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Dietary supplementation of *Polygonum chinense* improves the immunity of Asian seabass, *Lates calcarifer* (Bloch, 1790) against *Vibrio harveyi* infection

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ABSTRACT

Aquaculture plays a significant role in the overall fish production in Malaysia, contributing a substantial quantity of food-fish amounting to roughly 573,683 tonnes with an estimated economic value of US$860 million in 2022. However, diseases have become a significant limitation for aquaculture production. Therefore, herbal immunostimulant has been considered a natural and practical approach of preventing disease infection in fish. The ability of *Polygonum chinense* extract (PCE) on haemato-biochemistry parameters, immunomodulatory properties, and disease resistance of *Lates calcarifer* (Asian seabass) under *Vibrio harveyi* challenge was evaluated in this study, with a focus on dose-response associations and variability over various exposure durations (0-, 7- and 14-day post-infection). A total of 480 Asian seabass (9.5 ± 0.2 g) were distributed in 12 aquaria and fed four diets supplemented with 0 (control), 2, 5 and 10 g/kg diet for 60 days before being challenged with *V. harveyi*. Dietary PCE significantly improved ($P < 0.05$) survival, with the dose of 10 g/kg showing the highest survival rate (90 %) when compared to the control (60 %). Additionally, hematological (red and white blood cell counts, hemoglobulin, packed cell volume, and mean corpuscular volume) and immunological (activities of lysozyme, phagocytic activity and respiratory burst, and serum total immunoglobulin) properties were significantly increased ($P < 0.05$) in comparison to the control group. In contrast, serum aspartate aminotransferase and alanine aminotransferase levels, as well as glucose level were significantly reduced ($P < 0.05$) in PCE-fed fish compared to the control group. Conclusively, the current study discovered that supplementing fish feed with *P. chinense* extract improves fish haemato-biochemical profile, immunocompetence and disease resistance to *V. harveyi* infection.

1. Introduction

Due to rapid aquaculture growth, Southeast Asian countries have risen as worldwide fish producers [1]. Asian economies rely heavily on fish as a source of income [2] as fish is one of the significant components in the Asian people's diet [3]. Fish is high in protein, micronutrients and omega-3 fatty acids [4], accounting for nearly 20 % of the human average daily animal protein intake [5]. In addition, global per capita annual consumption of fish has increased from 18.9 kg in 2010 [6] to 20.5 kg in 2018 [7]. Global fish consumption is expected to rise more sharply as many countries face a high demand for fish as a food source in order to feed a 9 billion population by 2050 [8]. Fish is a regional commodity that is sold globally, and the Southeast Asia region is at the forefront of the global effort to meet the world's growing demand for seafood [1].

Farmers are boosting their fish production by cultivating fish more intensely to meet rising demand for fish. The aforementioned occurrence was witnessed in Malaysia, where the aquaculture industry has demonstrated a consistent upward trend in production, culminating in a noteworthy output of 573,683 tonnes in 2022 [9] with a substantial surge of 32 % when compared to the aquaculture production in 2018 [10]. However, intensive aquaculture with high feed inputs and stocking densities has increased the risk of pathogens and infectious diseases [11]. Infectious diseases caused by viruses, fungi, parasites, bacteria and other emerging pathogens [12] have become a limiting constraint for aquaculture intensification [13]. Infectious diseases are causing a significant devastating economic impact, whereby the annual loss attributable to diseases is predicted to rise up to USD$6 billion [14]. The

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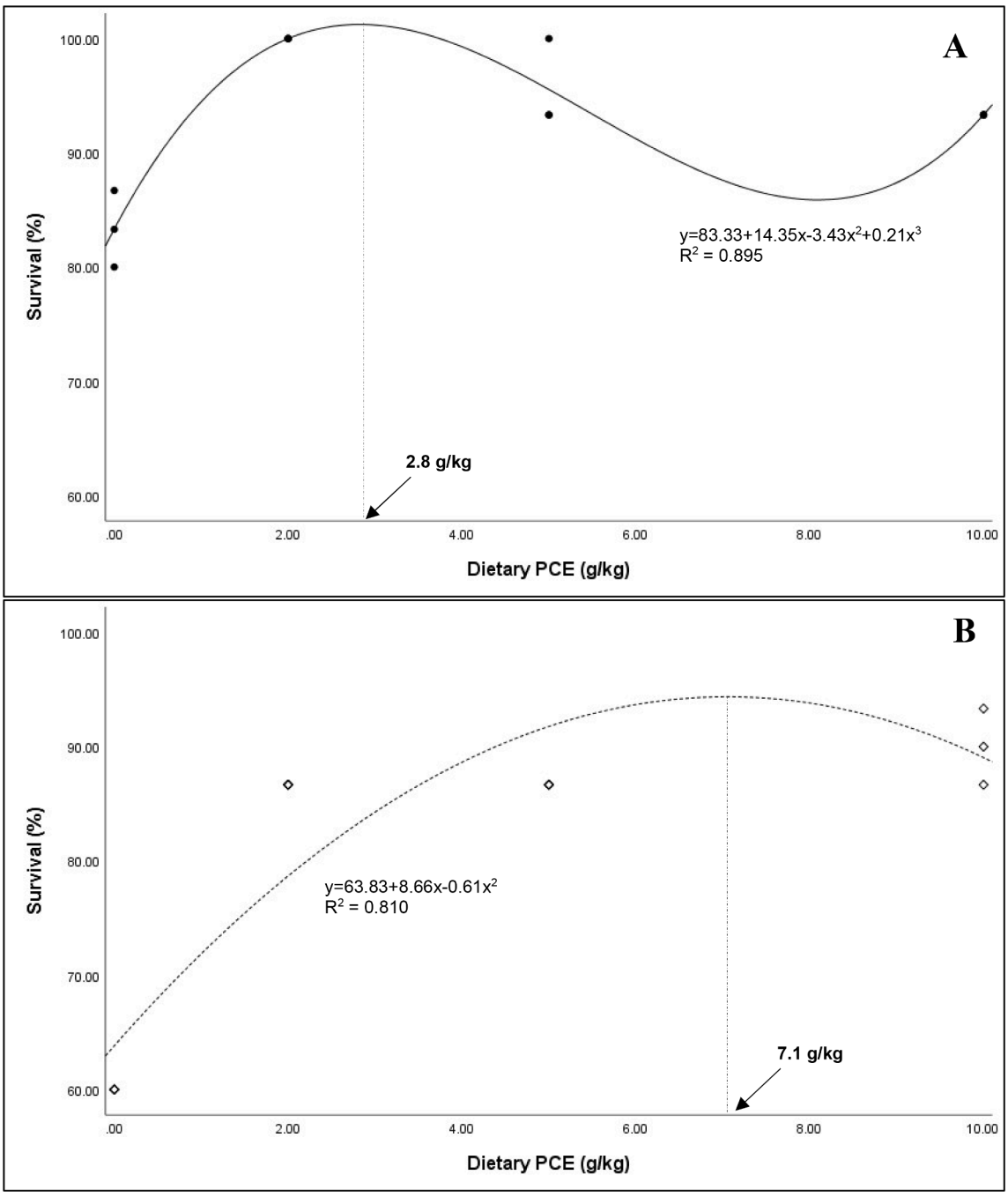

Fig. 1. Survival rates of Asian seabass fed with diets containing different *P. chinense* extract levels (0 (control), 2, 5 and 10 g/kg) after (post) *Vibrio harveyi* infection. The lines indicate the significant ($P < 0.05$) cubic relationship for 7-day post-infection (A, solid line) and quadratic relationship for 14-day post-infection (B, dashed line). Optimal requirements derived with the polynomial regression method for survival rate at day-7 and day-14 of post-infections are 2.8 and 7.1 g/kg PCE diet, respectively.

intensive culture of fish under stressful and crowded conditions has led to outbreaks of various bacterial diseases [15], resulting the aquaculture industry suffers economic losses each year [16]. Many aquatic animals have died as a result of a sudden outbreak of bacterial diseases, particularly those caused by *Vibrio* spp. [17] Many *Vibrio* species have caused vibriosis, a potentially severe disease [18], but *Vibrio harveyi* and *Vibrio anguillarum* are the most frequently isolated [19–21] Vibrio species exhibited a notable predominance amongst commercially farmed groupers [22], Asian seabass [23], and shrimps [24] in Malaysia.

To control the vibriosis in aquaculture, farmers take an immediate solution by applying various antibiotics and chemicals [25]. However, the indiscriminate antibiotics use resulted in the development of antibiotic resistance bacteria [26] and the accumulation of residues in fish products [27]. As the side effects of antibiotics and chemicals pose a risk to fish and humans, immunostimulants are being considered as an alternative in disease control in aquaculture [28]. Immunostimulants derived from medicinal plants can be used to stimulate the non-specific defence mechanisms of fish and increase the specific immune response [29]. Plant-derived products have the potential to be a promising alternative source of bioactive compounds because they are easily obtained, inexpensive and biocompatible [29]. Many studies have reported the use of plant parts, plant extracts and essential oils as immunostimulants and proven their efficacy in increasing fish immune responses, survival, growth rate and disease resistance [28]. *Malvae sylvestris,*

Table 1
Hematological parameters of Asian seabass, *Lates calcarifer* fed different levels of *Polygonum chinense* diet for 60 days and challenged with *Vibrio harveyi* for 14 days ($n = 15$).

