

Increased non-specific immune activity of vaname shrimp *Litopenaeus vannamei* using a leaf flour mixture from *Ocimum basilicum* and *Piper betle* and their characteristic compounds

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Abstract

Immunostimulants are more suitable for improving the health of shrimp than using drugs in the prevention of diseases. This study evaluated the efficacy of bioactive compounds from *Ocimum basilicum* and *Piper betle* in increasing the non-specific immune response of *Litopenaeus vannamei*. Samples were collected directly from a traditional market. Shrimp was fed: *O. basilicum* leaf flour- and *P. betle* leaf flour-un-supplemented diet (P1), *O. basilicum* leaf flour (44.84 g/kg feed)-supplemented diet (P2), *P. betle* leaf flour (31.57 g/kg feed)-supplemented diet (P3), and *O. basilicum* leaf flour (22.42 g/kg feed)- and *P. betle* leaf flour (16.79 g/kg feed)-supplemented diet (P4). Phytochemical screening tests were conducted using commonly used chemical reagents, including Dragendorff, Meyer, and Liebermann-Burchard reagents. Supplementation with *O. basilicum* and *P. betle* (P4) significantly enhanced the shrimp's immune response, namely the total hemocyte counts (THC), phagocytic activity (PA), phagocytic index (PI), and prophenoloxidase (proPO). The supplemented diet (P4) was synergistically able to improve shrimp health, with the highest values for THC (7.30×10^6 cells/mL), hyaline (61.33%), PA (84%), PI (2.19), and proPO (0.91). Flavonoids and alkaloids in the feed containing leaf flour from both plants were bioactive compounds suspected to be responsible for enhancing the immune response. These findings suggest that a leaf flour combination of *O. basilicum* and *P. betle* can be a new immunostimulant alternative to improve shrimp health for disease prevention.

Introduction

The Vaname shrimp (*Litopenaeus vannamei*) is one of the largest cultured shrimp species worldwide, with abundant production and high economic value. Vaname shrimp farming is in great demand owing to its many advantages, including high economic value and market demand (Azhar et al., 2021). *L. vannamei* is a source of proteins and minerals and contains eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, which are essential for human health (Gao et al., 2022). However, there are several obstacles in vaname shrimp farming, including vibriosis. Vibriosis is generally caused by *Vibrio* spp. (Abdel-Latif et al., 2022; Risjani et al., 2021). *Vibrio parahaemolyticus* is one of the *Vibrio* species that causes the highest mortality in vaname shrimp farming and is pathogenic in shrimp at a density of 10^4 CFU/mL (Soto-Rodriguez et al., 2015).

The proper prevention of diseases in shrimp farming is indispensable. Efforts to prevent shrimp diseases can be made by modulating immune responses using immunostimulants, vitamins, and hormones (Kilawati & Islamy, 2021; Vinay et al., 2019). Immunostimulants are chemical compounds, drugs, or other materials that enhance non-specific response mechanisms. The administration of immunostimulants is mainly done to activate the immune system of non-specific cells, such as macrophages in vertebrates and hemocytes in invertebrates. The increase in the body resistance of shrimp can be seen from the increase in total hemocyte counts (THC), differential hemocyte counts (DHC), phagocytic activity (PA), phagocytic index (PI), and prophenoloxidase (proPO). However, therapeutic agents that enhance the immune response of shrimp must consider safety aspects for consumers, the environment, and the shrimp itself.

Ocimum basilicum, also known as "basil" in Indonesia, is a plant commonly used as a herb in Asian countries. It is widely grown in tropical and temperate climates and is reported to be one of the most important sources of drugs and remedies for treating various diseases (Tabassum et al., 2022). *O. basilicum* has anti-inflammatory, antipyretic, anticancer, and neuroprotective properties of antimicrobial and cardioprotective agents (Beltrán-Noboa et al., 2022; Chanthavong et al., 2021; Kayathiri et al., 2022; Kustiati et al., 2022; Yadav et al., 2022). The stems and leaves of *O. basilicum* contain various secondary metabolites such as polyphenols, flavonoids, amino acids, unsaturated fatty acids, terpenoids, saponins, tannins, and essential elements (vitamins and minerals) (Kumari et al., 2022; Pradhan et al., 2022). The plant's two main compounds (polyphenols and flavonoids) are responsible for its various pharmacological activities, including anticancer, antioxidant, antimicrobial, and anti-inflammatory (Pradhan et al., 2022). In addition to basil leaves, *Piper betle* can also be a source of immunostimulant compounds since it contains compounds such as eugenol, chavicol, allylpyrocatechol, and chavibetol, which are antiseptic substances (Marchese et al., 2017; Sugumaran et al., 2011).

O. basilicum and *P. betle* can improve human health because of their efficacy as neuromodulators and immune stimulators (Singh, 2022). The use of these plants as natural antibacterial agents to prevent disease in shrimp farming is also an important need. In addition to being inexpensive and easy to obtain, the natural antibacterial ingredients of this plant are safe for use and do not cause resistance to the bacterium. Prevention of diseases due to the attack by *V. parahaemolyticus* bacteria is essential, given the malignant attack of this bacterium on most cultivated vaname shrimp. Preliminary studies have shown that the extracts of *O. basilicum* and *P. betle* inhibit the growth of pathogenic bacteria that cause vibriosis on shrimp farms (Harlina et al., 2022). In this study, we supplemented the vaname shrimp diet with *O. basilicum* and *P. betle* extracts (individually and combined) to investigate the efficacy of herbal supplements in increasing their non-specific immune responses. This study provides an excellent immunostimulant alternative for disease prevention in shrimp culturing.

Materials and methods

Animal testing

Vaname shrimp with an average weight of 7.80 ± 0.27 g/shrimp and a total length of 9.10 ± 0.31 cm were harvested from an intensively cultured pond in Pangkep Regency, South Sulawesi, Indonesia. The shrimps were transported to the Biota and Environmental Engineering Laboratory Program for Aquaculture Studies, Faculty of Fisheries and Marine Sciences, Universitas Muslim Indonesia (UMI), using plastic bags containing water and oxygen. Previously, PCR results indicated that the shrimp were free of the white spot syndrome virus (WSSV) and acute hepatopancreatic necrosis disease (AHPND). Therefore, 84 selected *L. vannamei* individuals were acclimatized in an indoor system using 12 aquariums containing 20 L of seawater, with a density of 7 shrimps/aquarium.

