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Original Article

The effects of *Excoecaria agallocha* extract on growth, innate immune responses, and resistance to Vibrio parahaemolyticus in white shrimp, *Litopenaeus vannamei*

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Abstract: The purpose of this study is to determine the effects of supplementary *Excoecaria agallocha* extract (EAE) on growth performance, innate immune responses, and resistance to *Vibrio parahaemolyticus* in white shrimp, *Litopenaeus vannamei*. Shrimp were separated into four groups, with each group fed diets containing different concentrations of 0 (control), 20 (EAE20), 30 (EAE30), and 40 (EAE40) g kg^{-1}. The results indicated that shrimp fed diet containing different concentrations of EAE did not affect growth performance and survival rate for a 56-day feeding trial. However, shrimp fed a diet containing EAE20 showed increased HC, GC, THC, PO activity, and Phagocytic rate on days 1, 3, 7, 14, and 28. Moreover, supplemented different concentrations of EAE in the diets showed increased resistance to *V. parahaemolyticus* infection of white shrimp after 56 days of feeding. The results indicate that EAE could be applied to shrimp feed to enhance immune response and resistance to bacterial disease.

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Introduction

Shrimp lack adaptive immunity; instead, they rely on an innate immunity to defend themselves against microbes. Three types of haemocytes, known as hyaline cells (HCs), semi-granular cells (SGCs), and granular cells (GCs), participate in immune reactions, including recognition, phagocytosis, melanization, encapsulation, and cytotoxicity (Lee and Söderhäll, 2002; Jiravanichpaisal et al., 2006). HCs are mainly involved in phagocytosis, whereas SGCs and GCs store and release proPO, peroxinectin, and other molecules (Johansson et al., 2000).

White shrimp, *Litopenaeus vannamei,* is the most important cultured penaeid species, with global production exceeding 5.8 million tons in 2020, and ranked as the top species in commercial value among fish, crustaceans, and mollusks (FAO, 2022). Shrimp farming frequently encounters disease threats caused by bacteria, viruses, fungi, and protozoa (Lightner and Redman, 1998). Acute hepatopancreatic necrosis disease (AHPND) and white faeces syndrome (WFD) are infectious bacterial severe diseases that have plagued many Asian countries (De Schryver et al., 2014; Sriurairatana et al., 2014; Thitamadee et al., 2016). Among them, *Vibrio parahaemolyticus* infection is the most concerning problem in shrimp aquaculture worldwide because it causes acute hepatopancreatic necrosis disease. The mortality rate due to AHPND is very high, at around 40-100% (De Schryver et al., 2014; Kua et al., 2016). This disease was first reported in China in 2009 and later observed throughout Vietnam, Malaysia, Thailand, Mexico, India, the Philippines, and the United States (Nguyen et al., 2021).

The application of immunostimulants to enhance the immune response of shrimp and their resistance to pathogens has received attention (Kumar et al., 2023; Smith et al., 2003). Many medicinal plants rich in phytochemicals can attenuate pathogenic microbes, and bioactive compounds that activate shrimp immune system are used as feed additives for disease control in aquatic organisms (Aminzare et al., 2018; Dewi et al., 2021; Liao et al., 2022; Chang et al., 2023; Maurus et al., 2023; Moh et al., 2024).

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Excoecaria agallocha extract (EAE) from leaves contains many bioactive compounds, such as alkaloids, flavanoids, tannins, saponin, steroids, triterpenoids, phenols, and glycosides (Sofia and Teresa, 2016). Some studies indicated that EAE was effective against human diseases. A phorbol ester obtained from its leaves is a potent inhibitor of HIV1 replication (Erickson et al., 1995). In contrast, polyphenols from the leaves showed inhibitory effects against hepatitis C virus (Li et al., 2012) and anticancer activity (Rifai et al., 2011). However, few studies have been conducted on the effects of EAE on aquatic animals. Therefore, this study aims to investigate the effect of EAE on immune response and resistance to the pathogen *V. paraheamolyticus* in *L. vannamei.*

Materials and Methods

Preparation of EAE: *Excoecaria agallocha* leaves were collected from Duyen Hai District, Tra Vinh province, Viet Nam. EAE was prepared using the method described by Parekh and Chanda (2007). Briefly, 100 g of *E. agallocha* powdered leaves were extracted in 500 mL ethanol at a ratio 1:5 for 7 days. Then, the crude extracts were filtered using Whitman No-1 filter paper, evaporated, and concentrated into solid extracts at room temperature. The EAE obtained was stored at 4°C for further experiments.

