Macrothink Institute™

Journal of Biology and Life Science
ISSN 2157-6076
2013, Vol. 4, No. 2

The Use of Active Compound in the Methanol Extract of *Alstonia Acuminata f*or the Improvement of Non-Specific Immune System in Tiger Grouper (*Epinephelus Fuscoguttatus*)

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Received: February 28, 2013 Accepted: March 14, 2013

doi:10.5296/jbls.v4i2.3682 URL: http://dx.doi.org/10.5296/jbls.v4i2.3682

Abstract

Tiger grouper (*Epinephelus fuscoguttatus*) was very susceptible to the poor environment and therefore, weak against infection. A solution to this problem was by using immune response of

fish body. The objective of research was to understand the ability *Alstonia acuminata* in improving immune system for the control of *Vibrio harveyi* disease and to acknowledge the effective dose to increase the survival rate of grouper. Parameters that were observed included macrophage, phagocytosis activity, leukocyte total, and grouper survival rate in the observation at day-1, day-3, day-5, and day-7. Result of research indicated that macrophage cells were increased at day-1 to day-5, but decreased at day-7. The dose of 200 ppm had the highest macrophage at day-5, precisely 15.5 x 10^5 cells/mL, and then, it decreased at day-7 to 15.2 x 10^5 cells/mL. The highest increase of phagocytosis was found at day-5 for 73.67%, and observed in the dose of 200 ppm, but it decreased at day-7 for 71%. The control fish had lowest means of leukocyte, precisely ranging from 24,433 cells/mL to 25,283 cells/mL. The highest leukocyte was obtained at day-5, precisely 72,666 cells/mL. Result of research concluded that *A. acuminata* extract could increase the non-specific immune system (number of macrophage cell, phagocytosis activity, and leukocyte) against *V. harveyi* bacteria. The highest survival rate was 94.4 %, which was obtained from dose 200 ppm.

Keywords: *Alstonia acuminate*, Tiger grouper, *Vibrio harveyi*, Immune response, Active compound

1. Introduction

Tiger grouper (*Epinephelus fuscoguttatus*) was widely distributed in tropical and sub-tropical areas. It was the most favorite seafood with good marketability in the domestic and international markets, and therefore, it had high sale value. Market demand for this commodity was very stable and even increasing from year to year. It was not surprising to say that the cultivation work of grouper would give good prospect. However, main barrier of this grouper cultivation was its high mortality from rearing stage to harvesting stage. Main causes of high mortality in the grouper cultivation were high cannibalism and mass death due to certain disease (Ninawe, 2006; Badrelin, *et al.*, 2008; Karthupandi, *et. Al.*, 2010.).

Diseases could attack fish when the water quality decreased and lead to poor health among fishes. Intensive cultivation system might cause such risks especially when the seed exceeded environmental carrying capacity. One of serious diseases in grouper cultivation was the infection from Vibrio pathogen bacteria, mainly *V. harveyi*. During peak of epidemic, fish immune was deprived, and fish was easily subjected to stress, being infected and then dead (Badrelin *et al.*, 2008).

The medication so far involved medicines and antibiotics, such as oxytetracycline, cananycine, chloramphenicol and terramycin (Selvaraj *et al.*, 2006; Lightner, 1996). One of these medication agents for fish disease, such as tetracycline, had been used (Jun *et al*, 2010) but without satisfying result. The use of chemicals was instead emerging new problems such as environmental pollution and the presence of antibiotic residue in fish tissue (Rairakhwada *et al.*, 2007; Khachatryan, 2006; Maqsood *et al.*, 2009). Avoiding the negative impact of antibiotics would require safer medication method, among other by increasing fish immune against disease (Selvaraj *et al.*, 2006).