Parameter	Period (Post-infection)	Treatment dose of *Polygonum chinense* (g/kg)				ANOVA	Polynomial contrasts		
		0	2	5	10		**Linear**	**Quadratic**	**Cubic**
RBC (x10^{12}/L)	0-day	3.61 ± 0.04^{a}	4.11 ± 0.09^{b}	4.41 ± 0.12^{b}	4.28 ± 0.13^{b}	0.017*	0.057	0.003*	0.017*
	7-day	3.37 ± 0.03^{a}	3.73 ± 0.20^{b}	3.48 ± 0.07^{ab}	4.16 ± 0.15^{c}	0.000*	0.001*	0.003*	0.000*
	14-day	3.24 ± 0.15^{a}	4.28 ± 0.11^{b}	4.33 ± 0.12^{b}	4.33 ± 0.09^{b}	0.000*	0.008*	0.000*	0.000*
Haemoglobin (g/L)	0-day	93.0 ± 1.0^{a}	110.5 ± 5.5^{b}	120.0 ± 4.0^{b}	115.0 ± 4.0^{b}	0.031*	0.079	0.008*	0.031*
	7-day	80.5 ± 3.2^{a}	88.5 ± 5.4^{b}	88.9 ± 1.7^{b}	103.3 ± 3.4^{c}	0.000*	0.000*	0.000*	0.000*
	14-day	83.3 ± 6.0^{a}	112.0 ± 3.4^{b}	108.5 ± 1.9^{b}	112.5 ± 5.3^{b}	0.001*	0.018*	0.005*	0.001*
Ht (%)	0-day	0.30 ± 0.01^{a}	0.34 ± 0.04^{b}	0.37 ± 0.01^{b}	0.36 ± 0.01^{b}	0.018*	0.042*	0.003*	0.018*
	7-day	0.23 ± 0.01^{a}	0.24 ± 0.02^{a}	0.25 ± 0.01^{a}	0.31 ± 0.01^{b}	0.000*	0.000*	0.000*	0.000*
	14-day	0.22 ± 0.01^{a}*	0.33 ± 0.01^{b}	0.33 ± 0.01^{b}	0.33 ± 0.01^{b}	0.000*	0.013*	0.001*	0.000*
MCV (fL)	0-day	83.1 ± 0.9^{a}	81.6 ± 0.7^{a}	82.8 ± 0.1^{a}	82.9 ± 0.1^{a}	0.673	0.773	0.839	0.673
	7-day	68.4 ± 1.5^{a}	69.6 ± 3.3^{a}	70.4 ± 3.5^{a}	77.6 ± 1.7^{b}	0.001*	0.000*	0.000*	0.001*
	14-day	68.8 ± 1.2^{a}	77.6 ± 1.2^{b}	76.0 ± 0.9^{b}	77.5 ± 1.0^{b}	0.000*	0.078	0.078	0.047*
MCHC (g/L)	0-day	310.0 ± 3.3^{a}	330.2 ± 8.7^{a}	328.7 ± 6.5^{a}	324.0 ± 2.4^{a}	0.620	0.358	0.077	0.047*
	7-day	350.0 ± 4.2^{bc}	339.8 ± 4.7^{ab}	363.3 ± 7.3^{c}	322.8 ± 6.7^{a}	0.003*	0.000*	0.000*	0.001*
	14-day	376.4 ± 3.5^{b}	340.5 ± 8.9^{a}	331.8 ± 8.9^{a}	340.4 ± 6.6^{a}	0.033*	0.090	0.016*	0.033*
WBC ($\times 10^{7}$/L)	0-day	2.9 ± 0.3^{a}	3.3 ± 0.5^{a}	3.1 ± 0.3^{a}	5.1 ± 0.7^{b}	0.042*	0.010*	0.017*	0.038*
	7-day	1.7 ± 0.1^{a}	2.5 ± 0.2^{b}	2.8 ± 0.1^{c}	2.9 ± 0.2^{c}	0.000*	0.001*	0.000*	0.000*
	14-day	1.6 ± 0.2^{a}	2.8 ± 0.3^{b}	2.9 ± 0.3^{b}	3.0 ± 0.1^{b}	0.000*	0.004*	0.000*	0.000*

Values are means ± SE of three replicates ($n = 3$). Values in the same row with different superscript letters are statistically different according to Duncan's test ($p < 0.05$).
Note: RBC, red blood cells; Ht, haematocrit; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; WBC, white blood cells.
* significant.

Origanum vulgare, Allium hirtifolium [30], *Aegle marmelos* [31], *Elaeagnus angustifolia* [32], *Plantago lanceolate* [33] and *Ocimum gratissimum* [34] are amongst the plant-derived immunostimulants that shown promising results in enhancing fish growth rate, antioxidant defence and immune parameters.

One of the Malaysian ethnic plants with various healing effects [35] was considered for use in aquaculture as an immunostimulant. *Polygonum chinense*, also locally known as Chinese knotweed, is a member of the family Polygonaceae [36]. A few *Polygonum* species are traditionally used as Chinese medical herbs and possess antioxidant properties [37]. Additionally, *P. chinense* and other *Polygonum* extracts, such as *Polygonum persicaria* and *Polygonum plejebum* have been reported to contain antibacterial and antifungal properties [38–41]. The current study aimed to determine the efficiency of *P. chinense* extract as a dietary supplement on survival, haemato-biochemical and immune parameters following *V. harveyi* challenge.

2. Materials and methods

2.1. Plant material and preparation of plant extract

The fresh leaves of *P. chinense* plant were procured from a nearby plant nursery in Johor, Malaysia and afterwards authenticated by personnel at University Agriculture Park, Universiti Putra Malaysia (UPM), located in Serdang, Selangor, Malaysia. The leaves underwent a triple washing process using distilled water, followed by drying in the shaded area and pulverised using an electric grinder.

The samples were subjected to extraction using 80 % methanol at a ratio of 1:10 (w/v) ratio and agitated at room temperature for 48 h [42]. After centrifugation at 3000 × g for 20 min, the crude extracts were separated and filtered (0.45 μm Whatman filter paper, Whatman, Maidstone, England). The aforementioned procedure was iterated thrice in order to accomplish the extraction process. The solvent underwent evaporation using a rotary vacuum evaporator (Rotavapor R-215 equipped with vacuum controller and pump, Buchi, Switzerland) at temperature range of 30 −35 °C. The resulting residues were subsequently freeze-dried into a powdered form (Freeze Drier, Labconco, USA). The extracts were stored at −20 °C until they were utilised.

2.2. Diet preparation

The study utilised a commercially available basal diet (Star Feed, Star Feedmills (M) Sdn Bhd, Malaysia) with the following composition: 42 % crude protein, 6 % crude fat, 13 % moisture, 5 % crude fibre, and 10 % crude ash. The basal diet was procured from a local fish pellet provider located in Rawang, Selangor, Malaysia. An electric household blender was used to roughly pulverise the feed pellets. The basal diet was supplemented with 2, 5 and 10 g/kg (w/w) PCE to create three experimental diets, while the control diet was not supplemented with PCE (0 g/kg) [43,44]. The dry ingredients were mixed in a laboratory food mixer with 40 % sterile distilled water to reach the correct pelleting consistency. Diets were hand pelleted in a cake mould and dried overnight at 35 °C before being stored at 4 °C until feeding.

2.3. Test microorganism

The *Vibrio harveyi* isolate used in the study was obtained from the bacterial collection of the Aquatic Animal Health Unit (AAHU), UPM, Selangor, Malaysia. This bacterial strain was isolated from diseased grouper (*Epinephelus* sp.) and identified using 16S rRNA sequencing, which generated fragments with high similarity (>90 %) (AB793708.1). *Vibrio harveyi* was passaged nine times through Asian seabass by intraperitoneal injection and re-isolated from the kidney or liver of moribund fish to resurrect its virulence. The bacterium was grown on TCBS and TSA agar with 1.5 % NaCl to obtain only pure colonies.

2.4. Ethical statements

Acclimatization, feeding, rearing and experimental procedures were carried out with the authorization at the AAHU, UPM, Selangor, Malaysia. The Institutional Animal Care and Use Committee of UPM (UPM/IACUC/AUP-R077/2017) approved all the experiments used in this study. They were performed in accordance with the Universiti Putra Malaysia code of conduct for the care and use of animals for scientific purposes (The Code).

2.5. Fish and experimental conditions

Four hundred eighty healthy Asian seabass juveniles (9.5 ± 0.2 g)

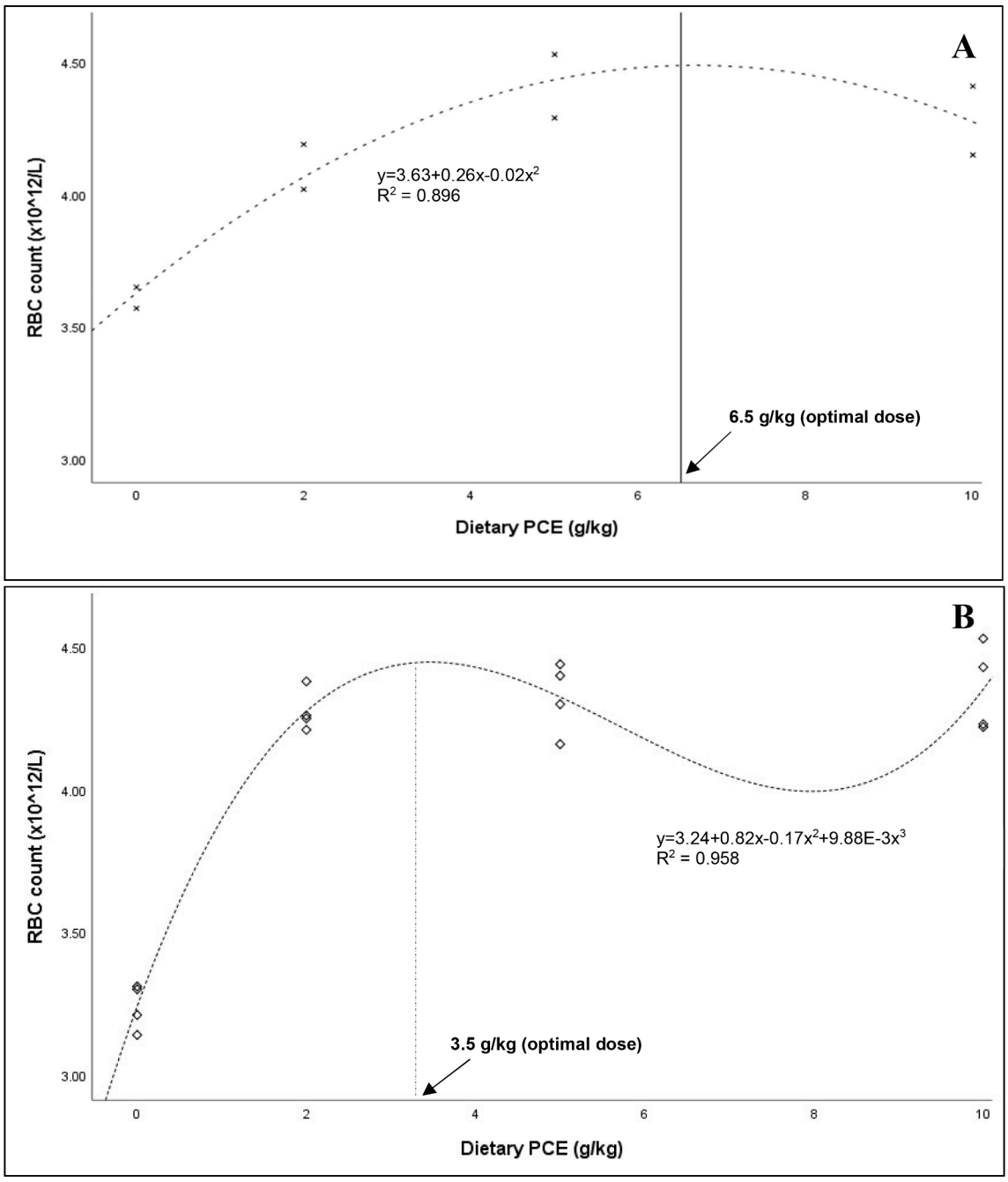

Fig. 2. Significant ($P < 0.05$) quadratic relationship for 0-day post-infection (A, loosely dashed line) and cubic relationship for 14-day post-infection (B, dashed line) between the red blood cell (RBC) count of Asian seabass and dietary *Polygonum chinense* supplementation levels.

were delivered from a local fish farm (Banting, Selangor, Malaysia) to the AAHU, UPM, Selangor, Malaysia. After two weeks of acclimatization, the fish were randomly distributed into 12 tanks (200 L), 40 fish per group in triplicate. Each tank was supplied with filtered saltwater, air diffuser stones provided continuous aeration, and a filter and protein skimmer were installed to maintain good water quality. Throughout the trial, the water in the tank was replaced in part (40–50 %) every evening. The fish were fed with 0, 2, 5, and 10 g/kg PCE supplemented diets at 4 % of their body weight twice daily (9.00 am and 4.00 pm) for 60 days.

