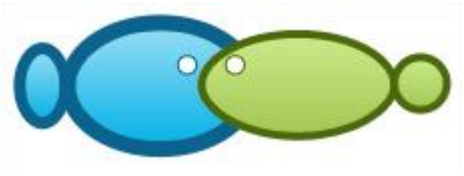

Hematology of *Vibrio alginolyticus*-infected humpback grouper *Cromileptes altivelis,* under treatment of *Alstonia acuminata* shoot extract

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Abstract. Natural material of terrestrial plants is one of the environmentally friendly alternatives in preventing *Vibrio alginolyticus*. This study was aimed to know the effect of crude extract application of *Alstonia acuminata* shoot on the hematological change of sick humpback grouper *Cromileptes altivelis*. Hematology is an indicator effectively and sensitively used to monitor the physiological and pathological changes in fish. *A. acuminata* is a traditional terrestrial plant from eastern Mallucas, Indonesia, used to cure hepatitis, diarrhea, and malaria. This fact indicates that this plant contains chemical compounds as anti-bacterial and immuno-stimulant being able to control diseases, particularly in fish. Based on the post hoc test, there was significant difference between treatments for erythrocytes, leukocytes, and neutrophils, but significant difference between treatments for lymphocytes. The concentration of 200 ppm was the best concentration capable of curing the infected fish.
Key Words: blood, fish, disease, plant, Mallucas.

Introduction. One of the major problems in humpback grouper *Cromileptes altivelis* culture is high mortality rate of the fry due to phatogenic infections. Most of the infections are caused by *Vibrio* sp. bacteria. This disease occurs from high stocking density and decreased water quality condition causing the fish health get worse (Murdjani 2002). Such a condition is largely caused by the application of intensive culture system through high density, fish stocking exceeding the environmental carrying capacity. One of the fish diseases that have become serious problem in grouper culture is infection of pathogen bacterium, particularly *Vibrio harveyi*.

Fish disease treatment can be done through drug and anti-biotic administration, such as oxytetracycline, cananycine, chloramphenicol, and terramycin (Jun et al 2010). The use of the chemical materials has also resulted in new problem in relation with environmental pollution development (Hameed et al 2003; Kerry et al 1997; Rairakhwada et al 2007; Khachatryan et al 2006). The accumulation of the antibiotic residues in fish tissue will affect the fish growth and resistance to drugs (Maqsood et al 2009).

Natural material of terrestrial plants is one of the environmentally friendly alternatives in controlling *V. harveyi*. This study was aimed to know the influence of *Alstonia acuminata* methanol extract administration on the fish hematological change of *Cromileptes altivelis* infected with *Vibrio alginolyticus.* Hematology is effectively and sensitively used as an indicator to monitor the fish physiological and pathological changes (Kori-Siakpere et al 2005).

One of the alternatives to overcome fish infection is the utilization of bioactive compounds from terrestrial plant, *A. acuminata* as immunostimulant, the substance that can increase the body resistance to disease infection, not from the acquired immune response, but from the non-specific immune response through humoral or cellular defense mechanisms (Sakai 1999; Galindo-Villegas & Hosokawa 2004). Non-specific defense system functions to fight against various invading pathogens. This defense system is permanent and does not require previous stimulation, so that it often determines if one fish species is more resistant to the pathogens than the other. Non-

specific defense consists of the first defense system (skin, scale, mucus) and the second defense system (blood). According to Irianto (2005), mucus has ability to inhibit the colonization of microorganisms on the skin, gill, and mucus. He also stated that fish mucus holds natural immunoglobin (Ig-M) that is able to pull down the invading pathogens.

A. acuminata is a traditional terrestrial plant from Southeastern Mallucas, Indonesia, which has empirically been utilized to cure hepatitis, diarrhea, and malaria. The medicinal source is taken from the stem skin with bitter taste indicating that this stem skin contains chemicals as immunostimulant for disease control, especially in case of fish disease. It is also supported by Dangeubun & Syahailatuan (2015) that methanol extract of the stem skin of *A. acuminata* contains alkaloid, phenol, flavonoid, steroid, and tannin that are potential to be an immunostimulant in grouper.

