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The comparative studies of Borneo plant extracts to increases vaccine efficacy in tilapia, *Oreochromis niloticus*

Studi perbandingan beberapa ekstrak tumbuhan dari Kalimantan Timur untuk meningkatkan efikasi vaksin pada ikan nila, *Oreochromis niloticus*

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ABSTRACT

This study investigated the adjuvant effect of *Boesenbergia pandurata* (BP), *Zingiber zerumbet* (ZZ), *Solanum ferox* (SF) on protection of tilapia with injection *Pseudomonas* sp. (Pseumulvacc) vaccination. The extract concentrations of BP (600 mg/L), ZZ (200 mg/L), and SF (900 mg/L) were combined with the vaccine, ratio between vaccine and extract was 1:1. Tilapia fish were injected with vaccine mix the extract and challenged at day 7 (d7), 14 (d14), and 21 (d21) post vaccination through injection with *A. hydrophila* and *P. fluorescens* (105 CFU/mL each bacteria). The results shown that the fish with BP+V were found in fin rot at d14 days challenge. The same symptom was found in ZZ+V at d14 challenge as much 11.11% and 42.86% while, in the vaccine groups (V), after the challenge, tilapia were found fin rot and darkness color until the last experiment. The BP+V and SF+ZZ+V groups shown reducing the number of bacteria in the fish body after challenge test on d7, d14, and d21. The efficacy of Pseumulvacc vaccine has increased after its administration with BP (BP+V) on day 7 and day 14 after challenge (90%) and 100% at the time of challenge test d21. The conclusion is *B. pandurata* extract was a promising adjuvant candidate, and the extract is the best plants as an adjuvant that mixed with the vaccine to against *A. hydrophila* and *P. fluorescens* infection.

Keywords: Adjuvant, plant extract, vaccine, fish pathogen bacteria

ABSTRAK

Penelitian ini bertujuan mengevaluasi efek adjuvan dari ekstrak tanaman temu kunci (*Boesenbergia pandurata*/BP), lempuyang (*Zingiber zerumbet*/ZZ), dan terung asam (*Solanum ferox*/SF) pada ikan nila yang diberikan bersama vaksin bakteri *Pseudomonas* sp. (Pseumulvacc). Dosis yang digunakan yaitu ekstrak BP 600 mg/L, ZZ 200 mg/L, dan SF 900 mg/L, dengan rasio antara vaksin dan ekstrak 1:1. Pengujian diawali dengan menginjeksi ikan nila dengan campuran vaksin dan ekstrak tanaman, dilanjutkan dengan uji tantang pada hari 7 (d7), 14 (d14) dan 21 (d21) pascavaksinasi dengan bakteri *A. hydrophila* dan *P. fluorescens* (kepadatan masing-masing 105 CFU/mL). Hasil pengujian menunjukkan ikan yang divaksin dengan penambahan ekstrak BP (BP+V) mengalami sirip gripis pada uji tantang hari ke-14, gejala serupa juga ditemukan pada pemberian vaksin yang dicampur dengan ZZ (ZZ+V) pada waktu uji tantang d14, sebesar 11,11 % dan 42,86%. Sedangkan, yang divaksin tanpa campuran ekstrak (V) mengalami sirip gripis dan menghitam pada uji tantang d14. Perlakuan BP+V dan SF+ZZ+V mampu mengurangi jumlah bakteri di dalam tubuh ikan nila pasca uji tantang d7, d14 dan d21, dan jumlahnya lebih rendah dibandingkan dengan perlakuan lain. Efikasi vaksin Pseumulvacc mengalami peningkatan pada BP+V pada hari 7 dan hari 14 pasca ujitantang (90%) dan 100% pada waktu uji tantang d21. Semakin lama uji tantang (d21), tingkat perlindungan vaksin plus ekstrak makin tinggi dibandingkan dengan hari d7 dan d14. Ekstrak *B. pandurata* adalah tanaman terbaik sebagai adjuvan untuk penanggulangan infeksi bakteri *A. hydrophila* dan *P. fluorescens.*

Kata kunci: Adjuvan, ekstrak tanaman, vaksin, patogen pada ikan

INTRODUCTION

The cultivation of tilapia grows rapidly, followed by the used for its rich source of protein (Wang *et al*., 2016), excellent gelatin source taken from the bone (Alfaro *et al*., 2013), or skin and scales (Jamilah & Harvinder, 2002). It triggers the increase of intensive aquaculture production, which leads to the increasing number of mortality caused by pathogens, disruption of cultivated land sustainability, and the slowing growth (Kibenge *et al*., 2012).