After 60 days of feeding, randomly 15 fish from each treatment group with varying levels of plant extract and control were tested for disease resistance. *Vibrio harveyi* was suspended in 50 mL of phosphate-buffered saline (PBS) and injected intraperitoneally into all fish (0.1 mL per fish) at the LD_{50} concentration (1.2×10^6 CFU/mL). The fish in the control group received the same bacteria as the fish in the treatment groups. The survival rate of each experimental and control group was examined and documented for 14 days. The following formulas used to calculate the survival rate and relative percent survival (RPS) [45];

1) $\text{Survival rate} = \frac{\text{Final number of fish (nf)}}{\text{Initial number of fish (ni)}} \times 100$

2) $\text{RPS} = 1 - \frac{\text{Percent mortality in treated group}}{\text{Percent mortality in control group}} \times 100$

2.6. Blood sampling and determination of hematological and biochemical parameters

Blood and serum samples were collected from randomly selected fish

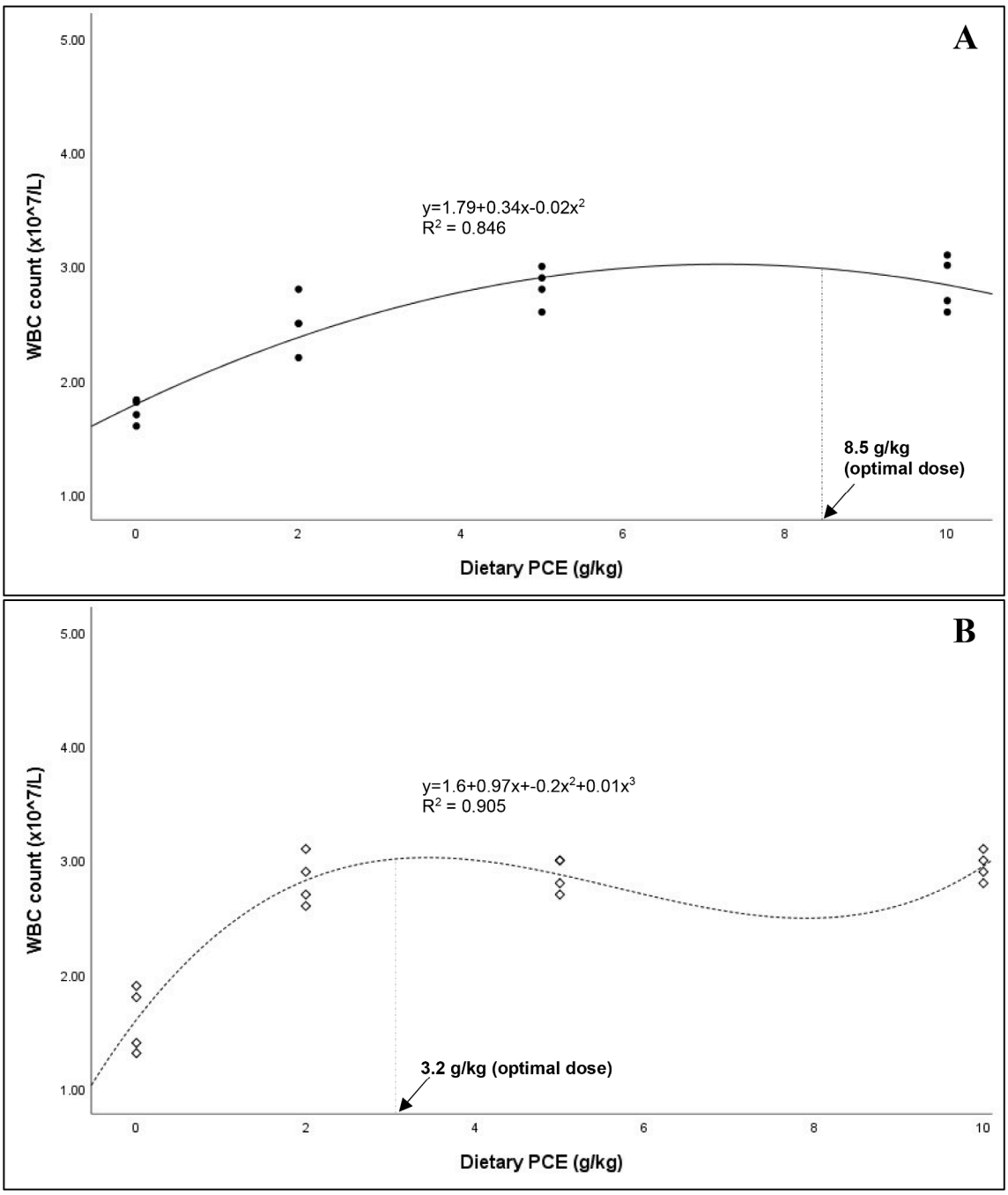

Fig. 3. Significant ($P < 0.05$) quadratic relationship for 7-day post-infection (A, solid line) and cubic relationship for 14-day post-infection (B, dashed line) between the white blood cell (WBC) count of Asian seabass and dietary *Polygonum chinense* supplementation levels.

($n = 15$ per group) in the challenge groups, as Talpur and Ikhwanuddin [46] described. Blood samples were promptly analysed hematologically utilising the Cell-Dyn® 3700 hematology analyser (Abbott Diagnostics, USA). The hematology parameters evaluated were red blood cell (RBC), white blood cell (WBC), haemoglobin (Hb) and packed cell volume (PCV). The following equations were used to compute the mean cell volume (MCV) and mean cell haemoglobin concentration (MCHC):

1) $\text{MCV (fL)} = \frac{\text{PCV} \times 1000}{\text{RBC}}$

2) $\text{MCHC (g/L)} = \frac{\text{Hb}}{\text{PCV}}$

The blood serum was used for the quantification of various biochemical parameters including total glucose (Glu), total protein (TP), albumin (Alb), alkaline transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP). This analysis was performed using an automatic chemistry analyzer (Hitachi 902, Japan) and optimised tests provided by Boehringer Mannheim GmBH, employing spectrophotometers. The globulin (Glo) concentration was calculated by subtracting the albumin (Alb) concentration from the total protein (TP) concentration. The albumin to globulin ratio (A:G) was derived by dividing the albumin concentration by the globulin concentration.

2.7. Immune response assays

2.7.1. Respiratory burst assay

The nitroblue tetrazolium (NBT) assay developed by Anderson and

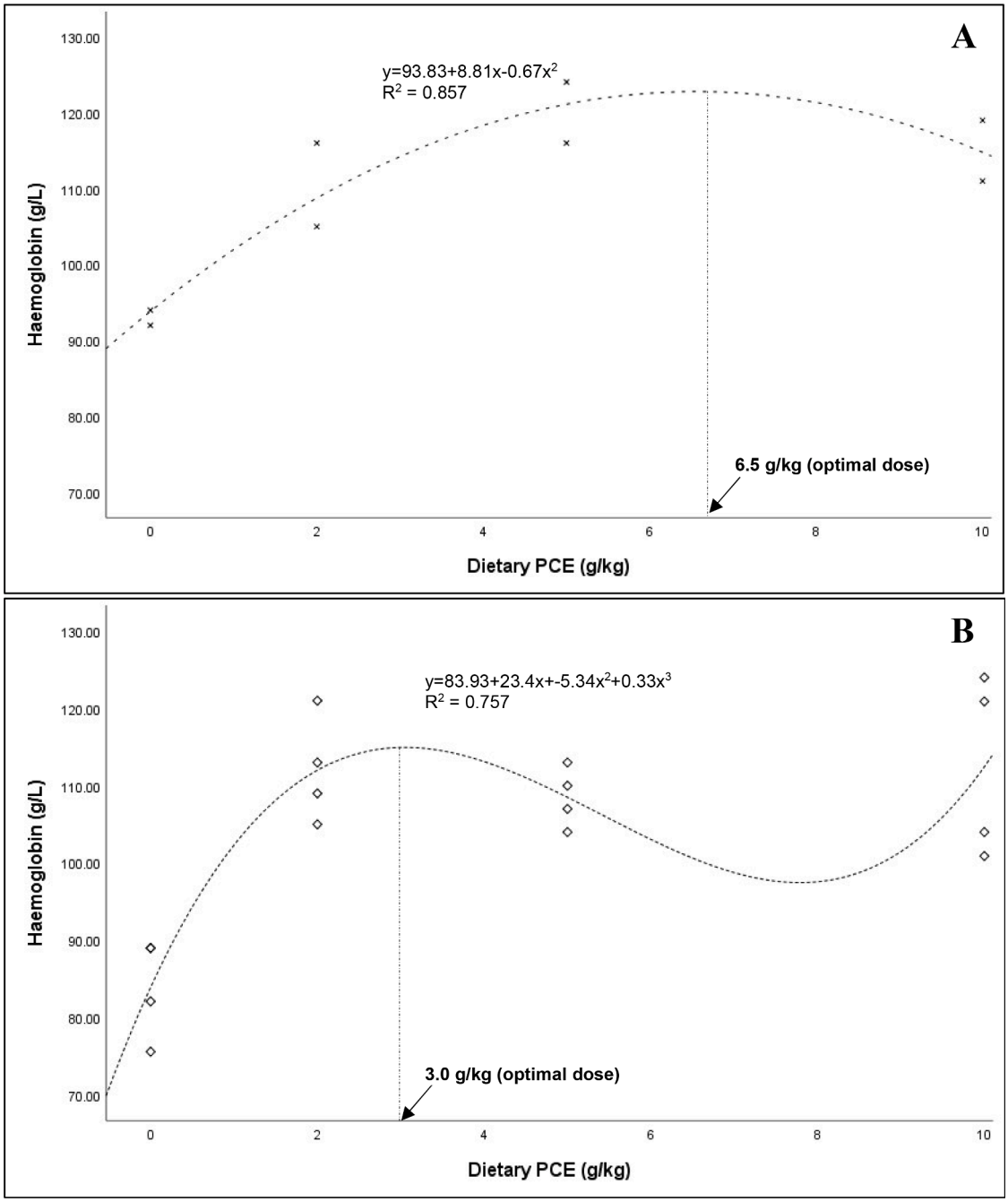

Fig. 4. Significant ($P < 0.05$) quadratic relationship for 0-day post-infection (A, loosely dashed line) and cubic relationship for 14-day post-infection (B, dashed line) between the haemoglobin of Asian seabass and dietary *Polygonum chinense* supplementation levels.

Siwicki [47] was employed to quantify the respiratory burst activity of phagocytes. In each individual well of a 96-well microtiter plate, a volume of 100 μL of blood was carefully dispensed, followed by the addition of 100 μL of a 0.2 % NBT solution. The plate was subjected to incubation at ambient temperature for a duration of 30 min. After the completion of the incubation period, a volume of 50 μL of the NBT-blood cell suspension was carefully transferred from the well of the plate to a glass tube. The glass tube contained 1.0 mL of N, N-dimethyl formamide (DMF). The suspension underwent centrifugation at a relative centrifugal force (RCF) of 3000 × g for a duration of 5 min. The optical density of the supernatant was measured by employing a glass cuvette in a spectrophotometer (Shimadzu, Japan) at a wavelength of 540 nm.

