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RESEARCH ARTICLE

Borneo herbal plant extracts as a natural medication for prophylaxis and treatment of *Aeromonas hydrophila* and *Pseudomonas fluorescens* infection in tilapia (*Oreochromis niloticus*) [version 1; peer review: 2 approved with reservations]

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Abstract

Background: This study aims to describe the antibacterial and immunostimulant abilities of *Boesenbergia pandurata* (BP), *Solanum ferox* (SF) and *Zingiber Zerumbet* (ZZ) plant extracts to treat and prevent *Aeromonas hydrophila* and *Pseudomonas fluorescens* infection on Tilapia (*Oreochromis niloticus*).

Methods: Tilapia (initial weight 15±2 g) were injected intramuscularly (0.1 ml/fish) with a combination of *A. hydrophila* and *P. fluorescens* at a density of 1×10^5 CFU ml^{-1} of each bacteria. Treatment trials were performed at day 7 post-injection with each combined extract, while the prevention trial was performed by including the combined extract into the diet for six and seven days prior to injection. Various combinations of extract—60 ml SF extract/kg feed with 40 ml ZZ/kg feed (SF60/ZZ40), SF50/ZZ50, BP90/SF10, and BP50/SF50—were mixed with a commercial diet and used in both treatment and prevention trials. Haematological and immunological parameters were performed every week for four weeks.

Results: In prevention trials, tilapia fed SF50/ZZ50 showed a significant increase of white and red blood cells from weeks 2 to 4. Similarly, significantly increased haematocrit was also found in tilapia fed SF50/ZZ50 in the treatment trial but not in the prevention trial. However, haemoglobin of tilapia in both trials was not affected by any of the various combinations of extract in the diet. Furthermore, phagocytic, respiratory burst, lysozyme activity indexes and survival rate of fish fed with combined extracts were found to be significantly higher than controls. Moreover, the amount of pathogenic bacteria in fish that were fed combined extracts was also lower than the control and was significantly different at week 4.

Open Peer Review

Referee Status: ✓ ✓

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REVISED version 2 published 13 Feb 2019	✓ report	✓ report
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Conclusions: This study indicates that the addition of combined extract into feed has a positive effect on the tilapia's immune system. The SF50/ZZ50 combination appears to improve the innate immune system of tilapia to treat and prevent bacterial infections.

Keywords
Imunomodulator, Concoction, Aeromonas hydrophila, Pseudomonas fluorescens, Prophylaxis

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Introduction

Tilapia (*Oreochromis niloticus*) is one of the most widely cultivated fish species in Indonesia. Tilapia is a freshwater fish that can be easily cultivated[1]. According to Pridgeon[2] and Harikrishnan *et al.*[3], freshwater fish culture is inseparable from bacterial infections which are caused by motile *Aeromonas* septicaemia, furunculosis, edwardsiellosis and *Aeromonas hydrophila*. Further, *Aeromonas* species have been identified as major causative bacteria and a serious pathogen in fish[4,5]. In Indonesia, particularly East Kalimantan, infection of *A. hydrophila* and *Pseudomonas fluorescens* in fish results in high mortality rates of up to 60–80%. In fish, both of these bacteria cause stresses, exophthalmia, ulcers, and watery-looking organs, particularly gallbladder rupture[6–8]. In addition, combined bacterial infection in fish is also common, such as infections found in tilapia caused by *Salmonella agalactiae* and *A. hydrophila*[9,10].

To reduce high mortalities of cultured fish, aquaculturists and researchers use antibiotics to prevent and treat infection. Nevertheless, due to concerns for maintaining eco-friendly environments, the application of antibiotics should be avoided, because they may enhance antibiotic-resistant pathogens, increase the accumulation of drugs in fish tissue and trigger immunosuppression[11]. Methods of controlling these infections should be developed as soon as possible because the pathogen disease type has significantly increased[12], while the type of pathogen that leads to edema in the cultivation area still cannot be overcome. One of the effective and safe methods for disease control in aquaculture is by improving the defence system of the fish through the provision of natural immunostimulants[13], through the use of several plant extracts.

Various plant extracts, such as Indian almond leaves (*Terminalia catappa*), oats (*Avena sativa*), oyster mushroom (*Pleurotus ostreatus*), nettle (*Urtica dioica*), sea grass (*Cymodocea serrulata*) and beetroot (*Beta vulgaris*) have been used as alternatives to antibiotics[5,14–16]. Plant extracts also contain levamisole[13] and saponin[17] which can enhance the work of nonspecific immune systems and increase the activation of phagocytosis[14]. Further, the plant extracts of *Boesenbergia pandurata* (BP) and *Zingiber zerumbet* (ZZ) from East Kalimantan have *in vitro* and *in vivo* antibacterial activity against *A. hydrophila* bacteria, while *Solanum ferox* (SF) has been found to be an antibacterial agent for *P. fluorescens* bacteria. Similarly, for the prevention and treatment of bacterial infections in tilapia, BP and ZZ are also effective for treating *A. hydrophila* and *P. fluorescens* infection[8,18].

