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EFFECTIVENESS OF DIFFERENT METHODS FOR PREVENTING *Aeromonas hydrophila* AND *Pseudomonas fluorescens* INFECTION IN TILAPIA

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Running title: Method for preventing *A. hydrophila* and *P. fluorescens* infection in Tilapia

ABSTRACT

This research evaluated a method involving provision of a concoction of *Boesenbergia pandurata, Solanum ferox* dan *Zingimber zerumbet* extracts for pathogen prevention in tilapia. The concentration of each extract was 600 ppm of *Boesenbergia pandurata*/BP, 900 ppm of *Solanum ferox*/SF and 200 ppm of *Zingimber zerumbet*/ZZ. The examination was performed by issuing two combinations of extracts (SF:BP, SF:ZZ) against *Aeromonas hydrophila* and *Pseudomonas fluorescens* (10^5 $CFUmL^{-1}$). Preventive trials were carried out by providing a concoction of extracts through intraperitoneal injection (0.1 mL/fish) in tilapia (15±2 g) and the immersion method was performed by bathing the fish in the extracts for 20 minutes, with pathogen challenging during the following 24 h being carried out. The composition of the used extract was by SF60:ZZ40; SF50:ZZ50; BP90:SF10; BP50:SF50; and fish without being given the extract. Haematology and immunology parameters were observed at the 4th week after challanges with pathogenic bacteria. The number of white blood cells (WBCs) increased significantly ($P < 0.05$) compared to controls without extract, with a similar increase observed for red blood cell (RBCs), but heamatocrit (Ht) and hemoglobin (Hb) values did not significantly increase compared to control. Phagocytic index, respiratory burst and lysozyme activities also experienced a significant increase in fish fed with combined extracts compared to controls. The numbers of pathogenic bacteria in the body of the fish given extract were also lower than the control and significantly different at the 4th week. The results of this study showed that the administration of a combined extract of SF50: ZZ50 and BP90: SF10 provided the best protection as indicated by relative survival percent (RPS) of 100% after being tested challenged with *A. hydrophila* and *P. fluorescent* by. This study indicates that providing combined extracts by injection and immersion in the ratio of SF50:ZZ50 has a positive effect in increasing the non-specific immune system of tilapia and increasing protection against bacterial infections.

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

INTRODUCTION

The increase of global fish farming has been occurring rapidly, with increased biomass production, species diversification, geographical expansion and enlarging methods to fulfill the protein needs of fish. The increase has continually faced challenges due to associated diseases and health problems of aquacultural animals. Another triggering factor is climate change and the development of affecting aquaculture technologies in the balance or imbalance of interactions

between pathogens, hosts and the environment. Almost every year, new aquacultural pathogens are being isolated and novel diseases continue to be identified in various areas of cultivation and many species in these areas (Rodger 2018).

Aeromonas hydrophila and *Pseudommonas fluorescens* are two pathogenic bacteria occurring throughout the year, with a mortality rate of 60–80% (Hardi *et al*. 2012, 2016, 2017). Aeromonas causes loss and destruction of the aquaculture industry around the world (Monette *et al.* 2006). Fish infected with these bacteria include (Janda & Abbott 2010) tilapia *Oreochromis niloticus* (Hardi *et al.* 2012), *Cyprinus carpio* (Sioutas *et al.* 1991; Monette *et al.* 2006), *Clarias gariepinus* (Chowdhury 1998) and indian major carps (Karunasagar *et al.* 1991). Combined bacterial infections are typically found in nature with heavier symptoms than in single bacterial infections. Combined infection of *A. hydrophila* and *P. fluorescens* leads to stressed fish, exoptalmia, ulcers and watery organs 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 the fish diseases or pathogens do not yet have suitable preventative or treatmnent options. For example, the use of vaccines, imonostimulants, 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 deriving 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 for fish include levamisole and saponins (Findlay & Munday 2000) being able to increase the non-specific immune systems activity (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). That extract can be used for prevention and treatment of infections of both bacteria in tilapia (Hardi *et al*. 2017, 2018b).

