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Increased THC, DHC, PA and Survival of White Shrimp (*Litopenaeus vannamei*) by Feeding *Porphyridium cruentum* Nanoparticle

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Abstract

A common disease in white shrimp is vibriosis, which can cause acute hepatopancreatic necrosis, also known as Acute Hepatopancreatic Necrosis Disease (AHPND). Immunostimulants are one alternative for defending against pathogen infections. *Porphyridium cruentum* is a natural substance that can be used as an immunostimulant. The development of nanotechnology is progressing rapidly today due to its important role in various fields. The use of *P. cruentum* nanoparticles enhances the absorption and efficiency of feed, making their application more effective. The purpose of this study is to determine the effect of the addition of *P. cruentum* nanoparticles in feed on the enhancement of the immune response of white shrimp after administration for one month of rearing. Shrimp were divided into four groups, one group was not fed with *P. cruentum* nanoparticles (K), three other groups were fed with *P. cruentum* nanoparticles at 0.8, 1.0, and 1.2% per kg of feed, respectively. The results showed that the treatment with the addition of *P. cruentum* nanoparticles provided an increase in Total Hemocytes Count (THC), Differential Hemocyte Cells (DHC), and phagocytic activity (PA) after administration for one month of rearing, and at the end of rearing provided a higher survival rate than the control.

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INTRODUCTION

Litopenaeus vannamei or commonly called white shrimp is one of the shrimp with high productivity. One of the limiting factors in shrimp farming is the emergence of bacterial and viral attacks. A common disease in white shrimp is Vibriosis caused by bacteria, one of which is *Vibrio parahaemolyticus,* which can cause epidermal degeneration and necrosis of the tissue. Shrimp does not have an adaptive immune system, only a non-specific immune system (innate immune system) that works cellularly, including phagocytic activity, encapsulation, and nodule formation (Mutmainnah *et al*., 2018). An increase in the shrimp's immune system can be observed through the increased number of hemocytes (Himzanah *et al*., 2023). Hemocytes can be increased by the addition of immunostimulants. The use of immunostimulants is an alternative to defend against pathogenic infections (Tseng *et al*., 2020).

Porphyridium cruentum is a type of red microalgae (Rhodophyta) that contains 39-56% protein in dry conditions (Torky *et al*., 2023). *P. cruentum* contains Extracellular Polysaccharide Sulfate (EPS) as an anti-bacterial and potential source of immunostimulants through antioxidant activity consisting of phenol, alkaloids, sterols, and flavonoid compounds (Mutmainnah *et al*., 2018). Administration of *P. cruentum* extract in white shrimp feed has been studied by Ozorio *et al*. (2015) increased shrimp weight gain by adding 1% and 1.5% of *P. cruentum* extract. Nanoparticles are known to have a small size (1-100 nm). The nanoscale size enhances the active properties of the material by enabling it to penetrate intracellular components (Mursal, 2018).

However, there are still limited studies available on the use of *P. cruentum* nanoparticles in feed as an immunostimulant for white shrimp, highlighting the need for further exploration of this topic. This study aims to enhance the absorption of *P. cruentum* nanoparticles in shrimp, which is expected to increase Total Hemocytes Count (THC), Differential Hemocyte Cells (DHC), Phagocytic Activity (PA), and the survival rate of white shrimp.

METHODOLOGY

Ethical Approval

During this study, no animals were harmed or subjected to improper treatment. The trial animals involved in the study were treated appropriately, ensuring an optimal environment, including water quality controls.

Place and Time

This study was conducted from August to October 2024. Microalgae cultivation, harvesting, and white shrimp maintenance were carried out at the Laboratory of Anatomy and Aquaculture, Faculty of Fisheries and Marine Sciences, Airlangga University. The *P. cruentum* nanoparticle was conducted at the Finder U-CoE Laboratory, Padjadjaran University. Immune response parameter testing was performed at the Microbiology Laboratory, Faculty of Fisheries and Marine, Universitas Airlangga.

