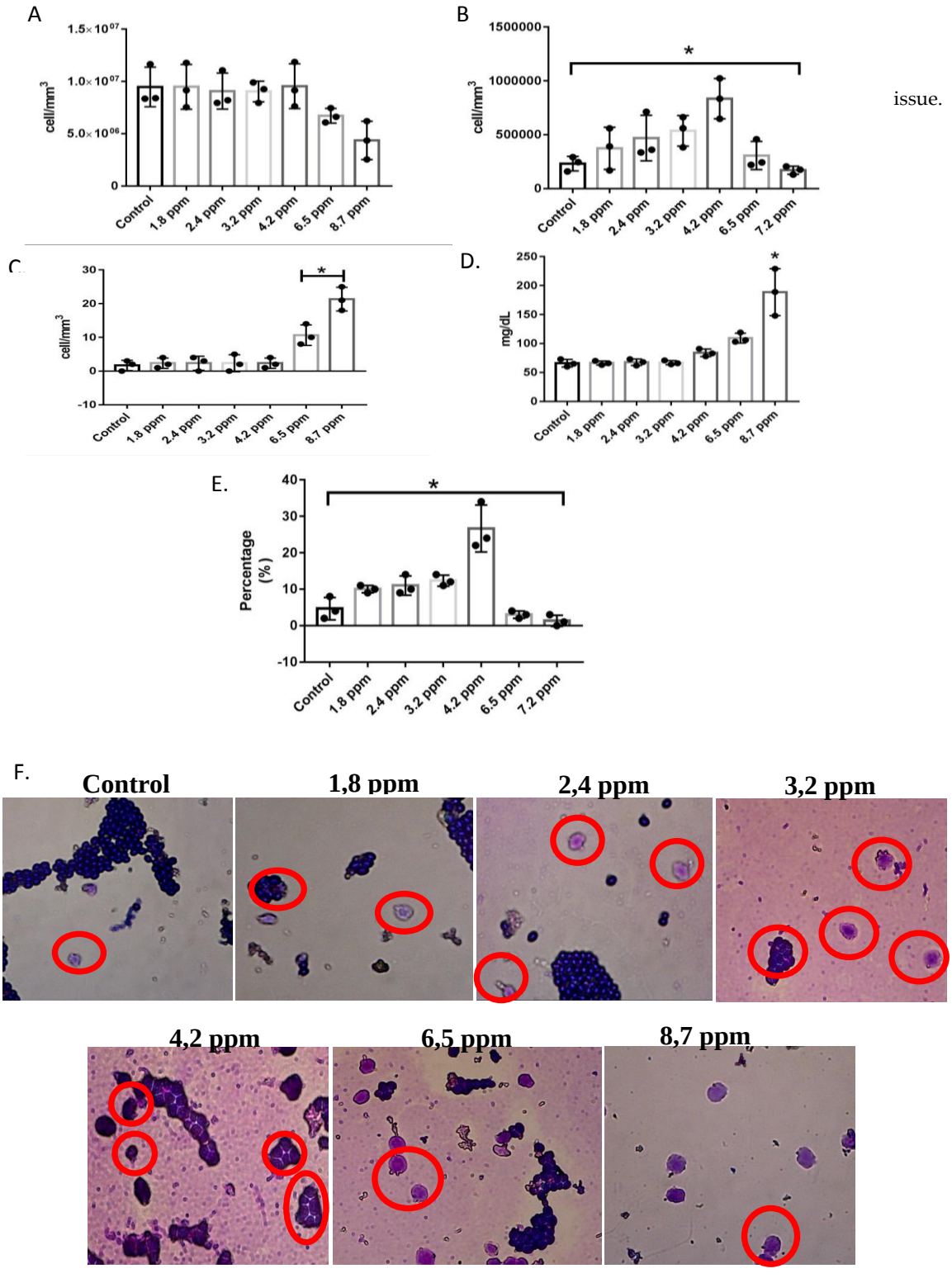

Fig. 2. Analysis of the effect of cinnamaldehyde toxicity on the hematological profile of zebrafish. A. Total erythrocytes, B. Total leukocytes, C. Total micronuclei, D. Blood Glucose, E. Total Phagocytosis, F Morphology of phagocytic activity. Results were the mean ± SD *P<0.05 was significant

issue.