The objective of the study is to know the influence of *A. acuminata* methanol extract application on the hematological change and the survival of the humpback grouper *C. altivelis,* infected with *V. alginolyticus*.

Material and Method

Preparation of bacteria V. alginolyticus. This study employed *V. alginolyticus* as test bacteria. Bacteria stock was aseptically collected and grown in SWC media, then incubated for 24-48 hours.

Test fish. The test subjects consisted of *C. altivelis* specimens. Individuals were reared under a density of 10 individuals in 45 L-aquarium.

Test fish infection. The test fish were infected with *V. alginolyticus* bacteria through injection of 10^6 cfu/mL bacteria for one week until the individuals exhibited clinical sings of infection, in which fish body color has changed. The extract of *A. acuminata* was used to treat the infected fish following different administration doses. This experiment used two types of control, healthy fish as negative control (K-) and infected fish as positive control (K+). The former was intended to present the normal hematological condition of the fish, and the later to show the effect of *A. acuminata* extract application. The infected subject was moved into tanks containing the *A. acuminata* extract treatment of different concentrations. The immersion was carried out twice for one-hour and repeated for 2 successive days. The individuals were then reared in the tanks for 7 days and the observations were done on the clinical symptoms and the fish mortality. Water quality parameters, such as temperature, pH, and dissolved oxygen, were also recorded at the beginning and the end of the study.

Leucocyte count. Fish blood previously mixed with anticoagulant was taken using a leucocyte pippet of 0.5 µL, then diluted with Turk's solution in the leucocyte pipette up to 101 µL, and shaken to be homogenous. The mixture was then taken about 20 µ. Before the blood solution was inserted into the Improved Neubauer counting chamber, two drops had been discarded that the blood solution became fully homogenous. Counting the number of cells started from upper right corner to the left, then went down and from the right to the left side (on 4 green-colored squares). Under 10x enlargement, the leucocyte cells were counted on 4 large squares in the corner of count space (Bijanti 2005).

Leucocyte differential count calculation. The object glass was dipped in 96% alcohol until use. The fish blood taken was added with anticoagulant solution and cannot be left longer than 15 minutes to prevent leucocyte distortion. To count the differential leucocyte, the blood smear was done. Blood sample was taken using a capillar pipette; one small drop was laced near the edge of the object glass on the flat surface. The other object glass was placed with the edge touching the surface of first object glass and made an angle of 30-40^o. The second object glass was pushed at the same angle to form a thin layer, left dry in the open air, and painted with Giemsa. Painting was done as follows: blood smear preparat was fixed in methanol for 2 minutes, removed and left dry in the

open air. It was then soaked in Giemsa paint for 30 minutes, washed with clean water and left dry on the shelf. It was then observed under the microscope.

Results and Discussion

Hematology of C. altivelis after treatment with crude extract of A. acuminate shoot as anti-bactericide on laboratory scale

Erythrocytes. ANOVA showed significantly different effect between treatments on number of erytrocytes (F_{hit} = 7.556 and $P \geq 0.001$ indicating that different treatment application significantly affect the number of erythrocytes (Table 1).

Table 1

Mean value, ANOVA, Duncan test notation of *Cromileptes altivelis* blood cells

Treatment	*Erythrocytes*		*Leucocytes*		*Neutrofils*		*Monocytes*		*Lymphocytes*	
Control -	$1,113.5x10^6$	bc	$112,240x10^3$	a	14.383	a	8.803	a	13.893	-
Control +	1,099,666,666	a	$136,067x10^3$	c	22.447	d	16.190	e	20.227	-
50 ppm	$1,115.5x10^6$	bc	$131,190x10^3$	c	21.407	d	13.450	d	19.170	-
100 ppm	$1,118x10^6$	c	$125,227x10^3$	b	19.670	c	11.853	c	16.483	-
150 ppm	$1,120,333x10^3$	c	$123,218x10^3$	b	18.867	c	10.500	b	16.290	-
200 ppm	$1,112,666.6x10^3$	a b	$121,157x10^3$	b	17.363	b	8.570	a	15.460	-
250 ppm	$1,122,666.6x10^3$	c	$133,123x10^3$	c	21.550	d	13.567	d	17.690	-
F	7.556*		20.702**		43,538**		66,658**		2.678	
P	0.001		0.000		0.000		0.000		0.060	

* - significant; ** - highly significant.