Research Materials

Several devices were used in this study, including an autoclave (Hirayama HVE-50, Japan), beaker glass (Gerhardt SEBMA-13-0050, Germany), Erlenmeyer flask (Iwaki 4980-250N, Indonesia), measuring cylinder (Iwaki CTE33, Indonesia), aerator (Resun LP100, China), dissolved oxygen (DO) meter (Lutron DO-5510, Taiwan), pH meter (Hanna HI 9813-5, Romania), refractometer (Atago EG PG 2930, Japan), syringe (Terumo, Japan), hemocytometer (Assistant WHI-30014, Germany), and microscope (Nikon Eclipse E100, Indonesia). The materials used included TSB (Merck 105459, Germany), PBS (Hyclone, USA), Giemsa stain (Onemed, Indonesia), and *P. cruentum* nanoparticles.

Research Design

This study used a Randomized Group Design with four (4) treatments, and each

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treatment had three replicates. The shrimp were divided into four (4) groups: one group, Control (shrimp was not fed with additional *P. cruentum* nanoparticles), and the other three groups, shrimp were fed with 0.8%, 1.0%, and 1.2% *P. cruentum* nanoparticles.

Work Procedure

Preparation of test animals

White shrimp used in this study were obtained from the Brackish Water Aquaculture Center, Situbondo, East Java, and were certified disease-free. The shrimp were acclimated for 7 days to reduce stress during the experimental period. The shrimp with the size of ± 3.266 g were maintained in aquariums at a density of 10 individuals per aquarium, and equipped with an aeration system.

Preparation of *P. cruentum* nanoparticles and mixing in shrimp feed

Porphyridium cruentum used in this study was obtained from mass cultivation at the Brackishwater Aquaculture Development Center, Situbondo, East Java. The biomass was then dried to produce powdered *P. cruentum*. The resulting dry powder was sent to the Department of Physics, Faculty of Mathematics and Natural Sciences (FMIPA), Padjadjaran University (UNPAD), Bandung, for nanoparticle processing.

The *P. cruentum* nanoparticles were prepared using the top-down method. This process involved the use of High Energy Milling (HEM) to reduce the particle size from micro to nano scale. *P. cruentum* powder was placed in a tube containing a ball mill, which had been pre-cleaned with ethanol to ensure sterility and avoid contamination. The HEM method was selected to produce smaller and more homogeneous particles. The duration of the grinding process directly influenced the particle size; the longer the milling, the finer and more uniform the nanoparticles obtained (Mosaddegh and Hassankhani, 2014).

The shrimp feed used in this study was a commercial feed formulated to match the mouth opening size of white shrimp. *P. cruentum* nanoparticles were incorporated into the feed at concentrations of 0.8%, 1.0%, and 1.2%. These dosages were selected based on previous studies that evaluated their safety and immunostimulatory efficacy under similar experimental conditions (Ozório *et al.*, 2015).

The incorporation of *P. cruentum* nanoparticles into the feed was carried out using the re-pelleting method. In this process, the commercial feed was first mashed, then mixed thoroughly with the nanoparticles according to the specified dosage. Water and 1% carboxymethyl cellulose (CMC) were added to aid binding and homogenization. The resulting mixture was then re-molded into pellets and dried before use.

SEM (Scanning Electron Microscope) of *P. cruentum* nanoparticles

After the dried *P. cruentum* nanoparticles were produced, they were analyzed using Scanning Electron Microscopy (SEM) to observe their shape and size. The SEM analysis was conducted at the Laboratory of the Institute of Life Sciences and Engineering, Universitas Airlangga.

Shrimp rearing

White shrimp were fed with the addition of *P. cruentum* nanoparticles according to the dose in each treatment, except for the control group, which was only treated with artificial feed for 1 month of rearing.