Vaccination is a more environmental friendly in pathogen prevention technology compared to the application of antibiotics and other drugs (Sommerset *et al*., 2005; Rodger, 2016; Sukenda *et al.,* 2018). In general, there are three methods for immunostimulant or vaccine application in fish include injection, immersion, and feed. Pasnik *et al.* (2005); Hardi *et al*. (2013); and Evensen (2016) stated that vaccination through intraperitoneal injection (IP) has a better efficacy rate than two other methods.

Giving the vaccine in combination with oil-based adjuvants would potentially improve vaccine efficacy in fish. Some of the adjuvant materials used in fish include Freund's adjuvants used with fish vaccine (Bøgwald & Dalmo, 2012; Dalmo *et al*., 2016), EseD a Putative T3SS (*Edwardsiella tarda* has a type III secretion system) (Wang *et al*., 2010; Jiao *et al*., 2010b); *Streptococcus agalactiae* and *Aeromonas hydrophila* (Pasaribu *et al*., 2018). In addition, the use of other adjuvant mineral oils reported being effective in increasing the performance of *Moritella viscosa* vaccine and *Aeromonas salmonicida* in salmon (Mutoloki *et al*., 2010). Furthermore, non-oil material which also acts as adjuvant is aluminum salts (Jiao *et al.,* 2010a), β-Glucans (Dalmo & Bøgwald, 2008; Mizel & Bates 2010); plant extract *Quillaja saponaria* saponin (Wang *et al*., 2016); a combination of *Ocimum sanctum* plant extract (Tulsi), *Withania somnifera* (Ashwagandha), *Tinospora cordifolia* (Guduchi), and *Emblica officianalis* (Amlaki) (Priyadarshini *et al*., 2012).

Saponin is steroids and terpenoid glycosides produced by several species of plants (Song, 2009; Sun *et al*., 2009; Tafalla *et al.,* 2013) and could serve as specific adjuvants (Dalmo *et al*., 2016). This material has immunostimulant capabilities for animals including fish; is able to increase macrophage cell phagocytosis activity, antibody production, and produce cytotoxic T-lymphocytes (CTLs) which can inhibit exogenous antigen (Zhang *et al.,* 2007; Xie *et al*., 2008; Tam & Roner, 2011;). According to Freitas *et al.* (2006) and Wang *et al.* (2016), the saponins from the *Q. saponaria* plant are non-toxic and capable of enhancing the specific immune system and tend to be immune protective in mixing with the *Leishmania donovani* vaccine. Saponin testing as an adjuvant has also been performed by Wang *et al.* (2016), the result indicated that the *Vibrio anguillarum* vaccine combined with *Q. saponaria* could enhance humoral antibody responses and increases the protective level of turbot (*Scophthalmus maximus*) fish after bacterial infection.

The Borneo plant extract of *Boesenbergia pandurata* (BP), *Solanum ferox* (SF), and *Zingiber zerumbet* (ZZ) are plants which easily grown in the yard and used as herbs plants by communities in east Kalimantan (Borneo). Based on previous research, that plant extracts containing saponins could serve as immunostimulants for tilapia (Hardi *et al*., 2016a,b and Hardi *et al*., 2017a,b). This study aimed to obtain information and test the associated potential adjuvant of *B. pandurata* extract, *S. ferox,* and *Z. zerumbet* in improving the efficacy of Pseumulvacc vaccine in tilapia.

MATERIALS AND METHODS

Experimental fish

The experimental fish sized about 15 g was tilapia originated from Seed Fishery Seedling Center Sebulu, Kutai Kartanegara District, which previously quarantined and isolated to ensure *A. hydrohila* and *P. fluorescens* free. The experimental fish was checked with *A. hydrophila* and *P. fluorescens* through isolating the gill and kidney in GSP media then incubated at 30°C for 18–21 hours. If the bacteria was not growth, the fish was ready to be used for this study, otherwise, if the bacteria was growth, the fish was immersed with formaline solutions 3% for five minutes (Kent *et al*., 2009).

