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Submission date: 04-Jan-2019 07:43AM (UTC+0700)
Submission ID: 1061388189
File name: 29.pdf
Word count: 7518
Character count: 41045

NUSANTARA BIOSCIENCE
Vol. 9, No. 2, pp. 220-228
May 2017

ISSN: 2087-3948
E-ISSN: 2087-3956
DOI: 10.13057/nusbiosci/n090219

Utilization of several herbal plant extracts on Nile tilapia in preventing *Aeromonas hydrophila* and *Pseudomonas* sp. bacterial infection

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Manuscript received: 7 November 2016. Revision accepted: 3 May 2017.

Abstract. *Hardi EH, Kusuma IW, Suwinarti W, Saptiani G, Sumoharjo, Lusiastuti AM. 2017. Utilization of several herbal plant extracts on Nile Tilapia in preventing Aeromonas hydrophila and Pseudomonas* sp. *bacterial infection. Nusantara Bioscience 9: 220-228.* This study described the effect of the ethanol extracts of temu kunci (*Boesenbergia pandurata)*, terong asam (*Solanum ferox*), and lempuyang (*Zingiber zerumbet*) in increasing the nonspecific immunity of tilapia fish (*Oreochromis niloticus*) as well as in inhibiting *Aeromonas hydrophila* and *Pseudomonas* sp. infections. The ethanol extracts concentrations used in this study were: 600 and 900 ppm for *B. pandurata*, 400 and 900 ppm for *S. ferox*, and 200 and 2000 ppm for *Z. zerumbet*. 0.1 mL of each extract was administered via intraperitoneal injection into the tilapia fish with a weight of about 15 g. Challenge test on day 6 was done via intramuscular injection of 0.1 mL *A. hydrophila* and *Pseudomonas* bacterial suspension (density 10^{10} CFU/mL^{-1}) into the fish. Thereafter, the fishes were kept for another 14 days to monitor the effectivity of the extract treatments in inhibiting pathogenic bacterial infection, by measuring several parameters such as hematocrit (He), hemoglobin (Hb), total erythrocyte (TE) on day 0 and day 14. Total leukocyte and phagocytic index were observed on day 0, 6, and 14. In addition, observations on the changes in swimming pattern, anatomic pathology, as well as fish mortality rate and relative percent survival (RPS) after challenge test were conducted as well. The results indicated that, overall, the three extracts were able to increase the total leukocyte, phagocytic index and maintain the blood parameters (He, Hb and TE) in a normal state. The protection rate against *A. hydrophila* observed in the fish treated with 200 and 2000 ppm *Z. zerumbet* extracts were 100%. Meanwhile, that of the fish supplemented with 600 and 900 ppm *B. pandurata* were 63% and 68%, respectively. In response to *Pseudomonas* sp. infection, the RPS of the fish receiving 900 ppm *S. ferox* was significantly higher than that treated with 400 ppm *S. ferox*. In conclusion, all concentrations *B. pandurata, S. ferox*, and *Z. zerumbet* extracts evaluated in this study was effective in preventing *A. hydrophila* and *Pseudomonas* sp. Infections, and had a positive impact on the nonspecific immune response which in turn enhance its capability in inhibiting pathogenic bacterial infection.

Keywords: Antibacterial, herbal, immunomodulator, *Oreochromis niloticus,* septicemia

INTRODUCTION

Utilization of plant extract is an alternative and an eco-friendly way to control pathogen infection in fish. This is due to the nature of plant-derived extract which is not harmful to the environment, water-degradable, safe, low in cost and do not cause a resistant effect on fish, thereby suitable for a sustainable aquaculture. The use of plant extract to prevent bacterial infection in fish is highly potential to be implemented because there are many of these prospective natural medicinal plants grow in Indonesia. The application of papaya (*Carica papaya*) leaf, water spinach (*Ipomoea aquatica*), and galangal (*Alpinia galanga*) extracts (Saptiani et al. 2016a, b, c) could boost the survival of the catfish infected with *Aeromonas hydrophila* and *Pseudomonas* sp. bacteria, and *Saprolegnia* sp. fungus.

Temu kunci (*Boesenbergia pandurata*), hairy-fruited eggplant (*Solanum ferox*) and lempuyang (*Zingiber zerumbet*) extracts also exhibited the antibacterial property that could suppress the growth of *A. hydrophila* and *Pseudomonas* sp. bacteria, which are the common pathogen of the Nile tilapia (*Oreochromis niloticus*) (Hardi et al. 2016a, b). In addition to having antibacterial property, plant extract can also function as an immunomodulator for fish. For instances, applications of *Curcuma longa*, *Ocimum sanctum*, and *Azadirachta indica* extracts through various methods on common carp could modulate the function of the nonspecific immune system of the fish during *A. hydrophila* infection (Harikrishnan and Balasundaram 2008; Harikrishnan et al. 2009; Harikrishnan et al. 2010). Furthermore, the extract of *Azadirachta* (Logambal and Michael 2001), *Viscum album*, *Urtica dioica* and *Zingiber officinale* (Dugenci et al. 2003), *Astragalus radix* and *Scutellari radix* (Yin et al. 2006), and *Achyranthes aspera* (Rao et al. 2004; Rao and Chakrabarti 2005) have been reported to boost fish nonspecific immunity. Mixing the extract of *B. pandurata* and *Z. zerumbet* with fish feed could enhance the Nile tilapia's nonspecific immunity upon challenge with *A. hydrophila*. Meanwhile, application of *S. ferox* extract by effective immersion method, prevented the infection of *Pseudomonas* sp. (Hardi et al. 2017).

