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The use of immunostimulant from phycocyanin of *Spirulina platensis* to control motile aeromonad septicaemia (MAS) disease in common carp *Cyprinus carpio*

Pemanfaatan imunostimulan fikosianin dari *Spirulina platensis* untuk mengatasi penyakit *motile aeromonad septicaemia* (MAS) pada ikan mas *Cyprinus carpio*

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ABSTRACT

Motile aeromonad septicaemia (MAS) is a major disease in common carp *Cyprinus carpio* caused by *Aeromonas hydrophila*. This study aimed to evaluate the performance of phycocyanin imunostimulant extracted from *Spirulina platensis* to control MAS disease in common carp. This study was conducted into two phases. First phase was conducted by adding 150 mg/kg, 250 mg/kg, 350 mg/kg feed phycocyanin dose, and 0 mg/kg feed phycocyanin dose as control treatment. Fish was challenged with pathogenic *A. hydrophila* after 14 days rearing. Second phase was conducted by applying the best dose obtained from the first phase added in the feed for feeding the fish in one week/month, two weeks/month, three weeks /month, and two weeks/month with one week interval. Fish was challenged with pathogenic *A. hydrophila* after 28 days rearing. First phase study result showed that the relative percent survival (RPS) for fish fed 150 mg/kg, 250 mg/kg, and 350 mg/kg phycocyanin dose were 87.50%, 81.25%, and 75.00% respectively. Total erythrocytes, hemoglobin, total leucocytes, phagocytic activity, and respiratory burst showed higher results than control treatment on all treated fish. The second phase study showed that fish fed one week/month, two weeks/month, three weeks/month, and two weeks/month with one week interval had RPS value 65.38%, 69.23%, 76.92%, and 69.23% respectively. The immune responses of treated fish were higher than control treatment, as well as the number of pathogenic *A. hydrophila* in the liver, kidney, and intestine. Fish fed with phycoyanin dose 150 mg/kg feed and three weeks/month administration had the highest RPS value.

Keywords: Phycocyanin, *Spirulina platensis*, *Aeromonas hydrophila*, *Cyprinus carpio*

ABSTRAK

Motile aeromonad septicaemia (MAS) adalah penyakit yang menyerang ikan mas *C. carpio* yang disebabkan bakteri *A. hydrophila*. Penelitian ini bertujuan menguji kinerja imunostimulan fikosianin dari *Spirulina platensis* dalam mengatasi penyakit MAS pada ikan mas. Penelitian ini terdiri atas dua tahap, pertama, pakan ikan dengan penambahan fikosianin 150 mg/kg, 250 mg/kg, dan 350 mg/kg pakan serta kontrol tanpa penambahan fikosianin. Setelah 14 hari, ikan diuji tantang dengan *A. hydrophila*. Tahap kedua, dosis terbaik dari penelitian pertama digunakan untuk pakan ikan masing-masing selama satu minggu/bulan, dua minggu/bulan, tiga minggu/bulan, dan dua minggu/bulan dengan interval satu minggu. Setelah 28 hari, ikan diuji tantang dengan *A. hydrophila*. Hasil penelitian pertama menunjukkan kelangsungan hidup relatif (RPS) ikan yang diberi pakan fikosianin 150 mg/kg, 250 mg/kg, dan 350 mg/kg pakan adalah 87,50%; 81,25%; dan 75,00%. Total eritrosit, hemoglobin, total leukosit, aktivitas fagositik, dan *respiratory burst* menunjukkan hasil yang lebih tinggi daripada kontrol untuk semua perlakuan pemberian fikosianin. Penelitian kedua menunjukkan nilai RPS ikan diberi pakan selama satu minggu/bulan, dua minggu/bulan, tiga minggu/bulan, dan dua minggu/bulan dengan interval satu minggu yaitu 65,38%; 69,23%; 76,92%; dan 69,23%. Respons imun ikan yang diberi fikosianin lebih tinggi daripada kontrol serta mampu menekan jumlah bakteri *A. hydrophila* di hati, ginjal, dan usus. Kesimpulan dari penelitian ini bahwa pemberian fikosianin sebanyak 150 mg/kg pakan selama tiga minggu/bulan memiliki nilai RPS tertinggi.