Experimental feed preparation: Commercial diets were ground and supplemented with different concentrations of EAE. The diet without EAE was used as the control group; the EAE was added to the test diets at levels of 20 (EAE20), 30 (EAE30), and 40 (EAE40) g kg^{-1} diet. Each diet was then passed through a mincer to form pellets of the experimental diets. It was then dried in a drying cabinet using an air blower at 40°C until the moisture levels were 10%. After drying, the finished pellets were stored in sealed plastic bins at 4°C until use.

Preparation of *V. parahaemolyticus*: A strain of *V. parahaemolyticus* isolated from diseased white shrimp was used for the study (Nhi et al., 2020). Stocks were cultured on thiosulfate citrate bile salt sucrose (TCBS; Merck) for 24 h at 37°C and then transferred to 10 ml of tryptic soy broth (TSB; supplemented with 2% NaCl, Merck) for 24 h at 37°C. The broth culture was centrifuged at 7155×g for 20 min at 4°C. The supernatant was removed, and bacterial pellets were re-suspended in a marine saline solution (MS) at 2.0×10^8 colony-forming units (CFU) ml^{-1} as suspensions for the challenged tests (Yeh and Chen, 2009).

Experimental animals: White shrimp were obtained from the Center for Aquaculture and Research, Tra Vinh University, Viet Nam. They were acclimatized to laboratory conditions in composite tanks and fed twice a day. During the experiments, the water temperature was 24±1°C, pH 8.06-8.34, and salinity 15‰.

Experimental design

Effect of dietary EAE on the growth performance of white shrimp: A total of 360 shrimp with an initial weight of 3.0±0.14 g was randomly divided into 4 tanks, each consisting of three replicates (30 shrimp/replicate), and the shrimp in each replicate were reared in cages. Shrimp in four experimental diets, 0 (control), EAE20, EAE30, and EAE40, were fed approximately 5% of their body weight at 9:00, 15:00, and 21:00. The individuals were weighted every two weeks over the experimental period of 56 days. Uneaten food and waste were removed before each feeding, and a third of the water was exchanged daily. The parameters such as weight gain percentage (WG%), specific growth rate (SGR), and survival rate (SR) were calculated using the following equations (Cárdenas et al., 2015).

WG (%) = [final weight (g) – initial weight (g)]/ initial weight (g) × 100

SGR (% day^{-1}) = [ln final weight (g) – ln initial weight (g)]/ days × 100

SR (%) = Final numbers/Initial numbers) × 100

Effect of EAE on immune response: 144 white shrimps (14.5±0.48 g) were randomly distributed into 4 tanks corresponding to four experimental groups with three replicates of each. The shrimp's immune parameters, total haemocyte count (THC), differential haemocyte count (hyaline cell-HC and granular cell-GC), phenoloxidase activity, and phagocytic activity

were measured on days 0, 1, 3, 7, 14, and 28 of the experiment. The immune parameters are described below.

Haemolymph (50 µL) was extracted from the ventral sinus cavities of shrimp using a 1 mL sterile syringe with a 25-gauge needle. The haemolymph was diluted in 450 µL of anticoagulant. A drop of the diluted haemolymph was placed in a haemocytometer to obtain THC using a microscope (CX23, Olympus Corporation Tokyo, Japan). All the haemocytes in both the upper and lower fields (1 × 1 mm) of the haemocytometer were counted (Yeh and Chen, 2009).

The HC and GC were measured based on the methods described by Le Moullac et al. (1997). Briefly, haemolymph was withdrawn from the ventral sinus of each shrimp into a 1-mL sterile syringe (25 gauge), and it was then 2-fold diluted with an anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, and 10 mM EDTA, 0.115 M glucose). The anticoagulant haemolymph mixture was centrifuged at 700×g for 5 min at 4°C, the supernatant discarded, and the pellet rinsed once with the formalin-anticoagulant (pH 4.6) and resuspended gently in the same solution. A drop of the haemocyte suspension was spread on glass slides, air-dried, fixed for 5 min in ethanol, washed in distilled water, and flooded with Giemsa stain for 30 min. The stained slides were rinsed in acetone and xylene.