Plant material extraction

Boesenbergia pandurata, *Z. zerumbet,* and *S. ferox* herbal materials were collected from the traditional market in Samarinda. The extraction process carried out using Limsuwan and Voravuthikunchai *et al.* (2008) and Hardi *et al*. (2016a) methods. The first step in extraction methods was cutting the plants into smaller pieces, oven-dried for four days or until dry and the air-dried plant samples were mashed using a blender. The dried samples were soaked in ethanol (96%) at room temperature with the ratio 1:1 for

48 hours. The extract solution was filtered with Whatman® filtration paper and the filtered sample was centrifuged for 24 hours at 50 rpm to obtain a crude extract. The last step was to keep the crude extract in the oven (30–40°C) until the ethanol lost from the extract, then the extract were kept in the refrigerator at -4°C until used. The extract concentrations used were 600 mg/L *B. pandurata*, 900 mg/L *S. ferox* and 200 mg/L *Z. zerumbet* (Sun *et al*., 2016a,b and Hardi *et al*., 2017a,b). The dose was achieved by diluting it using a sterile distilled water.

Bacteria test

Aeromonas hydrophila (EA-01) and *P. fluorescens* (EP-01) used as a bacterial test challenge, and *Pseudomonas* sp. as a bacterial vaccine came from the Microbiology Laboratory, Faculty of Fisheries and Marine Science, Mulawarman University, Indonesia. Bacteria were grown in the media brain heart infusion broth (BHIB DIFCO®) and brain heart infusion agar (BHIA, DIFCO®) for 24 hours at 30°C.

The vaccine (Pseumulvacc) for *Pseudomonas* sp. bacteria was produced by inactivating using 3% formalin for 24 hours. The density of bacteria vaccine used was 10^4 CFU / mL (Hardi *et al.,* 2014b).

The bacteria for the challenge test used were a combination of *A. hydrophila* and *P. fluorescens* 1:1 ratio with the density of each bacterium 10^5 CFU/mL (Hardi *et al.,* 2017a). The administration was intramuscularly injected of 0.1 mL/fish (Hardi *et al*., 2014a).

Effectiveness test of *B. pandurata*, *S. ferox,* and *Z. zerumbet* as an adjuvant of Pseumulvacc vaccine

Experiment test was conducted by mixing each extract with a vaccine ratio 1:1. This experiment consisted of 6 groups: (1) extract of *B. pandurata* mix with vaccine (BP + V); (2) *Solanum ferox* extract mix with vaccine (SF + V); (3) *Zingiber zerumbet* extract mix with vaccine (ZZ + V); (4) concoction *S. ferox* and *Z. zerumbet* mix with vaccine (SF + ZZ + V); (5) Vaccine without extract (V); (6) control group. This extract that using as an adjuvant in this research based on the pre-research, the concoction *S. ferox,* and *Z. zerumbet* was the best extract as an immunomodulatory extract than other concoction (*B. pandurata* and *S. ferox* or *B. pandurata* and *Z. zerumbet*).

Vaccine plus extract was injected to fish by intraperitoneal injection as many of 0.1 mL/fish. Challenge test was conducted on days 7, 14, and 21 post vaccination. The observations parameters were the percentage of fish undergoing changes in the anatomy of the external organs and internal organs, the total number of bacteria in the body of the tilapia fish, the total leucocyte, antibody titers, phagocytic index, and cumulative of mortality and RPS (relative percent survival).

Fish pathology anatomy

This parameter was done to evaluate the percentage of fish pathology anatomy post vaccination and challenge test. The observed fish pathology anatomy were fin rot, body darkness, and exophthalmia. The percentage of fish pathology anatomy was calculated according to Hardi *et al.* (2017 a,b) formula.

$$\%\ \text{Fish pathology anatomy} = \frac{\text{Fish pathology anatomy}}{\text{Alive fish in the end of treatment}} \times 100$$

Total bacteria

Record the results for the total bacteria in the fish body after challenges test, calculate the mean colony count of five sample. As much 1 g of sample organ was crushed, then put into 9 ml of sterile distilled water and continued by dilution 7, 8, 9, and 1 ml of suspense were grown on TSA agar medium, incubated at 30°C for 24 hours. The number of bacterial cells is calculated using colony number as Hardi *et al.* (2016a):

$$N = \frac{n \times 300 \times f}{m \times X} \times 100$$

Note:

N : The total colony number, (CFU/gram of dry mass
n : The mean colony count of five sample plates
F : The dilution factor
M : The weight of the test portion (gram)
X : The dry matter content of the sample (%)

Total leucocyte

Total leucocyte describes the number of leucocyte cells in the fish body after vaccine and challenge test with pathogenic bacteria (Blaxhall, 1972). The first step of total leucocyte examination has collected the blood from the fish, put into microtube and then the blood sample was sucked with a leucocyte pipette up to 0.5 and added Turk's solution into 11 scales, wiggling the pipette to

homogeneously. Remove the first droplet, the next inserted the blood mix Turk's solution into the hemocytometer and cover with a cover glass, put on the microscope and accounted the cells. The number of leucocyte cells calculated on the five large boxes of hemocytometer and calculation by using the formula:

$$\text{Total leucocyte} = \sum \text{leucocyte cells} \times 50 \text{ cell/mm}^3$$

Antibody titers

Serum preparation: fish blood was collected via caudal veins and put it in a micro tube, the next step was centrifuged at 3000 rpm for 3 minutes. After the serum was separated from the blood cells, incubated at 44°C for 20 minutes to activate the complement (Lumsden *et al*., 1993). The serum was stored in a refrigerator at 4°C for antibody titer observation.

The antibody titers measurements were carried out by taking 25 µL of PBS solution and inserted into the microplate at holes 1st through 12th, then inserting 25 µL blood serum at 1st hole and diluting the level up to 11th hole. As much as 25 µL of bacteria inserted into 1st hole to 12th, then the microplate homogenized by gently waving. Further, it stored for two hours in the incubator at 37°C, followed by storing into the overnight in refrigerator 4°C, the antibody titers were determined from the last remaining hole of the agglutination reaction.

Phagocytic index

Phagocytic index was measured using Anderson and Siwicki (1995) method as much 50 µL of blood put into the microtube, added 50 µL of a *Staphylococcus* aqueous suspension in PBS (10^7 cells/mL) was homogenized and incubated at room temperature for 20 min. Make a prepared on the glass object and dry it out. Next step was fixing with methanol for five minutes and air-dried, stained by immersion into Giemsa for 15 minutes, washed with running water and dried with tissue, and then observed and counted the number of cells showing the phagocytic process of 100 phagocyte cells observed.

Cumulative mortality & RPS

The effectiveness of the extract as an adjuvant vaccine was measured by observing the fish mortality after challenges with *A. hydrophila* and *P. fluorescens*. Cumulative of mortality and RPS method using Ellis (1988) method.

$$\%\ \text{Cumulative of mortality} = \frac{\sum \text{mortality fish at the end of the research}}{\sum \text{alive fish at the beginning of the research}} \times 100$$

$$\text{RPS} = 1 - \left[\frac{\text{Percent mortality in treated group}}{\text{Percent mortality in control group}}\right] \times 100$$

Data analysis

All results were presented in the average and standard deviation of three independent measurements. Cumulative mortality and RPS were analyzed using nonparametric ANOVA (SPSS 16 computer program) was used to determine whether there was a significant difference ($P<0.05$) compared to control and vaccine groups, while the hematological and immunological parameters were analyzed with description.

RESULTS AND DISCUSSION

Results

Fish pathology anatomy

Bacterial infections of *A. hydrophila* and *Pseudomonas* sp. causing exophthalmia symptoms in the eyes, fin rots and bleeding (Hardi *et al*., 2014a). The test results show that prevention of bacterial infection of *A. hydrophila* and *P. fluorescens* using vaccine mixed with plant extract reducing the fish symptoms such as fin rot, darkness and exophthalmia average decreased on day 14 post-challenge, and the results for all treatment were significantly different from vaccine without extract ($P< 0.05$) on test challenges d21 (Table 1).

Total bacteria in tilapia after the challenge test

The number of bacteria in the fish body also decreased in the vaccinated without and mixed with the plant extract on day 14 after the challenge test. The highest decrease was found in the treatment of SF+ ZZ+V reached 10^3 CFU / mL and the result was significantly different from the un extracted vaccine (V) ($P< 0.05$).