Mean number of erythrocytes of *C. altivelis* after anti-bacterial application of *A. acuminata* hoot extract was significantly different ($p<0.05$). The application of *A. acuminata* of 200 ppm gave the optimal mean value $1,112,666.6x10^3$ cells/mm^3. This value nearly similar to number of erythrocytes of healthy fish ($1,113.5x10^5$ cells/mm^3) and is significantly different from that of other treatments, while the application of 100 ppm, 150 ppm, 250 ppm did not show significant differences between them. Mean number of erythrocytes at the application of 50 ppm, 100 ppm and 150 ppm, 250 ppm, and positive control (K+) was $1,111.5x10^5$ cells/mm^3, $1,118x10^6$ cells/mm^3, $1,120,333x10^3$ cells/mm^3, 1,122,666,633 cells/mm^3 and 1,099,666,666 cells/mm^3, respectively (Figure 1).

Increased total erythrocytes at the application of 50 ppm, 100 ppm, 150 ppm, and 250 ppm indicates defense mechanism of the fish body to the pathogenic infection in which the body produces more erythrocytes to replace the lysis of the erythrocytes from infection. Decline in number of erythrocytes at the positive control dose of the infected fish without treatment (1,099,666,666 cells/m^3), reflects the presence of hemorrhage in liver and kidney due to infection causing the erythrocytes decline.

The condition of number of erythrocytes in healthy, infected, and test grouper after *A. acuminata* antibacterial extract application is presented in Figure 1.

High number of erythrocytes in healthy fish could result from good environmental condition and lack of stress. In unhealthy fish, the amount of erythrocytes is reduced because the body needs to fight against the pathogenic agents.

Number of erythrocytes of fish varies depending on species, environmental condition, stress condition, and water temperature (Fujaya 2004). Blood set concentration needs to be known to assess the body physiology. Sufficient erythrocytes help ensure enough oxygen content in the cells of various tissues that the cells can function at the optimum parameters. In contrast, if the amount of erythrocytes declines, there will be an indication of a foreign agent which attacks the organism (Sadikin 2002).

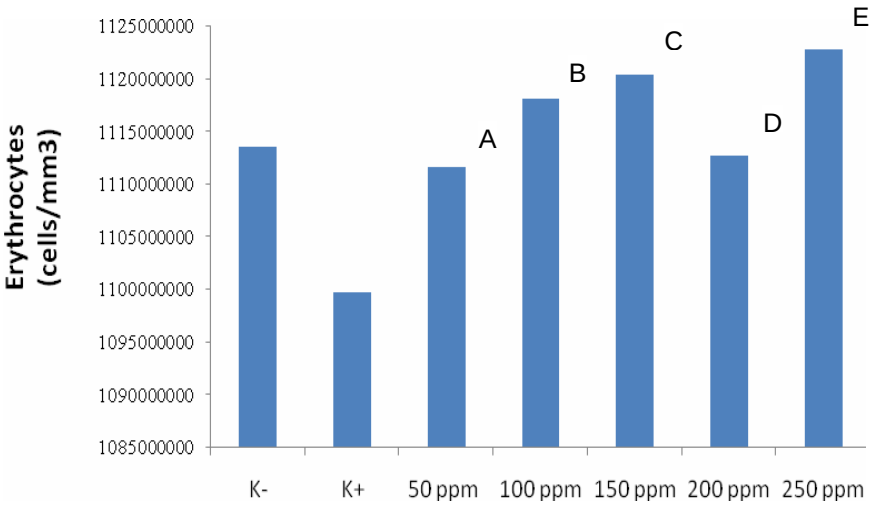

Figure 1. Change in mean number of erythrocytes (cells/mm^3) after *Alstonia acuminata* shoot extract application (K- - healthy fish, K+ - infected fish, and A, B, C, D, E - treatment application of 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm).