Kata kunci: fikosianin, *Spirulina platensis*, *Aeromonas hydrophila*, *Cyprinus carpio*

INTRODUCTION

Aeromonas hydrophila is one of the pathogenic bacteria that attacks fish both in the culture environment and common water (Shayo *et al*., 2012). These bacteria are opportunistic and can be deadly when environmental conditions are in a deteriorated state. The infection type of *A. hydrophila* is acute, chronic, until latent by forming a septic infection, which is better known as hemorrhagic septicaemia disease or motile aeromonad septicaemia (MAS) (Ismail *et al*., 2010). The clinical symptoms of acute infection are the occurrence of systemic inflammation, resulting in fish death after 24 to 48 hours of infection. *A. hydrophila* bacterial infection causes tissue swelling, dropsy, necrosis, ulcer, bleeding (hemorrhagic), then massive mortality reaching 90–100% in one to two weeks of the infection period (Lukistyowati & Kurniasih, 2011). *A. hydrophila* infection invades a variety of freshwater fish culture, including the common carp, *Cyprinus carpio*, which is one of the important fish species in fish culture industries (Jeney *et al*., 2009).

One of the preventive efforts in *A. hydrophila* infection that is safe, effective, and environmental friendly is immunostimulant utilization obtained from the natural ingredient. Immunostimulant is a chemical compound, drug, or other substances that is capable of enhancing non-specific immune responses. The use of immunostimulant from natural materials currently has demonstrated its ability to increase the disease resistance of fish through non-specific immune system mechanisms enhancement (Fauziah *et al*., 2015). One of the natural ingredients that have an immunostimulatory effect is *Spirulina platensis* (Satyantini *et al*., 2016).

Spirulina platensis is a filamentous unicellular cyanobacterium, which belongs to the prokaryote algal group containing chlorophyll-A. Cyanophyceae is also called as blue-green algae due to the presence of phycocyanin and phycoerithrin, which gives the chlorophyll color (Usharani *et al*., 2012). *Spirulina* has also contained high protein content, which is 50–70%, besides antioxidant, anti-inflammatory, and antibacterial properties, which can improve the immune function (Wu *et al*., 2016). Phycocyanin extract of *S. platensis* is an antibacterial agent against Gram-negative and Gram-positive bacteria, besides affecting the stem cells in the spinal cord (Sarada *et al*., 2011). Based on several studies, the administration of *S. platensis* successfully stimulated the non-specific immune to tilapia (Ragap *et al*., 2012), Rainbow trout (Yegfreak *et al*., 2015), and growth improvement of juvenile great sturgeon (Adel *et al*., 2016).

A study conducted by Satyantini (2014) showed that the administration of phycocyanin extract from *S. platensis* with the dose 250 mg/kg feed for 14 days increased growth, non-specific immune response, and the survival rate of juvenile humpback grouper against *Vibrio alginolyticus* infection. Further studies dealing with phycocyanin bioactive compound of *S. platensis* as fish immune system enhancement agent needs to be done. Applied administration of phycocyanin in feed for common carp culture is expected to improve health, immune system, and resistance to disease infections. This study was conducted to testify the immunostimulatory performance of phycocyanin obtained from *S. platensis* in overcoming MAS disease on common carp, *C. carpio*. This study will obtain the optimal administration dose and duration that is able to improve common carp immune system.

MATERIALS AND METHOD

Fish sample

Fish samples used in this study were common carp *C. carpio*, obtained from fish culturist in Ciseeng, Bogor, West Java, with average weight 8.28±0.19 g for the first phase and 8.72±0.22 g for the second phase. Fish were reared using aquarium sized 60×30×30 cm^3 in 20 cm water height with 10 fish/aquarium stocking density. Fish were acclimatized in the aquarium until showing good feeding response.