The incorporation of some extracts for the prevention and treatment of bacterial infections is likely to increase the effectiveness because some materials can work synergistically, so that the infection of both bacteria in the fish body can be controlled optimally. However, research regarding the combination of plant extracts to treat and prevent bacterial infection is limited. This study therefore aims to determine the effectiveness of the combination of three extracts (BP, ZZ and SF) to prevent and treat bacterial infections of *A. hydrophila* and *P. fluorescens* in tilapia.

Methods

Fish and bacteria

In total, 450 Tilapias (Initial weight 15 ± 2 g, age ±2.5 months, random sex) were obtained from Teluk Dalam Village in Tenggarong Seberang, Kutai Kartanegara, Indonesia. The fish were randomly distributed and assigned into five aquariums in triplicate, representing four treatments and one control. The fish were kept in the laboratory for two weeks for acclimatization in the aquarium (60×40×30 cm). Each aquarium was filled with 60 l of freshwater and the water was changed by as much as 50% every 2 days to remove remaining faeces and inedible feed. The average temperature of the water was 27°C. The feed given in the acclimation phase was a commercial feed (PT Rama Jaya Mahakam, Kutai Kartanegara East Kalimantan-Supplier) at a rate of 5% of the body weight of the fish per day. The bacteria used for the challenge test were *A. hydrophila* (EA-01) and *P. fluorescens* (EP-01), which was provided from the Aquatic Microbiology Laboratory, Faculty of Fisheries and Marine Sciences, Mulawarman University, Indonesia. To bring about bacterial challenge, a combination of bacteria at density of 10^5 CFU ml^{-1} of each bacteria was used. Each fish was injected intramuscularly with 0.1 ml of the suspension of the bacteria.

Plant materials

The plant materials, BP, SF and ZZ, were collected from a traditional market in Samarinda City, East Kalimantan, Indonesia. The plants were cleaned, cut and dried at 40°C for 48 hours in the oven, finely powdered and stored at -4°C for the further extraction stage. Ethanol solution (95%) was used to extract the plant materials, following a method described by Limsuwan & Voravuthikunchai[19].

Experimental design and challenge test

This treatment and prevention trials were carried out for 28 days. The treatment experiments were conducted with five combination treatments with the following stages: tilapia (average initial weight 15 ± 2 g, n = 30 fish per group, random sex) were injected intramuscularly (0.1 ml) with a mixture of *A. hydrophila* and *P. fluorescens* bacteria, each bacteria at density10^5 CFU ml^{-1}. At day 7 after injection, the fish were fed with feed combined with extract as follows (ml per kg feed): P1, 60 ml SF extract/kg feed with 40 ml ZZ extract/kg feed (SF60/ZZ40); P2, SF50/ZZ50; P3, BP90/SF10; P4, BP50/SF50; and P5, fed with no additional extract (control). All fish were fed twice a day *ad satiation*. The remaining feed was siphoned out before the next feeding.

Meanwhile, the prevention trial was performed by providing the same feeding combination and procedure for 6 days prior to intramuscular injection of the fish with 0.1 ml of mixed bacteria at day 7. After injection, feeding combination was continued until the 4th week. Haematological and immunological parameters were measured every week after the injection with bacteria until week 4.

Haematology and phagocytic index

At days 14, 21 and 28 following bacterial challenge, haematological profiles of fish (n=3 per treatment group) were observed.

Fish were anesthetized using 50 mg l^{-1} MS 222 (Sigma Aldrich, USA) per dm^3 water. The fish blood was taken through the caudal vein, using a 1 ml syringe rinsed with 10% trisodium citrate anticoagulant (fish were kept alive after blood withdrawal). Total red blood cells (RBC) (10^6 per mm^3) and white blood cells (WBC) (10^3 per mm^3) were determined manually using an improved Neubauer counting chamber. The number of WBC was calculated using the method of Blaxhall and Daisley[20]. Haemoglobin (Hb) was measured spectrophotometrically at 540 nm using the cyanmethemoglobin method[17]. The haematocrit (Htc %) was counted using the microcentrifuge and heparinized was used as a standard solution. Meanwhile, phagocytic activity was determined using a modification of previous methods[20,21].

Antibody titres

To obtain serum, the fish blood was taken from the caudal veins and collected in an Eppendorf tube and centrifuged at 3,000 rpm for 3 minutes. Serum was then incubated at 44°C for 20 min to activate the complement[22]. Serum was stored in the refrigerator at 4°C for the next antibody titre observation. Measurements of antibody titres were performed using 25 µl PBS and inserted into microplate at holes 1 to 12, with the serum being inserted into hole 1 (25 µl) and then diluted into 11 holes. A total of 25 µl of bacteria (*A. hydrophila* and *P. fluorescens*) were inserted into holes 1 to 12, the mixture homogenized, and stored for 2 hours in an incubator at 37°C, followed by storing at 4°C overnight in a refrigerator. For analysis, observing the antibody titre was carried out, indicated by the agglutination reaction in the last hole.

