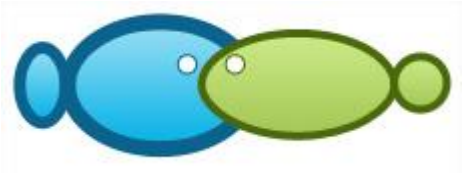

Study of *Holothuria scabra* effect on the immune activity of *Pangasianodon hypophthalmus* against *Aeromonas hydrophila*

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Abstract. *Holothuria scabra* or holothurians are one of the Echinodermata group which is specific and easily known. They have been known to be used as a drug to treat some diseases in humans. The aim of this study was to investigate the effect of *H. scabra* on the immune activity of *Pangasianodon hypophthalmus* by immersion and their protection against *Aeromonas hydrophila* invasion. In brief, *P. hypophthalmus* was immersed with *H. scabra* extract at 0, 50, 100 and 150 ppm by twice booster. Leucocyte level, number of phagocytes, phagocytosis, survival rate, and clinical symptoms were assessed to determine *P. hypophthalmus* immunity. The results revealed that the concentration of *H. scabra* extract at 100 ppm enhanced significantly the leucocyte level of *P. hypophthalmus* compared with the control. Concerning survival rate it was showed that all treatments worked better than the control. The highest percentage of survival rates was also observed at 100 ppm of *H. scabra* extract after challenged with *A. hydrophila*. The present discoveries suggest that *H. scabra* extract at the concentration of 100 ppm may raise the immune responses in *P. hypophthalmus* using immersion.
Key Words: holothurians, immersion, immune responses, iridescent shark, survival rate.

Introduction. Iridescent shark, *Pangasianodon hypophthalmus*, is one of the freshwater fish organisms having the fastest growth and potential economic value (Singh & Lakra 2012). In 2011, Indonesia was the second greatest *P. hypophthalmus* producer in the global manufacturing up to 16.1% of overall global production of Pangasius; however, the quantity is still below Vietnam which leads to world's Pangasius production amounting up to 80.9% (Ramadhan et al 2016). However, the intensive production of *P. hypophthalmus* with high density has posed a problem of infectious diseases (Afrianto & Liviawaty 1992). The most common disease-causing agents of *P. hypophthalmus* is bacteria (Griffiths et al 2010). In a recent study, *Aeromonas hydrophila* was observed infecting *P. hypophthalmus* (Griffiths et al 2010; Sarker & Faruk 2016). *A. hydrophila* is a gram-negative bacteria that cause motile Aeromonas septicemia in fish (Abdelhamed et al 2017). They produce exotoxins properties which cause hemorrhages on body, eyes, and fins, bloody ascites in the peritoneum, and contributing to swollen belly (Daskalov 2006; Griffiths et al 2010).

Today, antibiotics are still used to treat and prevent bacterial diseases (Igbinosa et al 2012; Serrano 2005). However, it is figured out that overuse of antibiotics can diminish the effectiveness of antibiotics (Sørum et al 2006), increase environmental hazards (Guo et al 2011) and generate residual antibiotics (Lakshmi et al 2013). One of the alternatives replaces antibiotics with natural products (Disch et al 2017). *Holothuria scabra* has been known to be used as a drug for treating several diseases in humans (Darsono 1993; Dhinakarana & Liptonb 2014; Manan et al 2016). Contains compunds as sterol (Chalorak et al 2017; Goad et al 1985) triterpenoid (Kerr & Chen 1995) sapogenin, alkaloid, glycosaminoglycan (Nagase et al 1995), lectins (Mojica & Merca 2005), phenol (Mamelona et al 2007) and flavonoid (Bordbar et al 2011; Nimah et al 2012). These compounds are used as an anticoagulant, antithrombotic, reducing cholesterol level and

blood fat, anticancer, antitumor, antibacterial, immune-stimulant, antifungal, antiviral, antimalarial and antiemetic (Bordbar et al 2011; Nimah et al 2012).

Therefore, the aim of this study was to examine leucocytes level, number of phagocytes, phagocytosis, survival rate and clinical symptoms of *P. hypophthalmus* and its tolerance to *A. hydrophila* post-immersion of *H. scabra* extract.