S. platensis phycocyanin extraction

The extraction of phycocyanin *S. platensis* was conducted based on Boussiba and Richmond (1979) with the modification of Hayashi *et al*. (2006). Dried *S. platensis* was dissolved with 0.1 M Na-phosphate pH as a solvent with 4% concentration. *S. platensis* was sonicated for 30 minutes and shaken for 24 hours with 140 rpm speed. The shaken solution was centrifuged on 12000 rpm speed at 4°C for 15 minutes. The supernatant was harvested and inserted into the test tube for subsequent precipitated in $(NH_4)_2SO_4$ 50%. The pellet was once again centrifuged on 12000 rpm speed at 4°C for 10 minutes, thus obtained the blue precipitate (blue pellets) after clearing the supernatant. Phycocyanin pellet was dissolved with 0.025 M Na-phosphate pH 7 as buffer solution and analyzed using Snakeskin

Dialysis Tubing 3500 MWCO (molecular weight Ccutoff) in 0.025 M Na-phosphate pH 7 buffer solution at 4–5°C for 24 hours. The analyzed phycocyanin was frozen in the freezer under -80°C and subsequently deployed in a freeze dryer for 24 hours.

Fish feed

The feed given to the sample fish was commercial floating pellet with 39% protein content. Feed in this study was produced by the coating technique. Four feed treatments were prepared, comprising the addition of phycocyanin with the dose 150 mg/kg feed, 250 mg/kg feed, 350 mg/kg feed, and 0 mg/kg feed as a control treatment. Feed treatments and control material were mixed using egg white binder as much as 2% (v/w) and water as a solvent with 6% concentration (v/w) on the first phase study. Mixed feed was dried on the room temperature. The feed used for the second phase study was obtained from the best feed treatment on the first phase study for inducing common carp immune system.

***A. hydrophila* pathogenic bacteria**

Bacteria used in this study was *A. hydrophila* (ATCC 49140) pathogenic bacteria. *A. hydrophila* bacteria was characterized using API 20E kit and grown on Rimmler-Shotts media. One bacterial colony from Ose needle was grown on Trypticase Soy Broth (TSB) with 5 mL volume and incubated in the waterbath shaker at 29°C with 140 rpm speed for 24 hours. *A. hydrophila* density obtained was 10^9 CFU/mL. This bacterial stock was serially diluted three times to obtain 10^6 CFU/mL (LD_{50} dose) for the challenge test.

Fish rearing

This study used six aquariums for each treatment, containing three aquariums for survival rate parameter, while other aquariums for blood sampling to obtain immune system and total plate count (TPC) parameter. Fish was fed using at satiation method with feed treatment three times at 08.00, 12.00, and 17.00 (GMT+7). Water quality condition was kept by 50% water exchange once every two days. Water quality value during the rearing period consisted of temperature 28–31°C, pH 7.2–7.6, DO 4.5–6.4 mg/L, and TAN 0.03–0.11 mg/L.

In vivo test

This study consisted of two phases, Phase one was conducted to determine the appropriate phycocyanin dose in feed, containing four treatments and three replications, namely control, 150 mg/kg feed (PF1), 250 mg/kg feed (PF2), and 350 mg/kg feed (PF3) phycocyanin dose. Fish were reared for 14 days, then intramuscularly injected (IM) with 10^6 CFU/mL *A. hydrophila* (ATCC 49140) on the 15th day of rearing as much as 0.1 mL/fish. The test fish were fed commercially and observed for seven days. Phase two study was conducted to analyze the period of proper phycocyanin administration, consisting five treatments and three replications, namely control (without phycocyanin administration), one week/month (F1), two weeks/month (F2), three weeks/months (F3), and two weeks/month treatment at one week interval (F4) treatment. Fish were reared for 28 days and challenged on the day 29 of rearing using the same procedure with phase one study.