Preparation of medicated dietormulation

The leaves of *O. basilicum* and *P. betle* were collected from a traditional market in Makassar. The leaves were washed under running water and dried in an oven at 50°C for three days. Dried leaves of *O. basilicum* and *P. betle* were homogenized using an electrical grinder to obtain *O. basilicum* and *P. betle* leaf flour. Finally, *O. basilicum* and *P. betle* leaf flours were mixed into the formulation feed with a protein content of 36%. The complete formulation and composition of the feed used in this study are summarized in Table 1.

Table 1
The formulation for artificial shrimp feed (% dry weight) and its proximate chemical compositions.

Ingredients		Composition (%) for feed formulation			
		Diet P1	Diet P2	Diet P3	Diet P4
Fish flour		38	38	38	38
Shrimp head flour		10	10	10	10
Soybean flour		20	20	20	20
Fine bran		14	14	14	14
Fermented copra meal		10	10	10	10
Vitamins		2.5	2.5	2.5	2.5
Starch flour		5	5	5	5
Carboxymethyl cellulose (CMC)		0.5	0.5	0.5	0.5
<i>Ocimum basilicum</i> leaf flour (g/kg feed)		0	44.84	0	22.42
<i>Piper betle</i> leaf flour (g/kg feed)		0	0	31.57	16.79
Amount		100	100	100	100
Ingredients	Proximate chemical analysis (%) on a dry matter basis				
	Protein	Fat	NFE	Ash	Coarse fibers (%)
Fish flour	55.6	5.0	4.1	25.9	1.1
Shrimp head flour	20.31	6.37	31.64	25.78	15.9
Soybean flour	41.7	23.5	20.8	4.9	9.1
Fine bran	11.6	8.9	56.7	10.5	12.3
Fermented Copra meal	32.4	6.6	35.6	7.7	17.7
Starch flour	1.3	0.3	87.9	6.2	4.6

All ingredients listed in Table 1 were ground and mixed using a food mixer (Legacy, USA). The treatment, *O. basilicum* leaf flour, and *P. betle* leaf flour were thoroughly dispersed in water (10% w/w), added to the basal diet ingredient mixture, and stirred for 15 minutes to form a dough. The dough was then placed in a perforated plastic and steamed for 15 minutes. After steaming was completed, the mixture was allowed to cool for 5 minutes. The cooled mixture was formed into pellets using a pellet machine with a 1.0 mm mesh size. Pellets were dried to a humidity of 10% in a drying cabinet covered with a lamp balloon at a temperature of 50°C for 12 hours. The feed was then stored at – 20°C until use.

Experimental design

The experiment used a completely randomized design consisting of four treatments and three replicates. The four treatments are summarized in Table 1. The artificial diets P1, P2, P3, and P4 were formulated to evaluate the effectiveness of *O. basilicum* and *P. betle* leaf flours in improving the immune response of vanamei shrimp. The doses of *O. basilicum* and *P. betle* leaf flours used were determined using the minimum bactericidal concentration (MBC) assay by converting them to 1 kg of feed (Harlina et al., 2022). During acclimatization for seven and feed treatment for 15 days, the shrimp were fed 5% of their weight per day. Feeding was done thrice daily, namely at 07.00, 13.00, and 16.00. The salinity, temperature, pH, and dissolved oxygen (DO) were maintained at 28 ppt, 30°C, 7.2, and 5.6 mg/L, respectively.

Collection of hemolymph and analysis

Hemolymph was collected at the beginning of the feeding trial (day zero) and after 2, 4, 8, and 15 days of this feeding period to determine the effect of feeding enriched with *O. basilicum* and *P. betle* leaf flours on the non-specific immune response of vaname shrimp. The observed immune response parameters were THC, DHC, PA, PI, and proPO activity in the three shrimp treatments. Before collecting hemolymph, the shrimp were anesthetized at cold temperatures. Haemolymph retrieval was performed according to the method reported by Parenrengi et al. (2022). A volume of approximately 100 µL of hemolymph was gently pulled from the pleopod base of the abdominal segment of the two shrimp using a 1 mL sterile syringe and gently mixed with 900 µL of clean anticoagulant solution (30 mM trisodium citrate, 338 mM NaCl, 115 mM glucose, and 10 mM EDTA, pH 7).

Total hemocyte counts (THC)

After hemolymph withdrawal, hemocytes were diluted with 0.5% trypan blue in 2.6% NaCl. Hemocyte counts were performed using a light microscope (Olympus DP21, Japan) at 40x magnification by placing 10 µL of diluted hemolymph in a Neubauer hemocytometer. THC was determined as follows: $\text{THC (cells/mL)} = \text{number of hemocytes (cells)} / \text{calculated volume (mL)} \times \text{dilution factor} \times 10^4$.

Differential hemocyte counts (DHC) (dup: abstract ?)

DHC observations were based on a previously described method (Chang & Chang, 2022). A total of 20 μL of hemolymph was spread on a microscope glass slide. The distribution of hemocytes on the slides was dried at room temperature and fixed with 100% methanol for 15 minutes. The revised hemolymph was redried at room temperature and stained with a 10% Giemsa solution for 15 minutes. The slides were washed under running water for 30 s and allowed to dry. To morphologically identify the cell type, the slide was covered with a cover glass and observed under a light microscope (Olympus DP2I, Tokyo, Japan) at 40x magnification. Shrimp hemocytes were divided according to their type: hyaline, granular, and semi-granular (Rowley & Pope, 2012) and calculated based on the composition of the hemocyte cell type. DHC was calculated as follows: $\text{DHC (\%)} = \text{number of different hemocyte types} / \text{total hemocyte cell count} \times 100$.

Phagocytic activity (PA) and phagocytic index (PI) (dup: abstract ?)

The PA and PI were determined following the method reported by Sudaryono et al. (2018) to determine the blood parameters after administering medicated feeds and vaccination. At the start of the treatment, formalin killed cell (FKC) was prepared by growing *Vibrio parahaemolyticus* in tryptic soy broth (TSB) supplemented with 1.5% NaCl for 24 hours at 27°C and then harvested by centrifugation for 10 min at 4,000 rpm and a temperature of 4°C. The supernatant was removed, and the precipitate was added to 40 mL phosphate-buffered saline (PBS) and re-centrifuged using the same settings. This procedure was repeated to obtain bacterial deposits that were free of TSB. Clean bacterial deposits were added to 25 mL of PBS and inactivation for 18–24 hours using 2% formalin. The inactivated bacterial deposits were incubated at 25°C for 72 h and then washed with 40 mL PBS by centrifugation at 3,000 rpm for 15 min. The PA of vaname shrimp was determined by pipetting 20 μL hemolymph into an Eppendorf tube and diluted with PBS in a volume ratio of 2:1, mixed evenly with 25 μL FKC, and incubated at 30°C for 20 minutes. A total of 5 μL of this hemolymph and FKC mixture was spread on a clean glass slide and air-dried. Dried slides were dipped in 100% methanol for 5 minutes and stained with 10% Giemsa (Himedia, S011) for 15 minutes. The slides were rinsed under running water, dried, and observed under a light microscope (Olympus DP2I, Tokyo, Japan) at 40x magnification. Phagocytic activity was measured based on the percentage of phagocytic cells exhibiting phagocytosis (Fernandes et al., 2021). PA was calculated as follows: $\text{PA (\%)} = \text{number of active phagocytes} / \text{number of phagocytes observed} \times 100$. PI was determined as $\text{PI} = \text{number of phagocyte cells that perform phagocytosis} / \text{number of phagocytic cells}$.