Material and Method

Fish and plant extraction. *P. hypophthalmus* (6±1.0 g) were obtained from a commercial farm from Tulungangung, East java and reared in Parasite Laboratory, Faculty of Fishery and Marine Science, Brawijaya University, Malang, East java, Indonesia. Fish (10 individuals/aquarium) were acclimatized at 25°C for one week before conducting an experiment and fed with commercial feed at 3% of fish body weight per day. Water quality was controlled to keep the environment in good condition.

H. scabra extraction followed the study of Aras (2013) and Dong et al (2008) with slight modifications. Briefly, *H. scabra* was minced to small pieces, then 400 g of *H. scabra* was added to 400 mL of methanol and macerated in a bottle for 24 hours. The mixture was then filtrated to get methanol extract, while the waste was added again with methanol to isolate the extract completely. The clear supernatant of *H. scabra* extract was evaporated using vacuum evaporator to evaporate the solvent. Eight gram of thick extract was fractionated with water and n-butanol (1:1). The n-butanol fraction was evaporated using vacuum evaporator to obtain *H. scabra* extract. The extract was kept at 4°C until use.

Effect of H. scabra extract on the immune level. First, the *H. scabra* extract was dissolved directly into each aquarium with different concentrations of 0, 50, 100 and 150 ppm (T0, T1, T2, and T3, respectively) by twice booster at 0 and 7 day. The immersion of *H. scabra* extract was conducted for 1 hour and then *P. hypophthalmus* was transferred to each aquarium. The blood collection was conducted at 2, 4, and 6-day post-immersion of *H. scabra* extract and 3rd day post-infection to observe leucocyte level. Blood collection was taken from the caudal vena using sodium citrate 3% as anti-coagulant (Nuryati et al 2010).

Based on a method of Kamaludin (2011), leucocyte level was calculated under a microscope with 400x magnification. The number of leucocytes was formulated by (Suhermanto et al 2011):

$$SDP = (A/N) \times (1/V) \times Df$$

Where:
SDP = Number of leucocyte
A = Counted leucocyte
N = Number of hemocytometer square observed
V = Volume of hemocytometer square observed
Df = Dilution factor

The total number of macrophage cells was calculated based on the method of Irianto (2005). Fish kidney gently was powdered using tissue grinder and diluted in RPMI medium contained Fetal Bovine Serum (FBS) 2% in order to facilitate calculating the macrophage cells. Total macrophage cells was determined using a microscope with 40X magnification (Irianto 2005), while phagocytosis activity was determined following the Irianto & Austin (2002) method. Briefly, macrophage suspension was centrifuged with 1,000 rpm for 5 minutes and the pellet was spread on object glass. It was incubated at 26°C for 60 minutes and washed using RPMI medium contained FBS 2%. *A. hydrophilia* was used for phagocytosis activity identification under a microscope with x100 magnification. The percentage of phagocytosis activity was calculated from 100 macrophage cells identified (Irianto & Austin 2002).

Challenge and evaluation of survival rate. The fish-pathogenic strain of *A. hydrophila* was kindly provided by Microbiology Laboratory, Brawijaya University and used in this study with $1x10^8$ (CFU) mL^{-1} for a challenge (LD 50). The challenge test was conducted at

day 8 of post-second booster (day 7). Ten fish were immersed with *A. hydrophila* ($1x10^8$ (CFU) mL^{-1}) into an aquarium. Survival rate of fish was monitored during 24, 48, 72, 96, and 144 hours post-challenge.

Statistical analysis. ANOVA (One-way analysis of variance) was applied to evaluate variations among groups. Multiple comparisons (BNT test) were carried out to determine significant differences among treatments using SPSS (version 17, USA). Data were displayed as the mean ±SD, $p<0.05$ was considered significant.

Results and Discussion

Phytonutrients test. Sea cucumber can produce highly active substances, saponins, the main secondary metabolites, which are the essential of their chemical defense (Zhao et al 2018). Based on Table 1, *H. scabra* extract was identified possessing compounds including saponin and triterpenoids. According to Zhao et al (2018), sea cucumber possesses some compounds which of the dominant compound are saponin (Table 1). Saponin is a glycoside triterpenoid obtained from secondary metabolite by membrane sterols which is linked by aglycone and sugar chains through the β-glycosidic bond (Bahrami et al 2014; Caulier et al 2016; Van Dyck et al 2011). This compound is known as a defense to predator, antimicrobial, and poison to invertebrate and algae (Van Dyck et al 2011).