Parameter

Parameters obtained were mortality rate (MR) and relative percent survival (RPS) (Choudhury *et al*., 2008), which were observed after seven days of challenge test. Blood profiles observation included total erythrocytes and leucocytes (Blaxhall & Daisley, 1973), hemoglobin levels (Wedemeyer & Yasutake, 1977), phagocytic activity (Anderson & Siwicki, 1993), and respiratory burst (Singh *et al*., 2013). First phase study observations were conducted on the 14th (prior to the challenge), 17th, and 21st day (after the challenging test) of rearing. The second phase study observations were conducted on the 28th (before the challenge test), 31st, and 35th day of rearing. The abundance observation of *A. hydrophila* was focused on the target organs, namely liver, kidney, and intestines (Madigan *et al*., 2014), which was performed two days after the challenge test. Here is the calculation formula of *A. hydrophila* bacterial abundance.

$$\text{Total plate count} = \Sigma \text{ Counted colony} \times \text{Dilution factor} \times \frac{1}{\text{Sample weight}}$$

Statistical analysis

Study design for the first and second phase used completely randomized design (CRD) with three replications on each treatment. Blood profiles, *A. hydrophila* abundance in the liver, kidney, and intestine obtained were analyzed with ANOVA test using SPSS.22 software program on 95% degree of confidence level ($P<0.05$). Significant result data was continually analyzed using

Duncan's multiple range test. RPS value obtained was observed using a descriptive method.

RESULT AND DISCUSSION

Phase one study result

Survival rate after the challenge test

The mortality rate on all phycocyanin treatments were lower than the control treatment at the end of the observation period. Relative percent survival (RPS) value after challenged with *A. hydrophila* indicated that phycocyanin administration with the dose 150 mg/kg feed (PF1) had the highest value with 87.50%, followed with the dose 250 and 350 mg/kg feed, which were 81.25% and 75.00%, respectively. Mortality rate (MR) and relative percent survival (RPS) of common carp after challenged with *A. hydrophila* are presented in Table 1.

Table 1. Mortality rate (MR) and relative percent survival (RPS) of common carp after challenged with *A. hydrophila*

Treatment	MR (%)	RPS (%)
K	50.00 ± 0.00	0.00
PF1	6.67 ± 5.77	87.50
PF2	10.00 ± 0.00	81.25
PF3	13.33 ± 5.77	75.00

Note: K= Control, PF1= phycocyanin dose 150 mg/kg feed, PF2= phycocyanin dose 250 mg/kg feed, PF3= phycocyanin dose 350 mg/kg feed.

Immune response

Total erythrocytes, hemoglobin level, and total leucocytes after 14 days of phycocyanin administration showed a significant difference among all treatments and control (P<0.05). The highest total erythrocytes, hemoglobin level, and total leucocytes were presented on PF1 treatment with 2.80 ± 0.06 (× 10^6 cells/mm^3); 9.00 ± 0.00 g%, and 8.30 ± 0.26 (× 10^5 cells/mm^3) respectively, showing a significant difference on each treatment and control. The total erythrocytes and hemoglobin levels decreased in all treatments on two days after the challenge test, however, phycocyanin administration in feed showed a higher value than control treatment (P<0.05). Total leucocytes increased in all phycocyanin treatments significantly than control treatment (Table 2).

Phagocytic activity of common carp administered with phycocyanin in feed showed increased level on the 14th and 17th day of rearing, then decreased on the 21st day on PF1, PF2, and PF3 treatment. The highest phagocytic activity on the 14th and 17th day was demonstrated by PF1 with 26.00 ± 0.82% and 36.00 ± 0.82% respectively, having a significant difference with control treatment (P<0.05). The highest phagocytic activity on the 21st day was shown by PF3 with 25.33 ± 1.25%.

Respiratory burst on the 14th day indicated that the administration of phycocyanin with the dose 150 and 250 mg/kg feed gave no significant difference at 0.31 ± 0.01 and 0.30 ± 0.01 absorbance level, contradictory with control and the dose 350 mg/kg feed (Table 2). The highest respiratory burst value was also shown on PF1 treatment, after *A. hydrophila* bacterial infection on the 17th day with 0.40 ± 0.01 absorbance level, significantly different from all treatments (P<0.05). The results of total erythrocytes, hemoglobin level, total leucocytes, phagocytic activity, and respiratory burst level are presented in Table 2.