Increasing fish immune was one favorable alternative to conventional medication for disease

alleviation, but it always involved immunostimulant. Indeed, immunostimulant represented a type of chemical, drug, stressor or activator which increased fish immune response to have direct interaction with immune system cells (Kumari and Sahoo, 2006; Wang,*et al*, 2009). Immunostimulant had been widely used in some fish species by embedding it into the food, by injection and/or by submersion. Some researches examined the possibility of this stimulus to increase body immune for the alleviation of more generalized diseases including those caused by virus, bacteria or dangerous organism and polution (Rao *et al*, 2006; Christybapita *et al.*, 2007; Divyagnaneswari *et al.*, 2007; Ji *et at,*, 2007;. Sahu *et al.*, 2007;. Harikrishnan *et al.*, 2009;. Pratheepa *et al.*, 2010; Risjani *et al.*, 2012; Maftuch *et al.*, 2012; Andayani *et al.*, 2006; Andayani *et al.*, 2007).

A naturally-based immunostimulant material could be taken from a plant with environmental friendly nature but useful for *V. harveyi* alleviation, respectively *Alstonia acuminata*. Crude extract of *Alstonia acuminata* was useful for being immunostimulant active substance to increase non-specific immune system of tiger grouper in controlling *V. harveyi*

2. Method

2.1 The Extraction of Active Substance, and The Experiment of Adding Crude Extract A. Acuminata into Tiger Grouper and of Infecting Fish with V. Harveyi

Fresh bark of *A. acuminata* was taken from the research site at Southeast Maluku District. It was dried, cut into small pieces, ground into fine dust, and macerated at room temperature with methanol solvent for 3x24 hours. This extract was then partitioned with n-hexane and ethyl acetate solvents by the ratio of solvent to material of 4:1 (Harborne, 1987).

The grouper was maintained in the 40 liters batch with clean water and 150 ppm chlorine. It was neutralized by 75 ppm chemical-grade natrium tiosulfate. Fish was acclimated for 7 days, with adjusted temperature and salinity. Fish was removed into submersion batch and given immunostimulant of *A. acuminata* crude extract at several doses, such as 0 ppm (normal control), 50 ppm, 100 ppm, 150 ppm, and 200 ppm for 1 hour (based on period length in the result of toxicity test). After submersion, fish was returned to maintenance batch and incubated for 3 days. At day-3, fish was placed again in the extract submersion batch and submerged for 1 hour. After this, the challenge test against the density of bacteria *V. harveyi* at 10^7 cells/mL was conducted. Observation and measurement were carried out for 7 days to acknowledge macrophage, phagocytosis, leukocyte total, plasma protein, and the number of living fish.

2.2 Parameters to Observe

Parameters to observe were number of macrophage (measured based on lrianto, 2005), phagocytosis, leukocyte total (based on Bijanti, 2005; Harikrisnan *et at*., 2010) and survival rate of grouper.

3. Result

3.1 The Change of Grouper Macrophage after the Addition of Crude Extract A. Acuminata

Macrophage change in tiger grouper after the adding of crude extract of *A. acuminata* was

shown in Figure 1.

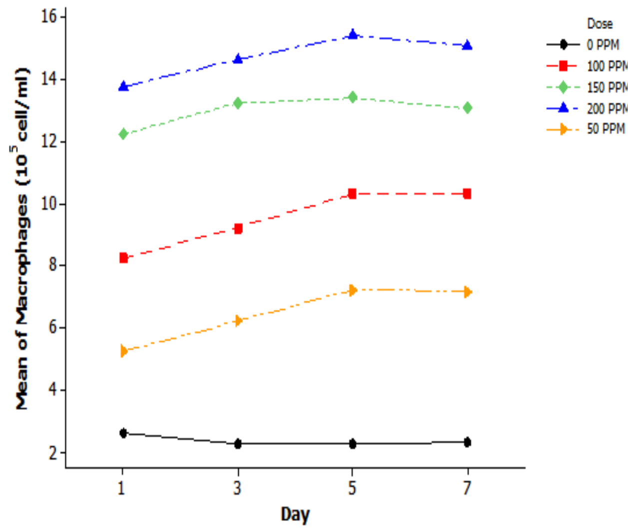

Figure 1. The change of tiger grouper macrophage based on *A. acuminata* crude extract doses

3.2 Phagocytosis

The change of phagocytosis in tiger grouper based on *A. acuminata* crude extract doses was indicated in Figure 2.