Respiratory burst and lysozyme activity

Respiratory burst activity test was performed using nitro blue tetrazolium (NBT) reagent, using the method outlined by Secombes and Olivier[23]. Meanwhile, lysozyme activity was performed using a microtiter plate ELISA reader at wavelength of 520 nm, following the method described by Soltani and Pourgholam[24].

Disease resistance

Both *A. hydrophila* and *P. fluorescens* (the pathogenic bacteria) were used for challenge testing (n = 10 fish per aquarium, in triplicates per group). The survival rate (SR) and relative percent survival (RPS) of the fish were recorded on a daily basis for 4 weeks[25].

Statistical analysis

Results are expressed as means ± standard error (SE) and the data were analysed using SPSS version 22 (SPSS, Inc., USA). The data of WBC, RBC, haematocrit, Hb, TPC, phagocytic index, respiratory burst and lysozyme activity were subjected to ANOVA, followed by Duncan's post hoc test to evaluate significant differences among the groups of treatments. The percentage of fish survival were arcsine-transformed. All tests were significant at $P < 0.05$.

Results

Haematological profile

The present results revealed that the total WBC count of tilapia in the treatment and prevention trials were significantly increased ($P<0.05$) from weeks 2–4 post-administration with combined extracts. The highest increase of WBC was found in tilapia fed with SF50/ZZ50. Similarly, total RBC and haematocrit of tilapia fed SF50/ZZ50 in the treatment trial showed a significant increase after week 2, while tilapia fed SF60/ZZ40 in the prevention trial led to a positively enhanced result from weeks 2–4. Further, haemoglobin of fish both in treatment and prevention trials were not affected by any various combination of extracts (Table 1).

Phagocytic index

All combination extracts fed to fish in the treatment (Figure 1) and prevention (Figure 2) trials increased the phagocytic index. The phagocytic index of fish fed SF50/ZZ50 in the diet, in both in treatment and prevention trials, were significantly higher than control and increased from the 2nd to 4th week of the post-challenge test.

Respiratory burst

The respiratory burst activity of infected fish fed with combination extract increased from week 2 to week 4 in the treatment trial (Figure 3). In addition, SF50/ZZ50 (ml per kg feed) combination extract resulted in a significantly different respiratory burst to other combinations of extracts and the control. Meanwhile, in the prevention test, infected fish fed SF50: ZZ50 combination extract in week 4 were significantly higher than control and other combinations of extracts ($P<0.05$) (Figure 4).

Lysozyme activity

This study revealed that lysozyme activity of infected tilapia fed SF60/ZZ40, BP90/SF10 and BP50/SF50 combinations of extract did not show a significant increase ($P<0.05$) at weeks 2 and 4 in the treatment test. However, starting from weeks 2–4, the addition of SF50/ZZ50 combination extract in the diet of fish resulted in significantly better lysozyme activity (Figure 5). Meanwhile, in the prevention test at weeks 2 and 4, the lysozyme activity of tilapia fed SF50/ZZ50 was significantly higher ($P<0.05$) (Figure 6) than in other combinations.

TPC

The overall combination of extracts administered to treat and prevent infection by *A. hydrophila* and *P. fluorescens* may decrease the number of bacteria in the fish body until the 4th week of observation (Table 2). The bacterial density, in both the treatment and prevention trials was lower than in the control. Total bacteria *of A. hydrophila* and *P. fluorescens* in tilapia fish fed combination extract in the treatment trial decreased from weeks 2–4. Further, the lowest bacterial density in tilapia was obtained from the fish fed SF 50/ZZ 50 combination extracts in their diet, which was also significantly different ($P<0.05$) compared to the control.

Survival rate

The administration of extract with different combinations on tilapia injected with *A. hydrophila* and *P. fluorescens* bacteria increased the SR and RPS when compared to those not given the extracts (Table 3 and Table 4). The percentage of survival of tilapia in treatment and prevention trials with combination extracts of SF 50: ZZ 50 had the highest SR compared to the other combinations of extract.

Table 1. Hematological profile of Tilapia (*Oreochromis niloticus*) fed different extract combinations in treatment and prevention trials.