To increase the immunomodulatory activities from plant extracts, several extracts were combined in aplication. A concocction of *Curcuma longa, Ocimum sanctum* and *Azadirachta 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 can increase the survival rate and inhibitory process due to infection by *A. hydrophila* bacteria in goldfish (*Carassius auratus*) (Harikrishnan *et al*. 2009). A concocction of *Boesenbergia pandurata, Solanum ferox,* and *Zingiber zerumbet* extract at a ratio 1:1:1 in tilapia has an immunomodulatory effect in tilapia and could increase protection and diseases recovery from *A.*

hydrophila and *Pseudomonas fluorescens* better than single extract (Hardi *et al*. 2019a). some research show that *B. pandurata* and *Z. zerumbet* extracts contains alkaloids, flavonoids, carbohydrates, and steroids (Hardi et al, 2016a). While the ekstract of *S. ferox* has higher levels of alkaloids that play an important role as antibacterial properties (Hardi et al, 2016a and 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. fluerescens* both in single and combined use (Hardi *et al.* 2018a; 2018b). This paper will discuss the effectiveness of these three extracts to prevent infection from *A. hydrophila* and *P. fluerescens* bacteria in tilapia using injection and immersion methods.

MATERIALS AND METHODS

Fish and Bacteria

Tilapia used in this research were of size 15±2 g, taken from the village of Teluk Dalam, Tenggarong Seberang Kutai Kartanegara. The tilapia fish had been kept at the laboratory for two weeks prior to use. The aquarium used for treatment was 60 x 40 x 30 cm, containing 60 L of water, 50% of which was changed every two days to remove remaining fish fesses and feed.

The bacteria used for the challenge trial were combination of *A. hydrophila* (EA-01) and *P. fluerescens* (EP-01) with ratio 1:1, derived from the Aquatic Microbiology laboratory, Faculty of Fisheries and Marine Sciences, Mulawarman University, Indonesia. Bacterial density was 10^5 $CFUmL^{-1}$ each bacteria, with 1 mL/fish being injected intramuscularly.

Extract Preparation for *B. pandurata, Z. zerumbet* and *S. ferox*

The rhizomes of plants used in the study were *B. pandurata* and *Z. zerumbet*, and fruit of *S. ferox*. All of them were collected from traditional markets in Samarinda City, East Kalimantan. Plants were cleaned of dirt, cut into slices, dried in an oven at 40 ^oC for 48 hours, blended in powder form and refrigrated at -4 °C until the extraction stage continued. The method for extraction uses ethanol solution and follows Limsuwan & Voravuthikunchai (2008). The concentrations of each extract were *B. pandurata* and *Z. zerumbet*, and *S. ferox* respectively 600, 200 and 900 mgL^{-1}. Comparison of combination of *Z. zerumbet* and *S. ferox* extracts with rasio 40:60 and 50:50 mL. Comparison of *B. pandurata* and *S. ferox* 90:10 mL and 50:50 mL.

Experiment

Extract was given to tilapia via use of injection and immersion methods to avoid bacterial infection of *A. hydrophila* and *P. fluerescens*. A preventive experiment was performed by issuing

combined extracts through intraperitional injection (IP) at a rate of 0.1 mL/fish and waiting for seven days; on the 8th day, fish were challenged with pathogenic bacteria. Regarding the immersion method for preventing pathogenic bacteria, fish were immersed for 20 minutes with a combination of extracts, and challenged with the combined bacteria through intramuscular injection (IM) 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 combination of 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 combination of 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 combination of 90 ml of *B. pandurata* extract and 10 ml of *S. ferox* (BP 90:SF 10) and challenged through IM injection with combination of pathogenic bacteria.