Phenoloxidase (PO) activity was measured using spectrophotometry by recording dopachrome formation produced from L-dihydroxyphenlalanine (HernándezLóp et al., 1996). PO activity was expressed as dopachrome formation per 50 µl of haemolymph. Briefly, 100 µl of haemolymph was withdrawn and diluted in 900 µl of anticoagulant. The diluted haemolymph in 1.5 ml Eppendorf was first centrifuged at 800×g at 4°C for 20 min. The supernatant fluid was removed, and the pellet was re-suspended again in 1 ml cacodylate citrate buffer solution (sodium cacodylate 0.01 M, sodium chloride 0.45 M, trisodium citrate 0.01 M, pH 7.0) and centrifuged again with the same setting as before. After the second centrifuge, the supernatant was disposed of, and the pellet was re-suspended with 200 µl caccodylate buffer solution (sodium cacodylate 0.01 M, sodium chloride 0.45 M, calcium chloride 0.01 M, magnesium chloride 0.26 M, pH 7.0). The 200 µl aliquot was divided equally into two parts: 100 µl for sample analysis and another 100 µl for background data analysis. The 100 µl of aliquot for sample analysis was incubated with 50 µl of trypsin (1 mg ml^{-1}) for 10 min at room temperature. Then, 50 µl of L-DOPA was added, followed by 800 µl cacodylate buffer 5 min later. The sample was then measured using a Hitachi U-2000 spectrophotometer (Tokyo, Japan) at 490 nm optical density. For the background of phenoloxidase, the other 100 µl of cell suspension was incubated with 50 µl of cacodylate buffer (to replace with trypsin), and 50 µl of L-DOPA as the control solution for the background PO activity.

Phagocytic activity was measured using a previously described method (Ngo et al., 2020). Briefly, 200 µl of the diluted haemolymph sample was spread on a glass slide and incubated for 60 min. Cells were washed with MCHBSS after incubation with 200 µl Latex beads (0.8 µm; 3×10^7 beads ml^{-1}, Sigma Aldrich) for 60 min. Next, cells were fixed by adding 200 µl of 100% methanol to the glass slide and then washing with diluted water. The slide was stained with Giemsa (5 %) for 20 min, washed with distilled water, air-dried, and finally observed under a light microscope. Two hundred haemocytes were counted. Phagocytic activity, defined as phagocytic rate (PR) and phagocytic index (PI), were calculated as:

PR % = (Phagocytic haemocytes)/ (Total haemocytes) × 100

PI = Beads in phagocytic cells/ Total phagocytic cells

Resistance to V. parahaemolyticus in shrimp fed diets containing EAE: Shrimp fed-diets containing 0 (control), EAE 20, EAE 30, and EAE 40 after the 56-day feeding were used for the experiment. A challenge test was conducted by injecting 20 µL of a bacterial suspension of 2.0×10^8 CFU ml^{-1}, resulting in 4.0×10^6 CFU per shrimp into the ventral sinus of the cephalothorax. In addition, 30 shrimp fed the control diet were injected with MS (20 µL) to serve as unchallenged controls. Experimental and control shrimp were kept in 90 L glass aquaria containing 70

Table 1. The initial weight, final weight, weight gain, SGR, and survival of white shrimp *Litopenaeus vannamei* fed a diet containing EAE at 0 (control), 20 (EAE20), 30 (EAE30) and 40 (PAE40) g kg^{-1} for 56-day feeding trial.

Diet	Initial Weight (g)	Final Weight (g)	Weight gain (%)	SGR (% per day)	Survival rate (%)
Control	3.0±0.14[a]	20.30±0.37[a]	598.99±29.15[a]	3.42±0.08[a]	84.44±2.22[a]
EAE20	3.0±0.14[a]	20.40±0.28 [a]	603,66±29.65[a]	3.44±0.08[a]	84.44±2.94[a]
EAE30	3.0±0.14[a]	20.35±0.31 [a]	600.54±27.41[a]	3.43±0.08[a]	85.56±2.94[a]
EAE40	3.0±0.14[a]	17.80±0.67[b]	519.71±35,63[a]	3.16±0.10[b]	81.11±2.94[a]

Data are presented as mean±SD. Values in the same column with different superscripts are significantly different from each other (P<0.05)

L of 15±1‰ sea water at 27±1°C, with three replicates of each. Each aquarium was provided with continuous aeration, and water was renewed daily during the challenge test. Mortality was counted on a daily basis for the total experimental period of 168 h. The survival rate (SR) was calculated using the following formula: SR (%) = (number of individuals at the end of experimental period/ initial number of individuals stocked) × 100

Statistical analysis: All data were subjected to a one-way analysis of variance (ANOVA), and Duncan's multiple-comparison test was conducted to examine significant differences among treatments using IBM SPSS (Version 20.0 SPSS). Significant differences were considered at P<0.05.

