PREVENTION METHODS AGAINST *Aeromonas hydrophila* and *Pseudomonas fluorescens* INFECTION IN TILAPIA

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ABSTRACT

This research evaluated a prevention method involving a concoction of *Boesenbergia pandurata, Solanum ferox* and *Zingiber zerumbet* extracts against pathogens in tilapia. The concentration of each extract was 600 ppm of *Boesenbergia pandurata* (BP), 900 ppm of *Solanum ferox* (SF) and 200 ppm of *Zingiber zerumbet* (ZZ). The investigation was performed by applying two combinations of extracts (SF:BP, SF:ZZ) against *Aeromonas hydrophila* and *Pseudomonas fluorescens* (10^5 cfu/mL) bacteria. The extract concoction was injected at 0.1 mL/fish through the peritoneum into the 15 ± 2 g tilapia, while the immersion method was performed by bathing the pathogen-challenged fishes with the extracts for 20 min. The extract composition were as follows; SF60:ZZ40; SF50:ZZ50; BP90:SF10; BP50:SF50; and fish without being given the extract. Hematology and immunological parameters were observed on the 4th week after injection with the pathogenic bacteria. The number of white blood cells (WBCs) significantly increased compared to controls without extract, with a similar increase observed for red blood cell (RBCs), but hematocrit (*Ht*) and hemoglobin (Hb) values did not significantly increase compared to control. Phagocytic index, respiratory burst and lysozyme activities also significantly increased among fishes fed with the combined extracts as compared to controls. The number of pathogenic bacteria in the body of the fishes given the extracts were also lower than the control and significantly different on the 4th week. The combined extract of SF50: ZZ50 and BP90: SF10 provided the best protection as indicated by the relative percent survival (RPS) of 100% for fishes being challenged with *A. hydrophila* and *P. fluorescens*. However, the combined extracts of SF50:ZZ50 administered by injection and immersion positively affected or increased the non-specific immune system of tilapia and increased its protection against bacterial infections.

Keywords: *Aeromonas hydrophila*, concoction, immunomodulator, *Pseudomonas fluorescens*

INTRODUCTION

The rapidly increasing global fish farming has resulted in increasing biomass production, species diversification, geographical expansion and methods enhancement to fulfill the protein needs of fish. This increase has continually been challenged with the associated diseases and health problems of aquacultural animals. Other triggering factors are the climate change and the development of aquaculture technologies affecting the balance interaction between pathogens, hosts and the environment. Almost annually, new aquacultural pathogens are being isolated and many novel diseases are continually identified in various areas of cultivation (Rodger 2018).

Aeromonas hydrophila and *Pseudommonas fluorescens* are two pathogenic bacteria occurring throughout the year, causing a mortality rate of 60 - 80% among aquacultured organisms (Hardi *et al.* 2012; 2016; 2017). *Aeromonas* has led to destruction and losses in the aquaculture industry worldwide (Monette *et al.* 2006). Fishes infected with these bacteria include tilapia *Oreochromis niloticus* (Hardi *et al.* 2012, Janda & Abbott 2010), *Cyprinus carpio* (Sioutas *et al.* 1991, Monette *et al.* 2006), *Clarias gariepinus* (Chowdhury 1998) and Indian major carps (Karunasagar *et al.* 1991). Typically, combined

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bacterial infections are naturally found with heavier symptoms than in single bacterial infections. Combined infection of *A. hydrophila* and *P. fluorescens* leads to stressed fish, exoptalmia, ulcers and watery otgans in the bile gland rupture. Likewise, combined infections of *Streptococcus agalactiae* and *A. hydrophila* cause tilapia and goldfish to die more rapidly than single bacterial infections (Sugiani *et al.* 2012; Sumiati *et al.* 2015).

Many of these fish diseases or pathogens have no suitable preventative or treatmnent options yet, i.e., the use of vaccines, immunostimulants, antibacterials and environmental management to minimize epidemics. The fish vaccines are particularly varied for freshwater fish because many strains infect these bacteria. The availability of commercial immunostimulants derived from natural ingredients is still limited due to the low level of immunomodulatory components contained in natural compounds (Pridgeon & Klesius 2012). Some beneficial immunomodulatory components in plants include levamisole and saponins for fish (Findlay & Munday 2000). These components were able to increase the non-specific immune systems activity, particularly the phagocytosis activation of leucocyte and WBC (Bricknell *et al.* 2005). The single extract of *B. pandurata* and *Z. zerumbet* plants from East Kalimantan have antibacterial activity *in vitro* and *in vivo* against *A. hydrophila* bacteria, while a single extract of *S. ferox* effectively inhibits *P. fluorescens* infection (Hardi *et al.* 2016a; 2016b). The extract was used for prevention and treatment of infections caused by both bacteria in tilapia (Hardi *et al.* 2017; 2018b).

To increase the immunomodulatory activities of plant extracts, several extracts were applied at one time as a concoction. A combination of *Curcuma longa*, *Ocimum sanctum* and *Azadiruchta indica* extracts at a ratio of 1:1:1 more effectively inhibits *A. hydrophila* bacteria *in vitro* compared to a single extract of each plant (Harikrishnan & Balasundaram 2008); the combined treatment of three extracts has increased the survival rate of goldfish and the inhibitory process due to infection by *A. hydrophila* bacteria in goldfish (*Carassius auratus*) (Harikrishnan *et al.* 2009). A concoction of *Boesenbergia pandurata*, *Solanum ferox* and *Zingiber zerumbet* extract at a ratio 1:1:1 had an immunomodulatory effect in tilapia and had increased protection and diseases recovery from *A. hydrophila* and *Pseudomonas fluorescens* better than single extract (Hardi *et al.* 2019a). *B. pandurata* and *Z. zerumbet* extracts contains alkaloids, flavonoids, carbohydrates, and steroids (Hardi *et al.* 2016a), while the *S. ferox* extract has higher levels of alkaloids that play an important role as an antibacterial substance (Hardi *et al.* 2016a; Huang *et al.* 2008).

A concoction of three extracts of *B. pandurata*, *Z. zerumbet* and *S. ferox* had *in vitro* antibacterial activity against *A. hydrophila* and *P. fluorescens* both in single and combined use (Hardi *et al.* 2018a; 2018b). This paper discussed the effectiveness of these three extracts to prevent infection from *A. hydrophila* and *P. fluorescens* bacteria in tilapia using injection and immersion methods.

MATERIALS AND METHODS

Fish and Bacteria

Tilapia weighing 15 ± 2 g, were taken from the village of Teluk Dalam, Tenggarong Seberang Kutai Kartanegara. The fishes had been in the laboratory aquarium for two weeks prior to use. The 60 x 40 x 30 cm treatmnent aquarium was filled wth 60 L of water, 50% of which was changed every two days to remove the remaining fish feces and feeds.