A. hydrophila abundance in target organs

The total abundance of *A. hydrophila* bacteria in the liver, kidney, and intestine of common carp indicated that each phycocyanin dose treatment was capable of suppressing *A. hydrophila* growth significantly compared to control treatment (P<0.05). *A. hydrophila* abundance after the challenge test is presented in Table 3.

Phase two study result

Survival rate after the challenge test

The mortality rate on F3 treatment was lower than other treatments. The highest relative percent survival (RPS) value after challenged with *A. hydrophila* was shown on F3 with 76.92%. mortality rate and RPS value of common carp are presented in Table 4.

Table 4. Mortality rate (MR) and relative percent survival (RPS) of common carp after challenged with *A. hydrophila*

Treatments	MR (%)	RPS (%)
K	86.67 ± 5.77	0.00
F1	30.00 ± 0.00	65.38
F2	26.67 ± 5.77	69.23
F3	20.00 ± 0.00	76.92
F4	26.67 ± 5.77	69.23

Note: K= without phycocyanin administration, F1=one week/month, F2= two weeks/month, F3= three weeks/month, F4= two weeks/month with a one-week interval.

Table 2. Total erythrocytes, hemoglobin level, total leucocytes, phagocytic activity, and respiratory burst level of common carp

Parameter	Day	Treatment			
		K	PF1	PF2	PF3
Erythrocytes ($\times 10^6$cell/mm^3)	14th	1.85 ± 0.05^d	2.80 ± 0.06^a	2.47 ± 0.36^b	2.23 ± 0.10^c
	17th	1.35 ± 0.09^d	2.31 ± 0.04^a	2.21 ± 0.03^b	2.16 ± 0.03^c
	21st	1.27 ± 0.05^d	2.69 ± 0.04^a	2.35 ± 0.10^b	2.18 ± 0.06^c
Hemoglobin (g%)	14th	6.67 ± 0.12^d	9.00 ± 0.00^a	8.40 ± 0.80^b	8.27 ± 0.50^c
	17th	4.13 ± 0.12^c	7.87 ± 0.59^a	7.53 ± 0.46^b	7.40 ± 0.20^b
	21st	4.47 ± 0.31^c	8.27 ± 0.50^a	7.73 ± 0.12^b	7.87 ± 0.59^b
Leucocytes ($\times 10^5$ cell/mm^3)	14th	4.17 ± 0.71^d	8.30 ± 0.26^a	7.27 ± 0.91^b	6.47 ± 0.87^c
	17th	3.96 ± 0.77^d	9.03 ± 0.68^a	7.93 ± 0.51^b	7.43 ± 0.31^c
	21st	2.73 ± 0.68^c	11.40 ± 0.62^a	10.87 ± 0.72^b	10.63 ± 0.76^b
Phagocytic activity (%)	14th	20.33 ± 1.89^d	26.00 ± 0.82^a	22.33 ± 0.47^c	22.67 ± 0.47^b
	17th	17.67 ± 0.47^d	36.00 ± 0.82^a	31.67 ± 0.47^b	28.00 ± 1.63^c
	21st	18.67 ± 0.94^d	22.33 ± 0.47^c	24.33 ± 0.47^b	25.33 ± 1.25^a
Respiratory burst (O.D. 630 nm)	14th	0.23 ± 0.01^c	0.31 ± 0.01^a	0.30 ± 0.01^a	0.27 ± 0.01^b
	17th	0.26 ± 0.01^d	0.40 ± 0.01^a	0.37 ± 0.01^b	0.29 ± 0.01^c
	21st	0.22 ± 0.01^d	0.33 ± 0.01^a	0.30 ± 0.01^b	0.27 ± 0.01^c

Note: Data on the same line with different superscript letter shows significant difference (P<0.05). K= Control, PF1= phycocyanin dose 150 mg/kg feed, PF2= phycocyanin dose 250 mg/kg feed, PF3= phycocyanin dose 350 mg/kg feed.