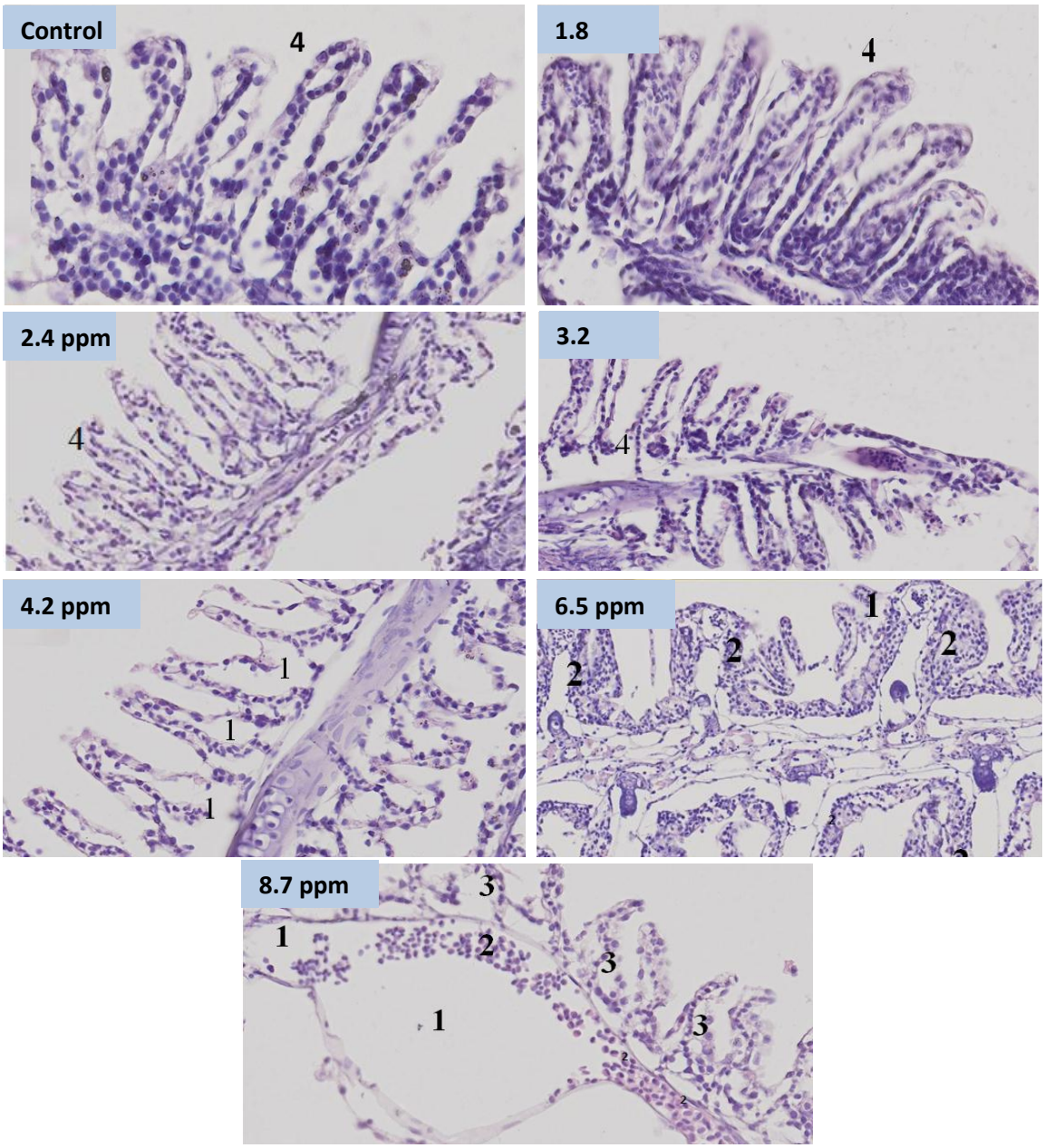

Fig. 3. Analysis of histology in the structure of gill tissue in zebrafish. A. Control; B. 1.8 ppm; C. 2.4 ppm; D. 3.2 ppm; E. 4.2 ppm; F. 6.5 ppm; G. 8.7 ppm. (1). Edema, (2). Hyperplasia or is a process of excessive tissue formation due to an increase in the number of cells; 3) Atrophy; 4) Normal. (400× magnification, Olympus BX 41 microscope, Olympus DP 20 camera)

Fig. (3) shows that at the concentration control to 3.2 ppm, the gill tissue structure of zebrafish shows normal gill lamellae. In contrast, an issue of alteration was observed in the structure of the gill lamellae tissue at concentrations of 4.2ppm to 8.7ppm. At a concentration of 4.2ppm in the gill lamellae, minor edema began to appear with a total damage value of only 1%. Furthermore, 6.5 ppm of cinnamaldehyde induced enhancing edema in gill lamellae, which began to enlarge so that it looked like as if its structure had shortened. Furthermore, a lot of hyperplasia was shown at the concentration of 6.5ppm.

It induced damage about 11%. Furthermore, at a concentration of 8.7ppm, cinnamaldehyde induced greater visible edema, followed by changes in the tissue structure of hyperplasia and a shrinkage of the tissue structure or an atrophy of the gill lamellae. The total damage produced by cinnamaldehyde at a concentration of 8.7ppm was about 45%.