The bacteria used for the challenge trial, a combination of *A. hydrophila* (EA-01) and *P. fluorescens* (EP-01) at a ratio 1 : 1, were taken from the Aquatic Microbiology Laboratory, Faculty of Fisheries and Marine Sciences, Mulawarman University, Indonesia. Bacterial density was at 10^5 cfu/mL of each bacteria, with 1 mL/fish being injected intramuscularly.

Extract Preparation for *Boesenbergia pandurata*, *Zingiber zerumbet* and *Solanum ferox*

The plant rhizomes of *B. pandurata* and *Z. zerumbet*, and fruits of *S. ferox* were used in the study. All of these were collected from traditional markets in Samarinda City, East Kalimantan. Plants were cleaned, cut into slices, dried in an oven at 40 °C for 48 hours, blended in powder form and refrigrated at -4 °C while

the extraction stage continued. The extraction method used ethanol solution following Limsuwan & Voravuthikunchai (2008). The concentrations of each extract were *B. pandurata* 600 mg/L, *Z. Zerumbet* 200 mg/L, and *S. ferox* 900 mg/L . Combined effects of *Z. zerumbet* and *S. ferox* extracts with ratio of 40 : 60 mL and 50 : 50 mL and of *B. pandurata* and *S. ferox* at 90 : 10 mL and 50 : 50 mL were compared.

Combination of Extracts

The extract was administered to tilapia by the injection and immersion methods to avoid bacterial infection of *A. hydrophila* and *P. fluorescens*. A preventive experiment was performed through Intra Peritional (IP) injection of combined extracts at a rate of 0.1 mL/fish. On the 8th day, the fishes were challenged with pathogenic bacteria. To protect them from pathogenic bacteria, the fishes were immersed for 20 min with a combination of extracts, and challenged with the combined bacteria through Intra Muscular (IM) injection on the 8th day. The experiment was carried out every week after injecting with bacteria until the 4th week. In addition, the applied research treatment comprised nine groups:

Group 1 = IP injected fish with combined 60 mL of *S. ferox* extract, 40 mL of *Z. zerumbet* extract (SF 60 : ZZ 40) and challenge through IM injection with combination of pathogen bacteria.

Group 2 = IP injected fish with combined 50 mL of *S. ferox* extract and 50 mL of *Z. zerumbet* extract (SF 50 : ZZ 50) and challenged through IM injection with combination of pathogenic bacteria.

Group 3 = IP injected fish with combined 90 mL of *B. pandurata* extract and 10 mL of *S. ferox* (BP 90 : SF 10) and challenged through IM injection with a combination of pathogenic bacteria.

Group 4 = IP injected fish with combined 50 mL of *B. pandurata* extract and 50 mL of *S. ferox* extract (BP 50 : SF 50) challenged through IM injection with a combination of pathogenic bacteria.

Group 5 = Fish immersed with combined 60 mL of *S. ferox* extract and 40 mL of *Z. zerumbet* extract (SF 60 : ZZ 40) challenged through IM injection with a combination of pathogenic bacteria.

Group 6 = Fish immersed with combined 50 mL of *S. ferox* extract and 50 mL of *Z. zerumbet* extract (SF 50 : ZZ 50) challenged through IM injection with a combination of pathogenic bacteria.

Group 7 = Fish immersed with combined 90 mL of *B. pandurata* extract and 10 mL of *S. ferox* extract (BP 90 : SF 10) challenged through IM injection with a combination of pathogenic bacteria.

Group 8 = Fish immersed with combined 50 mL of *B. pandurata* extract and 50 mL of *S. ferox* extract (BP 50 : SF 50) challenge via IM injection with a combination of pathogenic bacteria.

Group 9 = IP injected fish with PBS (Phosphate Buffer Saline) sterile and challenge through IM injection with the combined pathogen bacteria.

Group 10 = Fish immersed with PBS sterile and challenge via IM injection with the combined pathogenic bacteria.

To evaluate the effective prevention method, every group used 10 fishes from every aquarium and three replications, totalling to 30 fishes per group. A total of 300 tilapia (*Oreochromis niloticus*) tilapia were used to evaluate the effect of extract using the IP and immersion methods in non-specific immunity, survival rate (SR), and Relative Percent Survival (RPS).

Hematological Examination

After introducing the pathogenic bacteria, hematological observations were obtained weekly for one month. Before blood was taken, the fish were anesthetized using 50 mg/dm^3 MS-222, and blood was obtained through the base of the fish, with 1 mL of injection syringe being washed with anticoagulants (10% tri sodium citrate). Red Blood Cells (RBC) and White

Blood Cells (WBC) parameters were observed using a Neubauer hemacytometer. Observation of RBC begins by adding blood samples with Hayem's solvent and adding Turk's solvent for the observation of WBC. Hemoglobin examination involved the use of a sahli tube. Hematocrit (Ht%) was measured using the microcentrifuge method, and the standard solvent usedwas tri sodium citrate. The blood inserted into the micro hematocrit tube was centrifuged at 5,000 rpm for 10 min. Hematocrit is estimated by calculating the ratio of the column of packed erythrocytes to the total length of the sample in the capillary tube, and measured with a graphic reading device (Blaxhall & Daisley Methods 1973).

Phagocytic index

Fifty µL of blood was transferred into an Eppendorf tube containing 50 µL mixture of *A. hydrophila* and *P. fluorescens* suspension at a bacterial density of 10^5 cfu/mL), and then left for 20 min. The screw was prepared on a glass object and dried, fixed with alcohol (95%) for five minutes, then dried again. The screw was then dyed by soaking it in Giemsa dye (10%) for 15 minutes, washed with flowing water and dried. The screw preparations were then observed and the number of phagocytic cells demonstrating phagocytic processes were counted (total number of engulfed cells/total number of counted macrophages) × (number of macrophages containing engulfed cells/total number of counted macrophages) × 100, following the method of Anderson and Siwicki (1995). This parameter was observed on the 4th week after chalanges with *A. hydrophila* and *P. flourescens.*

Respiratory burst

The respiratory burst activity was tested using the nitro blue tetrazolium (NBT) reagent. The 50 µL blood derived from fish was transferred to a microplate, incubated for one hour at 37 °C. After the supernatant was removed, the cells were washed with 50 µL of PBS three times, added with 50 µL of 0.2% NBT and incubated for one hour at 37 °C. Plates were fixed with 100% methanol (50 µL) for 2 - 3 minutes, then rinsed with 30% methanol (50 µL) three times and air-dried. Then, 60 µL of KOH and 70 µL of DMSO were added, with the optical density then checked using an ELISA Reader at a wavelength of 540 nm. These paramethers were analyzed following the Secombes and Fletcher (1992) method. Like an index phagocity, this parameter was observed on the 4th week after challenges (immersion/IM) with *A. hydrophila* and *P. flourescens.*

Lysozyme activity

Moistened injection syringes with anticoagulants were prepared, while the blood samples of the fishes from the caudal vein were taken. Blood was stored at room temperatue for two hours and then maintained at 4 °C for 24 hours. Blood was centrifuged at 5,000 rpm for three minutes, then the separated clear liquid (serum) was removed. The lysozyme test for activity was performed based on the Lygren *et al.* (1999) method i.e., 10 µL of serum sample was placed into a micro titer plate and then 190 µL of lysodeikticus Micrococcus suspension was added (Sigma Aldrich Chemical) with 0.2 mg of lysodeikticus Micrococcus/mL PBS at pH 7.4, and shaken slowly at constant room temperature. After 90 minutes of incubation, a micro titer ELISA plate reader was used to take readings at a wavelength of 520 nm (Lie *et al.* 1989).