Variables	Trials	Groups	Weeks			
			1	2	3	4
WBC (10^4 cell/mm^3)	Treatment	SF60/ZZ40	1.53±0.1^a	1.68±0.1^a	1.62±0.1^a	2.07±0.2^b
		SF50/ZZ50	2.55±0.02^{ab}	3.60±0.1^b	3.90±0.2^b	8.85±0.2^c
		BP90/SF10	1.87±0.2^{ac}	1.88±0.5^a	2.00±0.1^a	2.10±0.1^a
		BP50/SF50	1.98±0.5^{ac}	1.89±0.5^a	2.02±0.2^a	2.20±0.1^b
		No extract	1.35±0.3^a	1.35±0.2^a	1.32±0.2^a	1.34±0.1^a
	Prevention	SF 60/ZZ 40	1.5±0.5^a	1.8±0.15^a	1.7±0.2^a	2.4±0.5^b
		SF 50/ZZ 50	2.8±0.3^a	3.9±0.2^b	4.0±0.1^b	7.9±0.2^c
		BP 90/SF 10	1.8±0.15^a	2.0±0.2^a	2.4±0.1^b	2.4±0.3^b
		BP 50/SF 50	2.0±0.25^a	2.0±0.3^a	2.2±0.2^a	2.5±0.1^b
		No extract	1.4±0.1^a	1.4±0.5^a	1.3±0.3^a	1.3±0.1^a
RBC (10^6cell/mm^3)	Treatment	SF 60/ZZ 40	6.9±0.1^a	5.4±0.3^a	4.2±0.2^b	6.0±0.1^b
		SF 50/ZZ 50	6.9±0.1^a	7.7±0.2^b	8.8±0.1^c	8.9±0.2^c
		BP 90/SF 10	5.1±0.2^a	6.3±0.1^b	7.2±0.1^c	5.4±0.2^a
		BP 50/SF 50	5.5±0.11^a	6.8±0.2^a	7.0±0.1^b	7.7±0.1^b
		No extract	2.4±0.3^a	2.7±0.1^a	2.7±0.2^a	2.4±0.1^a
	Prevention	SF 60/ZZ 40	6.9±0.15^a	6.4±0.25^a	5.2±0.2^b	6.0±0.5^b
		SF 50/ZZ 50	6.9±0.2^a	7.0±0.3^a	7.2±0.1^a	7.0±0.2^a
		BP 90/SF 10	5.1±0.1^a	6.2±0.1^a	5.5±0.1^a	5.4±0.2^a
		BP 50/SF 50	5.5±0.1^a	5.6±0.1^a	7.0±0.1^a	7.0±0.1^a
		No extract	2.4±0.2^a	2.7±0.2^a	2.7±0.2^a	2.4±0.1^a
Haematocrit (%)	Treatment	SF 60/ZZ 40	27±0.1^a	27±0.1^a	30±0.1^a	33±0.1^b
		SF 50/ZZ 50	22,5±0.5^a	27±0.2^b	33±0.1^c	36±0.2^c
		BP 90/SF 10	25,5±0.5^a	27±0.2^a	30±0.1^a	31±0.2^a
		BP 50/SF 50	27±0.2^a	27±0.2^a	27±0.2^a	30±0.2^a
		No extract	18±0.2^a	15±0.3^a	18±0.1^a	15±0.2^a
	Prevention	SF 60/ZZ 40	30±0.5^a	27±0.1^a	27±0.1^a	28±0.1^a
		SF 50/ZZ 50	22,5±0.1^a	27±0.2^a	33±0.2^a	33±0.1^a
		BP 90/SF 10	25,5±0.1^a	27±0.1^a	30±0.2^a	31,5±0.1^a
		BP 50/SF 50	27±0.2^a	27±0.1^a	27±0.1^a	30±0.2^a
		No extract	18±0.1^a	15±0.2^a	18±0.1^a	15±0.1^a
Haemoglobin (g dl^{-1})	Treatment	SF 60/ZZ 40	8±0.1^a	10±0.3^a	10±0.1^a	10±0.1^a
		SF 50/ZZ 50	10±0.2^a	10±0.3^a	10±0.2^a	10±0.1^a
		BP 90/SF 10	8±0.11^a	8±0.2^a	10±0.2^a	10±0.1^a
		BP 50/SF 50	8±0.1^a	8±0.2^a	8±0.2^a	10±0.1^a
		No extract	6.3±0.5^a	8±0.1^a	6±0.2^a	6±0.2^a
	Prevention	SF 60/ZZ 40	8±0.2^a	10±0.1^a	8±0.2^a	8±0.2^a
		SF 50/ZZ 50	10±0.2^a	10±0.1^a	8±0.2^a	10±0.1^a
		BP 90/SF 10	8±0.2^a	10±0.1^a	8±0.1^a	8±0.1^a
		BP 50/SF 50	8±0.2^a	8±0.1^a	8±0.1^a	8±0.2^a
		No extract	6.3±0.2^a	8±0.1^a	6±0.1^a	4±0.1^a

Data shown as mean±standard deviation. Different superscript letters (a,b,c) in the same column in each variable and each treatment or prevention trial showed significantly different at $P<0.05$. WBC, white blood cells; RBC, red blood cells; BP, *Boesenbergia pandurate*; SF, *Solanum ferox*; ZZ, *Zingiber zerumbet*; SF60, 60 ml SF extract/kg feed.

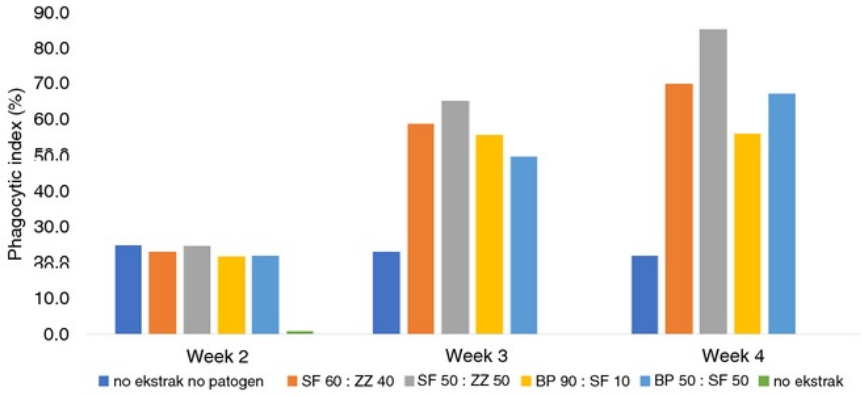

Figure 1. Phagocytic index (%) of Tilapia (*Oreochromis niloticus*) fed different extract combination in treatment trials. BP, *Boesenbergia pandurate*; SF, *Solanum ferox*; ZZ, *Zingiber zerumbet*.