Prophenoloxidase (proPO)

The proPO activity was measured using spectrophotometry to record dopachrome formation using L-dihydroxyphenylalanine (L-DOPA) as the substrate. The proPO test was performed as described by Chiu et al. (2021). First, 100 μL of hemolymph stock was centrifuged at 7,000 rpm and 40°C for 20 min. The

supernatant was removed. The pellets were added to 100 μ L of a cacodylate citrate buffer (0.1 M sodium cacodylate trihydrate, 0.45 M NaCl, and 0.01 M sodium citrate), followed by centrifugation using the same settings. Next, the pellets were added to 100 μ L of a cacodylate buffer and homogenized. Next, 100 μ L of the mixture was pipetted into a 96-well microplate (R-BioPharma Well Reader, Jerman) and mixed with 100 μ L trypsin (Sigma Aldrich), re-suspended, and incubated at 25°C for 10 min. Subsequently, 50 μ L of I-DOPA solution (1.6 mg/mL in trypsin) was added. The enzymatic activity was determined by measuring the absorbance of dopachrome at 490 nm against a blank containing 200 mL trypsin and 50 mL I-DOPA (Immanuel et al., 2012).

Phytochemical test

Approximately 10 g of grounded air-dried *O. basilicum* leaf and *P. betle* leaves were macerated in 200 mL of methanol at room temperature with shaking for 24 h. The macerates were filtered using filter paper (Whatman No.4), and the filtrate was concentrated to almost dryness in a rotary evaporator at 37°C. The tested phytochemical constituents included flavonoids, alkaloids, terpenoids, saponins, and steroids. The presence of these substances can be directly determined by visually evaluating the coloration profile of the reactions or the formation of precipitates. Phytochemical screening of the two methanolic extracts was performed using a standard method (Manzo et al., 2017; Nakaziba et al., 2022).

The flavonoid assay was performed by adding 1–2 mL of hot methanol (50%) to 1 mL of methanol extract, followed by the addition of Mg and 0.5 mL of concentrated HCl. Orange coloration indicates the presence of flavonoids. Dragendorff's and Maye's tests were performed for the alkaloids. The 1 mL of methanol extract was mixed with 0.5 mL hydrochloric acid (2%). The acidic solution was then divided into two tubes. Three drops of Dragendorff solution were added to tube I, and three drops of Meyer solution were added to tube II. A red or orange residue in tube I and a whitish precipitate in tube II indicated the presence of alkaloids. For the terpenoid test, 1 mL of the MeOH extract was diluted with chloroform (0.5 mL) and added to anhydrous acetic acid (0.5 mL). The mixture was then added to 1–2 mL of concentrated H₂SO₄ through the tube walls. The presence of triterpenoids was indicated by the formation of brownish or purple rings in both solvents.

The Liebermann-Burchard test was used for the steroid test. First, 1 mL of methanol extract was added to 2 mL of n-hexane and shaken. Next, the Liebermann-Burchard reagent was added to the n-hexane layer. A change in color to turquoise indicated the presence of steroids. The foam test was performed to determine the presence of saponins. The methanol extract (2 mL) was added to distilled water (2 mL), and the mixture was shaken for 1 minute. If foam formation occurred, two drops of HCl (1 N) were added and incubated for 10 minutes. If the foam remained stable, the extract contained saponins.

Statistical analysis

Data were analyzed using the SPSS software (version 21.0; IBM, Armonk, NY, USA). Bartlett's tests were used for the homogeneity of the data variances. One-way analysis of variance (ANOVA) was used to

compare the means of each treatment. Duncan's comparison tests were applied to determine differences between groups based on the mean difference between the treatments. All of the experiments described in this study were conducted in triplicates, and the results were expressed as mean $\pm$ standard deviation ($\bar{X} \pm SD$). In all statistical analyses, statistical significance was set at $p < 0.05$.

Results

Total hemocyte counts (THC)

The results of the THC observations showed that a higher increase in the THC of vaname shrimp (P2, P3, and P4) was established since the beginning of feeding supplemented with *O. basilicum* leaf flour and *P. betle* leaf flour than the THC of vannamei shrimp fed the unsupplemented diet (Fig. 1). Significant differences ($p < 0.05$) in THC were observed among shrimp being fed *O. Basilicum* leaf flour- and *P. Betle* leaf flour-supplemented diets (P2, P3, and P4) and the control diet (P1) after 2, 4, 8, and 15 days. No significant differences in THC were observed among shrimp that were fed diets P2, P3, and P4 after 2 and 4 days. A difference was observed on the fourth day; shrimp being fed diet P2 showed no significant difference in THC values ($p > 0.05$) from P3, but P2 was significantly different ($p < 0.05$) from P4. In contrast, the THC value of P3 differed significantly from that of P4. P4 had the highest THC value (7.30×10^6 cells/mL) on the 15th day and differed significantly from that of the other treatments (P1: 3.60×10^6 cells/mL, P2: 6.60×10^6 cells/mL, and P3: 6.37×10^6 cells/mL).

Values within the same column with different letters are significantly different ($p < 0.05$)

Differential hemocyte counts (DHC)

Table 2 shows that the number of hyaline cells (HC) in diets P2, P3, and P4 continued to increase with the feeding duration. Nevertheless, the highest increase was detected for the P4 diet and differed significantly ($p < 0.05$) from that of the other treatments on the fourth day. There was no significant difference on days 2, 8, and 15 among the P2, P3, and P4 treatments. In contrast, there was an increase in the percentage of hyaline values compared to that of the control (P1). P1 showed a decrease in hyaline on days 2 and 8, but there is an increase in hyaline on days 4 and 15 with hyaline percentage values lower than that of the *O. basilicum* leaf flour- and *P. betle* leaf flour-supplemented diets (P2, P3, and P4). The number of granular cells (GC) in the P2, P3, and P4 treatments decreased from days 2–15, and no significant difference was observed among the three treatments. In contrast, GC in P1 increased from day 2, with higher values that differed significantly from treatments P2, P3, and P4. The GC value of P4 was not significantly different from the GC values of P2 and P3 on day 8. All treatments experienced a decrease in semi-granular cell (SGC) values from days 2–15. However, the SGC count was the lowest in shrimps fed P4 and significantly differed ($p < 0.05$) from the control, except on day 2. Although the SGC count of P4 was the lowest, no significant differences were observed between P2 and P3. The percentage of hyaline cells was the highest among the three hemocyte cells in P2, P3, and P4 (Fig. 2). However, in P1, the

proportion of granular cells was the highest. The percentage of granular cells observed in P1 was higher than that for P2, P3, and P4.