Results

Effect of dietary EAE on growth performance and survival rate of white shrimp: At the beginning of the experiment, there were no significant differences in initial weight (IW) among all treatments. After 56 days of the experiment, no significant difference in final weight (FW), Weight gain (WG), or specific growth rate (SGR) was found in shrimp fed diets of EAE20, EAE30, and control diet. Notably, the FW and SGR of shrimp fed EAE40 were significantly lower than those with EAE20, EAE30 and control diet diets. There was no significant difference in the survival rate of shrimp with diets of EAE20, EAE30, EAE40, and the control diet (Table 1).

Immune responses of white shrimp fed diets containing EAE: The THC, HC, and GC of shrimp fed a diet of EAE20 were significantly higher than those of other diets (Fig. 1). The HC of shrimp fed a diet of EAE20 diet was significantly higher than that of other diets, except for day 1. No significant difference in HC was found in shrimp fed diets of EAE30, EAE30, and control diet (Fig. 1A). Similarly, the GC of shrimp fed diets of EAE20 and 30EAE was significantly higher than that of other diets on day 1. However, no significant difference in GC was found among diets on day 3. The GC of shrimp fed a diet of EAE20 was significantly higher than that of other diets on days 7, 14, and 28 (Fig. 1B). The THC of shrimp fed a diet of EAE20 diet was also significantly higher than that of other diets. No significant difference in THC was recorded among diets of EAE30, EAE40, and control diet from day 3-28 (Fig. 1C).

The PO activities of shrimps fed a diet of EAE20 were significantly higher than those of other diets from days 1-3. From day 7-28, the PO activities of shrimp fed a diet of EAE20 and EAE30 were significantly higher than those of EAE40 and control diets. No significant difference in PO activities was found between shrimp fed a diet of EAE20 and EAE30 and between the diet of EAE40 and a control diet (Fig. 2).

The PR and PI activity of shrimp fed different concentrations of EAE for 28 days is shown in Figure 3. The PR of shrimp fed a diet of EAE20 was significantly higher than that of a diet of EAE40 and a control diet on the 1st and 3rd days. There was no significant difference in the PR of shrimp fed a diet of EAE40 and a control diet on these days. The PR of fed a diet EAE20 was significantly higher than other diets on the 7th and 14th days. There was no significant difference in the PR of shrimp fed with diets of EAE30 and EAE40 and control diet these days. At the end of the experiment, no significant difference in the PR of shrimp was determined among diets (Fig. 3A). It was also found that no significant difference in the PI of