2.7.2. Serum lysozyme activity assay

The measurement of serum lysozyme activity was conducted using the Ellis method [48], with some modifications based on the lysis of *Micrococcus lysodeikticus*. A volume of 50 μL of fish serum was introduced into a solution containing 950 μL of *M. lysodeikticus* suspension. The suspension was prepared by diluting *M. lysodeikticus* to a concentration of 0.015 % (w/v) in a 50 mM potassium phosphate buffer (pH 6.2). The measurement of the decrease in absorbence at a wavelength of 450 nm was conducted at two time points, specifically after 1 min and 5 min of incubation using a spectrophotometer (Shimadzu, Japan). The quantification of lysozyme activity was established by defining a unit as the quantity of the sample that induced a reduction in absorbence of 0.001 per minute.

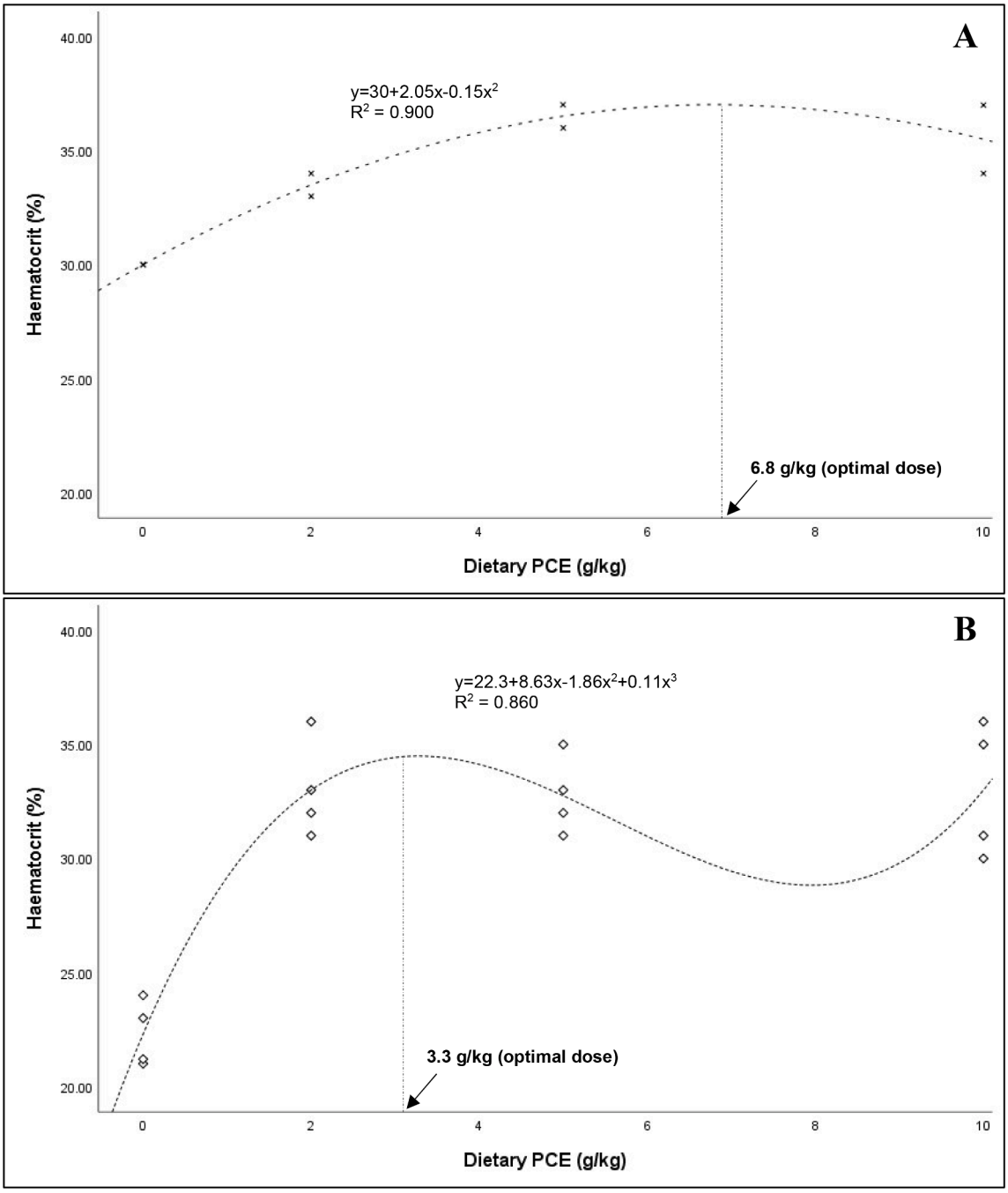

Fig. 5. Significant ($P < 0.05$) quadratic relationship for 0-day post-infection (A, loosely dashed line) and cubic relationship for 14-day post-infection (B, dashed line) between the haematocrit of Asian seabass and dietary *Polygonum chinense* supplementation levels.

2.7.3. Phagocytosis assay

The phagocytosis activity was determined using a modified Anderson and Siwicki method [47]. A microtiter plate well was filled with 100 μL blood, followed by the addition of 100 μL of *V. harveyi* (1×10^{-7}) cell suspension in phosphate-buffered saline (PBS). The blood-bacteria mixture was well mixed using a pipette to ensure bacteria contact with leucocytes. After 20 min of incubation at room temperature, a volume of 5 μL of the suspension was extracted and subsequently, a blood smear was meticulously produced and allowed to air-dry on glass slide. The smear was fixed for 5 min in ethyl alcohol (95 %), air-dried and stained for 10 min in 7 % Giemsa. The stained smear was observed under a compound microscope and cells with engulfed bacteria were counted. The results were expressed as a percentage (%).

2.7.4. Serum total immunoglobulin assay

The determination of serum total immunoglobulin was conducted using the biuret method as s described by Anderson and Siwicki [47]. A centrifuge tube was filled with 100 μL of serum and an equal volume of 12 % polyethylene glycol (PEG). Following a 2 h incubation period at room temperature with continuous agitation, the tube underwent centrifugation at 5000 × g for 10 min. The supernatant was collected and the protein concentration was measured as described in Section 2.6. The determination of the serum total immunoglobulin concentration was achieved by subtracting the value obtained from the analysis conducted from the total protein level.

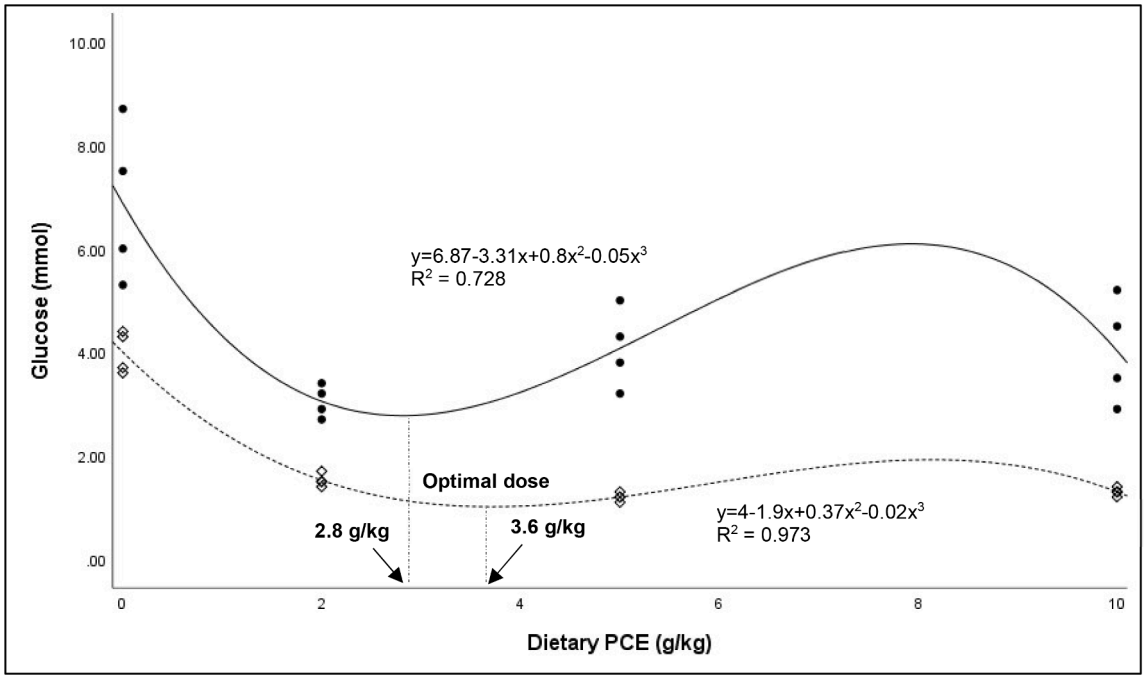

Fig. 6. Significant ($P < 0.05$) cubic relationships between the glucose level of Asian seabass and dietary *Polygonum chinense* supplementation levels during 7-day post-infection (A, solid line) and 14-day post-infection (B, dashed line).

2.8. Statistical analysis

The statistical analysis comparing the dietary groups was performed using one-way analysis of variance (ANOVA) and Duncan's multiple range comparison tests. In the case of substantial interaction effect, the polynomial contrast technique was used to investigate the polynomial trend or relationship (linear, quadratic or cubic response) that can be found between sets of means. This method allowed us to determine whether the distinguish effects were linear (directly proportionate to the varying amounts of additional *P. chinense* extract) or quadratic/cubic (dose-dependent). The best reflected function that connects the dependant (response) and independent variables (*P. chinense* extract supplementation levels) was also evaluated using polynomial regression analysis to determine the optimal dose. All results were presented as mean ± standard error (SE). All statistical analyses were carried out using SPSS Statistics version 23.0 for Windows (IBM Corporation, Armonk, NY, USA) and the level of significance was set at $P < 0.05$.

3. Results

3.1. Disease resistance – survival rate

There was no mortality of fish up to 48 h. On day 7 and onwards, the control group had significantly ($P < 0.05$) lower survival. Following the 7-day and 14-day post-infection periods, PCE supplementation in the fish diet significantly ($P < 0.05$) enhanced the fish survival rate after being challenged with *V. harveyi* as compared to the control group. In comparison to the control group, fish treated with dietary *P. chinense* extract appeared to have stronger resistance to *V. harveyi* infection, with the highest post-challenge survival rate (90.0 %) observed in the 10 g/kg PCE group, followed by 5 g/kg PCE and 2 g/kg PCE with 86.7 and 86.7 %, respectively (Fig. 1).

The relationship between dietary *P. chinense* levels and survival rate were best explicated by linear polynomial model on 0-day, and second-order polynomial models on 7-day and 14-day post-infections. Based on the quadratic regression analyses of survival, the dietary *P. chinense* requirements for optimum survival of Asian seabass on day-7 and day-14 post-infections were 2.8 and 7.1 g/kg, respectively (Fig. 1).