Phagocytic activity (PA) and phagocytic index (PI)

Phagocytic activity is essential for the control and destruction of salt particles. The phagocytic activity from days 0–15 ranged from 15–84% (Fig. 3). Figure 3 shows that PA increased drastically after shrimp were fed *O. basilicum* and *P. betle* leaf flour-supplemented diets (P2, P3, and P4), particularly in P4. However, PA in shrimp fed the unsupplemented diet did not increase. Phagocytic activity was the highest in the P4 treatment (84%) and showed a significant difference ($p < 0.05$) from that of the other three on days 2, 8, and 15. However, on day 4, there was no noticeable difference between treatments P4 and P3. Similar to the PA results, shrimp fed *O. basilicum* and *P. betle* leaf flour-supplemented diets (P2, P3, and P4) showed drastically increased PI values from the beginning of administration (Fig. 4). However, in contrast, the control (P1) shrimp did not show an increase in PI value during feeding. The highest PI value was obtained in the P4 treatment on day 15 and differed significantly from the other three treatments. On days 2 and 8, the P4 treatment also showed the highest PI values and differed significantly ($p < 0.05$) from that of the P1 (control), but P4 showed no noticeable difference from that of P2 and P3.

Table 2
The differential hemocyte counts (DHC) of vaname shrimp fed the experimental diets

Treatment	Cell type	Day post feeding				
		0	2	4	8	15
Diet P1	Hyaline (%)	34.67 ± 2.52	34.33 ± 1.53	37.67 ± 1.53	32.00 ± 2.00	36.33 ± 1.15
	Granular (%)	41.33 ± 0.58	42.67 ± 1.53	40.67 ± 2.08	44.67 ± 1.53	42.33 ± 1.53
	Semi-granular (%)	24.00 ± 2.00	23.00 ± 2.00	21.67 ± 2.08	23.33 ± 1.53	21.33 ± 1.53
Diet P2	Hyaline (%)	34.67 ± 3.08	43.00 ± 2.65	46.33 ± 0.58	55.00 ± 2.00	60.00 ± 2.00
	Granular (%)	42.67 ± 1.53	37.00 ± 2.00	31.33 ± 0.58	28.67 ± 3.06	26.33 ± 2.52
	Semi-granular (%)	22.67 ± 1.53	20.00 ± 2.00	22.33 ± 2.08	16.33 ± 2.52	13.67 ± 1.53
Diet P3	Hyaline (%)	33.00 ± 3.61	42.67 ± 2.52	48.00 ± 1.00	54.33 ± 1.53	58.33 ± 3.06
	Granular (%)	43.00 ± 1.00	35.33 ± 2.08	32.67 ± 1.53	24.00 ± 1.73	25.67 ± 1.53
	Semi-granular (%)	22.67 ± 2.00	20.00 ± 2.65	22.33 ± 2.52	16.33 ± 1.53	13.67 ± 2.00
Diet P4	Hyaline (%)	34.00 ± 1.00	45.00 ± 2.00	50.67 ± 2.08	54.67 ± 2.08	61.33 ± 1.53
	Granular (%)	43.33 ± 0.58	35.33 ± 2.52	31.67 ± 1.53	26.33 ± 1.15	22.67 ± 2.08
	Semi-granular (%)	22.67 ± 1.53	19.67 ± 3.21	17.67 ± 1.53	19.00 ± 1.73	16.00 ± 1.00

Prophenoloxidase (proPO) activity

The proPO activity was relatively stable until the 15th day after feeding for the control (P1), and increased in shrimp in the P2, P3, and P4 treatments from days 2–15 (Fig. 5). The highest proPO activity value was found in shrimp fed P4 and the lowest in P1 (control). Shrimp fed diet P4 showed significantly different proPO activity from those fed P1 and P2 on days 2 and 4 but did not differ significantly from those fed P3. However, shrimp in P2, P3, and P4 did not show significantly different proPO activity on days 8 and 14. The shrimp fed *O. basilicum* and *P. betle* leaf flour-supplemented diets (P2, P3, and P4) showed significant differences ($p < 0.05$) from that of the P1 (control).

Increased proPO activity in the hemolymph indicates an increased immune response to pathogens. The proPO activation cascade in shrimp plays an important role in the immune response to pathogenic infections (Amparyup et al., 2013). Activation of the proPO system involving PO enzymes in cellular melanotic encapsulation or melanization is one of the most effective immune responses against foreign particles (Tassanakajon et al., 2018). In contrast, reduced ProPO activity results in phagocytic failure and tissue injury.

Phytochemical analysis

Phytochemical analyses revealed the presence of flavonoids, steroids, and triterpenoids. Alkaloids were present only in the methanol extract of *O. basilicum*. Saponins were absent in the methanol extracts of *O. basilicum* and *P. betle*. The phytochemical screening results of the MeOH extract are presented in Table 3.

Table 3 Result of phytochemical test of <i>O. basilicum</i> and <i>P. betle</i> MeOH extrac						
MeOH Extract	Flavonoid	Alkaloid		Triterpenoid	Steroid	Saponin
		Dragendorff	Meyer			
<i>O. basilicum</i>	+	+	+	+	+	-
<i>P. betle</i>	+	-	-	+	+	-
Note: The sign (+) characterizes a positive reaction, whereas the sign (-) characterizes a negative reaction.						

Discussion

Modulation of the immune response through stimulation can help maintain a disease-free state. Agents that activate the host’s defense mechanisms against impaired immune responses may provide therapeutic solutions. According to Ghule (2006), the immune system is involved in the etiology and pathophysiology of various diseases. The results obtained in this study show that *O. basilicum* leaf flour- and *P. betle* leaf flour-supplemented diets contain powerful immunostimulants for stimulating specific and non-specific immune mechanisms in white shrimp, particularly in the P4 treatment. Higher THC in shrimps fed the diet P4 shows good shrimp health. The higher the THC value, the stronger the immune response, and the chances of creating phagocytic cells are significant in controlling microorganism attacks. Prayitno et al. (2022) reported that an increase in THC is closely related to incoming foreign materials. The body's defense system plays an important role in the body's defense mechanisms and hemocytes in shrimp. The spread and increase in the total number of hemocytes are assumed to be a form of cellular immune response in shrimp.