<u>*Leucocytes*</u>. ANOVA revealed that the use of different extract concentration of *A. acuminata* shoot extract significantly affected the leucocytes value (F = 20.702 and P<0.001) (Table 1). The use of 200 ppm of *A. acuminate* shoot extract gave the best number of leucocytes (121,157x10^3 cells/mm^3). Mean number of leucocytes at 50 ppm, 100 ppm, 150 ppm, 250 ppm and positive control was 131,190x10^3 cells/mm^3, 125,227x10^3 cells/mm^3, 123,218x10^3 cells/mm^3, 133,123x10^3 cells/mm^3, and 136,067x10^3 cells/mm^3 respectively (Figure 2). It was indicated with absence of bacteria *V. alginolyticus* in the blood samples.

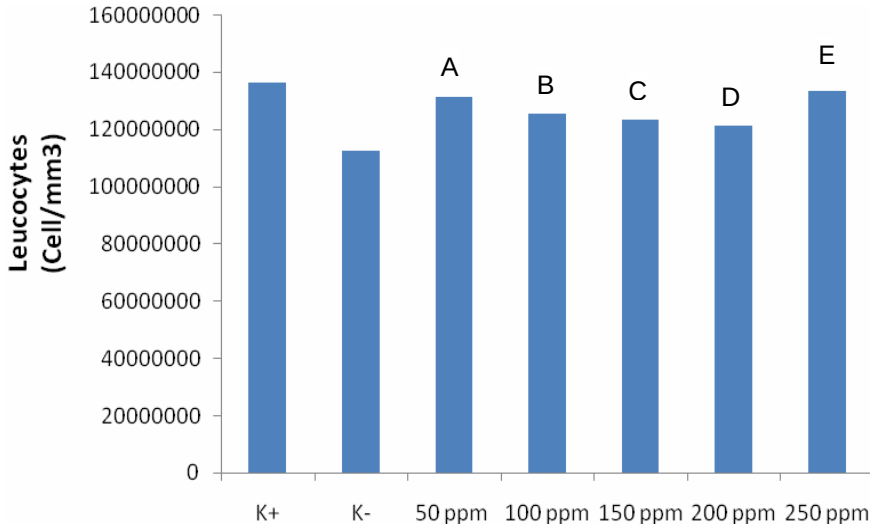

Figure 2. Change in mean number of leucocytes (cells/mm^3) after *Alstonia acuminata* shoot extract application (K- - healthy fish, K+ - infected fish, and A, B, C, D, E - treatment application of 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm).

The dose of 200 ppm was the highest dose of crude extract of *A. acuminate* skin given to *C. altivelis* and could increase the number of leucocytes so that the fish were able to eliminate the bacteria *V. alginolyticus* infection. It was indicated that the histological condition of the fish body suffered minor impairment from the administration of 200 ppm

compared with that from control dose, 50 ppm, 100 ppm, 150 ppm and 250 ppm that suffered severe damages on gill, liver, and muscle.

Leucocytes take important part in fish defense system to pathogen infection (Anderson et al 1995). When infection occurs, the leucocyte is pushed to the infected site to give quick defense against the infecting agent (Sadikin 2002). The administration of *A. acuminata* skin crude extract will help the defense system of the fish against the bacterial infection. The coumaric compounds contained in *A. acuminata* skin crude extract can destroy the cell membrane of the bacteria and can also bind the DNA of the bacteria that it does not express, and then kill the bacteria (Lou et al 2012). As a result, the leucocyte cells need not to be reduced in large number.

Leucocyte is a mobile or active unit of defense system of the body. After produced, the leucocytes are mostly transported particularly to the infected sites and serious inflammatory area, give quick and strong defense to any possibly existing infection matter (Sadikin 2002). According to Fujaya (2004), leucocyte is one of the blood groups possessing very crucial role in immune response system of the fish, and it will drastically increase when infection occurs.

This difference could result from that the leucocyte is only needed when conflict against foreign materials occurs. At this time leucocytes will be directed to inhibit the foreign material entering the body through blood vessels to the conflict area (Harikrishnan et al 2010). This point of view is supported by Fujaya (2004) that if the foreign materials infect the body, the leucocytes will respond by raising the number in order to maintain the body from the invasion of the foreign materials. Leucocytes are blood cells particularly functioning to self-defend from foreign invaders and cells. The function of leucocytes is reflected from the same origin as erythrocytes, the stem cells that continuously divide in the kidney, spleen, and thymus. When the foreign material enters the fish body, the stem cells will automatically produce great number of leucocytes to fight against the foreign materials that are always considered to be possibly resulting in hazard to the survivorship. If the foreign materials are numerous enough and need handling time, defense or resistance, the leucocytes could in part multiply by mitosis (Sadikin 2002).