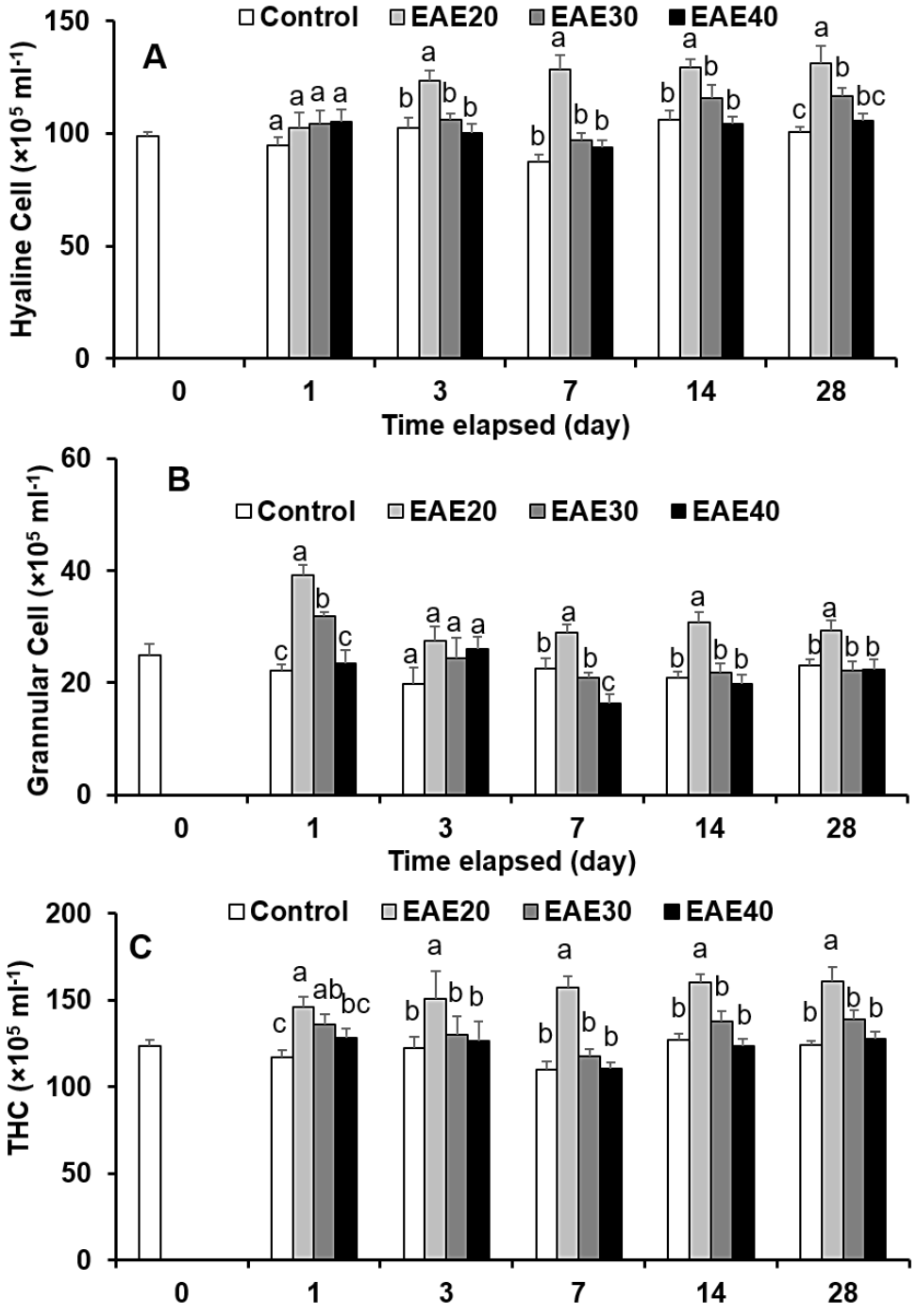

Figure 1. The hyaline cells (A), granular cells (including semi-granular cells) (B), and total haemocyte counts (C) of white shrimp *Litopenaeus vannamei* with diets of 0 (control), EAE20, EAE30, and EAE40 for 0, 1, 3, 7, 14, and 28 days. Data are expressed as mean±SE (n = 6) and significant differences (P<0.05) are indicated with different letters.

shrimp was observed among diets (Fig. 3B).

Resistance to *V. parahaemolyticus* in shrimp fed diets containing EAE: After 56 days of the experiment, shrimp were randomly selected for challenging with *V. parahaemolyticus*. No mortality was observed in the unchallenged control group over a 168-h period. For challenged groups, survival rates of shrimp that received the diet containing EAE20, EAE30, and EAE40 were significantly higher than the challenged control shrimp after 24-168 h. At the end of the challenge experiment, the cumulative survival rates of shrimp were recorded to be 75.0±5.77,