Table 2. Histology of gill tissue structure of zebrafish of LC_{50} acute toxicity

Concentration (ppm)	Replication	Hyperplasia	Edema	Athropy	Necrosis	Total (%)
control	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
1.8	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
2.4	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
3.2	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
4.2	1	0	1	0	0	1
	2	0	1	0	0	1
	3	0	1	0	0	1
6.5	1	6	3	0	0	9
	2	8	4	0	0	12
	3	4	3	0	0	7
8.7	1	12	18	13	0	43
	2	10	20	17	0	47
	3	13	16	12	0	41

DISCUSSION

The toxicity of cinnamaldehyde in aquatic organism is still lack of information. Cinnamaldehyde was selected for toxicity and carcinogenicity studies because of its widespread use as a flavoring and fragrance ingredient and its structural similarity to cinnamyl anthranilate and 3,4,5-trimethoxy-cinnamaldehyde, the two known rodent carcinogens **(Schoental and Gibbard, 1972)**. Cinnamaldehyde is used primarily to impart a cinnamon flavor to foods and beverages (including liquors, cordials, and medicinals) and to impart a cinnamon fragrance to medical products, cosmetics, and perfumes. In food and beverages, Cinnamaldehyde is found in concentrations that range from 7.7 ppm in ice creams and ices to 6400 ppm in fruits and juices **(Blakemore and Thompson, 1983)**. It has the potential to impair on the aquatic environment. In order to preserve the aquatic ecosystem from the potential effects of cinnamaldehyde toxicity, it is necessary to conduct the toxicity cinnamaldehyde study on aquatic organisms. In this

study showed that cinnamaldehyde toxicity starting at a concentration of 6.5 ppm, which was indicated by changes in swimming habits, fading color, and abnormal breathing of the operculum. However, this concentration is not a lethal dose due to the mortality rate does not reach 50%. Furthermore, based on the results of this study, cinnamaldehyde has a lethal dose of acute toxicity at a concentration of 7.2 ppm with a maximum lethal limit of 8.4 ppm. Acute toxicity of cinnamaldehyde has been reported in the LD_{50} assay. Cinnamaldehyde has a behavioral toxic effect (somnolence); gastrointestinal (hypermotility diarrhea) in the LD_{50} test of 2220 mg/kg in rats by oral administered; Cinnamaldehyde also has a behavioral toxic effect (coma at higher doses) reported in Guinea pigs at a concentration of 1160 mg/kg via oral administered **(Smith and Mehnert, 1986).**

In this study was found that cinnamaldehyde has toxic effect on the hematological profile of zebrafish. At concentrations above 6.5 ppm cinnamaldehyde caused a decrease in erythrocytes, leukocytes, and phagocytosis. Cinnamaldehyde induced physiological disturbance in the blood as indicated by an increase in the number of micronuclei at a concentration up to 6.5 ppm. At this concentration a significant increase in total blood glucose was observed. These results indicated that at concentrations up to 6.5 ppm the immune suppression was started. Leukocytes play main role in the immune system and regulating the amount of blood glucose. The suppression of total leukocytes induced the ability of phagocytosis activity. Blood glucose levels also correlate with mitochondrial transmembrane potential in leukocytes **(Matteucci *et al.,* 2011)**, an increase of which results in elevated superoxide production that may directly contribute to cell damage **(Brownlee, 2001)**. The reducing phagocytosis activity induced by cinnamaldehyde at a concentration of 6.5 ppm was thought to be due to suppression the number of macrophages. Macrophages are part of leukocytes, and it has been implicated in the pathogenesis of diabetes, wherein they display impaired phagocytic activity **(Khanna *et al.,* 2010)**, reduced release of lysosomal enzymes **(McManus *et al.,* 2001)**, and reduced chemotactic activity **(Khanna *et al.,* 2010 ; Raj *et al.,* 2018)** in diabetic patients. These traits are significantly correlated with increased blood glucose levels (Jakelic *et al.,* 1995) and reversed by decreasing blood glucose levels **(Alba-Loureiro *et al.,* 2006)**.

Cinnamaldehyde at concentrations up to 6.5 ppm indicated an abnormal issue in the breathing habit of the zebrafish operculum. In this study, histology analysis was conducted to determine whether cinnamaldehyde at different concentrations promoted gill tissue damage. Based on histology results, there was alteration in the structure of the gill tissue induced by cinnamaldehyde at a concentration up to 6.5 ppm. Figure "3" showed that gill lamella was swelling followed by hyperplasia in cinnamaldehyde-treated animals. The highest gill damage was occurs, which is indicated by atrophy. Impairment the lamellae structure induced alteration in the respiratory area. Edema promoted by increasing the hydrostatic pressure tends to force fluid into the interstitial spaces of the body **(Rennika *et al.,* 2013)**. Atrophy is the shrinkage of the cells in the primary lamellae of the gills due to the presence of toxic substances that enter the gills **(Sukarni *et al.,* 2012)**. This impairment absolutely promoted disruption of the process of respiration, osmoregulation and death **(Rennika *et al.,* 2013)**. Damage to the lamellae structure could occur if there is a change in environmental conditions in the fish habitat **(Wahyuni *et al.,* 2013)**.

CONCLUSION

In conclusion, cinnamaldehyde has acute toxicity (LC_{50}) at a concentration of 7.2 ppm in zebrafish after 96h. The higher concentration promoted some health tissues in zebrafish. It induced immunosuppression and alteration of the gill structure tissue in zebrafish. Interestingly, at concentration lower than 6.5 ppm promote immunomodulation as indicated by enhancing leukocyte and phagocytosis.

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