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

Group 5 = Immersion fish with combination of 60 ml of *S. ferox* extract and 40 ml of *Z. zerumbet* extract (SF 60:ZZ 40) challenged through IM injection with combination of pathogenic bacteria.

Group 6 = Immersion fish with combination of 50 ml of *S. ferox* extract and 50 ml of *Z. zerumbet* extract (SF 50:ZZ 50) challenged through IM injection with combination of pathogenic bacteria.

Group 7 = Immersion fish with combination of 90 ml of *B. pandurata* extract and 10 ml of *S. ferox* extract (BP 90:SF 10) challenged through IM injection with combination of pathogenic bacteria.

Group 8 = Immersion fish with combination of 50 mL of *B. pandurata* extract and 50 ml of *S. ferox* extract (BP 50:SF 50) challenge via IM injection with combination of pathogenic bacteria.

Group 9 = IP injected fish with PBS (phosphat buffer saline) sterile and challenge through IM injection with combination of pathogen bacteria.

Group 10 = Immersion fish with PBS sterile and challenge via IM injection with combination of pathogenic bacteria.

In this research, every group using 10 fish every aquarium and three replication, so 30 fish using every groups, to evaluate the effective method. Total tilapia were used in this research to evaluate the extract administration with the different method (IP and immersion) in non-specific immunity, susrvival rate (SR), and Relative Percent Survival (RPS) were 300 *Oreochromis niloticus*.

Hematological Examination

Every week (first, second, third and fourth) during one month after challenging with pathogenic bacteria, hematological observations were obtained. Before blood was taken, the fish were anesthetized using 50 mg MS-222 dm^{-3}, 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 haemocytometer. 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 use of a sahli tube. Hematocrit (Ht %) was measured using the microcentrifuge method, and the standard solvent employed was tri sodium citrate. The inserted blood into the micro hematocrit tube was centrifuged at 7,000 g 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, measured with a graphic reading device (Blaxhall and Daisley Methods, 1973).

Index Phagocytic

Fifty µL of blood was transferred into an eppendorf tube containing 50 µL, mixed of *A. hydrophila* and *P. fluorescens* suspension (the density of each bacteria were 10^5 $CFUmL^{-1}$), and left for 20 minutes. The preparation of the screw was made on a glass object and dried, fixed with alcohol (95%) for five minutes, then dried again. The preparation was then coloured by soaking it in Giemsa dye (10%) for 15 minutes, washed with flowing water and dried. The preparations were then observed and the number of cells demonstrating phagocytic processes were counted (100 phagocytic cells were observed), the method according to Anderson and Siwicki (1995). This parameter was observed in week 4^{th} after chalanges (IM) with *A. hydrophila* and *P. flourescens*.

Respiratory Burst

The test of respiratory burst activity involved use of nitro blue tetrazolium (NBT) reagent. Blood derived from fish (50 µL) was transferred to a microplate, incubated for one hour at 37 ° C, the supernatant removed; cells were washed with 50 µL of PBS three times, 50 µL of 0.2% NBT was added 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 the paramether analized by Secombes and Fletcher (1992) method. Likes a index phagocity, this parameter was observed in week 4th after chalanges (IM) with *A. hydrophila* and *P. flourescens*.

Lysozyme Activity

Moistened injection syringes with anticoagulants were prepared, with the blood of the fish from the caudal vein 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, with the separated clear liquid (serum) then being removed. The test for lysozyme activity was performed according to the method of Lygren *et al.* (1999) — 10 µl of serum sample was placed into a micro titer plate and then 190 µl of lysodeikticus Micrococcus suspension added (Sigma Aldrich Chemical) (0.2 mg of lysodeikticus Micrococcus / mL PBS (pH 7.4)) shaking slowly at constant room temperature. After 90 minutes of incubation, a micro titer ELISA plate reader at a wavelength of 520 nm (Lie *et al.* 1989) was used to take readings.