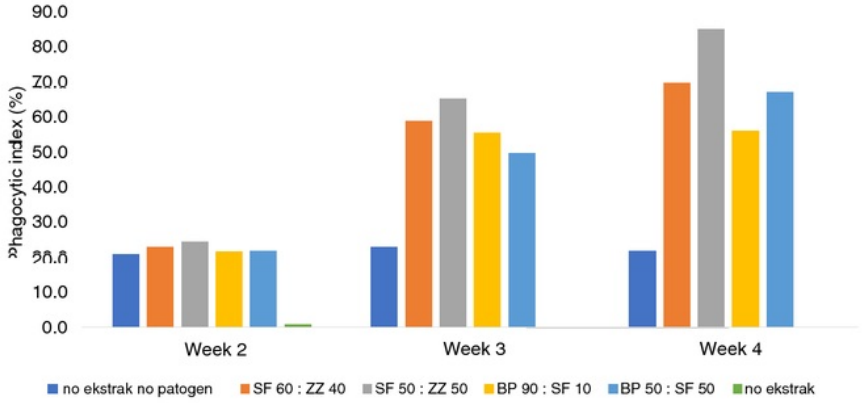

Figure 2. Phagocytic index (%) of Tilapia (*Oreochromis niloticus*) fed different extract combination in prevention trials. BP, *Boesenbergia pandurate*; SF, *Solanum ferox*; ZZ, *Zingiber zerumbet*.

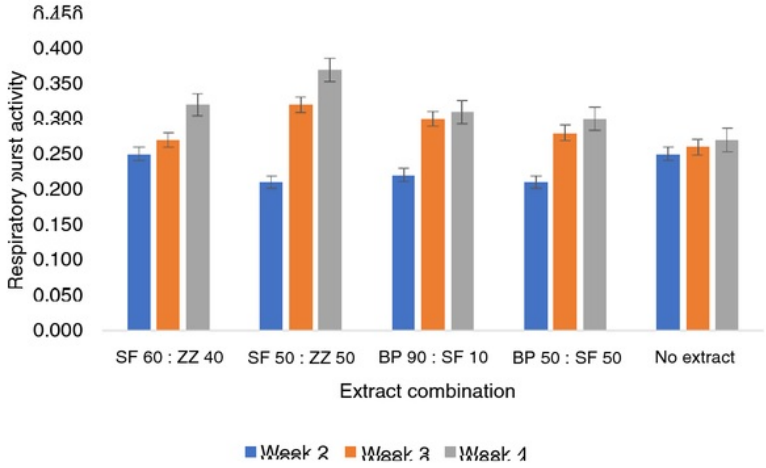

Figure 3. Respiratory burst activity of Tilapia (*Oreochromis niloticus*) fed different extract combination in treatment trials. BP, *Boesenbergia pandurate*; SF, *Solanum ferox*; ZZ, *Zingiber zerumbet*.

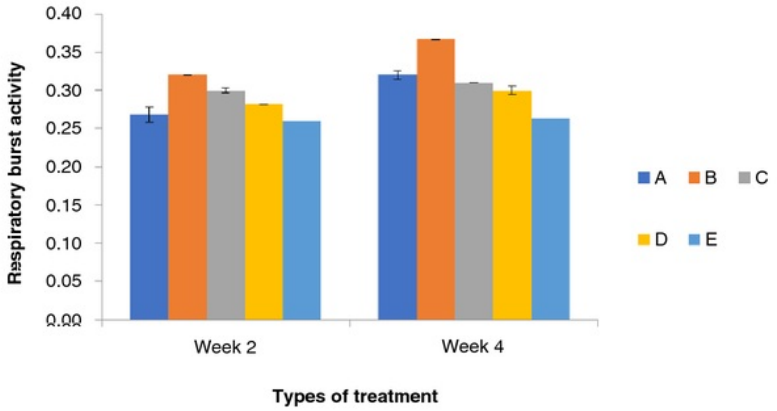

Figure 4. Respiratory burst activity of Tilapia (*Oreochromis niloticus*) fed different extract combination in prevention trials. BP, *Boesenbergia pandurate*; SF, *Solanum ferox*; ZZ, *Zingiber zerumbet*.