Neutrophils of C. altivelis (%). Neutrophil cells are oval-round with granular cytoplasms, and contain a nucleus divided into 2-5 lobus or even more. Lobus is nucleic material separated by thread-formed materials. Nucleus is filled with solid chromatin granules so that it strongly binds the base pigment to be blue or purple. The dense nucleus makes difficult to ensure the presence of nucleolus. The cytoplasm looks like dark blue-colored ring or unseen. The diameter of neutrophils range between 9.6 to 10.8 µm (Levengood et al 2000). According to Iwama & Nakanishi (1996), when pathogenic bacterial invasion occurs, the first cell coming to the infected site is neutrophil. This cell moves faster than the monocyte and can reach the infected area in 2-4 hours. At this stage, the defense cell or phagocytosis is dominated by neutrophils. Baratawidjaja (2006) claimed that neutrophils only occur in the circulation less than 48 hours before reaching the infected area.

Based on our observation and ANOVA test (Table 1), we found that mean number of neutrophils of *C. altivelis* treated with *A. acuminata* extract was significantly different ($p<0.05$). Duncan test indicated that the application of 200 ppm of *A. acuminata* shoot active extract gave the best mean neutrophils, 17.36%. Other concentration applications resulted in higher mean number, 21.41%, 19.67%, 18.86%, 21.55%, and 22.45% for 50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm, and positive control, respectively.

ANOVA test also revealed that the use of different treatment concentrations resulted in significantly different number of neutrophils ($F = 43.538$ and $P<0.001$).

The use of 200 ppm gave the best mean number of neutrophils (17.36%), while those at the concentration of 50 ppm, 100 ppm, 150 ppm, 250 ppm, and positive control were 21.41%, 19.67%, 18.86%, 21.55%, and 22.45%, respectively (Figure 3). Our observation demonstrated that the concentration of 50 ppm, 250 ppm, and the positive control caused increased number of neutrophils due to inflammatory stimulation, so that the neutrophils migrated to the blood vessel and entered the infected site. As a

consequence, the pathogenic bacteria will be phagocytosed by the neutrophils, and then inserted into phagosome wherein acidic hydrolase enzyme, myeloperoxidase and lysozyme will lyse and digest the cell of pathogenic bacteria. It is apparently clear that in fish treated at the dose of 50 ppm, 250 ppm, and the positive control, the injured part and the histology of liver and gill positively showed severe damages, while the fish treated at the dose of 200 ppm did not morphologically suffer any damage, but liver and gill got light damages and led to recovery.

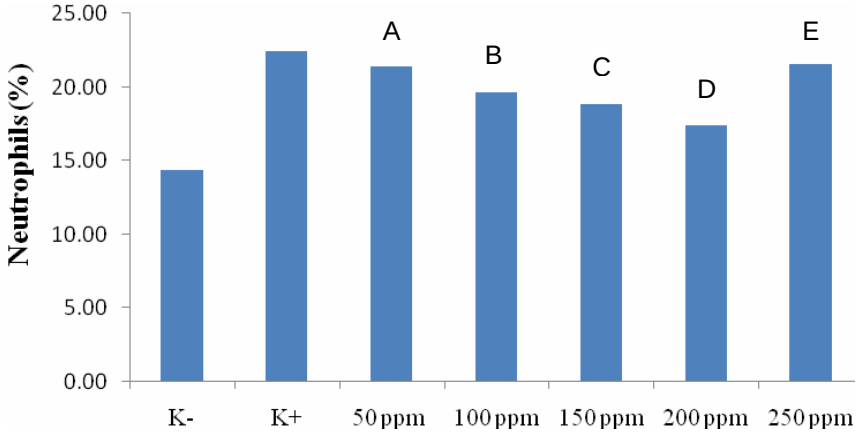

Figure 3. Change in mean number of neutrophils (%) after the application of *Alstonia acuminata* shoot extract (K- - healthy fish, K+ - infected fish, and A, B, C, D, E - treatment application of 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm).