Relative lysozyme activities (units) were calculated as follows: 1 Unit = 0.001 decrease in absorbance/min. If the calculation of lysozyme activity is absolutely necessary, a standard solution of chicken egg white with several concentrations can be used in order to ensure that the standard measurement procedure curve is the same. The paramethers were analyzed according to Lygren *et al.* (1999) and observed on the 4th week after chalanges (immersion/IM) with *A. hydrophila* and *P. flourescens.*

Total Bacteria in Fish Bodies using Total Plate Count (TPC)

The total *A. hydrophila* and *P. fluorescens* bacteria in the fish body was calculated to determine the antibacterial activity due to injection with the combined extracts. The measurement of total bacteria using the TPC method was performed by counting the number of bacterial colonies in the fish's organs using 10^{-2} to 10^{-6} dilutions. The initial bacterial

concentration was calculated using plates containing 30 - 300 colonies.

As the first step, petri dishes, test tubes and pipettes were sterilized using dried sterilization at 180 °C for two hours prior to use. Plate Count Agar (PCA) solid media were used as the growth substrate, wet-sterilized in an autoclave at 121 °C, 1 atm for 15 min. Samples of ten grams of fish (thymus, kidney, spleen, and liver), were mashed first, then dissolved in 100 mL of sterile diluent solvent to obtain a 10^{-1} dilution. One mL was then taken, and put in sample tubes containing 9 mL of sterile distilled water (10^{-2}) until a dilution of 10^{-6} was achieved. A total of 1 mL of each tube was transferred into a sterile petri dish and approximately 15 mL of PCA media was poured evenly; the petri dish was incubated upside down for 48 hours at 30 °C and growing colonies then counted, based on Mailoa *et al.* (2017) method. At the end of the incubation period, all the petri plates containing between 30 and 300 colonies were selected. Plates with more than 300 colonies cannot be counted and are designated as Too Many To Count (TMTC). Plates with fewer than 30 colonies are designated as Too Few To Count (TFTC). The colonies on each plate were counted using a quebec colony counter. This parameter was observed on the 4th week after immersion with *A. hydrophila* and *P. flourescens*.

$$\text{Number of colonies (CFU)/mL} = \frac{\Sigma \text{Coloni of bacteria}}{\textit{Dillution X amount plated}}$$

Protection Level against Pathogens

To determine the effectiveness of combined extracts in prohibiting *A. hydrophila* and *P. fluorescens* infection, the challenge-tested fishes were counted with respect to survival rate (SR) and the Relative Percent Survival (RPS) calculated on the 4th week after IM infection using the Amend (1981) and Ellis formula (1988), as follows:

$$SR = \frac{(\textit{alive fish at the end of the treatment})}{(\textit{alive fish at the beginning of the treatment})} \text{ X } 100$$

$$RPS = \frac{(\textit{percent mortality in treated group})}{(\textit{percennt mortality in control group})} \text{ X } 100$$

Statistical Data Analysis

To determine the effect of extract treatment on the observed parameters, the data were analyzed statistically using SPPS 16.0.

RESULTS AND DISCUSSION

Hematology

Under the immersion method, the total tilapia WBCs of Group 5 to Group 8 significantly increased starting on the 2nd week until the 4th week after administration of the combined extract, as compared to control (Group 10) without the extract (Table 1). The highest increase was experienced by tilapia given the combined extract of *B. pandurata* and *S. ferox* at a ratio of 50 : 50 (Group 6) from the 2nd week to the 4th week after bacterial infection by immersion/IM. The total RBC counts for Group 5 - Group 8 also significantly increased as compared with control fish since the second week of treatment, while hematocrit levels of Group 5 to Group 8, significantly increased in weeks 3 and 4. Post-treatment levels of tilapia hemoglobin in Group 5 to Group 8 increased but not significantly different from control/ Group 10.

Preventive test through the injection (IP) method (Table 2) showed that WBC had the highest increase in tilapia using the treatment ratio of SF 50 : ZZ 50 (Group 2). The WBC increased in Group 1 to Group 4 with IP treatment significantly differed from the control/Group 9 ($p < 0.05$) from 1st to 4th week of observations. Similarly, the RBC and hematocrit for fishes given the extracts significantly differed from control. Only the fish hemoglobin in Group 1 - Group 4 did not significantly differ from the fish control group as their preventive response against bacterial infections via the IP method.

Table 1 Hematology of tilapia appplied wth a combination of extracts against *A. hydrophila* and *P. fluorescens* bacterial infection through immersion methods