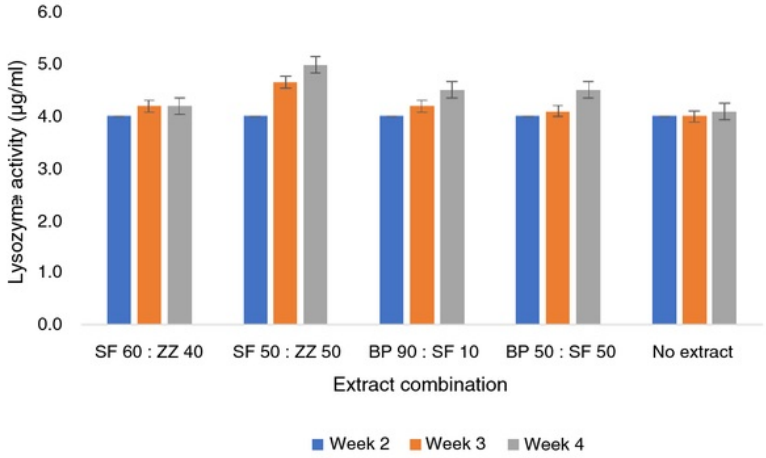

Figure 5. Lysozyme activity (µg ml^{-1}) of Tilapia (*Oreochromis niloticus*) fed different extract combination in treatment trials. BP, *Boesenbergia pandurate*; SF, *Solanum ferox*; ZZ, *Zingiber zerumbet*.

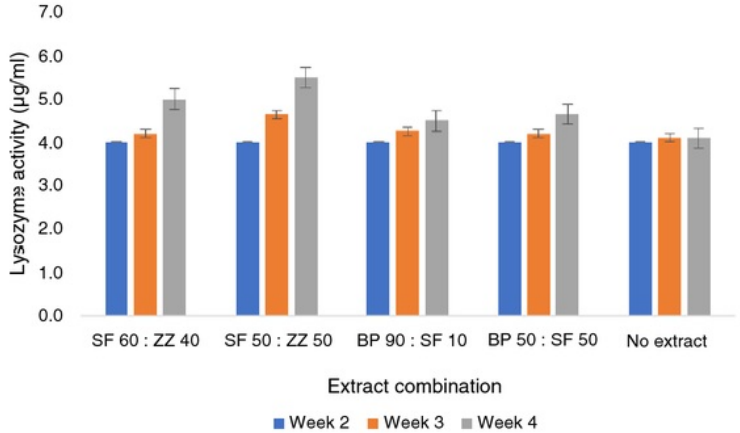

Figure 6. Lysozyme activity (µg ml^{-1}) of Tilapia (*Oreochromis niloticus*) fed different extract combination in prevention trials. BP, *Boesenbergia pandurate*; SF, *Solanum ferox*; ZZ, *Zingiber zerumbet*.

Table 2. Total plate count (10^6 CFU/ml) in Tilapia (*Oreochromis niloticus*) fed different extract combinations in treatment and prevention trials.

Trials	Groups	Week	
		2	4
Treatment	SF 60/ZZ 40	17.4 ± 5^{a}	10.86 ± 10^{c}
	SF 50/ZZ 50	22.4 ± 15^{a}	3.05 ± 10^{d}
	BP 90/SF 10	55 ± 10^{b}	16 ± 15^{c}
	BP 50/SF 50	47 ± 10^{b}	4.82 ± 10^{d}
	No extract	42.85 ± 15^{b}	30.9 ± 5^{b}
Prevention	SF 60/ZZ 40	78 ± 15^{a}	56 ± 15^{a}
	SF 50/ZZ 50	100 ± 5^{b}	32 ± 10^{d}
	BP 90/SF 10	65 ± 10^{a}	46 ± 5^{d}
	BP 50/SF 50	147 ± 11^{c}	85 ± 10^{a}
	No extract	157 ± 11^{c}	110 ± 10^{c}

Data shown as mean±standard deviation. Different superscript letters (a,b,c,d) in the same column in each treatment or prevention trial showed significantly different at $P<0.05$. BP, *Boesenbergia pandurate*; SF, *Solanum ferox*; ZZ, *Zingiber zerumbet*; SF60, 60 ml SF extract/kg feed.

Table 3. Survival rate (%) of Tilapia (*Oreochromis niloticus*) fed different extract combinations in treatment and prevention trials.

Trials	Groups	Week	
		2	4
Treatment	SF 60/ZZ 40	78.00^{a}	78.00^{a}
	SF 50/ZZ 50	90.00^{a}	86.00^{a}
	BP 90/SF 10	$83,30^{a}$	77.00^{a}
	BP 50/SF 50	86.00^{a}	85.00^{a}
	No extract	45.00^{b}	33.30^{b}
Prevention	SF 60/ZZ 40	85.00^{a}	75.00^{a}
	SF 50/ZZ 50	100.00^{b}	90.00^{b}
	BP 90/SF 10	90.00^{b}	80.00^{a}
	BP 50/SF 50	85.00^{a}	75.00^{a}
	No extract	45.00^{c}	29.00^{c}

Data shown as mean±standard deviation. Different superscript letters (a,b,c) in the same column in each treatment or prevention trial indicate significant differences at $P<0.05$. BP, *Boesenbergia pandurate*; SF, *Solanum ferox*; ZZ, *Zingiber zerumbet*; SF60, 60 ml SF extract/kg feed.