Boesenbergia pandurata rhizome extract contained alkaloid, flavonoid, and carbohydrate; *S. ferox* extract contained alkaloid and carbohydrate; *Z. zerumbet* extract had a much more amount of alkaloid, flavonoid, steroid and carbohydrate (Hardi et al. 2016a). A plant containing active compounds such as levamisole, flavonoid, steroid, and carbohydrate possessed a bacteriostatic property against pathogenic bacteria, *in vitro* and *in vivo* (Hardi et al. 2016a). Saponin (Ninomiya et al. 1995) and glycyrrhizin also function as natural antibacterial agents. Chelerythrine chloride and ellagic acid compounds contained in *Chelidonium majus* and *Zanthoxylum clava-herculis* extracts pose as antibacterial agents against *Edwarsiella ictaluri*. Whilst, chelerythrine chloride, ellagic acid, β-glycyrrhetinic acid, sorgoleone, and wogonin were toxic to *Flavobacterium columnare* (Schrader 2010). According to Harikrishnan et al. (2009), the application of plant extract via injection boosted the immune system more quickly than the administration by feeding, this is related to the absorption rate of the extract in the body. Therefore, this study will elaborate the effect of different dosages of *B. pandurata*, *S. ferox*, and *Z. zerumbet* ethanol-extract administered via injection on the Nile tilapia infected with *A. hydrophila* and *Pseudomonas* sp. pathogenic bacteria.

MATERIALS AND METHODS

Fish materials

The Nile tilapia (*Oreochromis niloticus*) weighted 15.1 ± 3.2 g (mean ± SD) were obtained from the Freshwater Fish Breeding Center (Balai Benih Ikan Air Tawar) of Kutai Kartanegara District, East Kalimantan, Indonesia. Sample fishes were transported by using plastic bags supplied with oxygen. Before used for analysis, the fish was kept in the laboratory for 7 days. The feeding with commercial feed pellet was done twice a day, with an amount of 3-5% of fish weight. Water renewal was conducted twice a week, by totally removing all leftover foods and fish feces from the bottom of the pond using a hose. Before being used for treatment analysis, fish samples were subjected to testing for bacterial infection by isolating the liver, eye, kidney and brain and incubated them in GSP medium (a specific medium for culturing *A. hydrophila* and *Pseudomonas* sp.). This bacterial culture test was done to ensure that fishes for further treatment were free from *A. hydrophila* and *Pseudomonas* sp. pathogenic bacteria. Each treatment was done in triplicate, with each test aquarium contained 10 fishes.

Figure 1. Herbal plants used in this study. From left to right: *B. pandurata, Z. zerumbet,* and *S. ferox*

Aeromonas hydrophila and *Pseudomonas* sp. bacteria

Pathogenic bacteria used in the treatment were *A. hydrophila* (EA-01) and *Pseudomonas* sp. (EP-01) obtained from Aquatic Microbiology Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Mulawarman, Samarinda, East Kalimantan, Indonesia. Bacteria were cultured in BHI (Brain Heart Infusion Broth, DIFCO®) media at 30°C, for 24 hours. The cultures were then centrifuged at 1000g for 10 minutes. The supernatant was removed, while the pellet was washed three times using PBS (phosphate-buffered saline) solution. For challenge test, the bacteria suspensions were diluted using sterilized distilled water until the bacterial density of 10^{10} CFU mL^{-1} was achieved, *i.e.* the LD50 density of the two bacteria (Hardi et al. 2014b).

Plant extract

Rhizomes of temu kunci (*B. pandurata*) and lempuyang (*Z. zerumbet*) as well as *S. ferox* fruit (Figure 1), were obtained from a local traditional market in Samarinda, East Kalimantan, Indonesia. The extraction process was conducted in the Wood Chemistry Laboratory, Faculty of Forestry, Universitas Mulawarman, Samarinda, East Kalimantan, Indonesia. The extraction followed the method of Harikrishnan and Balasundaran (2005) and Hardi et al. (2016a). The extraction began with the thorough washing of all the plant materials, followed by thin slicing the materials, and drying at 40-45°C for 48 hours in an oven. The dried materials were chopped and shredded into fine pieces using a blender. 100 g dry sample were mixed with 100 mL of 96% ethanol in an Erlenmeyer, at room temperature, for 72 hours. Separation of the ethanol fraction from the dregs was carried out by filtration using 0.5 μm Whatman paper. Separation of the plant extract from the ethanol was done using an evaporator for 2-5 hours. The crude extract was stored in an oven at 45°C for 24 hours for further solidifying the extract. Thereafter, the extract was stored in a refrigerator until the time of use.