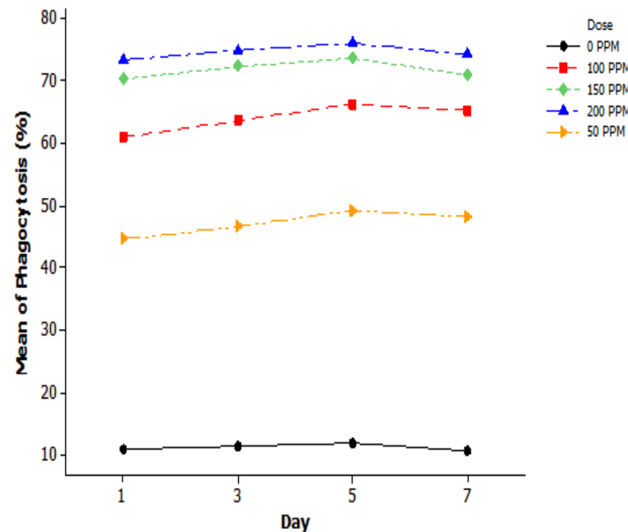

Figure 2. The change of tiger grouper phagocytosis based on *A. acuminata* crude extract doses

3.3 Leukocyte Total of the Trialed Fish

Leukocyte was a component of blood cells which functioned as non-specific defense to localize and to eliminate pathogen through phagocytosis process (Andayani, 2007). The

change of leukocyte total in tiger grouper based on *A. acuminata* crude extract was shown in Figure 3.

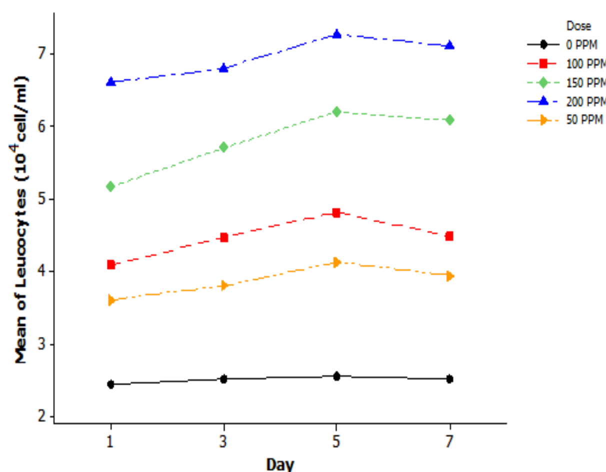

Figure 3. The change of tiger grouper leukocyte total based on *A. acuminata* crude extract doses

Figure 3 showed that leukocyte total was increased at day-5 for 72,666 cells/mL but decreased at day-7 to 71,100 cells/mL, while at 0 ppm dose (control), leukocyte total was 24,433 cells/mL. Appropriate immunostimulant dose could increase activities of complement substance, opsonine macrophage, phagocytosis, and PMn leukocyte. It also might facilitate the elimination of incoming antigens, thus preventing the fatalism of infection. The increased leukocyte total meant the presence of humoral and cellular responses of leukocyte in dealing with the bacteria (Erlinger, 2004; Finlay *et al*., 2006; Magnadottir, *et al*., 2005).

The production of leukocyte would be delivered to the infected part of the body, and it stimulated the fish body to produce defense. At day-7, leukocyte decreased and it seemed that fish body was only sustained until day-5. It was followed by leukocyte decrease as shown by histological damage in fish body at treatment of extract doses of 50 ppm, 100 ppm and 150 ppm.