Table 3. Total abundance level of *A. hydrophila* in the liver, kidney, and intestine on the 17th day of rearing (two days after challenge test)

Target organ	Treatment			
	K	PF1	PF2	PF3
Liver (Log CFU/g)	7.72 ± 0.14^a	6.11± 0.02^c	6.19 ± 0.10^{bc}	6.27 ± 0.03^b
Kidney (Log CFU/g)	8.35 ± 0.15^a	5.15 ± 0.01^c	6.32 ± 0.06^b	6.22 ± 0.12^b
Intestine (Log CFU/g)	8.44 ± 0.28^a	6.21 ± 0.19^b	6.37 ± 0.12^b	6.11 ± 0.03^c

Note: Data on the same line with different superscript letter shows significant difference (P<0.05). K= Control, PF1= phycocyanin dose 150 mg/kg feed, PF2= phycocyanin dose 250 mg/kg feed, PF3= phycocyanin dose 350 mg/kg feed.

Table 6. Total abundance level of *A. hydrophila* in the liver, kidney, and intestine on the 31st day of rearing (two days after challenge test)

Target organ	Treatment				
	K	F1	F2	F3	F4
Liver (log CFU/g)	7.81 ± 0.02^a	7.05 ± 0.02^c	7.18 ± 0.09^b	6.31 ± 0.09^d	7.22 ± 0.02^b
Kidney (log CFU/g)	8.77 ± 0.01^a	7.87 ± 0.08^b	7.47 ± 0.04^c	6.37 ± 0.10^e	6.76 ± 0.02^d
Intestine (log CFU/g)	6.53 ± 0.04^a	5.22 ± 0.03^c	5.20 ± 0.03^c	5.47 ± 0.08^b	5.46 ± 0.10^b

Note: Data on the same line with different superscript letter shows significant difference (P<0.05). K= without phycocyanin administration, F1=one week/month, F2= two weeks/month, F3= three weeks/month, F4= two weeks/month with a one-week interval.

Immune response

The observation result on the 28th day of rearing indicated that F3 had the highest total erythrocytes, hemoglobin level, total leucocytes, and respiratory burst among other treatments (P<0.05). F3 also showed significant difference with control treatment (P<0.05). Total erythrocytes, hemoglobin level, total leucocytes, and respiratory burst observation result are presented in Table 5.

A. hydrophila abundance in target organs

The total abundance level of *A. hydrophila* bacteria after challenge test showed the lowest abundance level in liver and kidney were observed at F3 with 6.31 ± 0.09 and 6.37 ± 0.10 log CFU/g, while the lowest abundance level in intestine was observed at F2 with 5.20 ± 0.03 log CFU/g, which was significantly different with control treatment (P<0.05). Bacterial abundance levels in the liver, kidney, and intestine are presented in Table 6.

Table 5. Total erythrocytes, hemoglobin level, total leucocytes, phagocytic activity, and respiratory burst level of common carp