Or results are in agreement with Fujaya (2004) that number of neutrophils, in general, raise in case of bacterial infection, since neutrophils exit the blood vessel and head to the infected site. They firstly go to the inflammatory area and start invading the area. The infectious substance is phagocytosed through digestion of proteolytic enzyme and lysozyme that will digest the bacteria. Moreover, Brown (2000) found that neutrophil cells in the blood rise when inflammation occurs due to invasion of disease agents or foreign object into the blood.

Neutrophils are mobilized to the injured or infected sites as early defense of leucocytes. In general, number of neutrophils increase when the body suffers from bacterial infection since neutrophils come out from the blood vessel and head to the infected site. Neutrophils work as follows: they firstly go to the inflammatory or infected site and start invading the area, and then the infectious materials are phagocytosized by proteolytic enzymes and lysozyme will digest the bacteria (Hrubec at al 2004).

According to Bijanti (2005), neutrophils are a strong phagocyte working by approaching the foreign particle and releasing the pseudopod to all directions around the particle. One neutrophil could phagocytosize 5-20 bacteria before then being inactive. Increase in number of phagocytes could be indicated by presence of bacterial or viral infection, and decline of number of neutrophils could result from inflammation.

Monocytes of C. altivelis (%). ANOVA test showed that different concentration application gave significantly different number of monocytes ($F = 66.658$, $P<0.001$) (Table 1). The use of 200 ppm of *A. acuminata* shoot extract had the best mean value, 25.71%. The mean monocytes occurring in the application of 50 ppm, 100 ppm, 150 ppm, 250 ppm and positive control were 40.35%, 35.56%, 31.55%, 40.7%, and 48.57%, respectively (Figure 4). Increased number of monocytes at the concentration of 50 ppm, 100 ppm, 150 ppm, 250 ppm, and positive control reflects immune response increment due to invasion of pathogenic bacteria in the form of phagocytotic activity development.

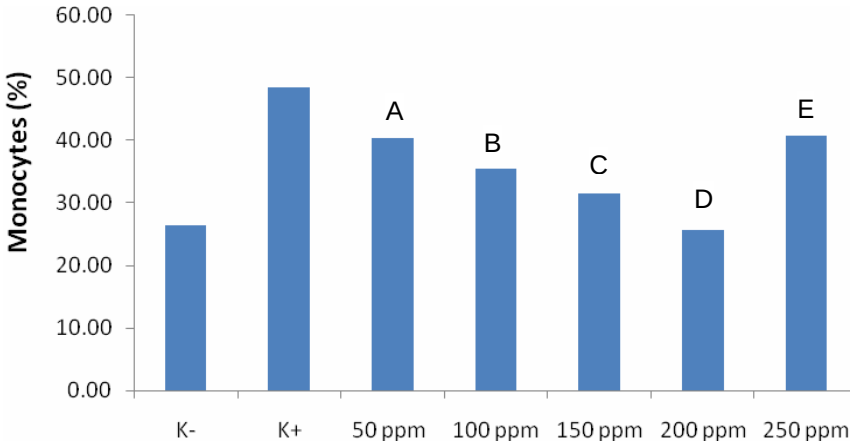

Figure 4. Change in mean value of monocytes (%) after *Alstonia acuminata* shoot extract application (K- - healthy fish, K+ - infected fish, and A, B, C, D, E - treatment application of 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm).

Our results are supported by Fujaya (2004) that number of monocytes in infected fish is higher than that of healthy fish and treated fish, since phagocytotic activity on the foreign substance occur in the infected fish. The phagocytosis of the macrophages is a similar process to the product of microorganism, the immune reaction product, and the damaged cells. The antigen is destroyed in the macrophage in the same way as neutrophils. Monocyte is stronger than neutrophil in bacterial phagocytosis (Fujaya 2004). Levengood et al (2000) added that increased number of monocytes could result from bacterial infection.