Variable	Groups	Extracts	Weeks			
			1	2	3	4
WBC (10^4 cell/mm^3)	5	SF 60 : ZZ 40	1.5 ± 0.1^a	1.4 ± 0.1^a	1.6 ± 0.1^a	2.0 ± 0.2^b
	6	SF 50 : ZZ 50	2.0 ± 0.2^b	3.2 ± 0.1^b	3.8 ± 0.2^c	7.6 ± 0.2^d
	7	BP 90 : SF 10	1.7 ± 0.2^b	1.7 ± 0.5^b	2.0 ± 0.1^b	2.0 ± 0.1^b
	8	BP 50 : SF 50	1.9 ± 0.5^b	1.7 ± 0.5^b	1.8 ± 0.2^b	2.0 ± 0.1^b
	10	No extract	1.3 ± 0.3^a	1.3 ± 0.2^a	1.3 ± 0.2^a	1.3 ± 0.1^a
RBC (10^6 cell/mm^3)	5	SF 60 : ZZ 40	5.9 ± 0.1^b	$5.0 \pm 0.b$	4.0 ± 0.2	5.3 ± 0.1
	6	SF 50 : ZZ 50	5.9 ± 0.1^b	7.0 ± 0.2^c	7.8 ± 0.1^c	6.9 ± 0.2^c
	7	BP 90 : SF 10	5.1 ± 0.2^b	6.0 ± 0.1^c	6.2 ± 0.1^c	4.4 ± 0.2^b
	8	BP 50 : SF 50	5.2 ± 0.1^b	6.0 ± 0.2^c	6.0 ± 0.1^c	6.7 ± 0.1^c
	10	No extract	2.0 ± 0.3^a	2.0 ± 0.1^a	2.7 ± 0.2^a	2.4 ± 0.1^a
Hematokrit (%)	5	SF 60 : ZZ 40	20 ± 0.1^a	23 ± 0.1^a	27 ± 0.1^b	30 ± 0.1^b
	6	SF 50 : ZZ 50	20.5 ± 0.5^a	23 ± 0.2^a	28 ± 0.1^b	30 ± 0.2^b
	7	BP 90 : SF 10	22.5 ± 0.5^a	23 ± 0.2^a	30 ± 0.1^b	31 ± 0.2^b
	8	BP 50 : SF 50	25 ± 0.2^a	23 ± 0.2^a	27 ± 0.2^b	30 ± 0.2^b
	10	No extract	20 ± 0.2^a	15 ± 0.3^a	$18 \pm 0.1a$	15 ± 0.2^a
Hemoglobin (g%)	5	SF 60 : ZZ 40	8 ± 0.1^a	8 ± 0.3^a	8 ± 0.1^a	8 ± 0.1^a
	6	SF 50 : ZZ 50	10 ± 0.2^a	8 ± 0.3^a	10 ± 0.2^a	8 ± 0.1^a
	7	BP 90 : SF 10	8 ± 0.11^a	8 ± 0.2^a	10 ± 0.2^a	10 ± 0.1^a
	8	BP 50 : SF 50	8 ± 0.1^a	8 ± 0.2^a	8 ± 0.2^a	10 ± 0.1^a
	10	No extract	6.3 ± 0.5^a	8 ± 0.1^a	6 ± 0.2^a	6 ± 0.2^a

Note: Means (± SD) with different superscripts in a row show significant differences at $p < 0.05$.

Table 2 Hematology of tilapia applied with the combined extract against *A. hydrophila* and *P. fluorescens* bacterial infection through the IP method

Variable	Groups	Extracts	Weeks			
			1	2	3	4
WBC (10^4 cell/mm^3)	1	SF 60 : ZZ 40	1.7 ± 0.5^a	2.2 ± 0.15^a	1.8 ± 0.2^a	1.8 ± 0.5^a
	2	SF 50 : ZZ 50	3.4 ± 0.3^b	4.3 ± 0.2^b	4.0 ± 0.1^b	4.9 ± 0.2^c
	3	BP 90 : SF 10	2.0 ± 0.15^a	2.7 ± 0.2^a	2.4 ± 0.1^a	2.4 ± 0.3^a
	4	BP 50 : SF 50	2.4 ± 0.25^a	2.8 ± 0.3^a	2.0 ± 0.2^a	2.5 ± 0.1^a
	9	No extract	$1.5 \pm 0.1a$	1.3 ± 0.5^a	1.3 ± 0.3^a	1.3 ± 0.1^a
RBC (10^6 cell/mm^3)	1	SF 60 : ZZ 40	7.4 ± 0.15^d	6.8 ± 0.25^c	5.9 ± 0.2^c	6.0 ± 0.5^c
	2	SF 50 : ZZ 50	7.9 ± 0.2^d	7.7 ± 0.3^d	6.0 ± 0.1^c	6.0 ± 0.2^c
	3	BP 90 : SF 10	5.5 ± 0.1^b	6.6 ± 0.1^c	5.0 ± 0.1^b	5.4 ± 0.2^b
	4	BP 50 : SF 50	5.8 ± 0.1^b	5.8 ± 0.1^b	7.7 ± 0.1^c	7.0 ± 0.1^c
	9	No extract	2.4 ± 0.2^a	2.6 ± 0.2^a	2.7 ± 0.2^a	2.4 ± 0.1^a
Hematokrit (%)	1	SF 60 : ZZ 40	31 ± 0.5^b	25 ± 0.1^a	22 ± 0.1^a	22 ± 0.1^a
	2	SF 50 : ZZ 50	22.2 ± 0.15^a	25 ± 0.2^a	20 ± 0.2^a	22 ± 0.1^a
	3	BP 90 : SF 10	25 ± 0.1^a	25 ± 0.1^a	30 ± 0.2^a	21.5 ± 0.1^a
	4	BP 50 : SF 50	25 ± 0.2^a	25 ± 0.1^a	25 ± 0.1^a	21 ± 0.2^a
	9	No extract	20 ± 0.1^a	15 ± 0.2^a	14 ± 0.1^a	15 ± 0.1^a
Hemaglobin (g%)	1	SF 60 : ZZ 40	10 ± 0.2^a	10 ± 0.1^a	8 ± 0.2^a	9 ± 0.2^a
	2	SF 50 : ZZ 50	10 ± 0.2^a	10 ± 0.1^a	8 ± 0.2^a	9 ± 0.1^a
	3	BP 90 : SF 10	10 ± 0.2^a	10 ± 0.1^a	8 ± 0.1^a	8 ± 0.1^a
	4	BP 50 : SF 50	10 ± 0.2^a	10 ± 0.1^a	8 ± 0.1^a	8 ± 0.2^a
	9	No extract	6.3 ± 0.2^a	7 ± 0.1^a	5 ± 0.1^a	4 ± 0.1^a

Note: Means (±SD) with different superscripts in a row show significant differences at $p < 0.05$.

Phagocytic index

The effect of the entire treatment of the combined extract increased and significantly differed from the control ($p < 0.05$) in phagocytic index parameter. All groups with the combined extract (Group 1 - Group 8) showed an increased in phagocytic index and were significantly higher than the controls in the 4th week of the challenge test (Fig. 1).

The fishes injected with the combined extract of SF 50 : ZZ 50 (Group 2) showed the highest phagocytic index improvement as compared with the other treatments on the 4th week after the challenge test and were significantly different from the controls/Group 9. Likewise, with the immersion method, prevention from *A. hydrophila* and *P. fluorescens* bacterial infections using the combined extract of SF 50 : ZZ 50 (Group 6) showed the highest increase of phagocytic index in the 4th week of the challenge test.

Respiratory burst

Given the combined extracts, the respiratory burst activity of tilapia generally increased during the 4th week either through injection or immersion methods (Fig. 2). A significant increase occured between the control group and those given the extracts with different combinations. However, only the ratio of SF 50 : ZZ 50 by IP method (Group 2) significantly differed ($p < 0.05$) among the combination of extracts (Group 9).