Total leucocytes

Post vaccination leucocyte types also increased, either with extracts or with no extracts. The increase in total leucocytes occurred from day 7 to day 21 after vaccination, and the highest increase occurred in indigo fish injected with a vaccine with a mixture of *S. ferox* and *Z. zerumbet* (Table 3).

Increased leucocytes occurred along with the formation of antibodies in fish after 5−10 days

Table 1. The anatomical pathology of the outer organ of tilapia observation in day 14th after post challenge test with bacteria *A. hydrophila* and *P. fluorescens*

Group	Pathology anatomy (%)	Challenges time		
		d7	d14	d21
BP+V*	Fit rot	0.00 ± 0.5	11.11 ± 0.5	0.00
	Darkness	20 ± 0.5	0	0
	Exophthalmia	0	0	0
SF+V*	Fit rot	16.67 ± 0.5	0	0
	Darkness	0	0	0
	Exophthalmia	0	0	0
ZZ+V*	Fit rot	28.57 ± 0.5	42.86 ± 0.5	0
	Darkness	0	0	10 ± 0.5
	Exophthalmia	0	0	0
SF+ZZ+V*	Fit rot	0	60 ± 0.5	40 ± 0.5
	Darkness	0	20 ± 0.5	0
	Exophthalmia	0	0	0
V*	Fit rot	50 ± 0.5	50 ± 0.5	30 ± 0.5
	Darkness	20 ± 0.5	10 ± 0.5	0
	Exophthalmia	0	0	0
Control	Fit rot	0	0	0
	Darkness	0	0	0
	Exop hthalmia	0	0	0

Note: BP (*Boesenbergia pandurata)*, SF (*Solanum ferox),* ZZ (*Zingiber zerumbet),* and V (Vaccine Pseumulvacc)

Table 2. Total bacteria in tilapia organ observation in day 14th after post challenge test with bacteria *A. hydrophila* and *P. fluorescens*

Group	Challenges time (CFU/mL)		
	Day-7	Day-14	Day-21
BP + V*	$4.50 \pm 0.1\times10^{4}$	$2.00 \pm 0.1\times10^{3}$	$2.2 \pm 0.1\times10^{3}$
SF + V*	$11.3 \pm 0.1\times10^{5}$	$4.00 \pm 0.1\times10^{5}$	$4.4 \pm 0.1\times10^{3}$
ZZ + V*	$8.9 \pm 0.1\times10^{4}$	$4.3 \pm 0.1\times10^{4}$	$4.3 \pm 0.1\times10^{4}$
ZZ+SF + V*	$4.5 \pm 0.1\times10^{4}$	$2.1 \pm 0.1\times10^{4}$	$2.2 \pm 0.1 \times10^{3}$
V*	$5.5 \pm 0.1\times10^{5}$	$3.3 \pm 0.1\times10^{5}$	$3.4 \pm 0.1\times10^{4}$
Control	1.00×10^{3}	1.00×10^{3}	1.00×10^{3}

Note: BP (*Boesenbergia pandurata)*, SF (*Solanum ferox),* ZZ (*Zingiber zerumbet),* and V (Vaccine Pseumulvacc)

Table 3. Total leukocytes at post-vaccination observation in day 14th after post challenge with bacteria *A. hydrophila* and *P. fluorescens*

Group	Challenges time (10^5 cells/mm^3)		
	Day-7	Day-14	Day-21
BP + V*	3.4 ± 0.05	4.5 ± 0.04	4.6 ± 0.02
SF + V*	2.9 ± 0.07	4.3 ± 0.05	4.4 ± 0.03
ZZ + V*	3.6 ± 0.1	3.9 ± 0.059	4 ± 0.05
ZZ+SF + V*	3.8 ± 0.06	4.9 ± 0.05	5 ± 0.05
V*	3.0 ± 0.04	4.0 ± 0.04	4.1 ± 0.05
Control	1.6 ± 0.05	1.8 ± 0.05	1.8 ± 0.03

Note: BP (*Boesenbergia pandurata)*, SF (*Solanum ferox),* ZZ (*Zingiber zerumbet),* and V (Vaccine Pseumulvacc)

Table 4. Tilapia's antibody titers (-log^2) at post-vaccination observation in day 14th after post challenge with bacteria *A. hydrophila* and *P. fluorescens*