The treatment with 200 ppm dose produced higher leukocyte total if it was compared to 0 ppm (control), 50 ppm, 100 ppm and 150 ppm doses. It meant that the higher concentration of *A. acuminata* crude extract that was given to tiger grouper was producing higher leukocyte total in the fish. Indeed, 200 ppm dose was the effective dose of *A. acuminata* crude extract because it could eliminate bacteria *V. harveyi* that infected the fish. It was supported by the fact that body histology of the fish that was given 200 ppm dose was only experiencing minor damage of body organ if it was compared to 0 ppm (control), 50 ppm, 100 ppm and 150 ppm doses.

3.4 Plasma Protein Characteristic with Electrophoresis

Result of research indicated that protein fraction was emerging in each sample. Testing with SDS PAGE electrophoresis had produced profiles and molecular weights of the protein in each blood plasma sample.

The description of protein profile of blood plasma after infection was using normal control, bacterial control and extract control, as displayed in Figure 4. The difference of protein pattern in each sample was found in the thickness rate and the number of protein ribbons. Electrophoresis count indicted that the extract concentration had more protein ribbons than the control.

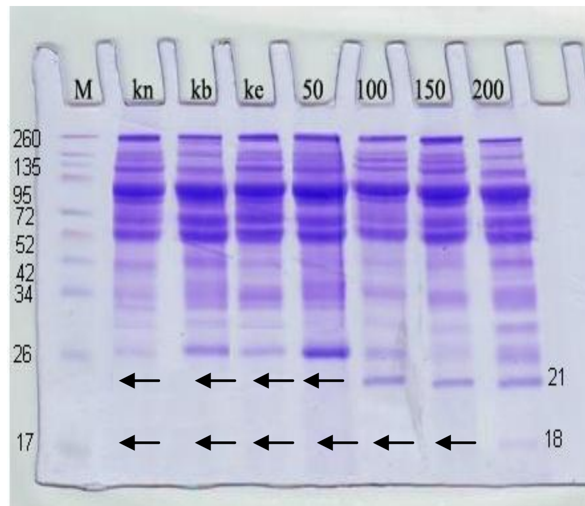

Figure 4. SDS Page over tiger grouper with *A. acuminata* crude extract

Notes: M = marker, kn = normal control, kb = bacterial control, ke = extract control, at 50, 100, 150 and 200 doses.

3.5 Survival Rate of Tiger Grouper

Survival rate was one of main parameters and success keys for aquaculture business. The providing of *A. acuminata* extract immunostimulant to tiger grouper fish could increase the body defense of the seed to control against *V. harveyi* disease, and therefore, it would improve the survival rate of tiger grouper. The survival rate of tiger grouper based on *A. acuminata* crude extract doses was shown in Figure 5.

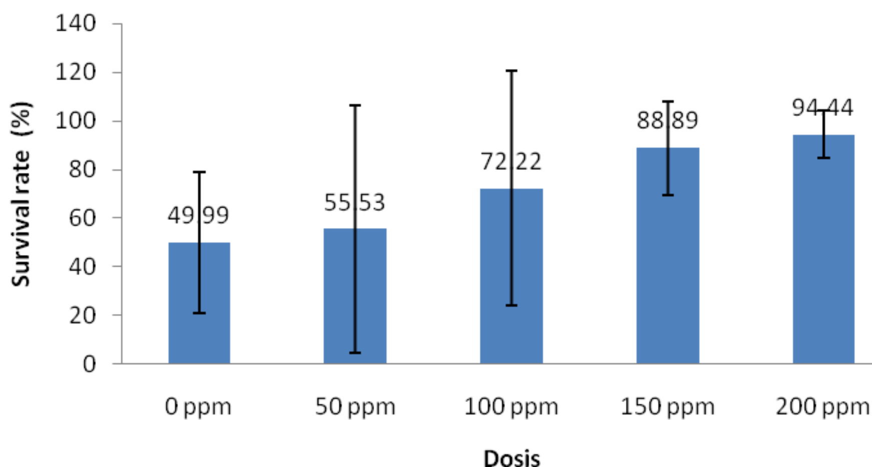

Figure 5. The survival rate of tiger grouper based on *A. acuminata* crude extract doses.