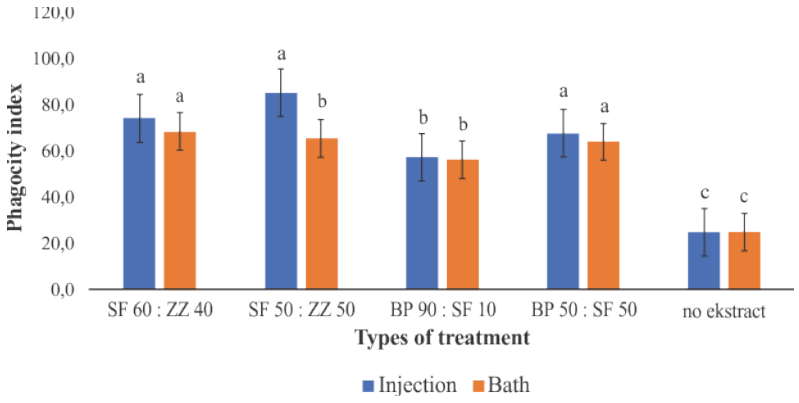

Figure 1 Phagocytic index of tilapia applied with the combined extract against *A. hydrophila* and *P. fluorescens* bacterial infection through injection and immersion methods
Note: Different superscripts on the same row indicate significant differences at $p < 0.05$.

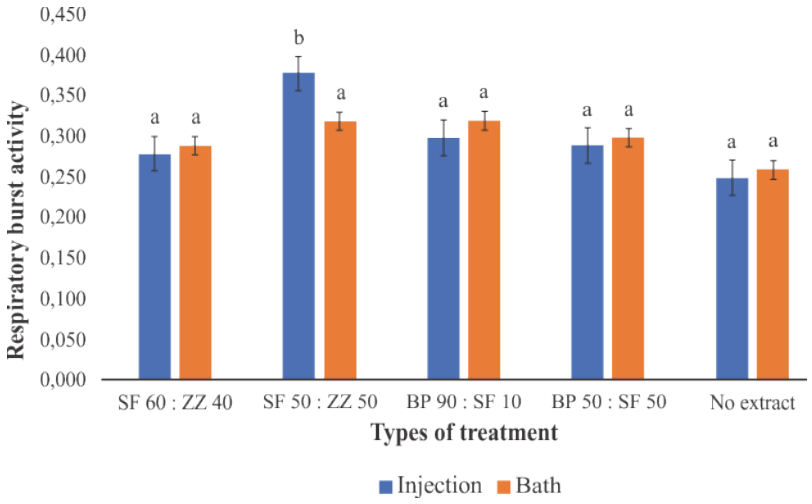

Figure 2 Respiratory burst activity of tilapia applied with the combined extracts against *A. hydrophila* and *P. fluorescens* bacterial infection through injection and immersion method

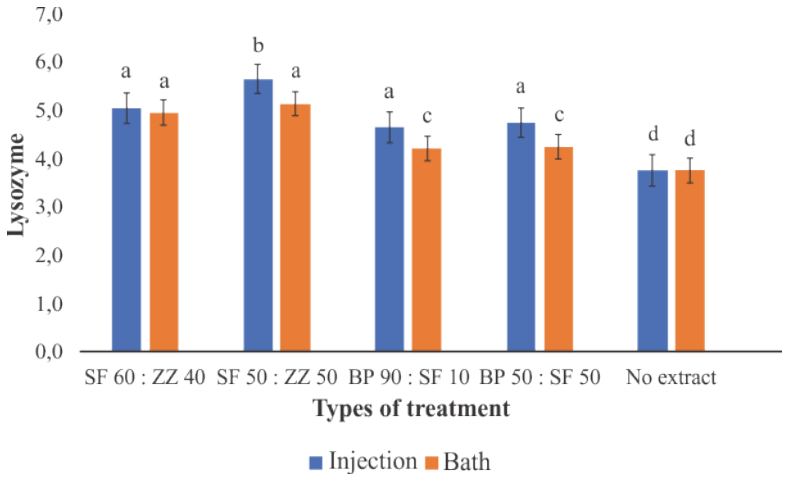

Figure 3 Lysozyme activity of tilapia applied with the combined extract against *A. hydrophila* and *P. fluorescens* bacterial infection through injection and immersion methods

Lysozyme activity

The activity of tilapia lysozyme given all the extract combinations (Group 1 - Group 8) also increased, and significantly differed from the control (Group 9 and Group 10) without extract in the 4th week of the challenge test. However, only Group 2 significantly differed from the other concoction extract (Fig. 3).

The total *A. hydrophila* and *P. fluorescens* bacteria in the body of tilapia treated through injection (IP) and immersion methods were lower than controls (Group 9 and Group 10) or without extracts at the 4th week of the challenge test (IM) with pathogenic bacteria (Table 3). The lowest bacterial density in tilapia was found in those fishes given a combination extract of SF 50 : ZZ 50 (Group 2) and significantly differed from control with no extract (Group 9) as well as for the combined extract of BP 90 : SF 10 (Group 3), in which the total value of bacteria was lower than the control (Group 9) and was significantly different. All the extract concoctions (Group 1 - Group 8) caused a decrease in total bacteria in the tilapia on the 4th week with *A. hydrophila* and *P. fluorescens* (IM). The decrease of total bacteria was significantly different from the control (Group 9 and Group 10). The concoction SF 50 : ZZ 50 and BP 90 : SF 10 (Group 2 and Group 3) administered by injection (IP) had significantly supress the bacterial growth in the fish body. However, only the concoction SF 50 : ZZ 50 (Group 6) administered by immersion significantly differed from the other concoction and the control/ Group 10 ($p < 0.05$).

Table 3 Total Plate Count (TPC) of tilapia applied with the combined extracts against *A. hydrophila* and *P. fluorescens* bacterial infection through injection and immersion methods

Groups	Extracts	Total bacteria (10^5 cfu/mL)
1	IP SF 60 : ZZ 40	120 ± 10^{c}
2	IP SF 50 : ZZ 50	32 ± 5^{b}
3	IP BP 90 : SF 10	55 ± 7^{b}
4	IP BP 50 : SF 50	117 ± 11^{c}
5	Immersion SF 60 : ZZ 40	140 ± 9^{c}
6	Immersion SF 50 : ZZ 50	45 ± 5^{b}
7	Immersion BP 90 : SF 10	98 ± 8^{c}
8	Immersion BP 50 : SF 50	110 ± 10^{c}
9	IP control	257 ± 11^{a}
10	Immersion control	300 ± 11^{a}

Note: Means (±SD) having different superscripts on the same row indicate significant differences at $p < 0.05$.