Phenol plays a role in increasing the number of hemocytes. Flavonoids are phenol-derived compounds that increase THC. In addition, *O. basilicum* and *P. betle* produce flavonoids that play roles in various

physiological functions, including development, growth, and protection from infections by bacteria, fungi, and viruses (Kumar et al., 2020; Pereira et al., 2016). The THC values detected in this study were lower than that reported by Jasmanindar et al. (2018), Prayitno et al. (2022), and Abdel-Tawwab et al. (2022), which resulted in THC of 59.5×10^6 cells/mL (*Gracilaria verrucosa*), 41.0×10^6 cells/mL (MOTIV™), and 11.8×10^6 cells/mL (*Withania somnifera*). Nevertheless, the shrimp-fed *O. basilicum* and *P. betle* leaf flour-supplemented diets (P4) showed a two-fold increase in THC compared to the control (P1).

In addition to THC, DHC indicates an increase in the immune response. Changes in the DHC during the study suggest that hyaline and granular cells are essential for the shrimp's immune response. In addition, hyaline cells, the smallest of the three types of hemocytes, were identified as active phagocytes. Granular cells contain proteases, agglutinins, antimicrobial peptides, and cell adhesion/degranulation factors (Kulkarni et al., 2021).

The high percentage of hyaline cells found in the P2, P3, and P4 diets in this study suggests that *O. basilicum* and *P. betle* leaf flour supplementation boosted the immune system by inducing an increase in hyaline hemocytes and lowering granular and semi-granular cells. Gudding et al. (2014) reported that when a pathogen attack occurs, the first response is that hyaline cells do not develop into semi-granular cells. There was a decrease in the number of semi-granular cells in hemocytes. All types of hemocytes can carry out phagocytic activities, but hyaline cells are generally more active in phagocytic activities. During phagocytosis, hyaline cells swallow and destroy pathogens and foreign particles that enter the shrimp bodies (Jasmanindar et al., 2018). The low total semi-granular content in the treatment with *P. betle* leaf flour was due to the low production of semi-granulated cells, which are mature hyaline cells. The average granular cell values observed in the shrimp during the study ranged from 22.67–44.67%, indicating that shrimp were as expected, as reported by Putri et al. (2013), who reported that the percentage of granulocyte cells in healthy shrimp ranged from 17–40% of the total hemocytes.

Phagocytosis is an important immune response mechanism in animals, including invertebrates such as shrimp, to control and destroy foreign particles such as pathogens (Xu et al., 2019). The results presented in Fig. 3 indicate that the phagocytic activity varied from 15–84% from days 0–15. Notably, shrimp being fed diets supplemented with *O. basilicum* and *P. betle* leaf flours (P2, P3, and P4) showed a significant increase in phagocytic activity compared to that of the control group (P1), particularly in the P4 treatment. Furthermore, the PI, a measure of phagocytic efficiency, showed similar trends. Shrimp fed with *O. basilicum*- and *P. betle* leaf flour-supplemented diets exhibited a drastic increase in PI from the beginning of the administration, with the highest PI value observed in the P4 treatment on day 15, which differed significantly from that of the other treatments.

These results suggest that the inclusion of *O. basilicum* and *P. betle* leaf flour in shrimp diets positively influences their phagocytic activity and efficiency in engulfing and destroying foreign particles. The significant differences observed between the treatments and the control group indicate that supplementation with *O. basilicum* and *P. betle* leaf flours in the shrimp diet may have immunostimulatory effects, enhancing the shrimp's immune response and potentially improving their

ability to defend against pathogens. Notably, the observed differences in phagocytic activity and phagocytic index between the treatment and control group were statistically significant ($p < 0.05$), indicating a strong association between supplementation of *O. basilicum* and *P. betle* leaf flours in the diet and the observed changes in the immune response. *These findings suggest that O. basilicum and P. betle leaf meal may have the potential as immunostimulant additives in shrimp feed.* The presence of immunostimulants in herbs can increase non-specific immunity by promoting phagocytosis and PO activity and enhancing resistance against pathogens (Chang et al., 2012; Hsieh et al., 2008). Previous studies have reported that herbal extracts can modulate the non-specific immune system of vanamei shrimps. For example, cacao pod husks (Lee et al., 2020), *Astragalus membranaceus*, and *Bupleurum chinense* (Angela et al., 2020) have been shown to increase shrimp immunocompetence.

The presence of terpenoid compounds in the methanol extracts of *O. basilicum* and *P. betle* leaves was characterized by reddish-brown rings. This discoloration is caused by the oxidation of the terpenoid group of the compounds via the construction of conjugated double bonds. The *O. basilicum* leaf extract used in this study contained various natural compounds, including flavonoids, alkaloids, triterpenoids, and steroids, as determined using phytochemical assays (Table 3). These findings highlight the rich phytochemical profile of *O. basilicum* and suggest that its pharmacological activity is partly attributable to the presence of triterpenoids and other bioactive compounds. Further research on the specific effects of triterpenoids in *O. basilicum* may provide insights into their potential health benefits and therapeutic applications.

Similar to the *O. basilicum* leaf extract, all classes of these compounds, except alkaloids, were present in the *P. betle* leaf extract. Saponins were not found in either *O. basilicum* or *P. betle* leaf extracts. Nur et al. (2020) reported that the presence of alkaloids, flavonoids, steroids, and other compounds (saponins, tannins, and β -carotene) in the seaweed extract of *Sargassum* sp. could protect vaname shrimp from vibriosis (a bacterial disease caused by *Vibrio* sp).

Flavonoids are essential in strengthening the immune system (Panche et al., 2016). Similar to flavonoids, alkaloids increase non-specific immune activity and can function as immunostimulants and antibacterial compounds (Andayani et al., 2018; Idris Affandi et al., 2019). Alkaloids increase the non-specific immunity of shrimp by increasing the activity of macrophages. Macrophages are phagocytic cells that feed on pathogens.