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

Total Bacteria in Fish Bodies using TPC

Calculation of total bacteria in the fish body was carried out to determine the antibacterial activity due to injection with combined extracts of *A. hydrophila* and *P. fluorescens*. 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 a first step, petri cups, test tubes and pipettes were sterilized using dried sterilization (180 ^{o}C for two hours) prior to use. PCA solid media were used as a growth substrate, wet-sterilized in an autoclave (121 ^{o}C for 15 minutes, 1 atm). 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 cup and approximately 15 mL of PCA media was poured evenly; the petri dish was incubated for 48 hours at 30 °C (the petri dish was placed upside down in the incubator) and growing colonies then counted,

based of Mailoa et al. (2017) method. At the end of the incubation period, select all of the petri plates containing between 30 and 300 colonies. Plates with more than 300 colonies cannot be counted and are designated too many to count (TMTC). Plates with fewer than 30 colonies are designated too few to count (TFTC). Count the colonies on each plate and a quebec colony counter should be used. This parameter was observed in week 4th after chalanges (IM) with *A. hydrophila* and *P. flourescens*.

$$\text{number of colonies (CFU)/mL} = \frac{\sum \text{Colony of bacteria}}{dilution\ X\ amount\ plated}$$

Protection Level for Pathogens

To determine the effectiveness of combined extracts to prevent *A. hydrophila* and *P. flurescens* infection, the challenge-tested fish were counted with respect to number survival rate (SR) and the protection level (RPS) was calculated on week 4th after IM infectio, and observations using the Amend (1981) and Ellis formula (1988).

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

$$\text{RPS} = 1 - \frac{(percent\ mortality\ in\ treated\ group)}{Percent\ mortality\ in\ control\ group} X\ 100$$

Statistics Data analysis

The data obtained were analyzed statistically using SPPS 16.0 to determine the effect of extract treatment on observation parameters.

RESULTS AND DISCUSSION

Hematology

The total tilapia WBCs to prevent bacterial infection of *A. hydrophila* and *P. fluorosence* using the immersion method (Table 1) significantly increased (P <0.05) starting from the 2nd week to the 4th week after being given the combined extract (Group 5-Group 8) compared with controls (Group 10) without extract. The highest increase was experienced by tilapia given the combined extract of *B. pandurata* and *S. ferox* at a 50:50 of ratio (Group 6) from the 2nd week to the 4th week after bacterial infection by IM. Likewise, total RBC counts (Group 5-Group 8) significantly increased with control fish (P <0.05) since the second week of treatment, while hematocrit levels (Group 5-Group 8) significantly increased (P <0.05) in weeks 3 and 4. Post-treatment tilapia hemoglobin levels in Group 5-Group 8 were increased but not significantly different to control/Group 10 (P <0.05).

Table 1. Haematology of tilapia in preventive method using a combination of extracts to bacterial infection of *A. hydrophila* and *P. fluorosence* 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±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±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

Notes: Values (means ± SD) with different superscript in a row show significant differences ($P < 0.05$).

Preventive test throug 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 increase was significantly different with the control/Group 9 ($P < 0.05$) in Group 1-Group 4 treatment by IP from 1st to 4th week of observations. A similar result was showed RBC and hematocrit, with results for fish given extracts being significantly different ($P < 0.05$) to control. Only fish heamoglobin (Group 1-Group 4) was not significantly different with the fish control in terms of prevention of bacterial infections via the IP method.

Table 2. Haematology of tilapia in preventive testing using a combined extract against bacterial infection of *A. hydrophila* and *P. fluorosence* through 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±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}	$21,5\pm0.1^{a}$
	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}

Notes: Values (means ±SD) with different superscript in a row show significant differences ($P < 0.05$).