3.2. Hematological and biochemical parameters

3.2.1. Hematological parameters

Dietary supplementation of PCE produced a significant ($P < 0.05$) influence on the RBC count, WBC count, haemoglobin and haematocrit of Asian seabass throughout the entire infection trial (Table 1). Fish RBC counts increased quadratically in response to increasing doses of supplemented PCE (0–10 g/kg) on 0-day ($P < 0.05$, $R^2 = 0.896$) but exhibited cubic relationships during 7-day ($P < 0.05$, $R^2 = 0.791$) and 14-day ($P < 0.05$, $R^2 = 0.958$) of post infection periods (Fig. 2). Throughout the challenge trial, the optimal dose of 3.5 g/kg yielded the highest estimated RBC count. In contrast, the polynomial regression analyses revealed linear trend for WBC count on 0-day ($P < 0.05$, $R^2 = 0.700$), quadratic trend on 7-day ($P < 0.05$, $R^2 = 0.846$) and cubic trend on 14-day ($P < 0.05$, $R^2 = 0.905$) of post infections (Fig. 3). The WBC count increased in treated fish, with the fish fed with 10 g/kg exhibiting the highest value throughout the post-infection period (Table 1). Fish fed with all supplementation diets presented significant ($P = 0.000$) increase in WBC count when compared to the control group on 7-day and 14-day of post infections. Based on regression analysis on WBC count, the optimum supplementation dietary PCE requirement that produced the highest WBC count in Asian seabass at 7-day and 14-day of post infections were estimated to be 8.5 and 3.2 g/kg, respectively (Fig. 3).

All PCE supplementation diets significantly ($P = 0.00$) increase the haemoglobin level on 7-day and 14-day of post infections, and haematocrit % only on 14-day of post infection, as compared to the control group. The orthogonal contrasts for haemoglobin and haematocrit levels of fish presented quadratic trends ($P < 0.05$, $R^2 = 0.857$; $R^2 = 0.900$) on 0-day, linear trends ($P < 0.05$, $R^2 = 0.733$; $R^2 = 0.736$) on 7-day and cubic trends ($P < 0.05$, $R^2 = 0.757$; $R^2 = 0.860$) on 14-day of post infections, respectively (Figs. 4 and 5). Moreover, the regression analyses indicate that the optimum inclusion level of *P. chinense* in diets of Asian seabass for highest level of haemoglobin and haematocrit levels were found to be 6.5 and 6.8 g/kg on 7-day, and 3.0 and 3.3 g/kg on 14-day of post infections, respectively.

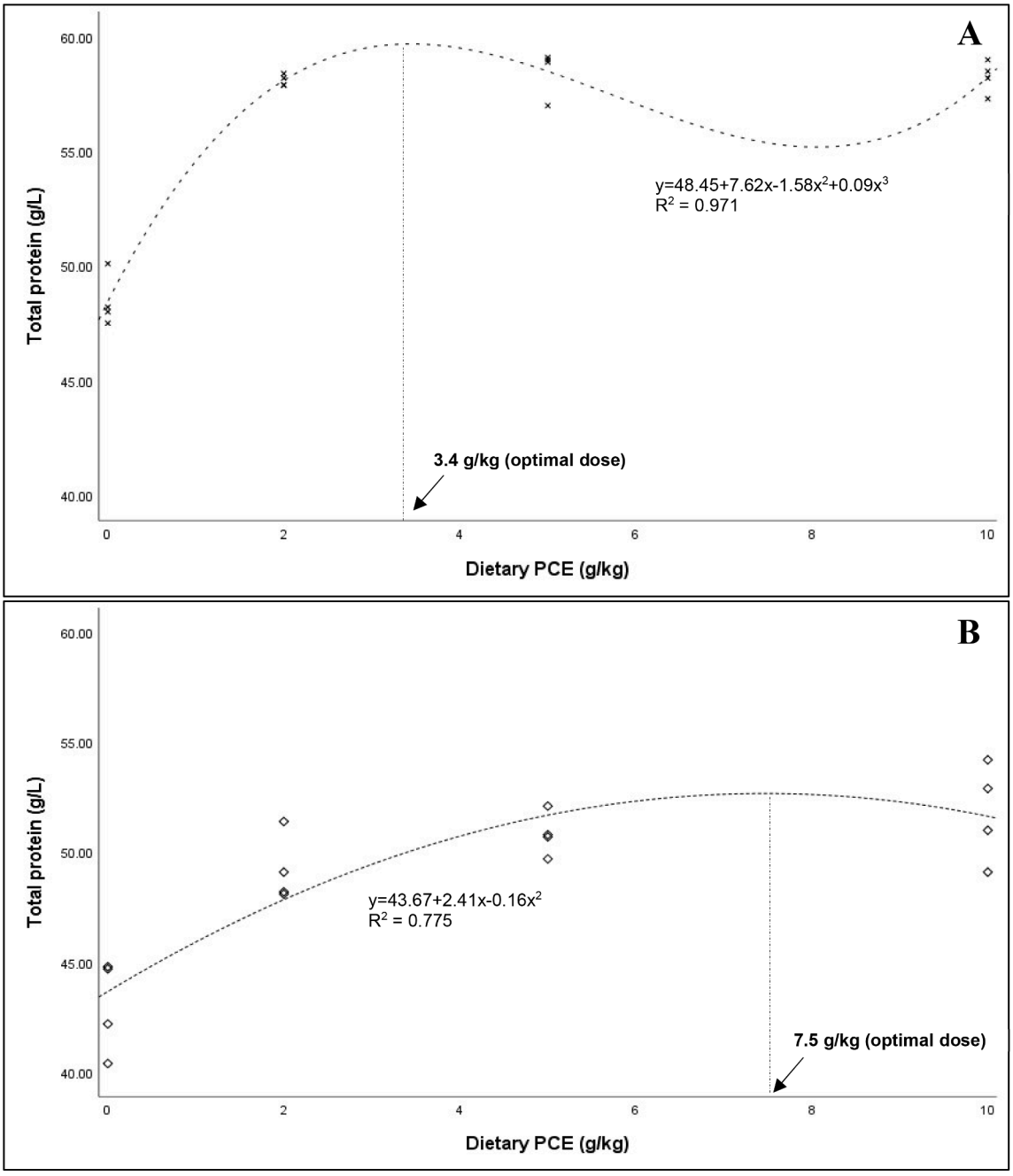

Fig. 7. Significant ($P < 0.05$) cubic relationships between the total protein level of Asian seabass and dietary *Polygonum chinense* supplementation levels during 7-day post-infection (A, solid line) and 14-day post-infection (B, dashed line).

3.2.2. Serum biochemical parameters

The dietary administration of *P. chinense* influenced fish serum glucose levels, which decreased linearly ($P < 0.05$, R2 = 0.834) as the initial supplementation level was increased from 0 to 10 g/kg diet at 0-day post infection. In contrast, the glucose level of fish showed cubic response ($P < 0.05$, $R^2 = 0.728$; R2 = 973) with the dietary levels of *P. chinense* during 7-day and 14-day post-infection periods (Fig. 6). Based on the second-order polynomial regression model, the optimum dietary *P. chinense* doses that resulted in the lowest glucose level in fish during the two previously mentioned periods were estimated to be 2.8 and 3.6 g/kg diet, respectively.

The supplementation diets also exhibited significant effect on the serum total protein levels ($P < 0.05$), almost increased as the levels of PCE rose across all challenge periods (0-, 7- and 14-day) (Fig. 7). Moreover, all the supplementation diets caused significant increase ($P < 0.05$) in the serum albumin and globulin levels through the different post-infection periods with compared to the control group (Table 2). In contrast, all supplementation diets (2–10 g/kg) demonstrated significant cubic effect ($P < 0.05$, $R^2 = 0.821$–0.957) on fish serum ALP concentration (Fig. 8) and quadratic effect ($P < 0.05$, $R^2 = 0.811$–0.855) on fish serum ALT concentration (Fig. 10) during all post-infection periods. However, the increase in the levels of *P. chinense* indicated significant cubic relationship ($P < 0.05$, $R^2 = 0.912$; R2 = 0.907) during the first two periods (0- and 7-days) of challenge trial, and quadratic relationship ($P < 0.05$, $R^2 = 0.859$) at 14-day of post-infection period between the PCE levels and the AST concentration (Fig. 9). The highest levels of ALP, AST and ALT were determined for fish fed with dietary doses of 7.2–7.8, 3.0–7.0 and 7–8.2 g/kg *P. chinense*, respectively after 14 days after

Table 2
Biochemical parameters of Asian seabass, *Lates calcarifer* fed different levels of *Polygonum chinense* diet for 60 days and challenged with *Vibrio harveyi* for 14 days ($n = 3$).

Parameter	Period (Post-infection)	Treatment dose of *Polygonum chinense* (g/kg)				ANOVA	Linear trend	Quadratic trend	Cubic trend
		0	2	5	10				
Glucose (mmol/L)	0-day	1.6 ± 0.1^{c}	1.2 ± 0.1^{b}	1.2 ± 0.1^{b}	1.0 ± 0.1^{a}	0.000*	0.000*	0.000*	0.000*
	7-day	6.9 ± 0.8^{b}	3.1 ± 0.2^{a}	4.1 ± 0.4^{a}	4.0 ± 0.5^{a}	0.001*	0.133	0.028*	0.001*
	14-day	4.0 ± 0.2^{b}	1.5 ± 0.1^{a}	1.2 ± 0.1^{a}	1.3 ± 0.1^{a}	0.000*	0.004*	0.000*	0.000*
Total protein (g/L)	0-day	48.5 ± 1.8^{a}	58.1 ± 0.1^{b}	58.5 ± 0.5^{b}	58.3 ± 0.4^{b}	0.000*	0.007*	0.000*	0.000*
	7-day	42.0 ± 1.3^{a}	44.8 ± 1.3^{c}	44.0 ± 0.3^{ab}	44.9 ± 1.3^{c}	0.109	0.105	0.171	0.109
	14-day	43.0 ± 1.1^{a}	49.2 ± 0.8^{b}	50.8 ± 0.5^{b}	51.8 ± 1.1^{b}	0.000*	0.001*	0.000*	0.000*
Albumin (g/L)	0-day	13.6 ± 0.4^{a}	18.4 ± 0.3^{b}	19.9 ± 0.4^{c}	18.9 ± 0.2^{b}	0.000*	0.006*	0.000*	0.000*
	7-day	11.2 ± 0.4^{a}	13.3 ± 0.1^{c}	12.1 ± 0.2^{b}	11.4 ± 0.2^{ab}	0.001*	0.510*	0.072	0.001*
	14-day	10.8 ± 0.3^{a}	13.7 ± 0.5^{d}	11.8 ± 0.7^{b}	12.7 ± 0.6^{c}	0.000*	0.290*	0.320	0.000*
Globulin (g/L)	0-day	35.0 ± 1.4^{a}	39.7 ± 0.2^{c}	38.7 ± 0.3^{b}	39.4 ± 0.2^{bc}	0.000*	0.016*	0.003*	0.000*
	7-day	30.9 ± 2.0^{a}	31.5 ± 3.5^{a}	32.0 ± 0.3^{a}	33.5 ± 1.1^{a}	0.253	0.037	0.123	0.253
	14-day	32.2 ± 0.4^{a}	35.5 ± 0.9^{b}	39.0 ± 0.9^{c}	39.1 ± 0.6^{c}	0.000*	0.000*	0.000*	0.000*
Albumin: globulin ratio (g/L)	0-day	0.39 ± 0.01^{a}	0.46 ± 0.01^{b}	0.51 ± 0.01^{c}	0.48 ± 0.01^{b}	0.000*	0.010*	0.000*	0.000*
	7-day	0.40 ± 0.01^{b}	0.40 ± 0.01^{b}	0.38 ± 0.01^{ab}	0.34 ± 0.01^{a}	0.001*	0.074	0.019*	0.001*
	14-day	0.34 ± 0.01^{b}	0.40 ± 0.01^{c}	0.30 ± 0.02^{a}	0.32 ± 0.01^{b}	0.000*	0.103	0.253	0.000*
Alkaline phosphate (U/L)	0-day	46.3 ± 2.3^{a}	67.8 ± 2.3^{c}	57.0 ± 2.4^{b}	61.3 ± 4.1^{b}	0.000*	0.161	0.100	0.000*
	7-day	40.0 ± 6.2^{b}	44.8 ± 2.8^{c}	34.8 ± 4.0^{a}	39.5 ± 2.6^{b}	0.000*	0.196	0.174	0.000*
	14-day	27.3 ± 0.6^{a}	34.8 ± 2.1^{c}	26.5 ± 1.4^{a}	32.3 ± 2.3^{b}	0.000*	0.435	0.693	0.000*
Aspartate aminotransferase (U/L)	0-day	111.8 ± 8.4^{b}	72.8 ± 3.9^{a}	77.8 ± 2.4^{a}	74.0 ± 0.4^{a}	0.000*	0.012*	0.001*	0.000*
	7-day	136.3 ± 3.7^{b}	57.0 ± 7.5^{a}	56.8 ± 5.0^{a}	55.3 ± 8.2^{a}	0.000*	0.008*	0.000*	0.000*
	14-day	139.5 ± 5.0^{c}	79.3 ± 7.4^{b}	60.5 ± 4.7^{a}	61.3 ± 9.7^{a}	0.000*	0.001*	0.000*	0.000*
Alanine aminotransferase (U/L)	0-day	50.8 ± 2.3^{c}	41.5 ± 0.6^{b}	34.3 ± 1.7^{a}	35.0 ± 2.5^{a}	0.000*	0.001*	0.000*	0.000*
	7-day	65.0 ± 4.4^{d}	46.3 ± 3.6^{c}	37.3 ± 4.4^{b}	31.8 ± 4.4^{a}	0.000*	0.000*	0.000*	0.000*
	14-day	77.0 ± 3.9^{d}	38.0 ± 3.9^{c}	35.3 ± 2.7^{b}	32.8 ± 2.6^{a}	0.000*	0.001*	0.000*	0.000*