The use of *O. basilicum* leaf flour and *P. betle* leaf flour-supplemented diet (P4) provided a higher phagocytosis index than feed containing only *O. basilicum* leaf flour or *P. betle* leaf flour. It is suspected that there is a difference in the concentrations of the compounds in the two leaf flours. Phytochemical tests revealed that the methanol extract of *O. basilicum* leaves contained alkaloids and flavonoids as their main components. In contrast, the methanol extract of *P. betle* leaves did not contain alkaloids but only flavonoids as the main components, in addition to steroids and triterpenoids, which are minor compounds. The combined use of these two bioactive compounds increases the activity of immunostimulants through synergistic effects.

However, when it used alone, its effectiveness was low. The dosage can be used to determine the immune response. It has been reported that if the minimum dose of an antigen is exceeded, the higher the amount used, the stronger the immune response. This suggests that dosing with a larger molecule may have a stronger immunostimulant effect. In this study, the phagocytic activity (84%), which is indicated by the application of *O. basilicum* leaf flour- and *P. betle* leaf flour-supplemented diet (P4), was higher than that of *Gracilaria* sp. ($53.258 \pm 1.89\%$) and *Padina* sp. ($63.474 \pm 2.14\%$) (Kilawati & Islamy, 2021).

In conclusion, shrimp fed with the combined diet of *O. basilicum* and *P. betle* leaf flour (P4) exhibited a significantly improved immune response. *O. basilicum* leaf flour (22.42 g/kg feed)- and *P. betle* leaf flour (16.79 g/kg feed)-supplemented diet was effective in significantly increasing THC, hyaline, PA, PI, and proPO in vaname shrimp *Litopenaeus vanname*. Flavonoids and alkaloids, present in the leaf flours of both *O. basilicum* and *P. betle*, were identified as potential bioactive compounds responsible for boosting the shrimp's immune response. The study suggests that using a combination of *O. basilicum* and *P. betle* leaf flour as an immunostimulant in shrimp diet could serve as an alternative strategy for improving shrimp health and preventing diseases.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Author Contributions

This study was conducted in cooperation between all authors. Harlina Harlina, Rosmiati Rosmiati: Conceptualization, Formal analysis, Investigation, Methodology, Resources, Visualization, Writing-original draft, and Writing-review and editing. Andi Hamdillah: Formal analysis, Validation, Project administration, and Writing – original draft, Syahrul Syahrul: Formal analysis, Validation, Visualization, and Writing – original draft. Alim Isnansetyo: Supervision, Validation, Writing-review and editing.

Ethical approval for animal use

All experimental procedures comply with the ARRIVE guidelines and carried out in accordance with the UK legislation under the Animals (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039) and associated guidelines, EU Directive 2010/63/EU for animal experiments. This study also followed the guidelines of the UMI Health Research Ethics Commission Number:205/A.1/KEPK-UMI/V/2022

Data availability statements

Upon reasonable request, the datasets of this study can be available from the corresponding author.

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Figures

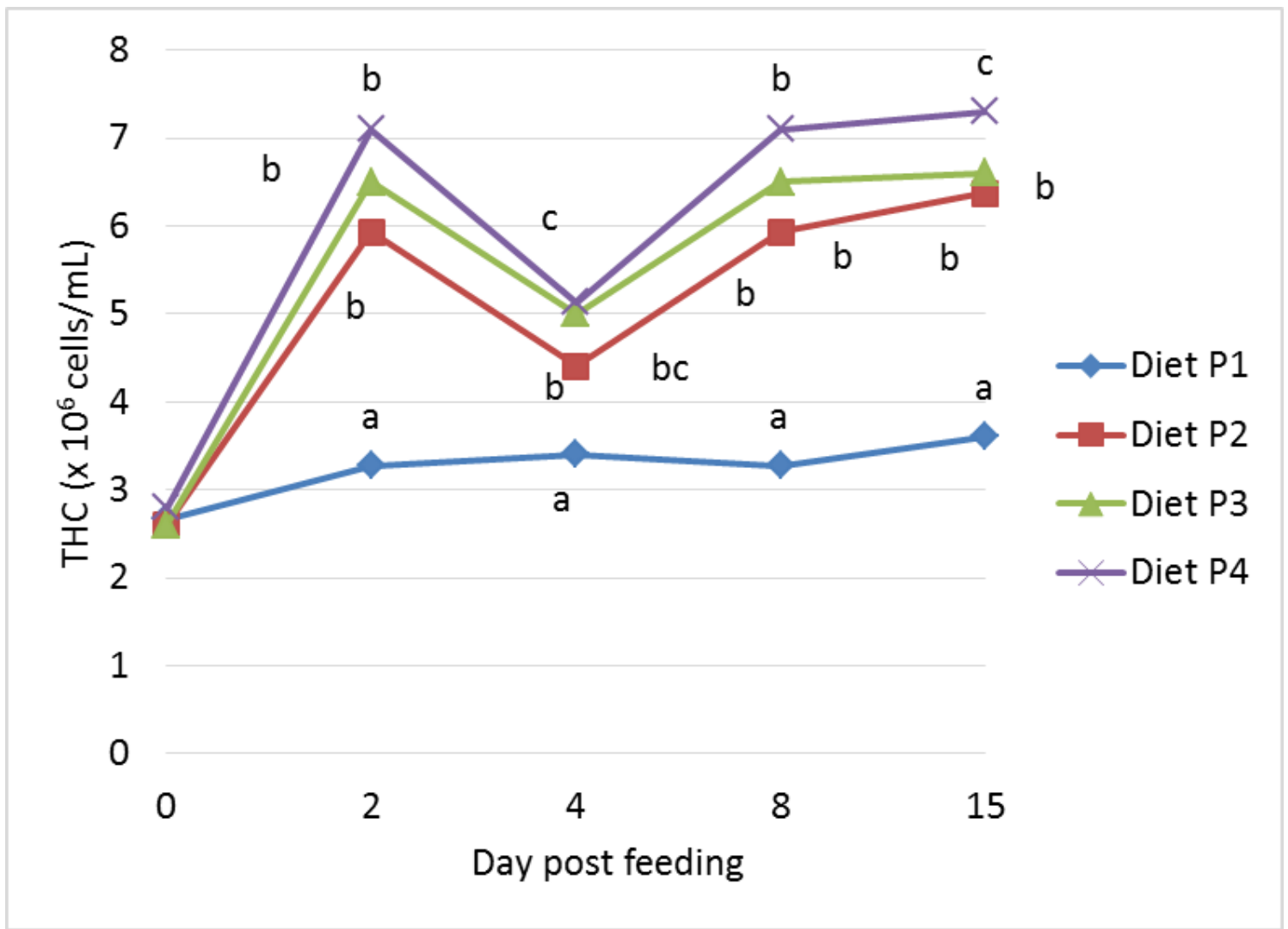


Figure 1

The effect of supplementation with *O. basilicum* leaf meal and *P. betle* leaf meal (P1, P2, P3, and P4) on total hemocyte counts (THC) of vaname shrimp.