Parameter	Day	Treatment				
		K	F1	F2	F3	F4
Erythrocytes ($\times 10^6$cell/mm^3)	28th	1.32 ± 0.08^c	1.46 ± 0.10^c	1.90 ± 0.16^b	2.24 ± 0.14^a	1.37 ± 0.05^c
	31st	0.66 ± 0.03^c	1.02 ± 0.02^b	1.04 ± 0.03^b	1.22 ± 0.10^a	1.06 ± 0.02^b
	35th	1.08 ± 0.03^d	1.39 ± 0.04^c	1.54 ± 0.07^b	1.73 ± 0.13^a	1.57 ± 0.05^b
Hemoglobin (g%)	28th	7.27 ± 0.12^d	7.73 ± 0.42^c	7.87 ± 0.31^{bc}	8.70 ± 0.17^a	8.03 ± 0.21^b
	31st	4.73 ± 0.42^c	6.27 ± 0.46^b	6.57 ± 0.40^b	7.87 ± 0.59^a	6.37 ± 0.32^b
	35th	6.47 ± 0.64^c	7.73 ± 0.12^b	7.80 ± 0.20^b	8.87 ± 0.23^a	8.53 ± 0.42^a
Leucocytes ($\times 10^5$cell/mm^3)	28th	2.90 ± 0.46^c	3.10 ± 0.36^c	3.90 ± 0.30^b	4.73 ± 0.21^a	3.73 ± 0.15^b
	31st	3.23 ± 0.25^d	4.43 ± 0.31^c	6.17 ± 0.76^b	7.60 ± 0.36^a	6.90 ± 0.36^{ab}
	35th	3.70 ± 0.26^d	6.37 ± 0.25^c	7.43 ± 0.31^b	9.03 ± 0.68^a	7.93 ± 0.51^b
Phagocytic activity (%)	28th	18.67 ± 0.58^d	21.00 ± 1.00^c	21.33 ± 0.58^c	24.00 ± 0.58^a	22.67 ± 1.00^b
	31st	18.00 ± 1.00^c	25.00 ± 1.00^b	24.67 ± 0.58^b	29.00 ± 0.58^a	25.67 ± 1.00^b
	35th	20.33 ± 0.58^c	22.33 ± 0.58^b	24.33 ± 0.58^a	25.00 ± 0.58^a	23.67 ± 1.00^a
Respiratory burst (O.D. 630 nm)	28th	0.18 ± 0.01^d	0.24 ± 0.01^c	0.26 ± 0.03^b	0.29 ± 0.01^a	0.24 ± 0.01^c
	31st	0.21 ± 0.01^d	0.78 ± 0.03^b	0.56 ± 0.07^c	0.95 ± 0.01^a	0.70 ± 0.02^b
	35th	0.17 ± 0.01^d	0.25 ± 0.02^c	0.28 ± 0.01^b	0.33 ± 0.05^a	0.28 ± 0.01^b

Note: Data on the same line with different superscript letter shows significant difference ($P<0.05$). K= without phycocyanin administration, F1=one week/month, F2= two weeks/month, F3= three weeks/month, F4= two weeks/month with one week interval.

Discussion

The survival rate observation result of the first phase study after challenge test showed that the administration of phycocyanin with the dose 150 mg/kg feed (PF1) had the highest relative percent survival (RPS) compared to other treatments with 87.5% (Table 1). This condition indicates that the feed containing phycocyanin is able to be optimally absorbed by common carp, stimulating the immune system to perform the body defense process in inhibiting the pathogenic bacterial proliferation. The administration of *Spirulina* sp. in feed was able to increase the non-specific immune response of carp as the initial defence against antigen attack before the specific immune systems formed (Bai *et al*., 2014). Increased fish immune system happened as phycocyanin stimulates cells in the spinal cord, affecting the spleen cells, granulocyte macrophage-colony stimulating factor (GM-CSF), and interleukin-3 (IL-3) to form erythrocytes and immune cells system modulated by erythropoietin for erythrocytes and cytokines for immune cells (Hayashi *et al*., 2006).

The optimization of phycocyanin with proper dose should be followed by the efficient duration and effective administration to provide the best results on improving the fish health status. Phase two study result showed the highest RP) after the challenge test was observed in F3 with 76.92% (Table 4). Feed containing phycocyanin at the dose 150 mg/kg feed with longtime administration duration (three weeks/month) gives the best results. Dosage and administration duration are essential to produce an optimal immunity response, because excessive immunostimulatory administration can suppress fish resistance to disease and growth (Mastan, 2015). Another study result illustrated the introduction of *S. platensis* on tilapia at the dose 10 mg/fish for four weeks and challenged with *A. hydrophila* could improve the immune system and produce 80% survival rate (Ragap *et al*., 2012). This result was in line with Satyantini (2014), who reported that the administration of phycocyanin at the dose 250 mg/kg feed with 14 days duration in juvenile humpback grouper demonstrated better growth, non-specific cellular and humoral immune response, and resistance capability against pathogenic bacteria *V. alginolyticus* with 81.83% RPS value.