Survival rate (SR) and relative percent survival (RPS) of tilapia applied with the combined extracts

The highest percentage of survival rate (SR) and relative percent survival (RPS) of tilapia applied with preventive methods against *A. hydrophila* and *P. fluorescens* were found at week 4 on tilapia that had been injected with extracts SF 50 : ZZ 50 (Group 2) and BP 90 : SF 10 (Group 3). Meanwhile, the average SR of tilapia that were given the extract concoction was generally higher than the controls/Group 9 and Group 10 (Table 4). The best SR and RPS of tilapia using immersion method was in extracts SF 50 : ZZ 50 (Group 6) and were higher than other concoctions (Groups 5, 7, 8) but, Groups 5, 7, and 8 had significantly higher SR than the control (Group 10) at $p < 0.05$.

RPS in all groups administered with the concoction extracts were more than 65 %, except in Group 7 (Immersion BP 90 : SF 10) that has the lowest RPS (58%). RPS of more than 60% showed that the vaccine or immunostimulant was effective protection against bacteria infection (Hardi *et al.* 2018a, Ellis 1988 and Osman *et al.* 2009).

The use of immunostimulants and antibacterials derived from plant extracts has been previously carried out for fish and shrimp cultures for *Aeromonas salmonicida, A. hydrophila, Vibrio anguillarum, V. vulnificus, V. salmonicida, Yersinia ruckeri* and *Streptococcus* spp. (Barman *et al.* 2013). To prevent pathogenic infections, immunostimulants are additional ingredients given to organisms and are able to increase the innate (non-specific) immune system (Sakai 1999; Findly & Munday 2002). Cells playing important roles in the non-specific immune system are WBCs and their activity is influenced by fish species, nutrition and the environment (Harrikrishnan *et al.* 2003; Mastan 2015). The increase in WBCs of tilapia aplied with the concoction extract (Group 1 - 8) was higher than the control without extract (Group 9 and 10), and the survival rate of tilapia infected with *A. hydrophila* and *P. fluorescens* has reached 100% under the combined SF 50 : ZZ 50 and BP 90 : SF 10 through injection (Group 2 and Group 3). The immersion method resulted in 58 - 72% RPS? and the best RPS (72%) was in Group 6 (immersion SF 50 : ZZ 50).

The combined extracts had improved the non-spatial performance of the fish immune system by producing more fish WBCs which subsequently inhibit the bacterial growth in the body. The different methods of administration (IP and immersion) resulted in different performances of the immune system. In another study, the addition of combined extract into feed had positively affected the tilapia's immune system and the SF50/ZZ50 combination had improved the innate immune system of tilapia against bacterial infections (Hardi *et al.* 2019b).

The administration of extract can be done via injection, bathing or oral administration, the latter seems to be the most practicable in fish (Yin *et al.* 2006, Jeney & Anderson 1993, and Mulero 1998). The injection and immersion methods have different advantages and disadvantages (Evensen 2016). The injection method are most potent, has little waste of immonostimulant and are cost-effective for high-value species. The immersion method is applicable on a large-scale possible, applies

Table 4 Survival rates (SR) and Relative Percent Survival (RPS) of tilapia applied with combined extracts against *A. hydrophila* and *P. fluorescens* bacterial infection through injection and immersion method

Groups	Extracts	SR	RPS
1	IP SF 60 : ZZ 40	88 ± 10^{b}	83 ± 10^{b}
2	IP SF 50 : ZZ 50	100 ± 10^{c}	100 ± 10^{c}
3	IP BP 90 : SF 10	100 ± 10^{c}	100 ± 10^{c}
4	IP BP 50 : SF 50	85 ± 10^{b}	79 ± 10^{b}
5	Immersion SF 60 : ZZ 40	75 ± 10^{b}	65 ± 10^{b}
6	Immersion SF 50 : ZZ 50	80 ± 10^{b}	72 ± 10^{b}
7	Immersion BP 90 : SF 10	70 ± 10^{b}	58 ± 10^{b}
8	Immersion BP 50 : SF 50	75 ± 10^{b}	65 ± 10^{b}
9	IP Control	29 ± 10^{a}	
10	Immersion Control	29 ± 10^{a}	

Note: Means (±SD) having different superscripts on the same row indicate significant differences at $p < 0.05$.

moderate stress to the fish, allows mass vaccination or immunostimulant of immunocompetent fishes. However, immersion method needs a large amount of immunostimulant and can be cost prohibitive, has low to moderate efficacy and inferior to injection method in terms of efficacy as it is cost prohibitive for large fish. Research shows that the injection method can increase RPS rather than immersion. This is due to immunostimulant delivery in the body of the fish. The injection method applies the immunostimulant directly into the blood, while in the immersion method, the immonostimulant must first penetrate the fish skin, so that more time is needed to improve the immune system (Midtlyng 2006).

The efficiency of immunostimulant admistration in fishes, is indicated by RPS, following Amend (1981) recommendations, the proposed acceptance criteria for immonostimulant potency in fish standardised RPS of 60% or above. In this research, All group applied with combined extracts had RPS above 60% except group 7 (Immersion with combination extract BP 90 : SF 10) was 58%.

The total RBCs of Tilapia in the preventive trial was significantly higher than the control or without extract at $p < 0.05$. Both *A. hydrophila* and *P. fluorescens* bacteria produce hemolysin protein which can lysis the RBCs, the number of which was therefore decreased in infected fish (Hardi *et al.* 2013). This decrease also occured in tilapia infected with *S. agalactiae* (Hardi *et al.* 2011), with *S. iniae* (Sugiani *et al.* 2012), with *A. hydrophila* (Dosim *et al.* 2006) and with *Pseudomonas* sp. The tilapia injected with extracellular and intracellular proteins from *A. hydrophila* (Hardi *et al.* 2013) and *Pseudomonas* sp. had degenerated and experienced necrosis and bleeding in kidney organs, subsequently affecting fish blood production (Hardi *et al.* 2014).

However, these symptoms were not observed in tilapia injected with and immersed in the combined extracts, as shown by the RBC values. Hemoglobin (Hb) and hematocrit (Ht) values did not change in the first week among the treatments wether by immersion or injection method. Decreased Ht and Hb occurred in controls without extracts from 2nd and 4th weeks after injection and immersion whereas in the fishes given the extract, Ht and Hb relatively increased but did not differ significantly among the different extract combination.

The decrease in RBC concentration, Hb and Ht, in tilapia that were not given extracts of *B. pandurata*, *S. ferox*, and *Z. zerumbet* at different proprtions was due to bacterial infections of *A. hydrophila* and *P. fluorescens* (Hardi *et al.* 2013). Ht is the proportion of the volume of RBC in the blood (Scott & Rogers 1981). The content of Hb in catfish was decreased due to swelling of RBCs and poor Hb mobilization of the spleen and kidneys. Spleen disorders can cause an increase of Ht levels due to the introduction of erythrocytes into the circulatory system (Scott & Rogers 1981).