Parameter measurement

Blood sampling (shrimp hemolymphs) was carried out once a week to determine the increase in THC, DHC, and PA of white shrimp during the administration of *P. cruentum* nanoparticles. Survival rate was measured at the end of shrimp rearing.

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Data Analysis

To examine the effect of each treatment on THC (Satyantini *et al*., 2016), DHC (Indraswati *et al.*, 2016), and PA (Rosyida *et al*., 2022), the data were analyzed using analysis of variance (One Way ANOVA) at an accuracy level of 95%. Differences between each treatment were determined using Duncan's test.

RESULTS AND DISCUSSIONS

P. cruentum nanoparticles

The results of nanoparticle measurement using SEM showed that particles which had not undergone the HEM (High Energy Milling) process ranged in size from 229 to 382 nm (Figure 1). In contrast, particles that had experienced the HEM process (Figure 2) were reduced to sizes below 100 nm, specifically between 48 and 89 nm. Therefore, it can be concluded that the processed particles qualify as nanoparticles, as their size is under 100 nm.

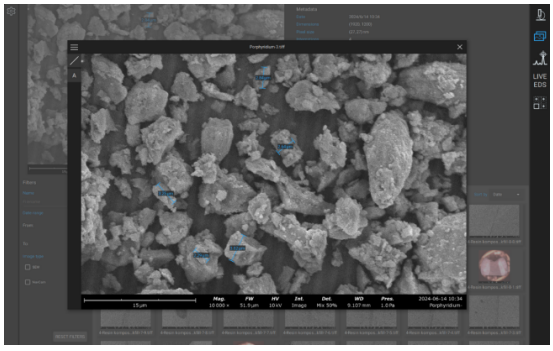

Figure 1. *P. cruentum* particles that have not undergone the HEM process.

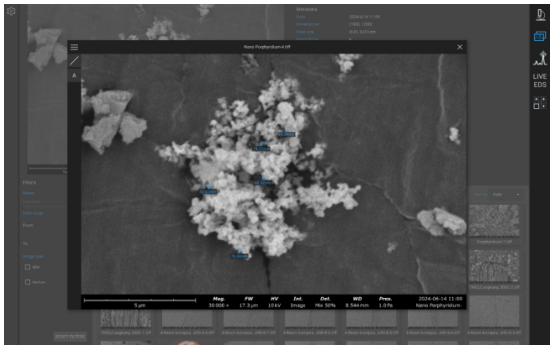

Figure 2. *P. cruentum* particles that have experienced the HEM process.

Total Hemocytes Count (THC)

The results of THC measurement in white shrimp fed with *P.cruentum* nanoparticles during the study are presented in Table 1.

Table 1. Total Hemocyte Count in white shrimp fed with *P.cruentum* nanoparticles.

Treatment	Total Hemocytes Count (x10^6 cell/mL)				
	Week				
	0	1	2	3	4
P0	0.817 ± 0.09	1.375 ± 0.01^{a}	1.652 ± 0.03^{a}	2.502 ± 0.01^{a}	3.677 ± 0.24^{a}
P1	0.847 ± 0.05	1.477 ± 0.07^{b}	1.837 ± 0.23^{a}	2.675 ± 0.19^{a}	4.135 ± 0.17^{b}
P2	0.850 ± 0.07	1.757 ± 0.06^{c}	2.542 ± 0.14^{b}	3.410 ± 0.05^{b}	5.415 ± 0.35^{c}
P3	0.850 ± 0.07	1.455 ± 0.09^{ab}	1.820 ± 0.25^{a}	2.660 ± 0.11^{a}	4.140 ± 0.11^{b}

Note : P0 control, without the addition of P. cruentum nanoparticles in feed, P1 addition of 0.8% P. cruentum nanoparticles, P2 addition of 1% P. cruentum nanoparticles, P3

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addition of 1.2% P. cruentum nanoparticles. Notations indicated by different superscript letters in the same column indicate the comparison between treatments has a significant difference ($p < 0.05$).