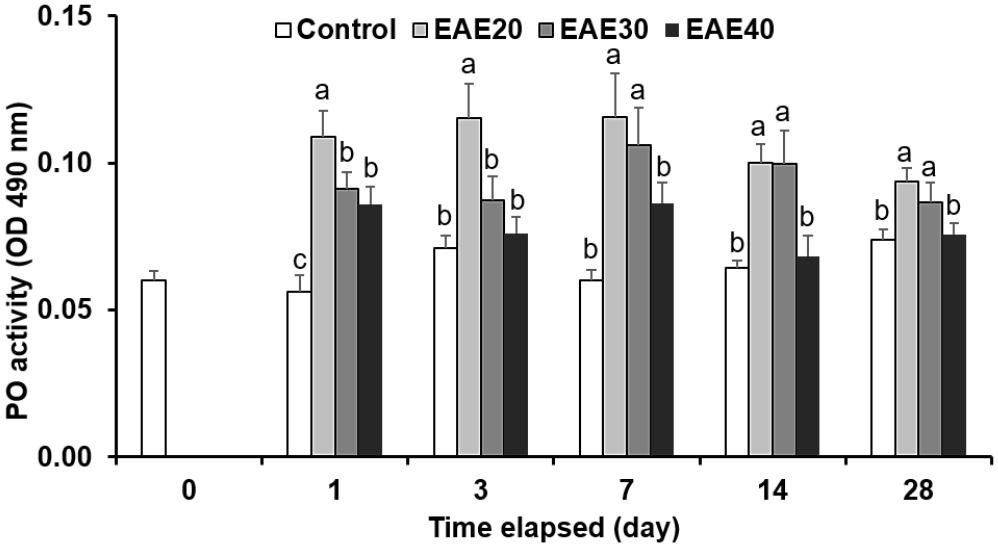

Figure 2. The phenoloxidase activity of white shrimp *Litopenaeus vannamei* fed diets of 0 (control), EAE20, EAE30, and EAE40 for 0, 1, 3, 7, 14, and 28 days. Data are expressed as mean±SE (n = 6), and significant differences (P<0.05) are indicated with different letters.

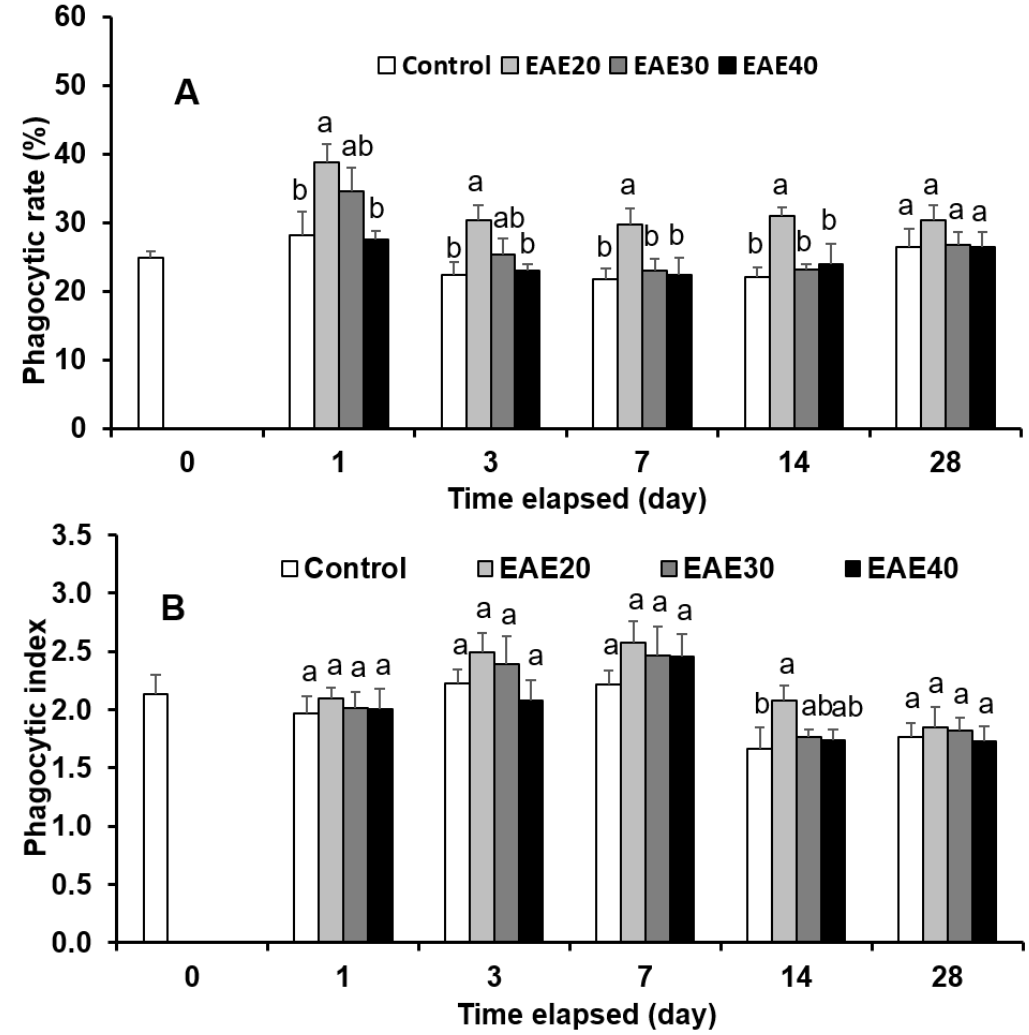

Figure3. Phagocytic rate (A) and phagocytic index (B) of white shrimp *Litopenaeus vannamei* fed diets of 0 (control), EAE20, EAE30, and EAE40 for 28 days feeding trial. Data are expressed as mean±SE (n = 6) and significant differences (P<0.05) are indicated with different letters.