Table 4. Relative Percent Survival (RPS) of Tilapia (*Oreochromis niloticus*) fed different extract combinations in treatment and prevention trials.

Trials	Groups	Week	
		2	4
Treatment	SF 60/ZZ 40	60^{a}	67^{a}
	SF 50/ZZ 50	82^{b}	79^{b}
	BP 90/SF 10	70^{a}	66^{a}
	BP 50/SF 50	75^{b}	78^{b}
	No extract		
Prevention	SF 60/ZZ 40	73^{a}	65^{a}
	SF 50/ZZ 50	100^{b}	86^{b}
	BP 90/SF 10	82^{a}	72^{a}
	BP 50/SF 50	73^{a}	65^{a}
	No extract		

Data shown as mean±standard deviation. Different superscript letters (a,b,c) in the same column in each treatment or prevention trial indicate significant differences at $P<0.05$. BP, *Boesenbergia pandurate*; SF, *Solanum ferox*; ZZ, *Zingiber zerumbet*; SF60, 60 ml SF extract/kg feed.

Discussion

The number of infectious diseases caused by pathogenic bacteria such as *A. hydrophila* have become a pivotal concern in fish culture, causing high economic losses owing to high mortality rates[5]. The use of plant-based extracts as immunodulators has been applied to increase survival and immune system of fish to prevent or cure bacterial pathogen. Several plant extracts that contain active phytochemicals have been found and used as supplements in the feed of fish[25–28].

The current study found that the WBC of tilapia infected by both bacteria in the prevention and treatment trials increased significantly ($P<0.05$), while the RBC of tilapia infected by both bacteria in the prevention and treatment trials decreased significantly ($P<0.05$). This result is similar to those of a previous study, which stated that the WBC increased in order to tackle the infection, while the RBC was decreased in tilapia infected with *Streptococcus agalactiae* bacteria[29], *S. iniae*[10], *A. hydrophila* and *Pseudomonas* sp.[7]. In contrast, tilapia fed with a combination of extracts SF60/ZZ40 showed a similar RBC value both in treatment and prevention trials. In addition, tilapia fed SF50/ZZ50 in treatment trial resulted the highest RBC at the end of the trial. The Hb and Htc values were unchanged during the first week of all treatments including control; the decrease in Htc and Hb values occurred in controls without extract from weeks 2–4 post-infection in the prevention and treatment trials. This result indicated that the combined administration of the extracts was capable of improving the performance of the fish immune system by producing more WBC, thus making the fish more able to suppress the growth of bacteria in the body.

RBC, WBC, Hb and Htc can be used as an indicator of the blood profile in fish with respect to the innate immune defence and regulation of immunological function[30]. WBC are particularly responsible for providing protection or resistance to disorders caused by infectious pathogens and non-infectious factors (nutrition, temperature and handling)[31]. Total value of WBC also

describes the health status and immune system of the fish. In addition to haematological statues, the Hb content decreases due to RBC swelling and poor Hb mobilization of the spleen and other haematopoesis organs[32].

Besides blood profiles, the phagocytic index, respiratory burst and lysozyme activity are good indicators for immunological status of fish during infection periods. The present results revealed that infected fish treated with a compound extract of SF50/ZZ50 showed the highest IP and increased from weeks 2–4 post-injection. These results are in line with the results of a previous study, which found that fish treated with immunostimulants usually show enhanced phagocytic cell activities[33]. Fish have several types of phagocytic leukocytes, which are part of WBC, in the peritoneal cavity, and various tissues. The phagocytic activity is also associated with the production of oxygen free radicals by using respiratory bursts, which are important events in bactericidal pathways in fish[34,35]. In addition, Secombes and Olivier[23] revealed that the release of superoxide anions, hydrogen peroxide and hypochlorous acid into the phagosome and extracellular space during the respiratory burst can be considered the pivotal mechanisms involved in the bactericidal activity of macrophages.

Total lysozyme level is a tool to measure the humoral component of the non-specific defence mechanism (innate immunity), which can be used to detect infections or injections of foreign material, including bacteria[36–38]. The present findings determined that tilapia fed SF 50: ZZ 50 had significantly higher ($P<0.05$) lysozyme activity. This finding is in line with past research, stating that the lysozyme activity of Jian carp (*Cyprinus carpio* var. Jian)[39] and large yellow croaker, *Pseudosciaena crocea*[40] were increased after being fed with traditional Chinese medicine formulated from Astragalus root (*Radix astragalin seu Heydsari*) and Chinese Angelica root (*R. angelicae Sinenesis*).

Conclusion

A combination of plant extracts was found to affect the health status of tilapia when compared with control. A combination of extracts of SF and ZZ (50:50) provides the optimum protection against bacterial infections of *A. hydrophila* and *P. fluorescens* in both prevention and treatment assays.

Data availability

Raw data for Tables and Figures can be accessed on OSF, DOI: https://doi.org/10.17605/OSF.IO/A42JB[41].

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

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Open Peer Review

Current Referee Status: ? ?