Table 1

Results of phytonutrients test

Test		*Sign*
Flavonoid	-	No color changed to red or orange
Saponin	√	Foam formed
Steroid	-	Blue or purple color
Triterpenoid	√	Red color
Fenol	-	No color changed to blue or green
Alkaloid	-	Brown sediment formed

- : negative expression; √ : positive expression.

Immunological effect post-immersion. The innate immune system is the first mechanisms defense of fish which is physiologically similar to greater vertebrates (Magnadóttir 2006; Uribe et al 2011). Their immune system is formed of physical barriers, cellular and humoral components which are used against foreign substances, such as microorganisms, toxins or malignant cells, responding to factors such as endogenous or exogenous components that stimulate this system (Biller-Takahashi & Urbinati 2014).

The inflammation is also recognized as an immune response, facilitated by complex interactions of cellular and humoral compounds (Biller-Takahashi & Urbinati 2014). The granulocytes are the initial cell that takes place at the inflammation site, being competent for eliminating pathogens. On the other hand, macrophage phagocytosis play an important role to remove the remaining pathogenic cells and cellular debris (Magnadóttir 2006).

Leucocytes level. Fish leucocytes could be used to determine fish health status or clinical evaluation of stress and diseases of fish (Palić et al 2011). According to Davis et al (2008), leukocyte studies are especially helpful to conservative physiology of fish because they are changed by stress and can be promptly connected to stress hormone levels. Leucocytes itself could be divided into three types such as monocytes, macrophages, and neutrophils (Mathias et al 2009; Neumann et al 2001). They play a role in intracellular and extracellular mechanisms to generate antimicrobial substances in defense against pathogens (Rieger & Barreda 2011).

In this study, the leucocytes level was enhanced post-immersion with *H. scabra* extract. The leucocytes level enhanced early 2th-day post-immersion by T1 and T2, while T3 showed no significant difference compared with the control group ($p<0.05$). Further, in another time point, leucocytes level was decreasing but it was still a significant difference comparing with control. In the 6th day, all treatments showed no significant differences, except T2 which still increased. Based on Suhermanto et al (2011) study, *H. scabra* extract also was applied for increasing leucocytes level of *Cyprinus carpio* pre-infection of *A. hydrophila*. Increased of leucocytes level was believed to be due to triterpenoids within *H. scabra* extract which have immune-regulatory function (Rascón-Valenzuela et al 2016). In another study, injection of triterpenoids enhanced white blood cells of mice (135.75±6.4%) at 3th and 6th-day post-injection (Raphael & Kuttan 2003). According to Aminin (2016), the small concentrations (0.1–6.0 mg mL^{-1}) of holothurians triterpene glycosides play to trigger a leukocyte movement that promote an increase in phagocytosis of *Staphylococcus aureus* by human polymorphonuclear leucocyte. Based on it, we assumed that increasing of leucocytes was caused by triterpenoid compound within *H. scabra* extract (Figure 1).

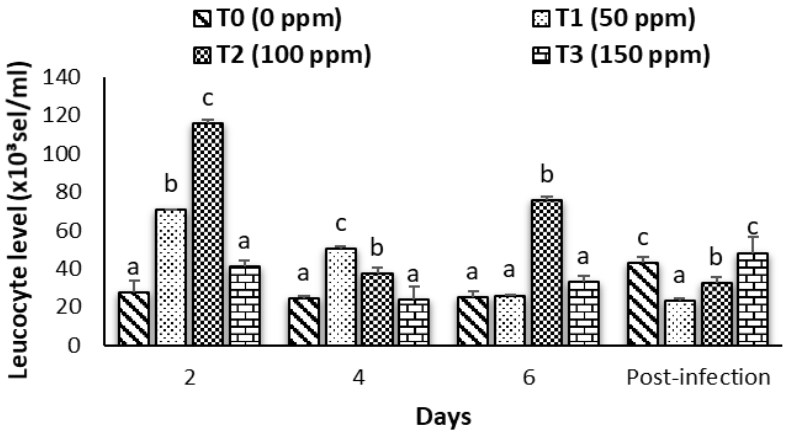

Figure 1. Total leucocyte pre- and post-infection with *Aeromonas hydrophila*.