Group	Titer antibody challenges time		
	Day-7	Day-14	Day-21
BP + V*	4	5	6
SF + V*	3	4	5
ZZ + V*	3	5	5
ZZ+SF + V*	4	5	6
V*	0	4	5
Control	0	0	0

Note: BP (*Boesenbergia pandurata)*, SF (*Solanum ferox),* ZZ (*Zingiber zerumbet),* and V (Vaccine Pseumulvacc)

Table 5. Phagocytic index (%) observation in day 14th after post challenge test with bacteria *A. hydrophila* and *P. fluorescens*

Group	Phagocytic index (%) challenges time		
	Day-7	Day-14	Day-21
BP + V*	20.0 ± 0.5	60.2 ± 0.2	63.2 ± 0.6
SF + V*	22.2 ± 0.2	64.4 ± 0.2	62.2 ± 0.5
ZZ + V*	20.1 ± 0.1	64.4 ± 0.5	55.3 ± 0.7
ZZ+SF + V*	22.2 ± 0.3	55.3 ± 0.2	55.3 ± 0.1
V*	20.0 ± 0.1	50.2 ± 0.1	49.3 ± 0.2
Control	10.1± 0.2	11.2± 0.5	12.1± 0.3

Note: BP (*Boesenbergia pandurata)*, SF (*Solanum ferox),* ZZ (*Zingiber zerumbet),* and V (Vaccine Pseumulvacc)

Table 6. Number of mortality and RPS of post-test indigo fish on day 7, 14 and 21 with bacteria *A. hydrophila* and *P. fluorescens*

Group	Challenges time					
	Day-7		Day-14		Day-21	
	Mortality (%)	RPS	Mortality (%)	RPS	Mortality (%)	RPS
BP + V*	10^{a}	90^{cde}	10^{a}	91^{bcde}	0^{abcd}	100^{bcde}
SF + V*	40^{bc}	48^{b}	40de	50^{a}	0^{abcd}	100^{bcde}
ZZ + V*	30^{b}	61^{bcd}	20^{abc}	72^{bc}	0^{abcd}	100^{bcde}
ZZ+ SF + V*	40^{bcd}	52^{bc}	20^{abc}	72^{bc}	0^{abcd}	100^{bcde}
V*	50^{cde}	28^{a}	30^{bcd}	77^{bcd}	20^{abcde}	80^{a}

Note: BP (*Boesenbergia pandurata)*, SF (*Solanum ferox),* ZZ (*Zingiber zerumbet),* and V (Vaccine Pseumulvacc). The same supercript letter indicated no significant difference (P>0.05).

post-vaccination. The formalin killed whole-cells of *S. agalactiae* and *A. hydrophila* bivalent mixed vaccine given through intraperitoneal injection in tilapia, *Oreochromis niloticus* was able to improve specific fish immune system and increase the value of RPS post-challenge test with *S. agalactiae* and *A. hydrophila*, or coinfection with both *S. agalactiae* and *A. hydrophila* (Pasaribu *et al*., 2018) .

Antibody titers

This research used a simple method to examine the antibody titer; Table 4 showed the result of value -log titer antibody. The results of measurement indicated that the value of fish antibody titer has already detected since 7 days after the test. However, the highest increase occurred on the 14th day of the 21st day test period increased up to 6 in the treatment using a mixture of *B. pandurata* as well as *S. ferox* and *Z. zerumbet* treatment, but the value was not significantly different between all treatments.

Phagocytic index (%)

The results showed the number of cells that performed phagocytosis in the mixing treatment between the vaccine and the extract increased post-challenge compared with the vaccine without extract (Table 5). The highest increase occurred in the treatment with the addition of *S. ferox* and *Z. zerumbet*. The phagocytic index increased on day 14 and day 21.

Cumulative mortality and RPS

Tilapia test fish injected with bacteria *A. hydrophila* and *P. fluorescens* on day 7, 14 and 21-post vaccination. Table 6 shows the cumulative mortality of tilapia. The entire tilapia fed with the mixture of extracts showed a decrease in the number of deaths compared to only vaccinated fish, ranging from seven days post-challenge test. When viewed from the post-challenge protective results, those seven days after the vaccination period has given the protection to the fish, and the better the protection as the challenge time (14 and 21 days post-vaccination) increases. Even

the vaccine-administered fish plus the *B. pandurata* extract was able to provide 100% protection against fish post bacterial infections.