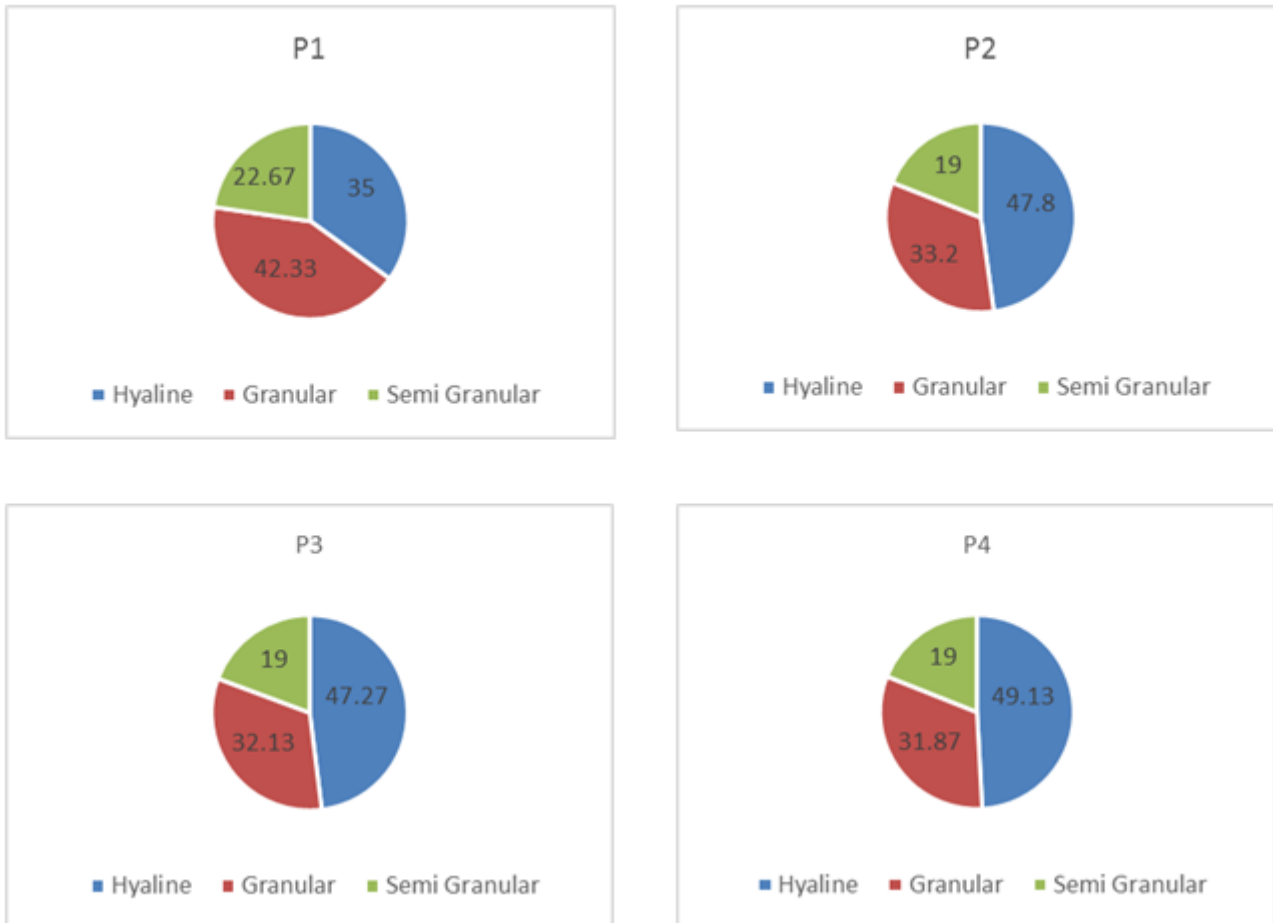


Figure 2

The effect of supplementation with *O. basilicum* leaf meal and *P. betle* leaf meal (P1, P2, P3, and P4) on the number of differential hemocytes (DHC) of vaname shrimp

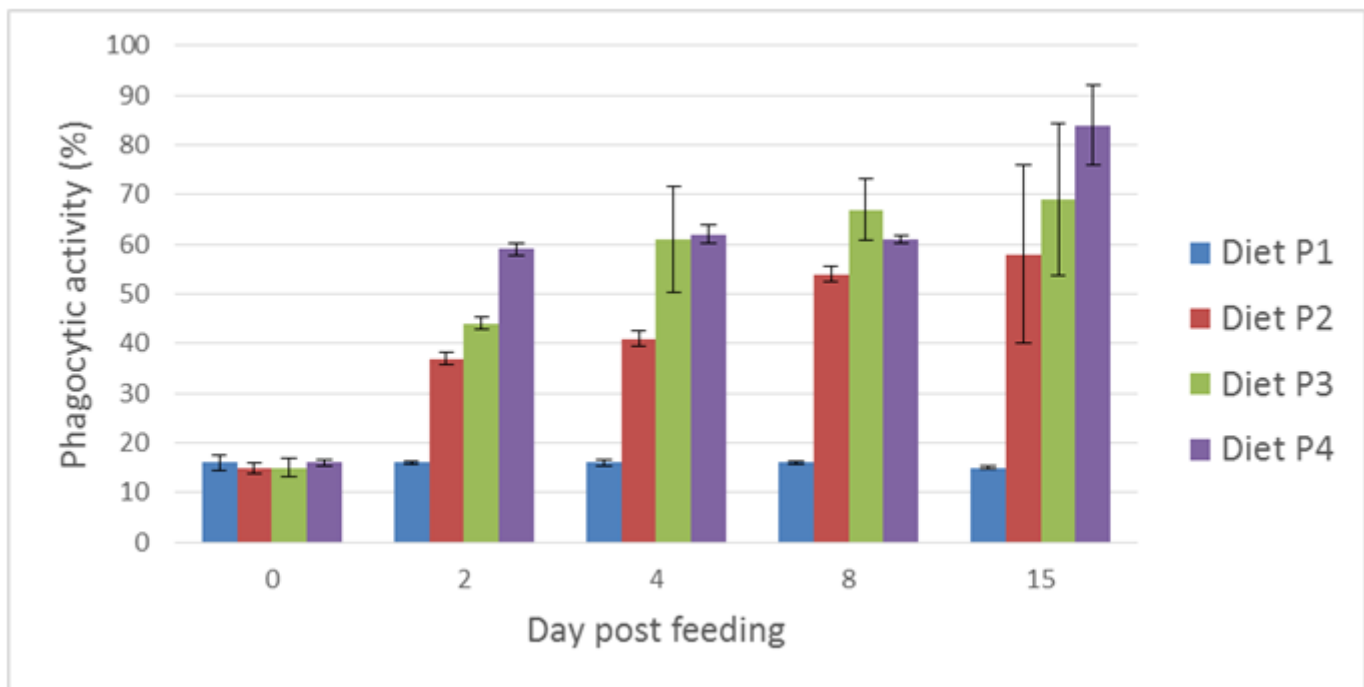


Figure 3

The effect of supplementation with *O. basilicum* leaf meal and *P. betle* leaf meal (P1, P2, P3, and P4) on the phagocytic activity of vaname shrimp