Tiger grouper was submerged into *A. acuminata* crude extract. Figure 5 showed that the highest survival rate of tiger grouper was 94 % achieved at 200 ppm concentration. The lowest survival rate was 56 % reached at 50 ppm concentration. At 0 ppm dose (control), the survival rate was 50 %. Based on these findings, it was said that the effective treatment of *A. acuminata* crude extract was at 200 ppm concentration, if it was compared to 0 ppm (control), 50 ppm, 100 ppm, and 150 ppm concentrations.

4. Discussion

Macrophage referred to a phagocyte cell that belonged to the precursor cells in the bone marrow. The divided premonocyte would produce monocyte, and monocyte circulated with blood to play a role as the reactor of body immunology which then had receptor to attach antibody and to destroy alien substance. Main function of macrophage in the body immune system was to swallow the particles, to subject it to the digestion by lisozom, and to deliver resultant substances for body defense. The macrophage could influence activity of immune response. It also processed and stored antigents, and sent the information to the immunologically competent nearby cells (such as lymphocyte and plasma cells). It also could be sectorial cells which released important substances such as enzymes, lisozymes, elastase, collagenase, protein and complement system, like interferon. (Sang *et al*., 2007; Mishra *et al.,* 2006;. Kumar and Mukhajee, 2007).

In study the number of macrophage was increased at day-5 for 15.5 x 10^5 cells/ml and decreased at day-7 to 15.2 x 10^5 cells/ml. The higher dose produced higher number of grouper macrophage. It proved that fish body was tolerant to the extract doses and still able to produce macrophage cell to suppress the growth of bacteria *V. harveyi*. Therefore, at day-7, the number of macrophage was decreased. This is supported by studies Risjani *et al*., (2012) using the extractas an ingredient immunostimulataoftory Gracillaria verucosa which showed an increase in macrophage cells are able to suppress the growth of bacteria *V.harveyi*.

This finding supported Anderson (1995) and Tizard (1998), which indicated that macrophage played important role in the fish body defense system against pathogen infection, and the number of macrophage increased in case of infection. It was supported by Lou et al (2012) who said that coumaric compound in the *A. acuminata* extract could destroy bacterial membrane cells and bind bacterial DNA such that this DNA could not express itself and thus, it was causing bacteria to death.

Fish with *A. acuminata* crude extract had greater non-specific defense ability if it was compared to 0 ppm dose (control). Immunostimulant-exposed dose at 200 ppm could stimulate immune warning of the trialed fish, and also influence the establishment of immunity response (Anderson 1995; Thompson *et al*., 1995; Mayer, 2008).

Macrophage as phagocyte cells could kill bacteria through 2 mechanisms: (1) One was oxidative process involving the increased use of oxygen and hexose monophosphate shunt (HMPS), the increased production of peroxide (H2O2) and some other compounds such as superoxide anion, radical hydroxyl, oxygen singlet, myeloperoxidase. All these substances

were interacting with enzymes. The reaction produced toxic oxygen metabolite which was useful to kill bacteria; (2) other was non-oxidative process where it involved the aid of proteins such as enzymatic hydrolytic, defenses, and lisozyme, in order to increase NO production from macrophage *(Galindo, 2004; Sang et al., 2007)***.**

The activity of phagocytosis was the ingestion of alien particles, mainly bacteria, into blood cell cytoplasm (Brown, 2000). Phagocytosis activity occurred with the contact between particles and the surface of phagocytosis cells. Cellular membrane was invaginated where two arms of cellular cytoplasm were protruded from membrane-coated vacuole (phagosom). Lisozom that was nearby phagosom was then blending with phagosom and producing enzymes that manufactured phagolyzosom or secondary lisozom to facilitate the killing of bacteria or alien particles in the phagocytosis cells.