Index Phagocytic

The fish given the combined extract of SF 50: ZZ 50 (Group 2) through the injection method (IP) showed the highest index phagocytic improvement compared to the other treatments (Group 1, 3,4,5,6,7,8) at the 4th week after the challenge test and were significantly different to the controls/Group 9 ($P < 0.05$). Likewise, with the immersion method, prevention from bacterial infections of *A. hydrophila* and *P. fluorescens* with a combined extract of SF 50: ZZ 50 (Group 6) showed the highest increase of index phagocytic in the 4th week after the challenge (IM) test. The entire treatment of the extract combination was increased and significantly different from the control ($P < 0.05$). All combination of extract (Group 1- Group 8) were increased in index phagocytic and significantly to the controls in the 4th week after the challenge test (Figure 1).

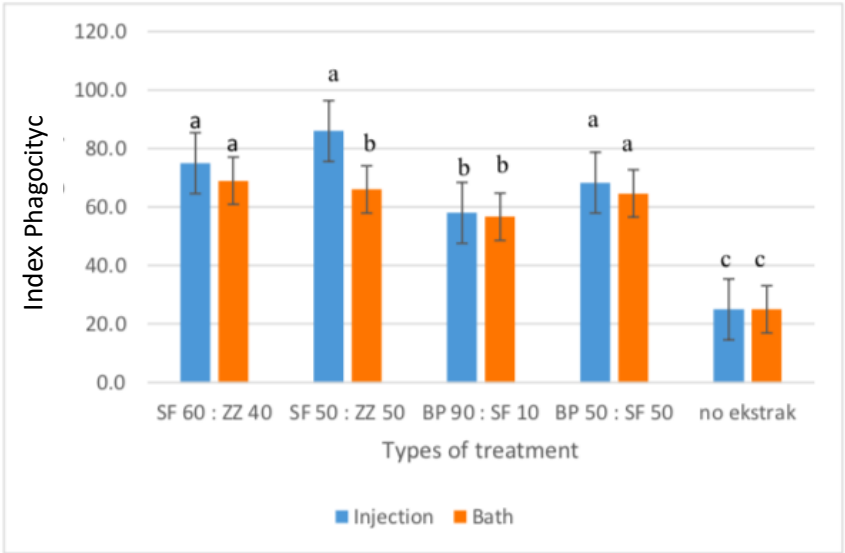

Figure 1. Index phagocityc of tilapia on challenge test using combined extract to bacterial infection of *A. hydrophila* and *P. fluorosence* through injection and immersion methods. Different superscript in a row show significant differences (P < 0.05).

Respiratory Burst

Respiratory burst activity of tilapia given a combination of extracts increased during the 4th week either through injection or immersion methods (Figure 2.). A significant increase compared to the control group occurred in all given extracts with different combinations. However, only the ratio of SF 50:ZZ 50 by IP method (Group 2) was significantly different (P<0.05) from the combination of other extracts and to the controls (Group 9).

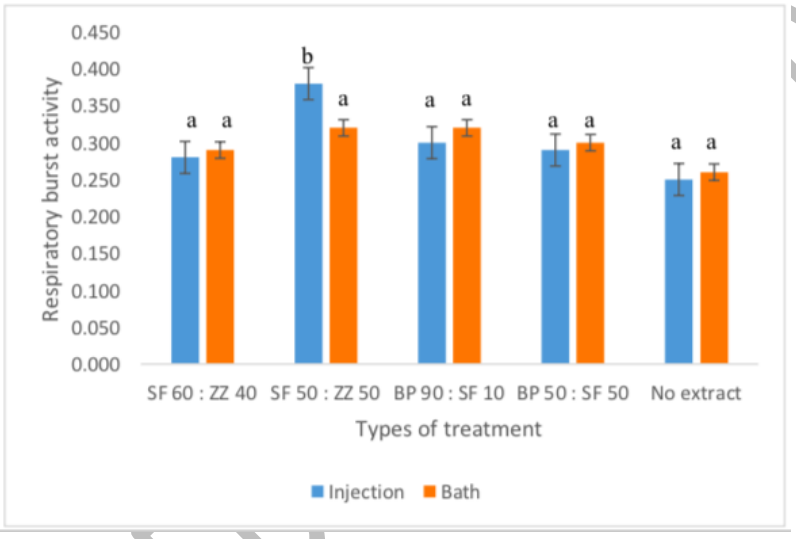

Figure 2. Respiratory burst activity of tilapia on preventive methods by using a combination of extracts to bacterial infection of *A. hydrophila* and *P. fluorosence* through injection and immersion method