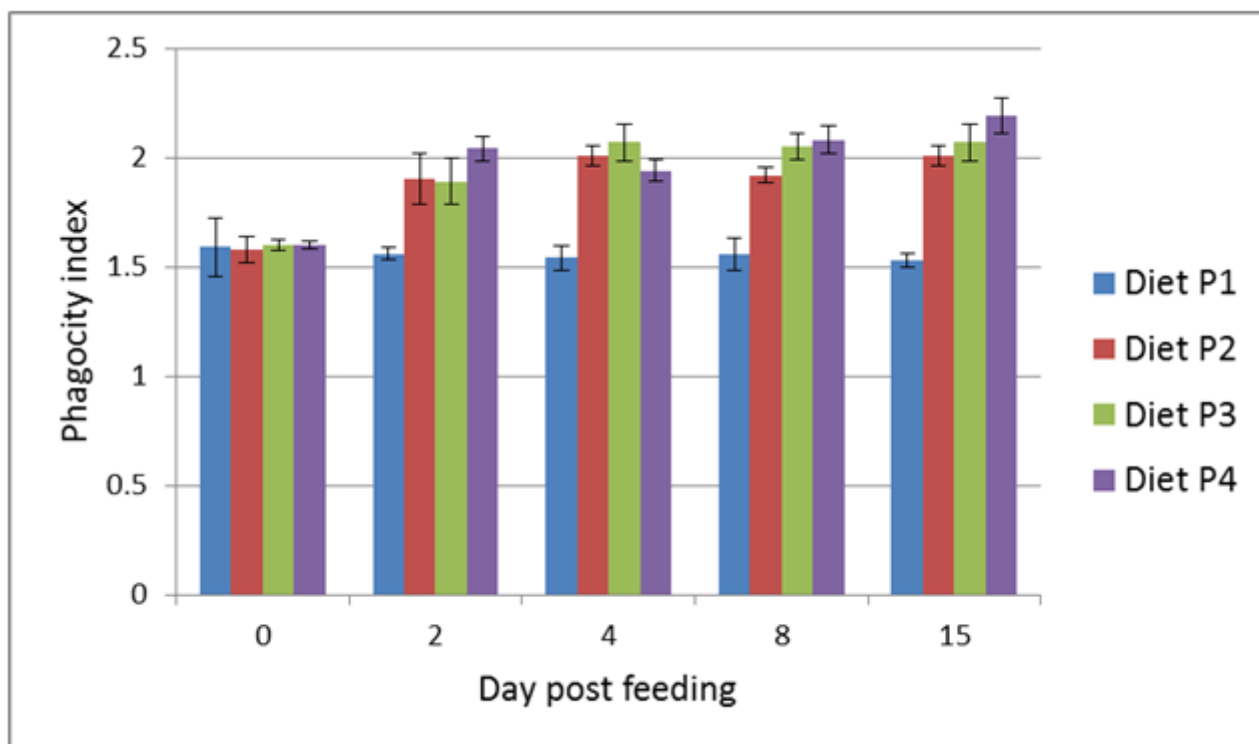


Figure 4

The effect of supplementation with *O. basilicum* leaf meal and *P. betle* leaf meal (P1, P2, P3, and (P4) on the phagocytic index of vaname shrimp

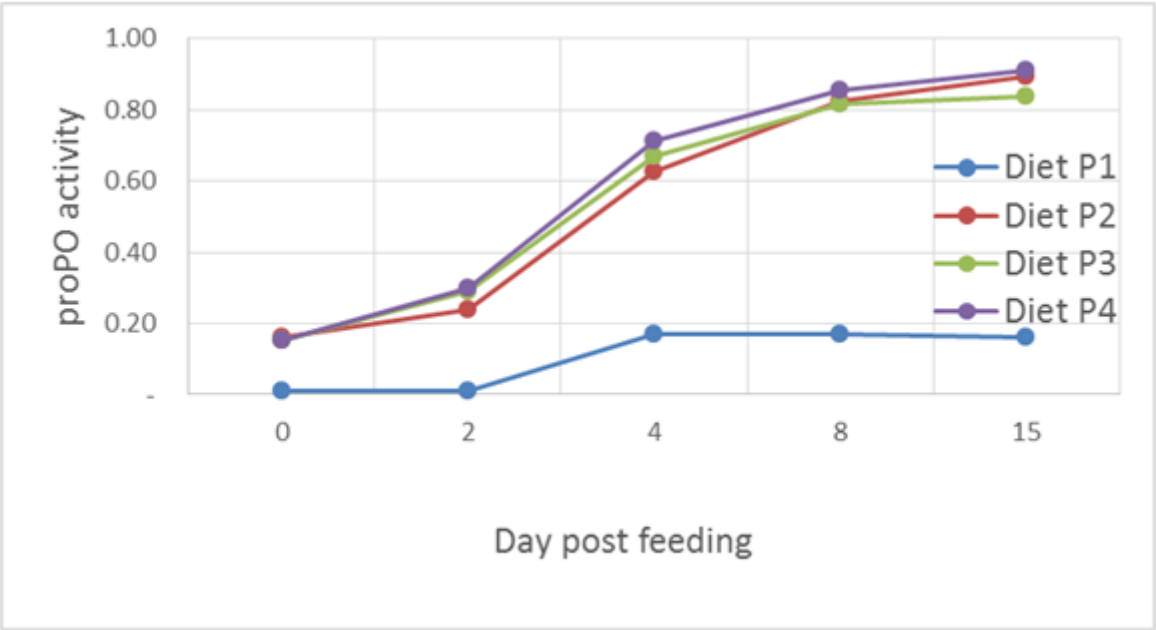


Figure 5

The effect of supplementation with *O. basilicum* leaf meal and *P. betle* leaf meal (P1, P2, P3, and P4) on Prophenoloxidase activity of vaname shrimp