The concentrations of the extracts used in this study were as follows: 600 ppm and 900 ppm *B. pandurata*, 400 ppm and 900 ppm *S. ferox*, and 200 ppm and 2000 ppm *Z. zerumbet*. These concentrations have been shown to exhibit the best antibacterial activities against *A. hydrophila* and *Pseudomonas* sp., based on the in vitro test conducted by Hardi et al. (2016a, b).

Design of experiment and challenge test

This study was conducted to identify the best concentration from each extract for modulating the fish nonspecific immune against *A. hydrophila* and *Pseudomonas* sp. This research consisted of nine experimental treatments, as follows: (i) The fish was injected neither with the extract nor with the pathogen. This treatment served as the medium control in the laboratory (no extract, no pathogen); (ii) The fish was injected with 600 ppm of *B. pandurata* extract and challenged with *A. hydrophila* (*B. pandurata* 600 ppm, *A. hydrophila*); (iii) The fish was injected with 900 ppm of *B. pandurata* extract and challenged with *A. hydrophila* (*B. pandurata* 900 ppm, *A. hydrophila*); (iv) The fish was injected with 400 ppm of

S. ferox extract and challenged with *Pseudomonas* sp. (*S. ferox* 400 ppm, *Pseudomonas* sp.); (v) The fish was injected with 900 ppm of *S. ferox* extract and challenged with *Pseudomonas* sp. (*S. ferox* 900 ppm, *Pseudomonas* sp.); (iv) The fish was injected with 200 ppm of *Z. zerumbet* extract and challenged with *A. hydrophila* (*Z. zerumbet* 200 ppm, *A. hydrophila*); (vii) The fish was injected with 2000 ppm of *Z. zerumbet* extract and challenged with *A. hydrophila* (*Z. zerumbet* 2000 ppm, *A. hydrophila*); (viii) The fish was injected with PBS solution and challenged with *A. hydrophila* (no extract, *A. hydrophila*); (ix) The fish was injected with PBS solution and challenged with *Pseudomonas* sp. (no extract, *Pseudomonas* sp.)

All treatments were performed in triplicate. 0.1 mL of each extract was administered to each fish via intraperitoneal injection. On day six after injection, the fish was challenged with *A. hydrophila* and *Pseudomonas* sp. The bacteria (density 10^{10} CFU mL^{-1}) was inoculated via intramuscular injection. The fish was then kept for another 14 days.

Blood drawing and preparation

0.2 mL of the fish blood was drawn from the caudal vein using a syringe containing 0.1 mL Titriplex-III anticoagulant. Blood tests, including hematocrit, hemoglobin, and total erythrocyte analyses were conducted on the first and last day of observation, *i.e.* day 0 and day 14. Whereas total leukocyte and phagocytic index analyses were performed on day 0, 6, and 14.

Hematocrit analysis (He)

The test was done following the method of Anderson and Siwicki (1995). The blood sample was added into a micro hematocrit tube until it reached 4/5 of the tube volume, the tube tip (marked by red color) was sealed using kretoseal and then centrifuged at 12.000 rpm for 10 minutes. Thereafter, the percentage of hematocrit was determined as a volume percentage of blood cells.

Hemoglobin test (Hb)

The test procedure was following the method of Wedemeyer and Yasutake (1977). A sahlinometer was filled with 0.1 N HCl until it reached the bottom most scale marks of the sahlinometer (scale 10). The tube was then placed in between the two tubes with standard color. 0.2 mL of fish blood was drawn from the microtube using sahli pipette and then put into the Sahli tube. The blood in the Sahli tube was left rested for 3 minutes, and the pipette tip was cleaned beforehand. Distilled water was added into the tube little by little and, at the same time, was stirred until the color was changed exactly the same as the standard color. The hemoglobin concentration was expressed as g%.

Total erythrocyte analysis

Following the method of Blaxhall and Daisley (1873), the blood sample was drawn using 0.5 scale pipette, followed by drawing hayem solution until it reached the scale 101. The mixture was homogenized by shaking the sample with a motion resembling the form of number 8. The first droplet was discarded and the next droplet was contained on a hemocytometer and covered with a coverslip. The counting was done on 5 small squares in a hemocytometer, and total cells counted was multiplied by the dilution factor and 10^4 cell.mm-^{3}.

Total leukocyte analysis

The test procedure followed the method of Anderson and Siwicki (1995). The blood sample was drawn using 0.5 scale pipette (a special pipette for leukocyte analysis), then the Turk's solution was drawn until it reached the scale of 11 of the pipette. The first droplet was discarded and the next droplet was contained on the hemocytometer and covered with a coverslip. The counting was done on 4 big squares in the hemocytometer, and the total cells counted was multiplied by the dilution factor and 50 cell. mm-3.