The second booster was conducted pre-challenge with *A. hydrophila* on 7th day. It was applied not only for increasing leucocyte again but also for showing the efficacy of *H. scabra* extract. In post-infection, the highest expression leucocytes level was showed by T0 and T3, while T1 and T2 showed no increase and dropped, respectively. In several cases, the leucocytes count increase post-infection of pathogen as a defense mechanism. According to Rajapakshe et al (2012) and Shahi et al (2014), an increase in the leukocyte level is the most reasonable results in an improvement of the non-specific defense, because macrophages and other phagocytic cells are main cells of the immune system. Interestingly, in our results, T1 and T2 did not enhance leukocytes level post-infection. Generally, a decreasing amount of leukocytes post-infection showed that leukocytes are believed active and come into blood vessel going through infected tissues (Nuryati et al 2010). Based on these findings, it is suggested that triterpenoids from *H. scabra* extract is recommendable to increase the immune response of *P. hypophthalmus*.

Total of macrophage cells. Macrophages can be simply collected from various sources such as blood (monocytes), lymphoid organs (specifically the kidney), and the peritoneal cavity (Secombes 1996). In this study, macrophages cells were isolated from kidney pre- and post-infection of *A. hydrophila.* The fish head kidney is believed equal of fish bone marrow-derived macrophages and has the capability of phagocytosis (Grayfer et al 2018), generate radicals (Grayfer & Belosevic 2009), and part of innate activation (Joerink et al 2006). Our results revealed that a total of macrophage cells increased significantly post-immersion with *H. scabra* extract and post-infection by all *H. scabra* concentrations. In pre-infection, T2 showed the most significant difference ($p<0.05$)

compared with control and even with all treatments, while after infection all treatments showed significant differences ($p<0.05$) compared control group (Figure 2).

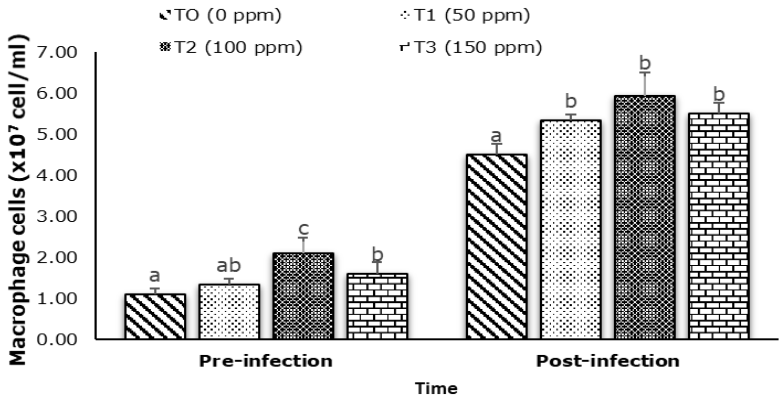

Figure 2. Total macrophage cells pre- and post-infection with *Aeromonas hydrophila*.

Increasing of macrophages post *H. scabra* extract immersion and *A. hydrophila* in *P. hypophthalmus* kidney could be possible due to leucocytes immigration. According to Grayfer et al (2018), Tissues macrophages were considered to result from circulating monocyte precursors in response to tissue entry and accompanying stimuli. An another study reported that phagocytic activity in tissues is facilitated by cytokines as macrophage stimulating factor released by peritoneal lymphocytes (Gordon & Plüddemann 2017; Graham & Secombes 1988). A similar result, reports that the use of 50 g *Euphorbia hirta* extract could enhance total macrophage in *C. carpio* kidney post- *E. hirta* feeding and *A. hydrophila* infection (Pratheepa & Sukumaran 2014). Based on our results, *H. scabra* extract is recomendable as immunostimulant to enhance total macrophage cells number in *P. hypophthalmus* kidney.

Phagocytosis activity. The fish kidney was considered as granulopoiesis site to produce granulocytes such as macrophages and neutrophils (Ainsworth 1992; Esteban et al 2015; Geven & Klaren 2017; Uribe et al 2011). They have been known as phagocytic cells that show a critical role in the fish nonspecific immunity, which have a phagocytosis activity (Hardi et al 2017; Romano et al 1998). Phagocytosis is typically a defensive reaction against invasion of foreign substances and, in the immune system, phagocytosis is a major mechanism used to remove pathogens and/or cell debris related function in engulfing and killing the pathogen (Esteban et al 2015).