It seen from the RPS level, on the 7th day of the challenge test, the highest reached 90% in vaccine treatment plus the *B. pandurata* extract (P<0.05) (Table 6). It followed on the treatment of the 14th day tested vaccine with a *B. pandurata* mixture was able to increase the best RPS reached 91%. However, in the 21st day of the 21st treatment, almost all vaccine treatments added with extracts were able to increase the RPS to 100% better than only the treated vaccine without the addition of extract (Table 6). The overall RPS results were significantly different from the non-extractive vaccine with P< 0.05. It turns out that the trial of using QSS as an adjuvant of the *V. anguillarum* vaccine in turbot fish indicating the same increasing in post-test RPS on days 14 and 28 (Wang *et al.,* 2016).

Discussion

The utilization of adjuvant in vaccination to human (Pasquale *et al*., 2015; Wang *et al*., 2016) and fish (Bøgwald & Dalmo, 2012) has already done for a long time, as it proved to increase the immunogenicity of the vaccine. Some ingredients which known as an adjuvant were aluminum, water-in-oil emulsions (Freund›s adjuvants), part of microorganism cell and components of plant extracts (Rajput *et al*., 2007; Pasquale, 2015).

Plant extracts containing saponins, flavonoids, carbohydrates have the ability to immunomodulate fish such as *B. pandurata*, *S. ferox* and *Z. zerumbet* (Hardi *et al*., 2017a,b). Furthermore, *Azadirachta indica* extract, *Ocimum sanctum*, and *Curcuma longa* were able to increase the activity of phagocytosis, respiratory burst, and alternative complement activity and lysozyme goldfish (*Carassius auratus*) (Harikrishnan *et al.,* 2009). Stratev *et al*. (2018) research showed that the treatment of fish pathogen infection using medicinal plants (secondary metabolites, fractions, or plant extracts) would be the best choice for sustainable aquaculture.

There are many of the phytocomponents of plant extracts, one of them is saponin which already proven to boost the fish's specific immune system when mixed with vaccines (Milgate & Roberts, 1995; Song & Hu, 2009; Bagherwal, 2011). The saponin component of *O. aponaria* extract was able to prevent rotavirus infection by inhibiting viral attachment of host cells, through the destruction of cell membrane proteins virus receptors (Tam *et al*., 2011). Saponin also stimulates growth and mucosal immune response that can prevent viral infections in humans (Wang *et al.,* 2016; Tafalla *et al*., 2017). The results of the experiment by Wang *et al*. (2016) showed that utilization of *Q. saponaria* saponin (QSS) (45 mg/L) mixed with *Vibrio anguillarum* formalin vaccine increased the antibody production of *Scophthalmus maximus* on the 28th day post vaccination. This indicated that QSS was capable as an adjuvant vaccine through immersion. QSS was able to increase the effectiveness of immune cells by increasing complement activity and macrophage cell phagocytosis capable of consequent antigen presenting activity, thereby initiating the downstream humoral adaptive immune responses of the immunized fish.

The results also showed that the administration of *B. pandurata* extract was able to increase antibody titer production, in accordance with the increasing of post-trial RPS with combined bacteria *A. hydrophila* and *P. fluorescens* extracts act as permeabilizing agents by which allowed the molecules penetrated into the cell, and have not been adequately addressed (Secombes & Belmonte, 2016).

The addition of plant extracts increased the ability of antibodies to react with antigenic epitopes so that antigens were unable to recognize host cell receptors which would lead to failure of the antigen attachment process on the host cell surface (antibodies act as an inhibitor). In addition, the extract was also able to accelerate the elimination of antigen by the opsonizing process (antibody as opsonin). Formalin-killed *E. tarda* vaccine mixed with the Quil-A saponin increasing the survival rates after *E. tarda* infection and showed better survival. Adjuvant utilization mix with the fish vaccine was strongly capable to induce a specific immune response and long duration time protection, therefore the impact increased the vaccine efficiency (Tafalla *et al*., 2013).

CONCLUSION

The conclusion of this research was the *B. pandurata* extract was a potential adjuvant in the application of vaccine in freshwater fish. It was shown by the RPS after challenges was significantly higher than other treatment. The fish protection to bacterial infection was faster increase with *B. pandurata* administration (D7 and D14) than control (vaccine without extract).

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