Phagocytic index analysis (IP)

The procedure followed the method of Anderson and Siwicki (1995). 50 µl blood in a microtube was added with 50 µl of *Staphylococcus aureus* suspension (10^7 cell.mL^{-1}). The mixture was homogenized and incubated at room temperature for 20 minutes. Thereafter, a smear preparation of the mixture was prepared on the glass slide and then air-dried. The smear preparation was fixed in methanol for 5 minutes and then dried. The preparation was stained by immersion in Giemsa staining for 15 minutes, washed in flowing water, and then dried using tissue papers. The slide was observed under a microscope. The amount of cell showing phagocytosis per 100 observed cells was counted.

Resistance to bacteria

In addition to blood analyses, another parameter observed in this study were the swimming pattern which includes gasping for air, weak movement, and aggressive behavior. The observation was done by monitoring fish swimming pattern for 30 minutes. Changes in the anatomic pathology of external organs were observed, including changes in fish fin, body darkening, and exophthalmos. The observed fish was not only the living fish but also the fish which died from suffering such symptoms. The scoring was categorized into four levels: normal, low, medium, and high. Normal meant that there was no change in the swimming pattern nor the anatomic pathology of the fish; low meant that the number of fish undergoing changes <20%; medium >20-50%; and high >50% from the total number of fish in each treatment. The observation of the change in fish swimming pattern and anatomic pathology was conducted on day 14 of treatment.

Fish death after the injection with pathogenic bacteria was monitored every day to day 14 of treatment. To ensure that the fish death was caused by the pathogenic bacterial infection, re-isolation of bacteria from the fish's liver and kidney was done in GSP medium (a specific medium for culturing *A. hydrophila* and *Pseudomonas* sp.). Furthermore, an observation of the change in fish swimming pattern and anatomic pathology of fish's external organ was conducted after the challenge test. The efficacy of plant extracts from each treatment was determined as relative percent survival (RPS) using the

Ellis formula (1988).

$$RPS = 1 - \frac{\text{(percent mortality in treated group)}}{\text{percent mortality in control group}} x100$$

Statistical analysis

Data (Mean±standard error, SE) was analyzed using one-way-analysis of variance (ANOVA) followed by Tukey's post hoc test (SPSS) by comparing each treatment ($P< 0.05$).

RESULTS AND DISCUSSION

Fish blood profile

Application of *B. pandurata*, *S. ferox*, and *Z. zerumbet* extracts could enhance the immunity of the Nile tilapia, judging from the total hematocrit and the normal hemoglobin level observed with a tendency to increase (Table 1). The hematocrit of fish supplemented with each of the three extracts was slightly higher than normal (28-30.4%), whereas the He of the control tilapia was normal (27.5%). The fish that was injected with bacteria without extract application showed a Hb level of 15-16 g%. The Hb level of the normal tilapia was 10%; that of the fish treated with the plant extracts was 10-12.5 g%; meanwhile, the Hb level of the fish infected with bacteria without any extract application decreased to 5-6 g%. These results were in line with the total erythrocyte. The total erythrocyte of control fish was $32.3x10^5$ cell.mm^{-3}. The total erythrocyte of the fish treated with each of the plant extracts was higher than that of control (ranged from 35-40.4 cell. mm^{-3}). On the other hand, the total erythrocyte of the fish that was not treated with any of the plant extracts decreased to 24.8-25 cell. mm^{-3} upon injection with the pathogen.

Total leukocyte (TL)

Total leukocyte (TL) of the tilapia fish supplemented with different dosages of each plant extract increased and differed significantly with that of the fish with no treatment but infected with the two bacteria ($P<0.05$). The normal tilapia had a TL ranged from 2.5 to 2.7 10^4 cell. mm^{-3}. In contrast, the fish supplemented with each of the extracts and infected with the pathogen showed an increase in TL which was ranged from 3.5 to 4.2 10^4 cell.mm^{-3}. Meanwhile, the fish that was not injected with any extract, but infected by the bacteria showed a lower TL of 2.9-$3.0x10^4$ cell. mm^{-3}.

Total leukocyte of the fish supplemented with the extracts at day 14 differed significantly ($P<0.05$) from that of the fish that was not treated with any of the plant extracts. This result indicated that *B. pandurata*, *S. ferox*, and *Z. zerumbet* extracts were able to significantly increase the production of leukocyte as a defense mechanism against pathogenic bacteria infection at the end of treatment. Each applied concentrations of *B. pandurata* and *Z. zerumbet* extracts did not differ significantly in inhibiting *A. hydrophila* infection. Likewise, both application of *S. ferox* concentrations (400 and 900 ppm) did not show a significant difference in inhibiting *Pseudomonas* sp. infection.