Monocyte is a stronger cell in phagocytosing particles or antigens than neutrophil (Fujaya 2004). It differentiates to be macrophage in the tissue and is capable of phagocytosing larger particles and high number up to 100 bacteria. Monocytes can enter the tissue and become macrophages. The role of monocytes is very important as main phagocytotic cells in breaking various incoming pathogens and as antigene presenting cells (APC) to provide antigens to lymphocyte cells (Siti 2001)

Lymphocytes of C. altivelis (%). ANOVA test also found no significant different effect on the lymphocytes as a result of different treatment application ($F = 2.678$ and $P>0.05$) (Table 1). The application of *A. acuminata* shoot extract at the concentration of 50 ppm, 100 ppm, 150 ppm, 250 ppm, positive control that gave 19.17%, 16.48%, 16.29%, 17.69%, and 20.23% of lymphocytes, respectively, did not show significant differences (Figure 5). Mean percent of lymphocyte in healthy fish is 13.89%, and this value is nearly similar to that at the treatment concentration of 200 ppm, 15.46%, in which the fish have started to be recovered. Furthermore, decreased number of lymphocytes at the treatment concentration of 50 ppm, 100 ppm, 150 ppm, and 250 ppm may also result from disruption of the lymphocyte-producing organ function, because injured fish body supports the development of pathogenic bacteria and presses the body defense, so that the fish endurance declines and makes the lymphocyte, as antibody cell, reduce as well. Decline in percent number of lymphocytes occurs, since when getting infected number of monocytes and neutrophils increased, and these three white blood cell components are influencing each other. It is supported by Alamanda et al (2007) that declined number of lymphocytes at the infection makes neutrophils and monocytes, as the foremost defense, work effectively against the infection. After infection, neutrophils will be more active than lymphocytes since the antibody of the lymphocyte will be activated after the neutrophils have worked.

According to Fujaya (2004), lymphocyte circulating in the blood and tissues comes from thymus and peripheral lymphoid organs, such as kidney and lymph. Destruction of

this producer organ will inhibit the lymphocyte formation, and insufficient lymphocyte can reduce the antibody concentration and increase the disease infection.

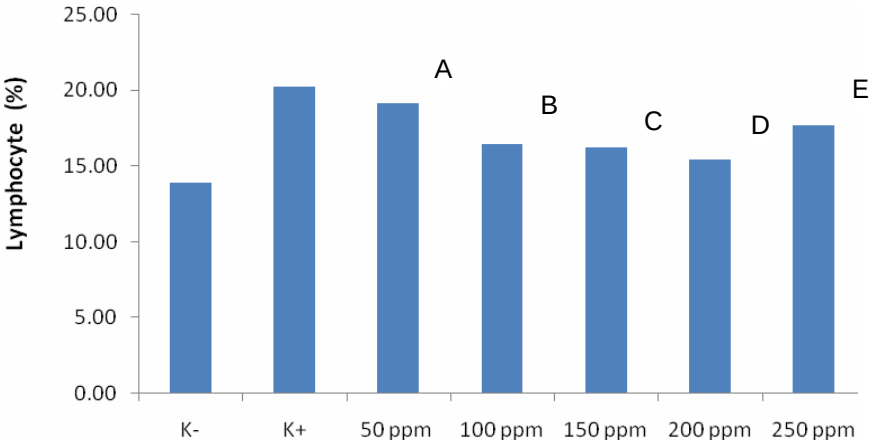

Figure 5. Change in mean lymphocytes (%) after *Alstonia acuminata* shoot extract application (K- - healthy fish, K+ - infected fish, and A, B, C, D, E - treatment application of 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm).

The mechanism of the lymphocyte number development is as follows: lymphocyte cells occur in the blood and will migrate to the lymph and stay there. When encountering foreign substances, the lymphocyte will develop and undergo mitosis to become plasmoid as antibody producer (Sadikin 2002).

Number of differential leucocytes of each type of blood cells, neutrophils, monocytes, and lymphocytes, will rise in sick fish, since the leucocyte component will fight against the pathogens. It is in agreement with Fujaya (2004) that leucocytes will enter the blood in great number after infection. Leucocytes are believed acting as protein detoxication before causing damages in the organism.

Survival rate of C. altivelis at the end of study. Figure 6 shows that the application of 200 ppm of the shoot extract gave the highest survivorship of *C. altivelis*, 100%, as recorded in healthy fish (K- = no treatment and no infection), while the lowest survivorship was found at the bacterial control positive dose (44.44%). It also indicates that the application of 200 ppm of *A. acuminata* shoot extract is the ideal concentration that is capable of reducing the bacteria *V. algynoliticus* population from *C. altivelis* and resulting in 100% survivorship. Bacterial infection can occur through injured body surface, food, or gills, and then the bacteria enter the blood vessel, disperse, and poison the blood.