The total bacteria in the fish body were lower in fishes applied with extract than those in the control groups. Flavonoids, alkaloid, and steroids are antibacterial substance or metabolic secunder, that have ability to inhibit the growth of bacteria. Extracts of *B. pandurata* contains alkaloids, flavonoids and carbohydrates and *Z. zerumbet* contains alkaloids, flavonoids, steroids and carbohydrates, which are able to suppress the bacterial growth (Hardi *et al.* 2016a) and (Wink 2010). Morover, the extract of *S. ferox* has higher levels of alkaloids that are antibacterial (Hardi *et al.* 2016a; Huang *et al.* 2008). Flavonoids and alkoloids can damage the wall surface of the growing bacteria, particularly at low temperatures and fatty acids are believed to damage the structure and function of the bacterial cell wall and membrane (Hayes & Berkovitz 1979). This current research showed that the extracts of *B.Pandurata* and *Z. Zerumbet* have improved the non specific immunity, supressed the bacteria growth, and increased the the fishes protection against bacterial infection.

CONCLUSION

Through injection, the extract concoction of *B. pandurata* and *Z. zerumbet* with a ratio of SF 50 : ZZ 50 and BP 10 : SF 10 provided the best protection against *A. hydrophila* and *P. fluorescens* bacterial infections in tilapia. SF 50 : ZZ 50 is the best ratio to prevent both bacterial infection using the immersion methods. However, injection is the better method to increase the tilapia's innate (non-specific) immune system

and protection against *A. hydrophila* and *P. fluorescens* infection; it is quicker than immersion. The concoction extracts ratio of SF 50 : ZZ 50 significantly increased the immunity of non-specific tilapia and protect them against bacterial infection through injection or immersion.

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REFERENCES

Amend, DF. 1981. Potency Testing of Fish Vaccines. International Symposium on Fish Biologics: Serodiagnostics and Vaccines. Developments in Biological Standardization proceedings 49:447-54.

Anderson DP, Siwicki AK. 1995. Basic hematology and serology for fish health programs. Phuket (TH): Symposium on Diseases in Asian Aquaculture Aquatic Animal Health and the Environment, 25-29 October 1995. p.25-41.

Barman D, Nen P, Mandal SC, Kumar V. 2013. Immunostimulants for aquaculture health management. Journal of Marine Science: Research and Development 3:134.

Blaxhall PC, Daisley KW. 1973. Routine haematological methods for use with fish blood. J Fish Biol 5: 577-81.

Bricknell I, Dalmo RA. 2005. The use of immunostimulants in fish larval aquaculture. Fish Shellfish Immunol 19:457-72.

Chowdhury MBR.1998. Involvement of Aeromonads and Pseudomonads in diseases of farmed fish in Bangladesh. Fish Pathol 33(4):247-54.

Dosim, Hardi EH, Agustina. 2006. Efek penginjeksian produk intraseluler (ICP) dan ekstraseluler (ECP) bakteri *Pseudomonas* sp. terhadap gambaran darah ikan nila (*Oreochromis niloticus)*. [Effect of ECP and ICP *Pseudomonas* sp. injected to Haematology of Nile Tilapia (*Oreochromis niloticus*)]. Jurnal Ilmu Perikanan Tropis 19(1):24-30. (in Indonesian).

Ellis AE. 1988. General principles of fish vaccination. London (UK): Academic Press. p.25-7.

Evensen Ø. 2016. Development of fish vaccines: focusing on methods. In: Adams A (eds.) Fish Vaccines. Birkhäuser Advances in Infectious Diseases. Basel (CH): Springer. pp.53-74.

Findlay VL, Munday BL. 2002. The immunomodulatory effects of levamisole on the nonspecific immune system of Atlantic salmon, *Salmo salar* L. J Fish Dis 23:369-78.

Hardi EH, Pebrianto CA. 2012. Isolasi dan uji postulat Koch *Aeromonas* sp. dan *Pseudomonas* sp. pada ikan nila (*Oreochromis niloticus*) di sentra budidaya Loa Kulu Kabupaten Kutai Kartanegara. [Isolation and postulat Koch test *Aeromonas* sp. and *Pseudomonas* sp. in tilapia (*Oreochromis niloticus*) in Loa Kulu aquaculture Kutai Kartanegara]. Jurnal Ilmu Perikanan Tropis 16:35-9.

Hardi EH, Pebrianto CA, Agustina. 2013. Histopatologi ikan nila (*Oreochromis niloticus*) asal Loa Kulu Kutai Kartanegara Kalimantan Timur yang diinjeksi produk ekstraselular (ECP) dan intrasellular (ICP) bakteri *Aeromonas hydrophila*. [Histopathology of The Tilapia (*Oreochromis niloticus*) from Kutai Loa Kulu in Kartanegara of East Kalimantan Injected Extracellular (ECP) and Intracellular (ICP) Products of Bacteria of *Aeromonas hydrophila*]. Solo (ID): Konferensi Akuakultur Indonesia 2013. p.153-7.

Hardi EH, Pebrianto CA, Saptiani G. 2014. Toksisitas produk ekstraseluler dan intraseluler bakteri *Pseudomonas* sp. pada ikan nila (*Oreochromis niloticus*). [Toxicity of Extracellular and Intracellular Product of *Pseudomonas* sp. in Tilapia (*Oreochromis niloticus*)]. Jurnal Veteriner 15(3):312-22.

Hardi EH, Kusuma IW, Suwinarti W, Agustina, Abbas I, Nugroho RA. 2016a. Antibacterial activities of some Borneo plant extracts against pathogenic bacteria of *Aeromonas hydrophila* and *Pseudomonas* sp. AACL Bioflux 9(3):638-46.

Hardi EH, Kusuma IW, Suwinarti W, Agustina, Nugroho RA. 2016b. Antibacterial activity of *Boesenbergia pandurata*, *Zingiber zerumbet* and *Solanum ferox* extracts against *Aeromonas hydrophila* and *Pseudomonas* sp. Nusantara Bioscience 8(1):18-21.

Hardi EH, Saptiani G, Kusuma IW, Suwinarti W, Nugroho RA. 2017. Immunomodulatory and antibacterial effects of *Boesenbergia pandurata*, *Solanum ferox*, and *Zingiber zerumbet* on tilapia, *Oreochromis niloticus*. AACL Bioflux 10(2):182-90.

Hardi EH, Saptiani G, Nurkadina, Kusuma IW, Suwinarti W. 2018a. Uji *in vitro* gabungan ekstrak *Boesenbergia pandurata, Solanum ferox, Zingiber zerumbet* terhadap bakteri patogen pada ikan nila. [*In Vitro* Test of

Concoction Plant Extracts from Borneo Island, Indonesia Against Fish Pathogenic Bacteria in Tilapia]. Jurnal Veteriner 19(1):35-44.