Phagocytosis index (PI)

The phagocytosis index of the Nile tilapia at day 14 increased after being supplemented with different concentrations of each extract. The highest increase observed in the fish supplemented with 600 ppm *B. pandurata*, and 200 and 2000 ppm *Z. zerumbet* extracts (Figure 3). The mean PI of the Nile tilapia before the extract treatment was 20.67-23.80%, whereas, 6 days after the treatment, the PI of the fish supplemented with extracts increased and differed significantly with that of the fish without supplementation ($P<0.05$), with the highest increase of 55.32% observed in the tilapia supplemented with 600 ppm *B. pandurata*. A slower increase in fish PI also occurred in the fish group treated with 900 ppm *S. ferox* (44.33 %). In the fish supplemented with *B. pandurata* extract, the PI still exhibited a further increase to 62.28% at 600 ppm and 61.40% at 900 ppm, after being challenged with *A. hydrophila* bacteria. The increase observed in the two *B. pandurata* extract concentrations was not significantly different. However, the increase in the PI of the fish supplemented with *B. pandurata* of any concentration differed significantly from that of the fish receiving no extract supplementation upon challenge test to *A. hydrophila*.

Table 1. Blood profile of the Nile tilapia fish treated with herbal plant extracts for prevention from *A. hydrophila* and *Pseudomonas* sp. infection on day 14

Treatments	Hematocrit (%)		Hemoglobin (g%)		Total erythrocyte (10^5 cells mm^{-3})	
	D0	D14	D0	D14	D0	D14
No extract no pathogen	22	27.5	9	10	29.2	32.3
Boesenbergia pandurata 600 ppm, *A. hydrophila*	22	30.4	10	11	29.5	40
Boesenbergia pandurata 900 ppm, *A. hydrophila*	25	30	8	10	18.3	40,4
Solanum ferox 400 ppm, *Pseudomonas* sp.	26	29	10	11.5	18.0	36
Solanum ferox 900 ppm, *Pseudomonas* sp.	27	30	8	11	29.1	38
Zingiber zerumbet 200 ppm, *A. hydrophila*	20	28	9	12.5	30.6	35
Zingiber zerumbet 2000 ppm, *A. hydrophila*	20	30	9	11	29.2	38
No extract, *A. hydrophila*	22	15	8	5	28.8	25
No extract, *Pseudomonas* sp.	24	16	10	6	30.0	24.8

Note: D0: day 0; D14: day 14

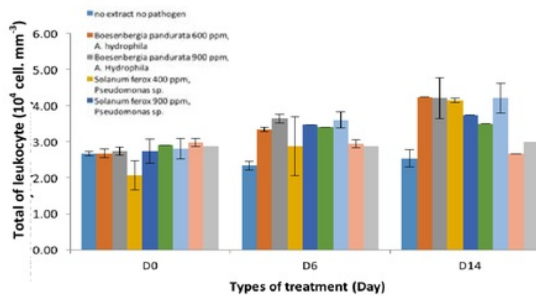

Figure 2. Effect of different dosages application of *B. pandurata, S. ferox* and *Z. zerumbet* extracts to total leukocyte of the Nile tilapia (*Oreochromis niloticus*)

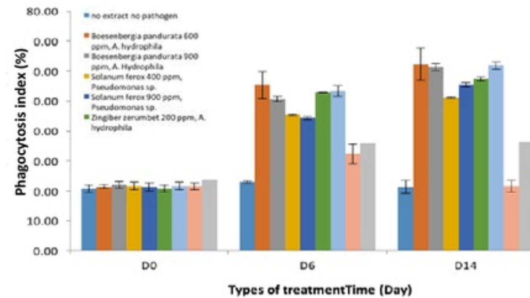

Figure 3. Effect of different dosages application of *B. pandurata, S. ferox* and *Z. zerumbet* extracts to phagocytic index of the Nile tilapia (*Oreochromis niloticus*)

In the fish treated with *Z. zerumbet*, there was no significant difference in the PI increase of the fish treated with 200 and 2000 ppm concentrations. Similarly, in response to *A. hydrophila* infection, there was no significant difference observed in the PI increase between the treatments of 200 and 2000 ppm *Z. zerumbet* concentrations; whereas the increase in PI was significantly noted between the fish treated with any of the two *Z. zerumbet* treatment concentrations and the fish group supplemented with no extract. These results indicated that *B. pandurata* and *Z. zerumbet* applications effectively increase phagocytosis of the fish, thus inhibiting the growth of *A. hydrophila* inside the fish.

On day 14, the phagocytosis index of the Nile tilapia supplemented with different dosages of each plant extract differed significantly from that of the fish receiving no extract supplementation ($P<0.05$). Meanwhile, such significant difference was not observed on day 0 and day 6.

Anatomic pathology

Fish infected with *A. hydrophila* and *Pseudomonas* sp. generally undergo changes in its external organs such as rotting fins, darkening of the skin, and exophthalmos. In this study, such changes in the external organs did not occur in the fish treated with different concentrations of each of plant extract. The only change observed was the darkening of the fish skin on day 14. The application of *B. pandurata* and *Z. zerumbet* extracts effectively prevented *A. hydrophila* infection from happening in the fish, which was indicated by the absence of the symptoms at day 14 after extract supplementation. Similarly, application of *S. ferox* for the prevention of *Pseudomonas* sp. infection (Table 2).