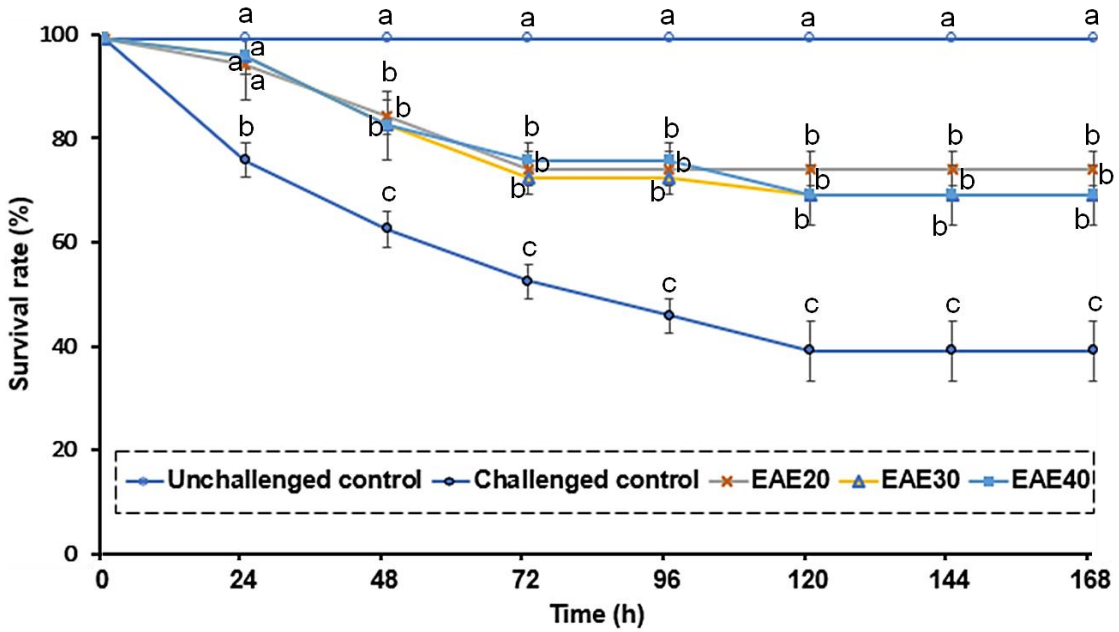

Figure 4. The cumulative survival rate of white shrimp *Litopenaeus vannamei* fed diets of 0 (control), EAE20, EAE30, and EAE40 for 56-day feeding trial, and then challenged with *Vibrio parahaemolyticus*. Data (mean±SE) at the same time with different letters significantly differ (P<0.05) among treatments.

70.0±0.00, and 70.0± 10.00% for a diet containing EAE20, EAE30, and EAE40, respectively (Fig. 4).

Discussions

Several studies have demonstrated the efficiency of using medicinal herbs to promote many aquatic animals' growth rate, survival, and nutrient absorption ability (Ahmad and Abdel, 2011; Ghosh et al., 2021; Huang et al., 2022). The main benefit of using herbs as sources of nutrients is that they enhance growth and improve physiological functions, resulting from an increase in energy flow that occurs upon application of the herb (Galina et al., 2009). However, along with nutrients, tannin, and saponin, which were anti-nutrients from plant extract, negatively affect shrimp's growth and survival rate. A previous study indicated that higher concentrations of tannin might be toxic to shrimp in aquaculture systems (Fitzgerald, 2000). Similarly, tannin from some leaves of mangrove species, *E. agallocha, Rhizophora apiculata*, *Avicennia officinalis*, and *Acacia auriculiformis* can negatively impact shrimp growth and survival rate of tiger shrimp (Hai and Yakupitiyage, 2005; Rejeki et al., 2019). In this study, the growth performance of shrimp fed with diets containing EAE was not significantly higher than that of the control diet. Especially, FW, WG, and SGR of shrimp fed a diet of EAE40 were significantly lower than those of other diets. The explanation for this result could be that shrimp fed a diet of EAE40 accumulated high concentrations of saponin and tannin in its body and water. Therefore, it had a negative effect on the growth performance of white shrimp.