Values are means ± SE of three replicates ($n = 3$). Values in the same row with different superscript letters are statistically different according to Duncan's test ($p < 0.05$).
Note:.
* significant.

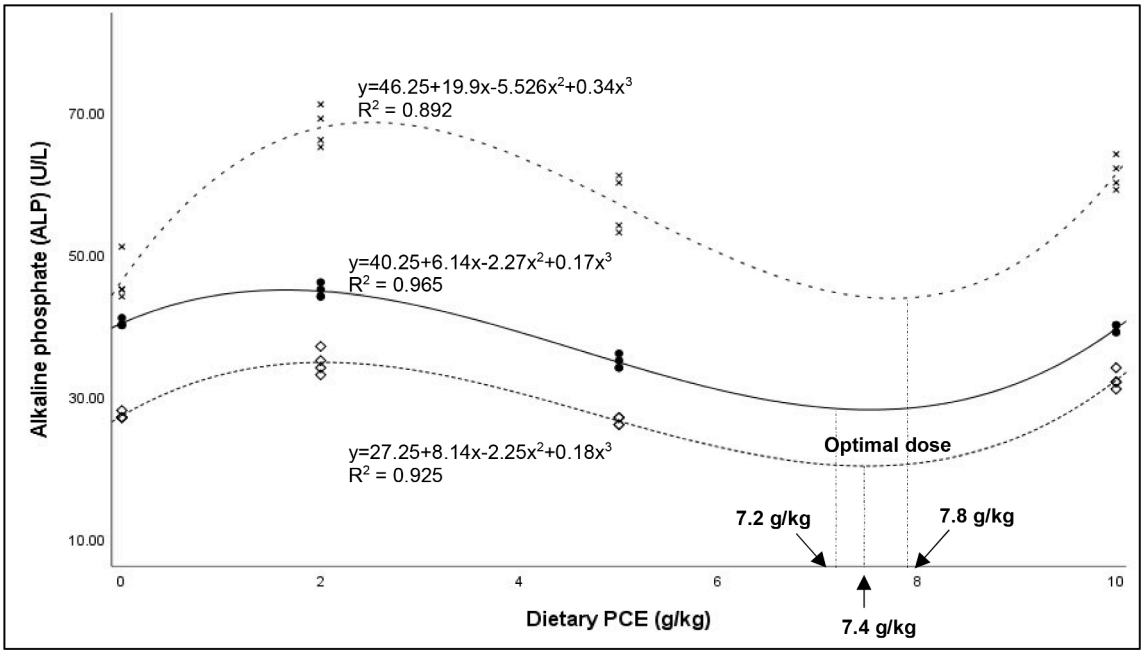

Fig. 8. Significant ($P < 0.05$) cubic relationships between the alkaline phosphate (ALP) levels of Asian seabass and dietary *Polygonum chinense* supplementation diet during the 0-day (loosely dashed line), 7-day (solid line) and 14-day post-infections (dashed line).

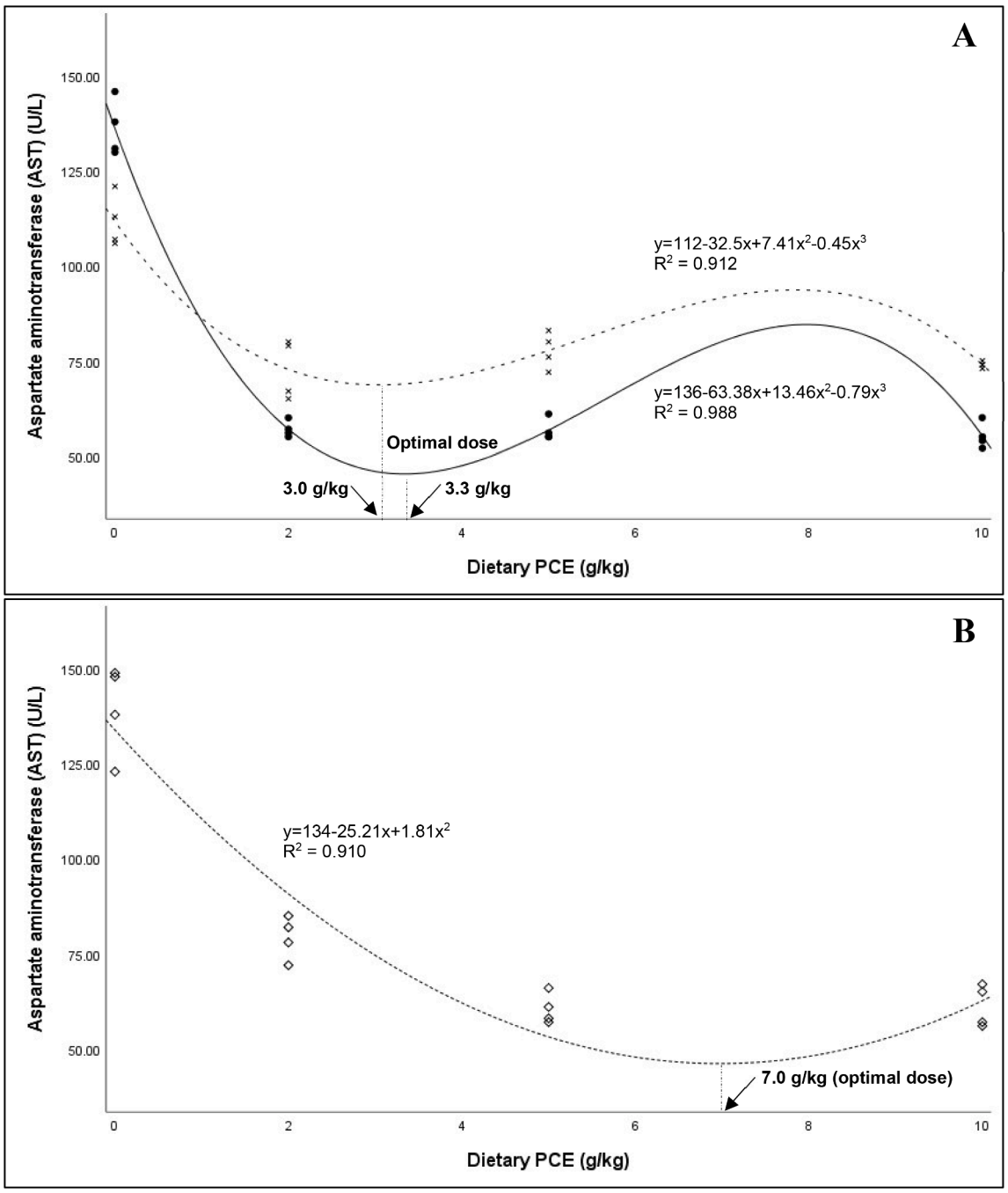

Fig. 9. Significant ($P < 0.05$) cubic relationships between the aspartate aminotransferase (AST) levels of Asian seabass and dietary *Polygonum chinense* supplementation diets at 0-day post-infection (A, loosely dashed line) and 7-day (A, solid line), and quadratic relationship at 14-day post-infection (B, dashed line).

infection.

3.3. Immunological parameters

Throughout all challenge periods (0-, 7- and 14-days post-infection), the activities of lysozyme, phagocytic, respiratory burst and total serum immunoglobulin were significantly higher ($P < 0.05$) in fish groups fed diets supplemented with 2–10 g/kg PCE compared to the control (Table 3). In addition, the orthogonal contrasts exhibited significant cubic relationships for lysozyme activity ($P < 0.05$, $R^2 = 0.941$; $R^2 = 0.957$; $R^2 = 0.991$), serum respiratory burst activity ($P < 0.05$, $R^2 = 0.928$; $R^2 = 0.944$; $R^2 = 0.796$), and total serum immunoglobulin ($P < 0.05$, $R^2 = 0.953$; $R^2 = 0.905$; $R^2 = 0.926$) during the three separate phases of post-infection, respectively. In contrast, phagocytic activity was best explicated by quadratic order polynomial model ($P < 0.05$, $R^2 = 0.940$; $R^2 = 0.969$; $R^2 = 0.983$) throughout different phases of challenge periods, respectively (Table 3). The highest lysozyme activity was determined for fish fed 10.0 g/kg of dietary *P. chinense* at all period of the challenge trial (Fig. 11a), while the optimal inclusion level for phagocytic activity was estimated to be 6.1, 6.8, 6.9 g/kg (Fig. 11b), 2.8, 3.1, 10.0 g/kg for respiratory burst activity (Fig. 11c) and 2.7, 3.1, 3.9 g/kg for total serum immunoglobin (Fig. 11d) at 0-, 7- and 14-day of post-infections, respectively.