Change in swimming pattern

Fish infected with *A. hydrophila* and *Pseudomonas* sp. exhibited abnormal swimming patterns, including gasping, weakened swimming ability, and aggressive behavior towards touching. Interestingly, gasping and aggressive response were not observed in fish treated with the extracts and only a few of the fish showed a weakened swimming behavior and rested on the bottom of the aquarium (Table 2).

Table 2. Anatomic pathology and swimming pattern changes of the Nile tilapia after challenge test to *A. hydrophila* and *Pseudomonas* sp.

Treatment	Anatomic pathology			Swimming pattern changes		
	Fin rot	Skin darkening	Exo-phtalmos	Gasping	Weakened	Aggressive
No extract no pathogen	-	-	-	-	-	-
Boesenbergia pandurata 600 ppm, *A. hydrophila*	-	+	-	-	-	-
Boesenbergia pandurata 900 ppm, *A. hydrophila*	-	+	-	-	-	-
Solanum ferox 400 ppm, *Pseudomonas* sp.	-	+	-	-	+	-
Solanum ferox 900 ppm, *Pseudomonas* sp.	-	-	-	-	+	-
Zingiber zerumbet 200 ppm, *A. hydrophila*	-	+	-	-	-	-
Zingiber zerumbet 2000 ppm, *A. hydrophila*	-	+	-	-	-	-
No extract, *A. hydrophila*	++	++	++	++	+++	-
No extract, *Pseudomonas* sp.	++	++	+	+	++	+

Note: (-) normal, (+) low, (++) medium, (+++) high

Figure 4. Effect of different B. pandurata, *Z. zerumbet*, and S. ferox extracts dosages to the cumulative mortality percentage in the Nile tilapia (*Oreochromis niloticus*) prior to pathogenic bacterial infection

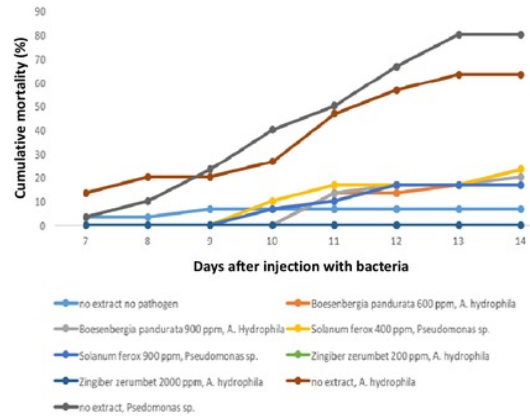

Figure 5. Effect of different *B. pandurata, Z. zerumbet*, and *S. ferox* extracts dosages to the cumulative death percentage in the Nile tilapia (*Oreochromis niloticus*) after the pathogenic bacterial infection

Cumulative mortality

On day 6 after the extract injections, just before the challenge test, 900 ppm *B. pandurata* extract treatment resulted in 30% fish death, whereas 2000 ppm *Z. zerumbet* extract treatment resulted in 25% mortality. Meanwhile, 400 ppm and 900 ppm *S. ferox* and 600 ppm *B. pandurata* only caused death as much as 5-10%. All of the extracts dosages were safe to be applied on the fish because the resulting mortality rate upon the extract application at those concentrations was < 30 % (Figure 4). Therefore, the experiment proceeded to the challenge test to *A. hydrophila* and *Pseudomonas* sp.

The mortality rate of the Nile tilapia supplemented with each of the three extracts in response to the challenge test to *A. hydrophila* and *Pseudomonas* sp. were lower than that of the fish without any extract supplementation treatment (Figure 5). In fact, there was no death in the fish treated with 200 and 2000 ppm *Z. zerumbet* extracts, which differed significantly from the fish without extract application (P<0.05). Similarly, the application of each of the two *B. pandurata* concentrations could suppress the death rate after the infection of *A. hydrophila* up to 20% (the death rate of the fish without extract application reached 60%). The death rate of the fish supplemented with 400 and 900 ppm *S. ferox* after *Pseudomonas* sp. injection was 10-15%, which was significantly different from the fish treated with no extract (P<0.05). The fish death rate after challenge test in all treatments was significantly different from that in no extract treatment. However, the cumulative fish mortality observed upon the treatment of each plant extract treatment was not significantly different (P<0.05). At day 14, the cumulative death observed upon *A. hydrophila* in the fish supplemented with 600 and 900 ppm *B. pandurata* concentrations was not significantly different. Likewise, that of the fish treated with 200 and 2000 ppm *Z. zerumbet* dosages was not significantly different as well (P<0.05). Thus, *B. pandurata* and *Z. zerumbet* extracts were protective agents that prevent *A. hydrophila* infection. The cumulative death occurred upon *Pseudomonas* sp. Infection in the fish treated with 400 and 900 ppm dosages of *S. ferox* was significantly different (P<0.05). In response to *Pseudomonas* sp. infection, the death rate of fish supplemented with *S. ferox* was higher than that of fish supplemented with other plant extracts.