Hardi EH, SaptianiG, Kusuma IW, Suwinarti W, Sudaryono A. 2018b. Inhibition of fish bacteria pathogen in tilapia using a concoction three of Borneo plant extracts. OP Conf Ser: Earth Environ. Sci 144:012015 (p.8).

Hardi EH, Nugroho RA, Kusuma IW, Suwinarti W, Apriza. 2019a. Immunomodulatory effect and disease resistance from of three Borneo plant extracts to *Aeromonas hydrophila* and *Pseudomonas fluorescens* in tilapia, *Oreochromis niloticus*. Aquacultura Indonesiana 20(1):41-7.

Hardi EH, Nugroho RA, Kusuma IW, Suwinarti W, Sudaryono A, Rostika R. 2019b. Borneo herbal plant extracts as a natural medication for prophylaxis and treatment of *Aeromonas hydrophila* and *Pseudomonas fluorescens* infection in tilapia (*Oreochromis niloticus*). F1000Research. 7:1847 Last updated: 28 MAR 2019.

Harikrishnan R., Balasundaram C. 2008. *In vitro* and *in vivo* studies of the use of some medicinal herbals against the pathogen *Aeromonas hydrophila* in goldfish. J Aquat Anim Health 20:165-76.

Harikrishnan R, Balasundaram C, Kim MC, Kim JS, Han YJ, Heo MS. 2009. Innate immune response and disease resistance in *Carassius auratus* by triherbal solvent extracts. Fish Shellfish Immunol 27: 508-15.

Janda JM, Abbott SL. 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clin Microbiol Rev 23:35-73.

Hayes ML, Berkovitz BKB. 1979 The reduction of fissure caries in Wistar rats by a soluble salt of nonanoic acid. Arch Oral Biol 24:663-6.

Huang W. H., Hsu C. W., Fang J. T., 2008 Central diabetes insipidus following digestion *Solanum indicum* L. concentrated solution. J Clin Toxicol 46:293-6.

Jeney G, Anderson DP. 1993. Enhanced immune response and protection in rainbow trout to *Aeromonas salmonicida* bacterin following prior immersion in immunostimulants. Fish Shellfish Immunol 3:51-8.

Lie O, Evensen O, Sorensen A, Froysada E. 1989. Study on lysozyme activity in some fish species. Dis Aquat Org 6:1-5.

Limsuwan S, Votavuthikunchai SP. 2008 *Boesenbergia pandurata* (Roxb.) Schltr., *Eleutherine americana* Merr. and *Rhodomyrtus tomentosa* (Aiton) Hassk. as antibiofilm producing and antiquorum sensing in *Streptococcus pyogenes*. FEMS Immunology and Medical Microbiology 53:429-36.

Lygren B, Hamre K, Waagbo R. 1999. Effects of dietary pro and antioxidants on some protective mechaniams and health parameters in Atlantic Salmon. J Aquat Anim Health 11(3):211-21.

Karunasagar I, Rosalind G, Karunasagar J. 1991. Immunological response of the Indian major carps to *Aeromonas hydrophila* vaccine. J Fish Dis 14: 413-7.

Mailoa MN, Tapotubun AM, Theodora EAA, Matrutty. 2017. Analysis Total Plate Counte (TPC) on fresh steak tuna applications edible coating *Caulerpa* sp during stored at chilling temperature. IOP Conf. Series: Earth and Environmental Science 89:0012014 (p.6).

Mastan SA. 2015. Use of Immunostimulants in aquaculture disease management. Int J Fish Aquat 2(4):277-80.

Midtlyng PJ. 2016. Methods for Measuring Efficacy, Safety and Potency of Fish Vaccines. In: Adams A (eds) Fish Vaccines. Birkhäuser Advances in Infectious Diseases. Basel (CH): Springer. p.119-41.

Monette S, Dallaire AD, Mingelbier M, Groman D, Uhland C, Richard JP, Paillard G, Johannson LM, Chivers DP, Ferguson HW, Leighton FA, Simko E. 2006. Massive mortality of common carp (*Cyprinus carpio*) in the St. Lawrence River in 2001: diagnostic investigation and experimental induction of lymphocytic encephalitis. Vet Pathol 43(3):302-10.

Osman KM, Mohamed LA, Rahman EHA, Soliman WS. 2009. Trials for Vaccination of Tilapia Fish Against *Aeromonas* and *Pseudomonas* Infections Using Monovalent, Bivalent and Polyvalent Vaccines. WJ FMS1(4):297-304.

Pridgeon JW, Klesius K. 2012. Major bacterial diseases in aquaculture and their vaccine development. CAB Rev 7:1-16.

Rodger HD. 2016. Fish Disease Causing Economic Impact in Global Aquaculture. A. Adams (ed.): Fish Vaccines, Birkhäuser Advances in Infectious Diseases, DOI 10.1007/978-3-0348-0980-1_1.

Sakai M. 1999. Current research status of fish immunostimulants. Aquaculture 172:63-92.

Scott AL, Rogers WA. 1981. Hematological effects of prolonged sublethal hypoxia on channel catfish *Ictalurus punctatus* (Rafinesque). J Fish Biol 18: 591-601.

Secombes CJ, Fletcher TC. 1992. The role of phagocytes in the protective mechanisms of fish. Ann Rev Fish Dis 2:58-71.

Sioutas S, Hoffmann RW, Pfeil-Putzien C, Fischer-Scherl T. 1991. Carp erythrodermatitis (CE) due to an *Aeromonas hydrophila* infection. Casuistic and experimental results. Zentralb Veterinarmed B 38(3):186-94.

Sugiani D, Sukenda, Harris E, Lusiastuti AM. 2012. Haemato responses and histopathology of tilapia

(*Oreochromis niloticus*) to co-infection *Streptococcus agalactiae* and *Aeromonas hydrophila*. J. Riset Akuakultur 7:85-91.

Sumiati T, Sukenda, Nuryati S, Lusiastuti AM. 2015. Development of ELISA method to detect specific immune response in Nile tilapia (*O. niloticus*) vaccinated against *A. hydrophila* and *S. agalactiae*. J. Riset Akuakultur 10:243-50.

Wink M. 2010 Annual plant reviews volume 40: biochemistry of plant secondary metabolism. 2nd ed. Iowa (US): Blackwell Publishing Ltd. p.445.

Yin G, Jeney G, Racz T, Xu P, Jun X, Jeney Z. 2006. Effect of two Chinese herbs (*Astragalus radix* and *Scutellaria radix*) on non specific immune response of tilapia, *Oreochromis niloticus*. Aquaculture 253: 39-47.