In our results, phagocytic activity results showed significant differences among treatments ($p<0.05$) in *P. hypophthalmus* kidney by all concentrations post-immersion of *H. scabra* extract and even post-infection of *A. hydrophila* (Figure 3). Based on it, all concentrations of *H. scabra* extract worked in order to increase phagocytosis activity. Accordin to (Hohn et al 2009), increasing of phagocytosis activity is considered by diverse factors such as pollutants (Weeks & Warinner 1986), diet (Blazer 1991), temperature (Hardie et al 1994), pathogens (Ainsworth & Dexiang 1990) and genetic variation (Sarder et al 2001). Phagocytic activity ratio (%) noted the highest value within T2 post-challenge with 41.89% activity followed by T1 and T3, respectively.

Aminin et al (2008) discovered that a small dosage of sea cucumber triterpenoid could enhance the antibody production to stimulate monocyte phagocytosis of *S. aureus* and the number of antibody such as IgM and IgG antibody levels in mice. An another study showed phagocytic activity increase in groups fed with 100 ng prolactin and 50 ng β-glucan per kg *Salmo salar* during the 20 days period (Paredes et al 2013). Based on it, we assumed that increase of phagocytic activity is enhanced by leucocytes migration from blood which goes through tissues.

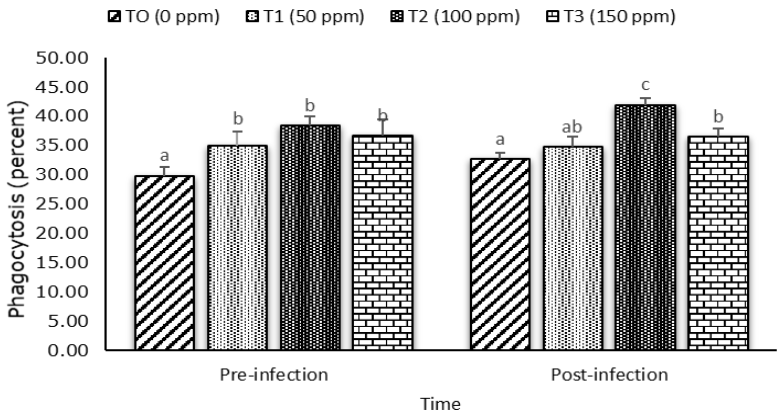

Figure 3. Phagocytosis activity cells pre- and post-infection of *Aeromonas hydrophila*.

Survival rate of P. hypophthalmus. The survival rate was monitored for 144 hours post challenge with *A. hydrophila.* Challenge was conducted day 2 post-immersion of *H. scabra* extract (second booster) by bathing of *A. hydrophila*. All treatments showed better results than control ($p<0.05$). The highest expression of survival rate was recorded by T2 with 76.7%, while the lowest survival rate was shown by T0 with 16.7% (Table 2).

Table 2

The survival rate of *Pangasianodon hypophthalmus* against *Aeromonas hydrophila*

Bacterial dose (cells/mL)	*H. scabra extract (ppm)*	*No. of fish*	*Survival rate (%) at different times (hour)*				
			24	*48*	*72*	*96*	*144*
No infection	0 (Control)	30	100	100	100	100	100
10^8	0	30	100	83.3	50	33.3	16.7
10^8	50	30	100	90	73.3	60	50
10^8	100	30	100	86.7	83.3	80	76.7
10^8	150	30	100	86.7	66.7	40	33.3

According to a study of Alipiah et al (2016), sea cucumber extract worked well to enhance survival rate of grouper post-challenge with several infectious bacteria. In a review, triterpenoid in *H. scabra* has a function as improvement of health status and can be used as a food supplement (Iñiguez-Martinez et al 2005). According to Nugroho et al (2016), triterpene was identified in *Terminalia catappa* which could enhance percentage of *Betta* sp. survival rate during cultivation. Based on the present study, *H. scabra* could improve health status of *P. hypophthalmus* post-immersion with *A. hydrophila.*

Conclusions. The results demonstrated that the immersion in *H. scabra* extract improved the survival rate of *P. hypophthalmus* after challenged with *A. hydrophila* and also enhanced the innate immunity of *P. hypophthalmus* by increasing the WBC, total macrophages, and phagocytosis activity that play crucial role in fish immunity.

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