Lysozyme Activity

The activity of tilapia lysozyme given all combination of extracts (Group 1-Group 8) with different comparisons were increased, and significantly different to control without extract (P> 0.05) in the 4th week after the challenge test. Only Group 2 was significantly different to others concocction extract (Figure 3). But the all conccoction of extract (Group 1- Group 8) were significantly different to contol (Group 9 and Group 10) without extract (P > 0.05).

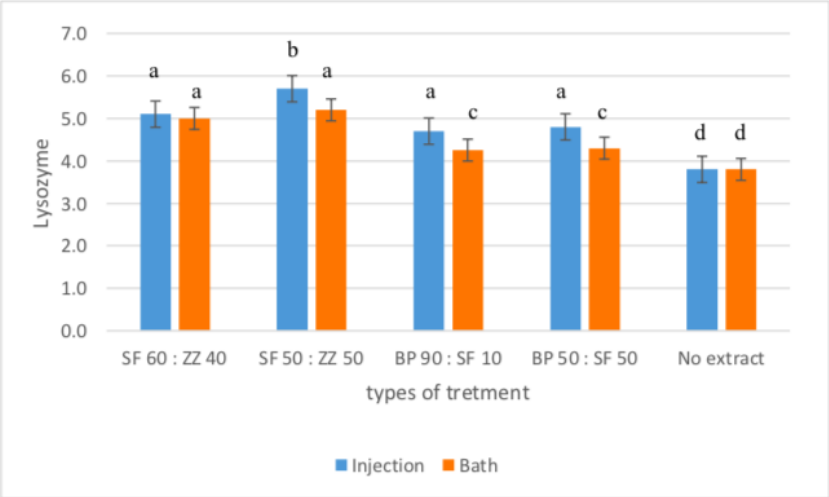

Figure 3. Lysozyme activity of tilapia in preventive method by using a concocction extract to bacterial infection of *A. hydrophila* and *P. fluorosence* through injection and immersion methods

The total bacteria of *A. hydrophila* and *P. fluorescens* in the body of tilapia in prevention through injection (IP) and immersion methods were lower than controls (Group 9 and Group 10) without extracts at the 4th week after the challenge test (IM) with pathogenic bacteria (Table 3). The lowest bacterial density in tilapia was found for those fish given a combination extract of SF 50: ZZ 50 (Group 2) and different control with no extract/ Group 9 ($P < 0.05$), 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 ($P < 0.05$). All concocction of extract (Group 1-Group 8) coused total bacteria decreased in tilapia on week 4th after chalanges with *A. hydrophila* and *P. fluorescent* (IM). The decreased of total bacteria was significantly to the control (Group 9 and Group 10) with $P < 0.05$. The concocction SF 50 : ZZ 50 and BP 90 : SF 10 (Group 2 and Group 3) were injection (IP) administration can supress the bacterial growth in the fish body and significantly to the others cocccoction. While, only the concocction SF 50 : ZZ 50 (Group 6) by immersion administration was significantly with the others coccoction and to the control/Group 10 ($P < 0.05$).