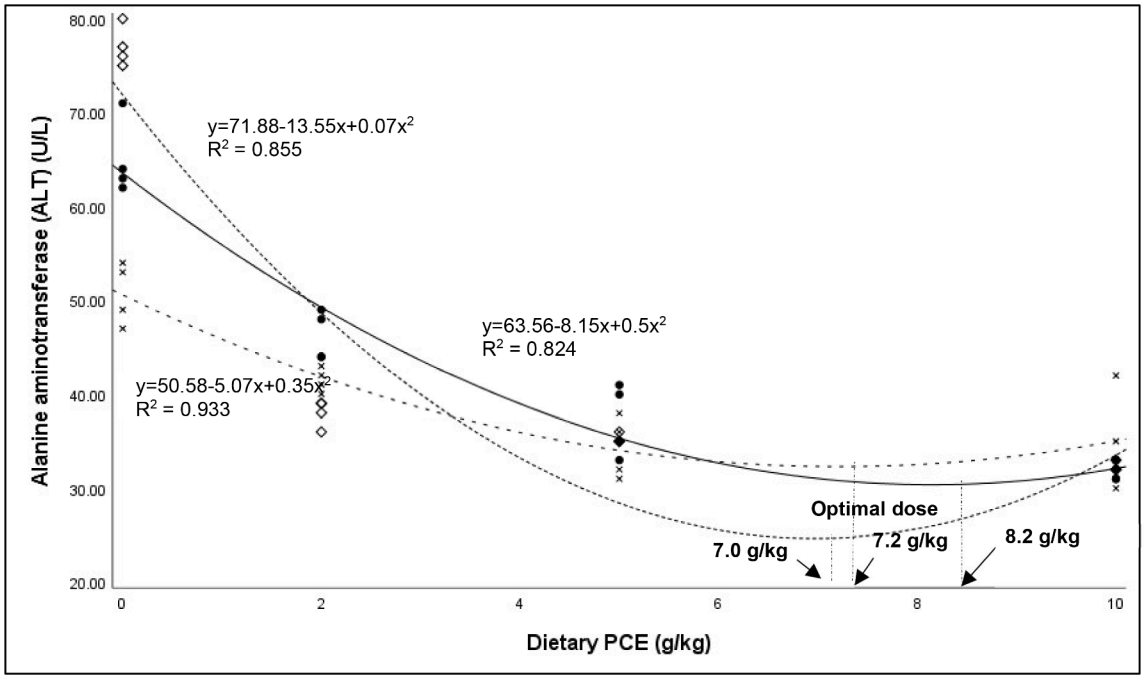

Fig. 10. Significant ($P < 0.05$) quadratic relationships between the alanine aminotransferase (ALT) levels of Asian seabass and dietary Polygonum chinense supplementation diets during the 0-day (loosely dashed line), 7-day (solid line), and 14-day post-infection periods (dashed line).

Table 3
Immunological status of Asian seabass, *Lates calcarifer* fed different levels of *Polygonum chinense* diet for 60 days and challenged with *Vibrio harveyi* for 14 days ($n = 3$).

Parameter	Period (Post-infection)	Treatment dose of *Polygonum chinense* (g/kg)				ANOVA	Linear trend	Quadratic trend	Cubic trend
		0	2	5	10				
Lysozyme activity (U/mL)	0-day	216.3 ± 5.2^{a}	301.1 ± 3.8^{b}	285.3 ± 3.0^{b}	327.6 ± 2.6^{c}	0.000*	0.000*	0.000*	0.000*
	7-day	164.7 ± 4.9^{a}	241.9 ± 3.6^{b}	241.3 ± 4.5^{b}	272.9 ± 6.1^{c}	0.000*	0.000*	0.000*	0.000*
	14-day	153.2 ± 3.2^{a}	252.1 ± 2.7^{b}	246.3 ± 3.1^{b}	291.9 ± 2.2^{c}	0.000*	0.000*	0.000*	0.000*
Phagocytic activity (%)	0-day	12.5 ± 0.9^{a}	22.3 ± 0.2^{b}	34.0 ± 0.5^{c}	24.5 ± 0.7^{b}	0.000*	0.032*	0.000*	0.000*
	7-day	11.3 ± 0.5^{a}	27.3 ± 0.9^{b}	38.8 ± 1.1^{d}	35.0 ± 1.5^{c}	0.000*	0.001*	0.000*	0.000*
	14-day	10.3 ± 0.5^{a}	28.8 ± 1.1^{b}	41.8 ± 0.8^{d}	38.0 ± 0.9^{c}	0.000*	0.001*	0.000*	0.000*
Respiratory burst activity (OD at 540 nm)	0-day	0.3761 ± 0.0049^{a}	0.4811 ± 0.0051^{b}	0.4731 ± 0.0015^{b}	0.4969 ± 0.0053^{b}	0.000*	0.002*	0.000*	0.000*
	7-day	0.2965 ± 0.0025^{a}	0.3259 ± 0.0006^{c}	0.3263 ± 0.0013^{c}	0.3052 ± 0.0022^{b}	0.000*	0.911	0.000*	0.000*
	14-day	0.3463 ± 0.0104^{a}	0.4147 ± 0.0040^{b}	0.4101 ± 0.0078^{b}	0.4246 ± 0.0118^{b}	0.000*	0.005*	0.002*	0.000*
Total serum immunoglobulin (g/L)	0-day	22.5 ± 2.3^{a}	34.5 ± 1.9^{c}	37.3 ± 0.7^{d}	28.8 ± 1.1^{b}	0.000*	0.408	0.000*	0.000*
	7-day	20.3 ± 0.9^{a}	32.1 ± 1.1^{d}	28.0 ± 0.6^{c}	24.3 ± 0.2^{b}	0.000*	0.904	0.009*	0.000*
	14-day	17.8 ± 1.4^{a}	31.4 ± 0.3^{b}	29.4 ± 0.9^{b}	29.5 ± 0.3^{b}	0.000*	0.032*	0.001*	0.000*

Values are means ± SE of three replicates ($n = 3$). Values in the same row with different superscript letters are statistically different according to Duncan's test ($p < 0.05$).
Note:.
* significant.

4. Discussion

Both wild and farmed fish are vulnerable to various bacterial infectious pathogens including *Vibrio harveyi, Shigella flexneri, Enterobacter* sp., *Salmonella* sp., *Flavobacterium* spp., *Aeromonas* spp. and *Pseudomonas aeruginosa* [49]. These pathogenic bacteria have the ability to infiltrate the immune system of a fish and iniatiate disease infection [50]. Consequently, stimulating fish immune system can provide protection against these infectious diseases. It has been observed that plant-derived immunostimulants activate the immune response and enhance innate immunological mechanisms, thereby increasing fish resistance to infectious diseases [51]. This is the first study to report the effects of *P. chinense* extract (PCE) on disease resistance in fish. However, these findings are consistent with the previous studies that demonstrated that feed supplementation with different plants such as *Allium sativum* [46], *Musa paradisiacal* [52], *Cissus quadrangularis* [53], *Mentha piperita* [54], *Azadirachta indica* [55], and *Zingiber officinale* [56] significantly increase the survival rate of Asian seabass after bacterial infection. The immunostimulatory capability of PCE-supplemented diet was established in this study, as fish given PCE diet showed superior resistance to the challenge *V. harveyi* infection in terms of survival rate and immunological response. After *V. harveyi* challenge, all fish fed PCE had a significantly greater survival rate than the control.

The findings of this study clearly reveal that oral administration of PCE could improve health status, as evidenced by a higher heamatological response than in the unsupplemented fish. Hematological parameters of fish cultivation can serve as clinical indications of the fish's physiological status, health, and diseases conditions, all of which affect

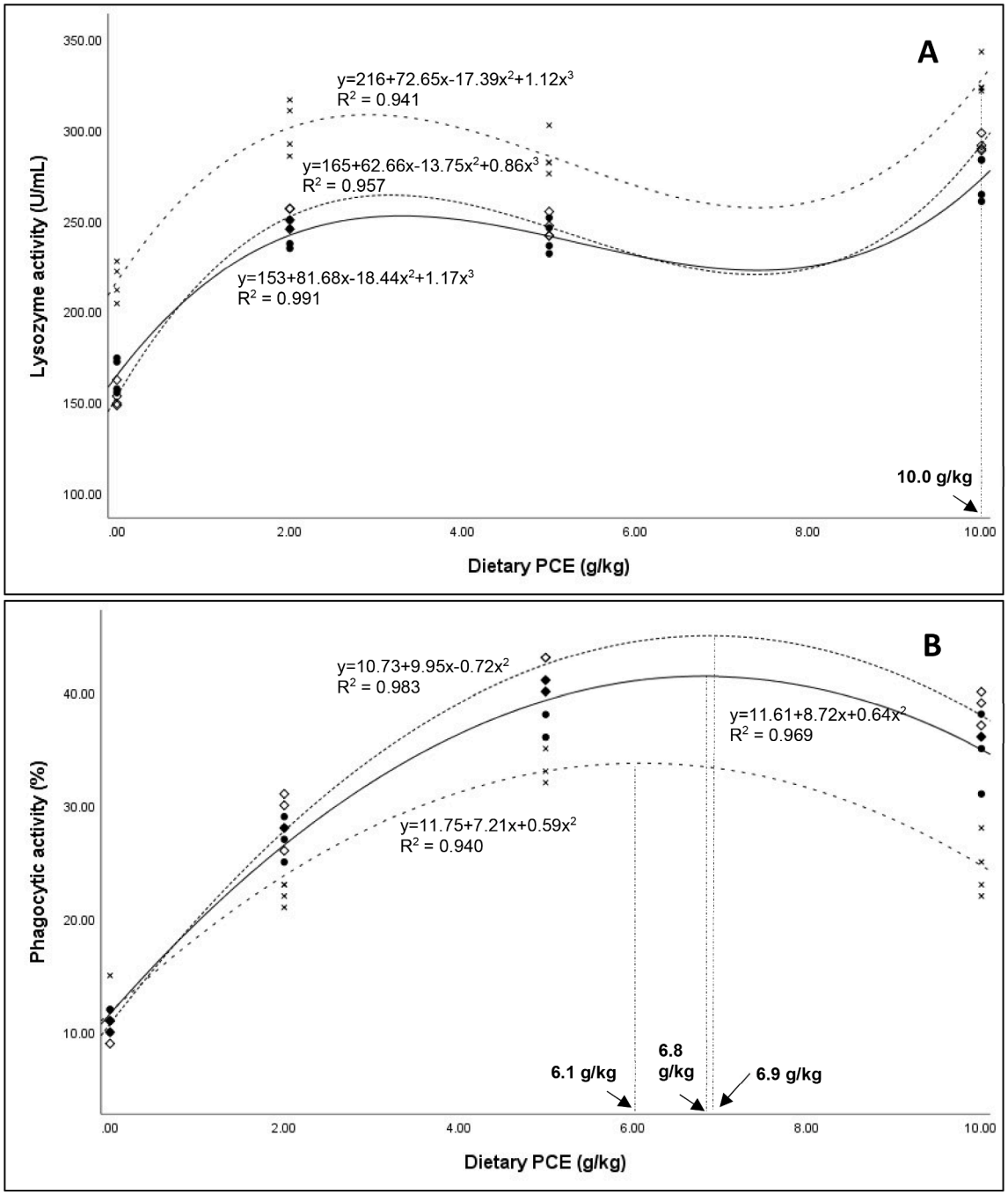

Fig. 11. Polynomial regressions of (A) lysozyme, (B) phagocytic, (C) respiratory burst activities and (D) total serum immunoglobulin of Asian seabass fed diets supplemented with graded levels of *Polygonum chinense* extract and subjected to *Vibrio harveyi* infection at 0-day (loosely dashed line), 7-day (solid line), and 14-day (dashed line) post-infection periods.