A. acuminata contains phenolic compounds, coumaric acid, that function as immunostimulant and anti-bacterial coumaric acid will block the biosynthesis of the cell wall through inhibition of enzymatic activity in synthesizing different components of the cell wall (Kanazawa et al 1995). High concentration penetration of phenolic compounds into the cell of bacteria causes protein coagulation and lysis of the cell membrane. Phenolic compound inhibition through hydrogen bond formation between the hydroxyl group of the phenolic compound and the cell membrane protein result in the membrane permeability disruption so that the essential cell components are removed from the cell and the bacteria being killed (Deasywaty 2011).

According to Ingram (1981), phenolic compounds are capable of break the cross-linkage of peptidoglycan in the effort of penetrating the cell wall. After penetrating the cell wall, phenolic compounds cause leakage of cell nutrients by destroying the hydrophobic bond of the cell membrane-forming components, such as protein and phospholipid, and the hydropobically-binded dissolved components make membrane permeability rise. The cell membrane destruction inhibits the activity and the biosynthesis of specific enzyme needed in metabolic reactions.

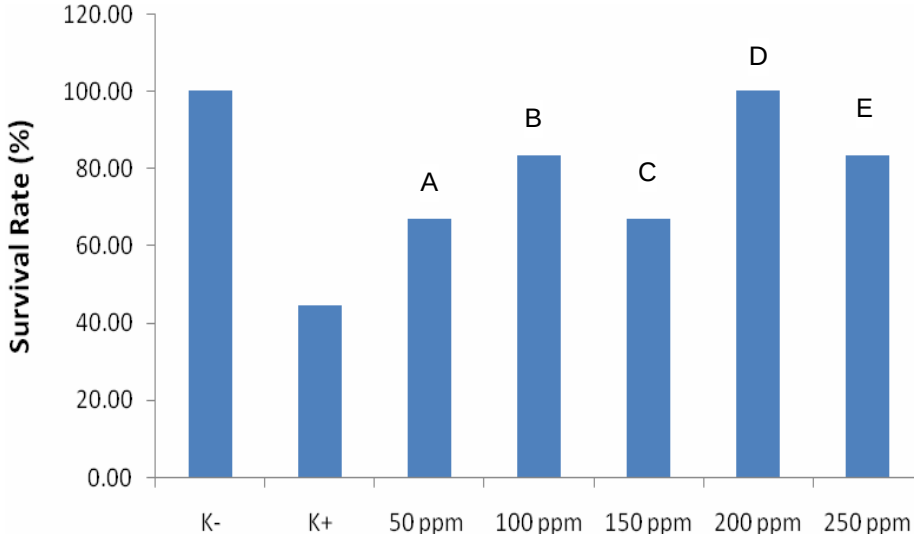

Figure 6. The survivorship of *Cromileptes altivelis* after treatment with *Alstonia acuminata* shoot active extract (K- - healthy fish, K+ - infected fish, and A, B, C, D, E - treatment application of 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm).

Phenolic compounds of *A. acuminata* can damage microbial cells by altering the permeability of cytoplasmic membrane to make intracellular substance leakage, then distorts and activate protein and break the peptidoglycan cross-linkage to penetrate the cell wall. Afterwards, phenolic compounds will cause the cell nutrient leak by damaging the hydrophobic bond of the cell membrane-producing components of cells, such as proteins and phospholipids, as well as dissolving the hydrophobically binded components so that the membrane permeability rises (Ingram 1981).

Conclusions. These results showed that *A. acuminata* shoot active extract application of 200 ppm gave the best mean number of erythrocytes, leucocytes, and differential leucocytes, while the use of 50 ppm, 100 ppm, 150 ppm and 250 ppm resulted in severe damages of the body organ of *C. altivelis*. It appears that the administration of 200 ppm of *A. acuminata* shoot active extract is capable of reducing *V. algynoliticus* population from *C. altivelis* and producing 100% of survivorship.

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