Table 3. TPC of tilapia bacteria in preventive method by using combination of extracts to bacterial infection of *A. hydrophila* and *P. fluorosence* through injection and immersion methods

Groups	Extracts	Total Bacteria (10^5 CFUmL^{-1})
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]

Notes: Values (means SD) with different superscript in a row show significant differences (P < 0.05)

Prevention against *A. hydrophila* and *P. fluorosence*

The highest percentage of SR and RPS of tilapia in preventive methods against *A. hydrophila* and *P. fluorosence* use were found on tilapia that had been given extracts SF 50:ZZ 50 (Group 2) and BP 90:SF 10 (Group 3) by injection until week 4. Meanwhile, the average SR of tilapia that were given a concoction of extract was demonstrated to be higher than controls/ Group 9 and Group 10 (Table 4). The best SR and RPS of tilapia preventive using immersion method had given extracts SF 50:ZZ 50 (Group 6) than others concocction (Groups 5, 7, 8) but, Groups 5, 7, and 8 ware increase the SR and significantly diferent to the control (Group 10) with P<0.05.

Relative percent survival in all groups that administration with the concocction extracts were more than 65 %, only on Group 7 (Immersion BP 90 : SF 10) that lowest RPS (58%). Hardi et al (2018a), Ellis (1988), Osman *et al* (2009) says that RPS more than 60% showed the vaccine or immunostimulant ware effective in protection bacteria infection.

Table 4. Survival Rate and RPS of tilapia bacteria in preventive method by using combination of extracts to bacterial infection of *A. hydrophila* and *P. fluorosence* 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]	

Notes: Values (means SD) with different superscript in a row show significant differences (P < 0.05)

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). According to Sakai (1999) and Findly & Munday (2002), immunostimulants are additional ingredients given to organisms and are able to increase the innate (non-specific) immune system to prevent pathogenic infections. Cells playing important roles in the non-specific immune system are

WBCs; their activity is influenced by fish, nutrition and the environment (Harrikrishnan *et al*, 2003; Mastan, 2015). The increase of WBCs of tilapia given all concoction extract (Group 1-8) was higher than the control without extract (Group 9 and 10), and the survival rate of tilapia after *A. hydrophila* and *P. fluorescens* infection reached 100% using a combination of SF 50: ZZ 50 and BP 90: SF 10 through injection (Group 2 and Group 3). Immersion administration method, give a infection protection around 58-72%, and the best protection agains bacteria shown in Group 6 (immersion SF 50: ZZ 50) was 72%.

The difference methods in administration of extracts in fish affects on protection against bacteria infection. These results demonstrate that giving a combination of extracts can improve the non-spatial performance of the immune system of fish by producing more WBCs, subsequently inhibiting bacterial growth in the body (as can be seen in the lowest bacterial TPC data in this treatment compared to other combined extracts or control), and the different methods (IP and immersion) of administration show differences in performance of the immune system. Hardi et al (2019b) explain that the that the addition of combined extract into feed has a positive effect on the tilapia's immune system and the SF50/ZZ50 combination appears to improve the innate immune system of tilapia to treat and prevent bacterial infections throug feed.

Based on Yin et al (2006), Jeney and Anderson (1993), and Mulero (1998) researchs, the administration of extract can be applied via injection, bathing or oral administration, the latter seems to be the most practicable in fish. Both injection and immersion methods have a different advantages and disadvantages (Evensen, 2016). Advantages the injection method are most potent, little waste of immonostimulan, Cost-effective method for high-value species. And the immersion method advantages are large-scale application possible, moderate stress to the fish, allows mass vaccination or immunostimulant of immunocompetent fish. Evensen (2016) exlpaint abaout the disadvantages using immersion method than injection. Immersion method need a large amount of immonostimulan is needed, can be cost prohibitive, low to moderate efficacy and inferior to injection delivery in terms of efficacy Cost prohibitive for large fish. Based on research shows that the injection method can increase RPS rather than immersion at the same time. This is due to immonostimulant delivery in the body of the fish. The injection method, immunostimulant directly into the blood, while the immersion method, immonostimulan must penetrate the fish skin, so that more time is needed to improve the immune system, the same statement was explain by Midtlyng (2006).