Version 1

Referee Report 13 December 2018

https://doi.org/10.5256/f1000research.18481.r41178

? **Alim Isnansetyo**
Department of Fisheries, Faculty of Agriculture, Gadjah Mada University, Yogyakarta, Indonesia

This article presented the finding of Indonesian herbal extracts for preventing and treating fish diseases. This article might be indexed after several major issues are addressed:

1. Are *Boesenbergia pandurata* (BP), *Solanum ferox* (SF) and *Zingiber Zerumbet* (ZZ) typical plants in Borneo? Are the plants not found in the other parts of Indonesia? If yes, please replace the "Borneo" with "Indonesia" in the title and throughout the article.

2. Write one sentence of background in the Abstract.

3. Describe systematically in the Abstract: how to prepare the extracts, design experiment, feed preparation, infection, data collecting (hematology, non-specific immune etc.) and data analysis.

4. Write the exact concentration for the extract in mg/kg feed instead of ml/kg. Using units of ml/kg feed is not appropriate as the exact concentrations are not known.

5. Write systematically the results in the Abstract as described in the Methods.

6. The units are written inconsistently: format (.../...., ... per ...., ... $....^{-1}$).

7. "Antibody titre" is a term to evaluate the effect of vaccines. To evaluate the effect of immunostimulants, we should use the term "Natural Agglutination" as we are not evaluating the specific antibody. No data are presented for Antibody titre/Natural Agglutination, even though this parameter is described in the Methods.

8. The authors are confused by the terms of phagocytic activity and phagocytic index. Phagocytic index is not described before either in the Abstract or Materials and Methods. However, the authors describe phagocytic activity in Materials and Methods. Phagocytic index and phagocytic activity are two different parameters. Please refer to some of the recommended references. Add deviation standard for each bar in all graphs.

9. Add the notation in each bar of all graphs and values in tables to show insignificant or significant difference based on DMRT test results.

10. Discussion: please interpret properly and add additional explanation about why the extracts affect the immune system of fish and increase the SR and RPS. Describe the possible constituents

in the extracts by citing the previous publications.

11. Add these references in the Introduction, Materials and Methods, and Discussion: Yudiati *et al.* (2016[1]), Isnansetyo *et al.* (2016[2]) and Isnansetyo *et al.* (2015[3]).

12. Some grammatical errors were found.

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunology, microbiology, fish diseases, natural products

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 29 Jan 2019

Rudy Nugroho, Mulawarman University, Indonesia

Dear Dr. Alim Isnansetyo,

Thank you for your valuable comment on our article. We have added some important information based on your comment and suggestions. We really appreciate it. For further details of our responses to your comment, please download the details of our responses from this link below: https://osf.io/qwy8z/download [direct download link]

Competing Interests: No competing interests

Referee Report 28 November 2018

https://doi.org/10.5256/f1000research.18481.r41177

Vishnu K. Venugopal
Centre for Marine Living Resources & Ecology, Kochi, Kerala , India

After checking the research article "Borneo herbal plant extracts as a natural medication for prophylaxis and treatment of Aeromonas hydrophila and Pseudomonas fluorescens infection in tilapia (Oreochromis niloticus)" by Dr. Rudy *et al.*, I reached the following suggestions to be made for the article's acceptance:

1. The overall structure of the manuscript is satisfactory, though some changes are recommended.
2. In the introduction the mechanism of action of plant extracts and its medical importance could have been added.
3. The authors didn't mention the composition of feed.
4. There is a possibility of residual ethanol in the sample. How can you conclude the results with this concern?
5. The nature and source of chemicals (Materials) used in this experiment are not mentioned.
6. Footnotes can be much clearer and the legends used in the figure should be mentioned properly. Also, in some graphs standard deviation is missing.
7. Give enough information about the figures in figure legends.
8. The Discussion part can be much stronger.

In conclusion, the content of the manuscript has value for indexing. The mentioned suggestions can be considered and resubmitted.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Clinical biochemistry, Lipid chemistry, Bioactive compounds characterization

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 29 Jan 2019

Rudy Nugroho, Mulawarman University, Indonesia

Dear Dr. Vishnu Venugopal,

First of all, we would like to say thank you for your valuable review and comment. We have revised our article according to your review. We have also made some responses to your review - for the details of our responses, please see the link below:
http://osf.io/vzsqe/download [direct download link]

Competing Interests: No competing interests

Comments on this article

Version 1

Author Response 13 Dec 2018

Rudy Nugroho, Mulawarman University, Indonesia

Dear Dr. Angela Lusiastuti,

Thank you for your valuable comments. We will improve our article.

Competing Interests: No competing interests

Reviewer Response 10 Dec 2018

Angela Lusiastuti, Research Institute for Freshwater Aquaculture and Fisheries Extension, Indonesia

After reading and checking the manuscript, I cannot find in the Methods the Total Plate Count (TPC) procedure and what kind of sample was used for the TPC. Please add it.

In the Methods, it was shown that the antibody titres were measured, however I cannot find the antibody titre in the Results and Discussion as well. Please add it.

Please add in the Discussion how the active content of Borneo herbs plant extracts acts to prevent and as a therapy for bacterial infection.

Competing Interests: No competing interests were disclosed.

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