Leukocyte played important role in the fish defense system against pathogen infection (Anderson, 1995). During infection, leukocyte was delivered into the infection system to manufacture fast defense against infectious genetic (Sadikin, 2002). The presence of *A. acuminata* crude extract was definitely helping defense system of fish to challenge bacteria infection. Coumaric compound in the *A. acuminata* crude extract could damage bacterial membrane cells and bind bacterial DNA such that DNA was not expressed, then causing bacteria to death (Lou *et al*., 2012). After this, leukocyte cells were not produced anymore in greater number.

The increased leukocyte population was caused by the increased rate of activities such as the division of cells and the production of mitogenic immunostimulant. Mitogenic compounds would activate defense cells toward differentiation. It caused DNA in the lymphocyte cells to produce synthesis, thus triggering the increase of leukocyte population (Rorstad *et al.*, 1993).

In study indicated that the phagocytosis rate increased at day-5 for 76.67% but decreased at day-7 to 74.33%. Meanwhile, 0 ppm dose (control) had phagocytosis means of 11%. The adding of *A. acuminata* extract influenced the increase of phagocytosis activity, where the higher dose would produce higher phagocytosis activity. Result of Dangeubun, J (2012) indicated that the isolation of *A. acuminata* methanol crude extract was containing a compound methyl 6-hydroxy-2-methoxy3-(2 oxohexy) benzoate. This compound was part of phenolic which containing OH cluster and interacting with bacterial protein. Macrophage cells might recognize and stimulate macrophage to activate T-lymphocyte cells. Indeed, T-lymphocyte cells produced interferon (lymphokin) and reactivated macrophage such that the ability of macrophage to phagocyte the bacteria was increased, thus increasing also phagocytosis rate.

Research about non-specific immune response was also carried out by Invadens and Dhanuskodi (2011). It used methanol extract of nigrum solanum leaf and the result showed that phagocytosis was significantly increased and non-specific response was induced to help preventing epizootic ulcerative syndrome in fish.

Above figure indicated that 200 ppm extract had 11 protein ribbons. Two of them were missing, that was protein ribbon 21 kDa and 18 kDa, during normal control, bacterial control, extract control, and 50 ppm treatments. The change in the molecular weight was estimated as

happening during the infection of the fish. It related to the presence of bacterial activity in the fish body with negative impact such as damaging certain proteins or erasing some proteins. The missing of protein ribbons was caused by degradation of protein and synthesis of protein. Both also caused the vanish of cellular-based important proteins such as anti-body enzyme or cytoskeleton matrix maker protein, whereas it accelerated destruction and elimination of cells (Andayani, 2007; Albert *et al*., 2002; Nayer & Reza, 2007).

It was assumed that protein played important role in the cellular defense mechanism. It was favorably expressed after treated with *A. acuminata* doses. The *A. acuminata* dose of 200 ppm could increase body defense and survival of tiger grouper. Andayani (2007) admitted that result of protein plasma electrophoresis had showed that protein ribbons were thickened during the infection in fish.

High survival rate of tiger grouper after treated with *A. acuminata* crude extract might be explained by the presence of bioactive substance in *A. acuminata* crude extract, precisely phenol compound with its anti-bacterial nature. Phenol compound interacted with component of bacterial cell walls such that bacterial cell was permeable and phenol was diffusing into the bacterial cell, and then, prohibiting and harming bacterial growth. Phenol could also penetrate into membrane and interact with genetic material such that the bacteria were mutating (Salosso, 2011).

5. Conclusion

The submersion of tiger grouper in the *A. acuminata* crude extract solution was able to increase non-specific immune of the fish (number of macrophage cells, phagocytosis activity, and leukocyte total) at day-5 against bacteria *V. harveyi*.

Result of SDS PAGE showed that the 200 ppm extract consisted of 11 protein ribbons. Two protein ribbons were 18 kDa and 21 kDa, that were missed from normal control, bacterial control, extract control and 50 ppm.

Doses between 0 ppm, 50 ppm, 150 ppm, and 200 ppm that were producing the highest survival rate were *A. acuminata* crude extract at 200 ppm dose, with survival rate of 94.44 %.

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