The efficiency of method in immunotimulant admistration in fish, can shown by RPS. The vaccine or immonostimulant potency and efficacy testing methods in fish and proposes detailed recommendation of test setup, challenge conditions and outcome acceptance criteria for controlled trials: exposure by bath challenge in two concentrations; maximum 10% non-specific mortality and 20% within-group variation after challenge; control mortality ≥60 %, vaccinate mortality ≤24 %; and

following the Amend (1981) recommendations, the proposed acceptance criteria for potency equate to a standardised RPS of 60 % or above.

The total value of Tilapia RBCs in the preventive trial was higher than the control without extract, and significantly different ($P < 0.05$). Both *A. hydrophila* and *P. fluorescens* bacteria produce hemolysin protein which can lyse RBCs, the numbers of which are therefore decreased in infected fish (Hardi *et al*. 2013). This decrease has also been noted to occur in tilapia infected with *S. agalactiae* (Hardi *et al*, 2011), *S. iniae* (Sugiani *et al*. 2012), *A. hydrophila* (Dosim *et al.* 2006) and *Pseudomonas* sp. Tilapia being injected with extracellular and intracellular proteins from *A. hydrophila* (Hardi *et al.* 2013) and *Pseudomonas* sp. has been found to lead to degeneration, necrosa and bleeding in kidney organs, subsequently affecting fish blood production (Hardi *et al*. 2014).

However, similar observations were not observed for tilapia given combination of extracts (both methods administration), with RBC values being noted after infection. Hemoglobin (Hb) and hematocrit (Ht) values did not change in the first week with all treatments and the immersion or injection method administration including controls, and decreased values of Ht and Hb occurred in controls without extracts from 2^{nd} and 4^{th} weeks after injection and immersion application methods, whereas in the treatment fish given extract, Ht and Hb values were relatively increased but not significantly different between the difference in extract comparison.

The concentration decrease in RBC, Hb and Ht in tilapia that were not given extracts of *B. pandurata, S. ferox*, and *Z. zerumbet* with different concoctions in this study was due to bacterial infections of *A. hydrophila* and *P. fluorescens* (Hardi *et al*. 2013). According to Scott and Rogers (1981), Ht is the proportion of the volume of RBC in the blood. For a further explaination, the content of Hb in catfish has been found to decrease due to swelling of RBCs and the presence of poor Hb mobilization of the spleen and kidneys. Scott and Rogers (1981) noted that spleen disorders can cause an increase of Ht levels due to the introduction of erythrocytes into the circulatory system.

The total bacteria in the fish body were decrease in fish extract groups than control groups. flavonoids, alkaloid, and steroids are antibacterial substance or metabolic secunder, that have ability to inhibit the growth of bacteria. Extract of *B. pandurata* contains alkaloids, flavonoids and carbohydrates and *Z. zerumbet* contains alkaloids, flavonoids, steroids and carbohydrates, which are able to suppress the bacteria growth (Hardi et al, 2016a) and Wink (2010). Ekstract of *S. ferox* has higher levels of alkaloids that play an important role as antibacterial properties (Hardi et al, 2016a and Huang et al 2008). Flavonoids and alkoloids could damage the wall surface of the bacteria that grow, 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 reseach showed that the extract improve the non specific immunity, supress the bacteria growth, and increase the bacterial infection protection.

CONCLUSION

This study demonstrates that a concoction of an extract with a ratio of SF 50: ZZ 50 and BP 10:SF 10 provides the best protection against *A. hydrophila* and *P. fluorescent* bacterial infections through injection and SF 50:ZZ 50 is the best ratio to protect the both bacteria infection in immersion methods. But, the injection is the better method to increase the innate (non-specific) immune system and protection againts *A. hydrophila* and *P. fluorescens* infection quicly than immersion. However, the concoction extracts ratio SF 50: ZZ 50 were increase immunity of non-specific tilapia and protect bacterial infection througt injection or immersion.

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