aquaculture production performance [57]. The leukocyt count (WBC) is an important hematology parameter in determining fish immune status [58]. Leukocytes are regarded to be the initial line of defence against foreign diseases and a marker for non-specific immunity, which indicates fish health [59]. In the present study, the leukocytes was considerably greater in the treatment groups after *V. harveyi* post-challenge, indication of the immunostimulatory effect of *P. chinense* extract on WBC production in *L. calcarifer*. Through the current infection trial, an escalate in WBC counts in the fish fed PCE diets indicated the cell-mediated immunity can be strengthened to counteract bacterial invasion, and recovery is possible. A continuous decrease in the control fish, on the other hand, may indicate an impaired or weakened immune system, making fish more susceptible to bacterial pathogens and, as a result, worsening the infection [60]. Similar increases in WBC were observed in *Oreochromis niloticus* fed *Excoecaria agallocha* extract and challenged with *Streptococcus agalactiae* [61] and in *O. niloticus* fed propolis and *Aloe barbadensis* and challenged with *Aeromonas hydrophila* [62]. Furthermore, the gradual increase in RBC count in the PCE-treated groups could be attributed to PCE's ability to induce an antioxidant response in RBC, which could protect their membrane from hemolysis. Increased RBC levels in the treated groups indicate fish defence mechanism against pathogenic infection in which the body produces more

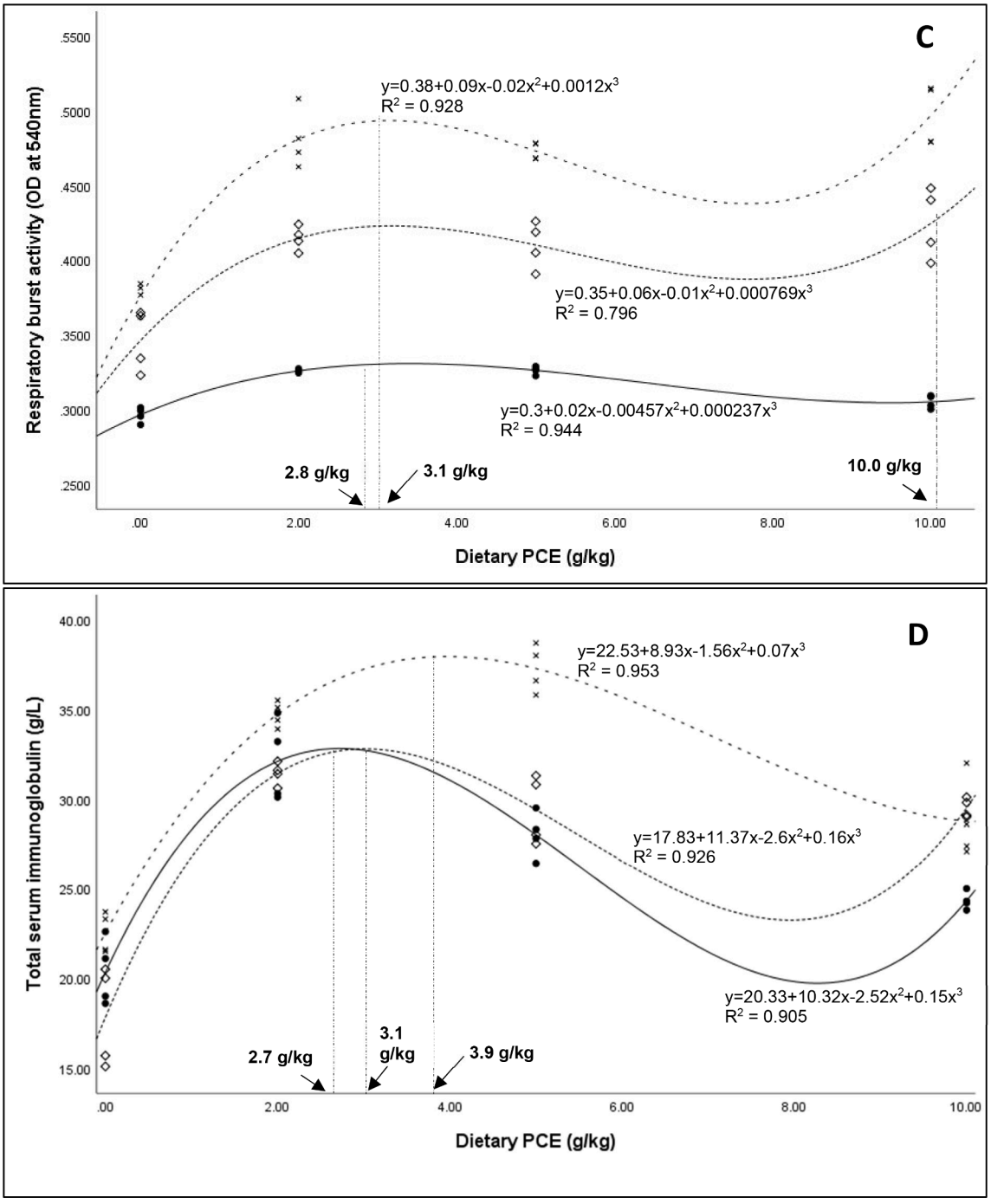

Fig. 11. (*continued*).

RBC to replace RBC lysis caused by infection.

The PCE-supplemented groups exhibited increase on the serum total protein and globulin concentrations. Previous studies by Das et al. [63] and Talpur and Ikhwanuddin [55] discovered that increasing total protein and globulin with feed supplementation of *Ocimum sanctum* and *A. indica* after bacteria post-challenge yielded comparable results. As serum total protein and globulin are amongst the non-specific humoral defence mechanisms [64] they can be used to detect the activation of immune system [65]. This higher level of total protein could be attributed to these extracts' ability to boost the antibody production [66] and repair damaged tissues in infected fish [67]. In addition, the fact that fish have higher levels of immunologically active globulin proteins in their blood [68] may explain the rise in globulin levels observed in these studies as a response to a more robust innate immune system in fish. Another common physiological index of energy regulation in aquatic organisms is the concentration of serum glucose, which rises in response to stressors of increasing magnitude [69]. Herein, the control-infected group had elevated serum glucose level, implying that glycogenolysis was enhanced with a decline in glycolytic pathway as a regulatory mechanism in response to glycogen mobilization into glucose to satisfy the increased energy demand in combating the stress caused by the bacterial infection [70]. Surprisingly, the groups supplemented with PCE presented lowered glucose levels, reflecting the potential of PCE to relieve the infection stress by improving insulin sensitivity and glucose metabolism. The levels of the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in fish are important indicators of

hepatic health [71]. As a result of hepatic cell damage caused by *V. harveyi*, the control group showed a significant increase in liver enzymes AST and ALT. Nonetheless, analysis of these hepatic enzymes revealed an improvement in the fish health status of the PCE-supplemented groups, with significant reductions in ALT and AST levels. The presence of some bioactive compounds in *P. chinense* may have promoted hepatic function, since other *Polygonum* species have been shown to have potential hepatoprotective properties against liver injury in mice [72–74].

Phagocytic cells are one of the most significant cellular components of the innate immune system of fish that may eliminate invading pathogens [75]. These phagocytic cells can produce reactive oxygen species (ROS) that is harmful to bacterial pathogens when stimulated during a period of intense oxygen consumption. A process known as respiratory burst occurs when phagocytes recognise and engulf a pathogen. Pathogen elimination is aided by ROS produced by phagocytes during respiratory burst [76]. In the current investigation, fish fed *P. chinense* extract had considerably higher phagocytic and respiratory burst activity than control. This study reveals that *P. chinense* extract increased phagocytosis and reactive oxygen species generation in response to *V. harveyi* infection. This conclusion is consistent with the finding of Laith et al. [56], who found that feeding *O. niloticus* different concentrations of *E. agallocha* extract significantly boosted phagocytic and respiratory burst activities.

Lysozyme is an antimicrobial enzyme that can lyse the cell walls of both Gram-positive and -negative bacteria [77], serving as a fish defence against bacterial infection [78]. Enhanced lysozyme activity can regulate and prevent infection, as well as keep mortality rates in aquaculture low [79]. After being challenged with *V. harveyi*, the fish fed on diets containing *P. chinense* extract in the current study displayed considerably increased lysozyme activity. After *A. hydrophila* challenge, lysozyme activity increased in *Labeo rohita* fed a diet containing single or mixed *Psidium guajava* and *Mangifera indica* extracts [80]. *Oreochromis niloticus* infected with *A. hydrophila* and fed a diet containing *Withania somnifera* extract showed increased lysozyme activity as well [81]. All of these findings suggested that plant extract supplementation diets can benefit fish with non-specific immune responses [82].

In addition to humoral innate immune responses, total immunoglobulin plays a significant function in the host's defence systems and serves as a biomarker for the adaptive immunological response in fish [83]. Because immunoglobulin is an antibody that plays an important part in the immune response of fish, measuring total immunoglobulin in the serum can disclose a lot about the fish's humoral immunological status [84]. Immunoglobulins serve an important role in fish health, allowing them to fight off bacterial, parasitic, and viral infections as well as recover from them [85]. In the current investigation, serum total immunoglobulin levels increased considerably in infected fish fed *P. chinense*-enriched diets. Similar increases in total immunoglobulin levels were seen in *Epinephelus brunneus* after they were fed *Lactuca indica* following a challenge with *Streptococcus iniae* [86] and in *L. rohita* after they were fed *O. sanctum* following a challenge with *A. hydrophila* [63]. An increase in the number of these specific antibodies' aids in the neutralisation and rapid elimination of antigens delivered into the host body [87].

5. Conclusion

This study confirms the beneficial effects of *P. chinense* supplementation on the hematological, biochemical, and humoral immune responses of Asian seabass, which improved in a dose-dependent manner. Dietary administration of *P. chinense* extract at doses of 5 and 10 g/kg showed the greatest potential for enhancing haemato-biochemical and immune response parameters in Asian seabass, as well as providing greater protection against *V. harveyi* infection. Nonetheless, the bioactive components involved in *P. chinense* extract's protective action have yet to be identified. More research into the method of action and pharmacokinetics of the identified compounds is needed to better understand and develop therapeutic and immunostimulant drugs for aquaculture.

CRediT authorship contribution statement

Abdul Razak Rashidah: Writing – original draft, Investigation, Formal analysis. **Mohamed Shariff:** Conceptualization, Writing – review & editing, Resources, Supervision. **Fatimah Md. Yusoff:** Conceptualization, Supervision, Validation. **Intan Safinar Ismail:** Supervision, Validation.

Declaration of Competing Interest

The authors declare that they do not have any known competing financial interests or personal relationships that could appear to have influenced the work reported in this study.

Data availability

Data will be made available on request.

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