Relative Percent Survival (RPS)

The protective property of each plant extract against pathogenic bacteria was good, which meat that there was no death observed in the fish supplemented with *Z. zerumbet* upon injection of *A. hydrophila*, *i.e.* 100% protection from *A. hydrophila*. The protective rate of *B. pandurata* and *S. ferox* were more than 80%. The increase in the nonspecific immunity enhancement of the fish supplemented with different dosages of each plant extract held an important role in reducing the mortality and protecting the tilapia fish after *A. hydrophila* and *Pseudomonas* sp. bacterial infections.

Discussion

Enhancement of fish immune system can be achieved by the administration of immunostimulants and vaccines. The mode of action of the two agents are different: an immunostimulant boosts the nonspecific immune system (Selvaraj 2005; Logambal et al. 2001), whereas a vaccine enhances the specific immune system (Pasnic et al. 2005). The nonspecific immune system of a fish consists of cellular and humoral components. The cellular component includes monocyte, lymphocyte, neutrophil, macrophage; while the humoral component includes lysozyme and complements (Secombes and Fletcher 1992; Magnadottir 2006). Phagocytosis in fish plays a pivotal role in

eliminating pathogens. The use of immunostimulant, antibacterial agent, and vaccine on fish is preferable to the use of chemically-synthesized medicines and antibiotics. The use of probiotic bacteria to suppress the growth of pathogenic bacteria (Hardi et al. 2016c) to control a particular disease is also preferred since the probiotic application is easy, economical, and effective. A vaccine only targets a specific type of bacteria (Hardi et al. 2013) and its application has to be repeated to boost its effect. Meanwhile, the use of antibiotics could lead to pathogen resistance and potentially harmed the environment (Kesarcodi-Watson et al. 2008; Nugroho and Fotedar 2013). Some plant extracts possess immunostimulant and antibacterial activities that can enhance the nonspecific immunity and thereby inhibits bacterial and virus growth. (Joseph and Carnahan 1994; Venkatalakshmi and Michael 2001; Hardi et al. 2016a, b; Saptiani et al. 2016a, b).

The total erythrocyte and leukocyte of the Nile tilapia exhibited an increase on 14 days after the extract injections compared with that of the fish supplemented with no extract in response to pathogenic bacterial infection. The increase in erythrocyte level was associated with the oxygen transportation and distribution throughout the fish body, while the increase in leukocytes was linked to phagocytosis and defense against the pathogen. Thus, the increase in total erythrocyte and total leukocyte will positively impact the fish's health. The Hb and He level of the tilapia fish supplemented with plant extracts upon challenge test to *A. hydrophila* and *Pseudomonas* sp. were slightly higher than the normal range. Meanwhile, the Hb and He level of the fish without any extract supplementation tended to be lower than the normal range. These results indicated that the application of *B. pandurata* and *Z. zerumbet* extracts increased the fish immunity to suppress the bacterial growth in order to prevent the total erythrocyte from decreasing. It is known that *A. hydrophila* and *Pseudomonas* sp. are gram-negative bacteria that produce hemolysin enzyme, an enzyme that digests the fish erythrocyte, thus causing reduced levels in the total leukocyte and Hb. (Harikrishnan et al. 2010; Hardi et al. 2014a, b, c).

In addition to increasing the total leukocytes, 600 and 900 ppm *B. pandurata* extract supplementation to the Nile tilapia also increased the fish phagocytic index at day 14 after extract administration. This finding, was in parallel with the growth inhibitory activity towards *A. hydrophila* in the fish (Hardi et al. 2016b) in which the protection rate was 83%. Likewise, supplementation of 200 and 2000 ppm *Z. zerumbet* extract increased the total leukocyte and PI, thereby allowing the fish to inhibit the bacterial growth, as indicated by the 100% protection rate after *A. hydrophila* infection. Fish treated with 900 ppm *S. ferox* showed a higher protection rate (83%) than those treated with 600 ppm dosage (67%). All the extract treatment concentrations were highly acceptable since the RPS value was above 60%.

Phagocytic cells play a pivotal role in the fish nonspecific immunity. Various phagocytic cells such as monocyte, neutrophil, and lymphocyte hold a similar function in engulfing and destroying the pathogen. Herbal plant extract can function as an immunostimulant in fish and has been shown to increase phagocytosis for example in golden fish (Jeney and Anderson 1993; Suhu 2004; Harikrishnan et al. 2009; Mastan 2015). Besides plant extracts, spirulina (Satyantini et al. 2014) supplementation can increase fish phagocytic index, thereby allowing the fish to eliminate the pathogen more effectively. Several plant extracts such as those from *Ocimum sanctum*, *Emblica officinalis*, *Cynodon dactylon*, and *Adhatoda vasica* exhibit immunostimulant activity in aquaculture and are able to suppress the bacterial infection in goldfish (Carassius auratus) (Selviraj et al. 2005). Similarly, Harikrishnan et al. (2009) showed that ethanol extract of *Azadirachta indica,*