Blood profiles describe the fish health status. Total erythrocytes, hemoglobin level, total leucocytes, phagocytic activity, and respiratory burst can be used as indicators of the immune response. Total erythrocytes, hemoglobin

level, total leucocytes, phagocytic activity, and respiratory burst showed higher value after phycocyanin administration treatments than control treatment in phase one study on the 14th day and phase two study on the 28th day before challenge test (Table 2; Table 5). This indicates that phycocyanin is effective as an immunostimulant material, the same as reported by Satyantini (2014). Immunostimulant has specific receptors against phagocytic cells (neutrophils, monocytes, and macrophages), which binds to receptor molecules on the circulatory surface and phagocytic tissue. This binding can increase the phagocytic activity for immune cell adaptation, attack, and digestion against pathogenic bacteria (Elala *et al*., 2013). Phagocytic cells have an important function in the fish body defence. Along with these activities, there is a release of molecular signals (cytokines) that can stimulate the formation of phagocytic cells. This causes elevated leucocytes, although there has not been any infection. This condition is in accordance with Kozenko and Henson (2010), who mentioned that phycocyanin affected cells in the spinal cord to produce erythrocytes and leucocytes. Satyantini *et al*. (2014) also added that the administration of phycocyanin with the dose 250 mg/kg feed enhances the number of leucocytes and phagocytic activity in juvenile humpback grouper.

Reduced total erythrocytes and hemoglobin level were observed on the 17th day in phase one study and 31st day in phase two study after the challenge test (Table 2; Table 5). Kumar and Ramulu (2013) stated that *A. hydrophila* bacteria has aerolysine and β-hemolysine toxin which can suppress total erythrocytes and hemoglobin levels, resulting in anemia and hematopoetic organ disorders. There was a strong correlation between erythrocytes and hemoglobin level, whether decreased erythrocyte number would result decreased hemoglobin level. However, the phycocyanin treatment in both phases showed significant better value than control treatment ($P<0.05$). Enhanced total erythrocytes and hemoglobin level occurred again on the 21st day in phase one study and day 35th in phase two study. This indicates the occurrence of the recovery process after bacterial infection in fish with the help of phycocyanin compound.

Phase one and two studies after the challenge test occurred increased level of total leucocytes, phagocytic activity, and respiratory burst (Table 2; Table 5). This was due to the resistance mechanism from the fish body against *A. hydrophila* infection by suppressing the bacterial proliferation. This was demonstrated in the phase one study which showed lower abundance level of bacteria in the liver, kidney, and intestine observed on all treatments compared to control treatment (Table 3). Phase two study also showed reduced bacterial abundance observed on all duration treatments compared to control (Table 6). According to Janda and Abbot (2010), *A. hydrophila* systemic infection that causes acute death occurs at 24−48 hours after bacterial exposure, showing clinical symptoms, such as septicaemia, ascites, ulcer, followed with the liver, spleen, and kidney damages in postmortem phase. Abdel-Tawwab and Ahmad (2009) implied that there was a decreased number of *A. hydrophila* in the liver and kidney organ of tilapia, after the administration of *S. platensis*.

Increased total leucocytes after the challenge test is an attempt to phagocytize pathogenic bacterial cells entering the fish body of fish, thus reducing bacterial growth and development (Kurniawan *et al*., 2014). Control treatment showed lower total leucocytes compared to phycocyanin administration treatments. This shows that the administration of phycocyanin in common carp provides non-specific immune system enhancement characterized by elevated leucocyte production to attack pathogens along with increased phagocytic activity and respiratory burst. Increased respiratory burst value indicated that there is a lot of pathogen exposure in the fish body, triggering the phagocytic activity to attack the pathogenic microbes. Phagocytic cells that perform phagocytosis activity produce H_2O_2 anions and superoxide (O_2^-) that are highly toxic to bacteria, thereby increasing the ability of phagocytes to destroy pathogenic bacteria (Rawling *et al*., 2012).

CONCLUSION

Phycocyanin administration with the dose 150 mg/kg feed and three weeks/month duration provides the best results for improving survival rate and non-specific immune response of common carp against *A. hydrophila* bacterial infection.

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