Haemocytes play the most important role in cellular immune response and produce and release various humoral immune factors (Bachère et al., 2004). White shrimp receiving guava leaf extract showed increased THC, RB, PO, phagocytic activity (Dewi et al., 2021). Similarly, THC, DHC, PO activity, and RBs of shrimp fed diet containing cacao pod husk extraction were significantly higher than those of other groups after 30 days of feeding trial (Chang et al., 2023). Our study indicated that the THC, GC, and HC of shrimp fed a diet of EAE20 were significantly higher than those of other diets. This can be attributed to the accelerated maturation of haemocyte precursors in the haematopoietic tissue, followed by the release of new cells into the circulation system to maintain the haemocyte population and functionality in the shrimp (Sequeira

et al., 1996).

PO, a crucial enzyme of the proPO system in invertebrates, promotes humoral defense mechanisms and subsequently eliminates pathogens (Cerenius and Söderhäll, 2004). In this study, the PO activity of shrimp-fed diets of EAE20 and EAE30 was significantly higher than that of other diets (Fig. 2). The result of this study is similar to some previous research. A previous study indicated that the PO activity of white shrimp fed a diet containing *Phyllanthus amarus* extract at concentrations of 20 and 40 g kg^{-1} was significantly higher than that of the control diet (Ngo et al., 2020). The similar effects were also observed in white shrimp, in which PO activity increased when they received a diet supplemented with guava leaf extract at a concentration of 5 g kg^{-1} and cacao pod husk extraction at concentrations of 12.5 and 25 g kg^{-1} (Chang et al., 2023; Dewi et al., 2021).

Phagocytosis is an important cellular defense mechanism in crustaceans (Ratcliffe et al., 1985). Previous studies have shown that bioactive compounds from plant extracts can increase the phagocytic activity of haemocytes (Rattanavichai and Cheng, 2015; Wu et al., 2015). The PR of shrimp fed a diet of EAE20 was significantly higher than that of other diets from day 1 to 14. Similarly, the PR of white shrimp fed diet containing *P. amarus* extract at concentrations of 20 and 40 g kg^{-1} were significantly higher than those from the control group from day 4 to 28 of the feeding trial (Ngo et al., 2020). Also, a work noted that the PR of white shrimp fed a diet supplemented with guava leaf extract at the concentration of 5 g kg^{-1} was significantly higher than that of the control diet from day 7-28 (Dewi et al., 2021). Recently, research indicated that shrimp-fed diets supplemented with cacao pod husk powder (25 g kg^{-1}) and cacao pod husk supernatant (12.5 g kg^{-1}) group had higher phagocytic activity than the control diet at 30 and 90 days (Chang et al., 2023).

It is known that *V. parahaemolyticus* is the causative agent of AHPND in shrimp and has caused large economic losses for shrimp farming worldwide (Nguyen et al., 2021). Recently, immunostimulants were used as an alternative to antibiotics. They were found to enhance the immune system of shrimps in eco-friendly manner (Kumar et al., 2023). EAE is rich in phytochemical compounds, such as alkaloids, flavanoids, tannins, saponin, steroids, triterpenoids, and phenols, which inhibit bacterial activity *in vitro* (Sofia and Teresa, 2016). The survival rate of shrimp fed diet containing EAE was significantly higher than that of the control group during the challenge test. This finding is similar to previous studies in which white shrimp fed a diet containing plant extract was effective against *V. parahaemolyticus* (Dewi et al., 2021; Maurus et al., 2023). Additionally, the previous study indicated that the survival rate of shrimp fed diet containing aqueous extracts from *E. agallocha* was effective against white spot syndrome disease in 15 days, getting a 75% survival rate (Sudheer et al., 2011).

Conclusions

The present study demonstrated that EAE-supplemented diets did not enhance white shrimp's survival rate and growth performance. However, shrimp fed a diet of EAE20 showed increased immune response by increasing HC, GC, THC PO activity, and phagocytic rate. In addition, supplemented different concentrations of EAE in the diets showed increased resistance to *V. parahaemolyticus* infection of white shrimp after 56 days of feeding. This result indicates that the potential of EAE can be used to manage aquatic bacterial disease.

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