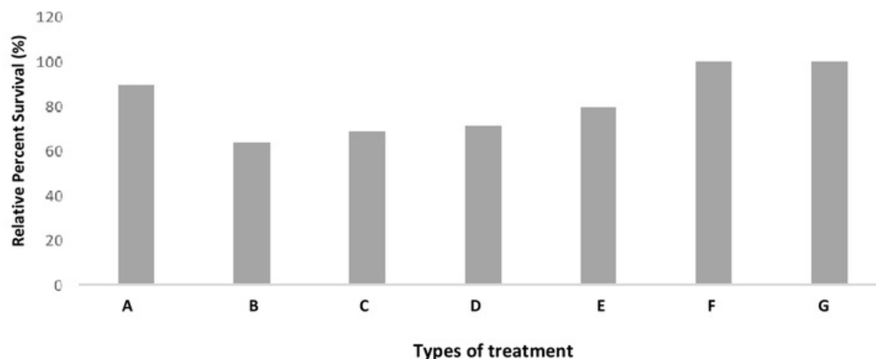

Figure 6. Effect of different dosages of B. pandurata, *Z. zerumbet*, and S. ferox extracts to the Relative Percent Survival (RPS) of the Nile tilapia (*Oreochromis niloticus*). Note: A. No extract no pathogen, B. *Boesenbergia pandurata* 600 ppm, *A. hydrophila,* C. *Boesenbergia pandurata* 900 ppm, *A. hydrophila,* D. *Solanum ferox* 400 ppm, *Pseudomonas* sp., E. *Solanum ferox* 900 ppm, *Pseudomonas* sp., F. *Zingiber zerumbet* 200 ppm, *A. hydrophila,* G. *Zingiber zerumbet* 2000 ppm, *A. hydrophila*

Ocimum sanctum and *Curcuma longa* supplemented with the fish feed in different concentrations of 5, 50 and 100 mg. kg^{-1}feed enhanced the phagocytic index of goldfish at week 2 after treatment. Meanwhile, in this study, all dosages of the plant ethanol-extracts were shown to boost the phagocytic index of the Nile Tilapia as early as 6 days after administration compared with the fish without any extract supplementation treatment. The fish phagocytic index will naturally increase upon any pathogenic infection. However, in the fish that receive no extract supplementation, such natural increase in phagocytosis was too low to effectively eliminate all the pathogen and to adequately support the fish survival after infection. In agreement with the high mortality rate (85.71 %) upon *A. hydrophila* injection, which was observed in the fish treated with no herbal plant extracts, Satyantini et al. (2004) suggested that the high phagocytic activity was in parallel with the ability to produce phagocytic cells in blood, thus upon the onset of pathogen infection, the cells were ready to undertake phagocytosis. An effective phagocytosis in the onset of a disease will prevent the disease from fully progressing.

After the challenge test to *A. hydrophila* and *Pseudomonas* sp., the tilapia receiving each the three plant extracts showed a suppressed mortality rate compared with that receiving no extract supplementation. This result was linked to an increase in the nonspecific immune system performance of the Nile tilapia, which was an important component of the fish immune system. This finding was in agreement with the previous report, in which the injection of *Ocimum sanctum* (Logambal et al. 2000) increase *Oreochromis mossambicus* resistance to *A. hydrophila*. Application of *Azadirachta indica*, *Ocimum sanctum* and *Curcuma longa* extracts (Harikrishnan et al. 2009) via injection in *Carassius auratus* enhanced protection against *A. hydrophila*. Similarly, Abutbul (2004) reported that supplementation of fish feed with *Rosmarinus officinalis* extract increased the fish survival after being infected with *A. hydrophila* and *Edwardsiella tarda* (Fujiki et al. 1994). Furthermore, application of *Achyranthes* extract also resulted in an increase in the resistance of *Labeo rohita* to the disease caused by *A. hydrophila* (Joseph and Carnahan 1994).

The survival rate of the tilapia supplemented with each of *B. pandurata* and *Z. zerumbet* extracts after *A. hydrophila* infection was higher than that of the fish supplemented with no extract and so was the survival rate of the fish treated with *S. ferox* extract upon *Pseudomonas* sp. infection. These results indicated that the ethanol extract from the three plants effectively increased the performance of the tilapia nonspecific immunity and was able to inhibit the growth of *A. hydrophila* and *Pseudomonas* sp. and increased the fish survival after infection.

ACKNOWLEDGEMENTS

This research was funded by the Indonesian Ministry of Research, Technology and Higher Education (RISTEK-DIKTI) through National Strategic Research 2015 and 2016 (No. 248/UN 17.16/PG/2015). The authors also wish to thank the Faculty of Fisheries and Marine Sciences, Universitas Mulawarman, Samarinda, East Kalimantan, Indonesia and the Department of Marine Affairs and Fisheries, Kutai Kartanegara District, East Kalimantan, Indonesia for their supports and assistance during this study.

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