Based on the results of observations and measurements of the THC in white shrimp during the study, shrimp fed with feed addition of nanoparticles (treatments P1, P2, and P3) showed a statistically significant increase in THC values each week ($p < 0.05$). The treatment without the addition of *P. cruentum* nanoparticles (Control) showed a lower increase compared to the treatment with *P. cruentum* nanoparticle addition. The results indicated that feeding white shrimp with *P. cruentum* nanoparticles can enhance the shrimp's immune system. This improvement was evident from the increase in the total number of hemocytes, which ranged from 4.140 to 5.415 × 10^6 cells/ml, observed in shrimp fed with the addition of *P. cruentum* nanoparticles at a dose of 0.8–1.2% per kg of feed.

Differential Hemocyte Count (DHC)

The results of DHC measurements in white shrimp fed with the addition of *P. cruentum* nanoparticles during the study are presented in Table 2.

Table 2. Differential Hemocyte Cells in white shrimp fed with addition of *P.cruentum* nanoparticles.

Treatment	Hemolymph Cells	Hyaline Cells				
		Week				
		0	1	2	3	4
P0	Hyaline Cells	43.25 ± 1.89	45.25 ± 1.70^{a}	43.50 ± 1.29^{a}	44.75 ± 1.25^{a}	45.75 ± 1.70^{a}
P1		43.00 ± 1.82	48.25 ± 1.70^{ab}	49.75 ± 1.70^{b}	52.50 ± 1.91^{b}	53.25 ± 2.21^{b}
P2		43.50 ± 1.91	51.25 ± 2.36^{b}	56.00 ± 1.63^{c}	59.50 ± 1.29^{c}	64.25 ± 1.70^{c}
P3		42.50 ± 1.91	48.25 ± 1.70^{ab}	50.50 ± 2.08^{b}	53.25 ± 1.70^{b}	54.25 ± 1.25^{b}
P0	Semi Granular Cells	29.25 ± 0.95	28.50 ± 1.91	29.00 ± 1.41	28.50 ± 1.91	27.75 ± 2.50
P1		28.75 ± 0.95	27.75 ± 2.21	27.75 ± 2.21	28.00 ± 1.63	28.00 ± 2.44
P2		28.75 ± 1.70	29.75 ± 2.06	30.00 ± 2.16	30.25 ± 2.21	30.75 ± 2.06
P3		28.75 ± 0.95	27.75 ± 1.70	27.75 ± 1.25	28.00 ± 1.41	28.25 ± 1.70
P0	Granular Cells	27.50 ± 2.51	26.25 ± 1.25^{b}	27.50 ± 0.57^{c}	26.75 ± 1.25^{c}	26.50 ± 1.00^{c}
P1		28.25 ± 1.50	24.00 ± 1.41^{b}	22.50 ± 1.91^{b}	19.50 ± 3.00^{b}	18.75 ± 0.95^{b}
P2		27.75 ± 2.50	19.00 ± 2.44^{a}	14.00 ± 0.81^{a}	10.25 ± 1.25^{a}	5.00 ± 1.63^{a}
P3		28.75 ± 1.50	24.00 ± 1.63^{b}	21.75 ± 3.30^{b}	18.75 ± 1.89^{b}	17.50 ± 1.00^{b}

Note : P0 control, without the addition of *P. cruentum* nanoparticles in feed, P1 addition of 0.8% *P. cruentum* nanoparticles, P2 addition of 1% *P. cruentum* nanoparticles, P3 addition of 1.2% *P. cruentum* nanoparticles. Notations indicated by different superscript letters in the same column indicate the comparison between treatments has a significant difference ($p < 0.05$).

In the first week, the hyaline cell count in treatment P0 was significantly different ($p < 0.05$) from treatment P2. However, there were no significant differences ($p > 0.05$) between P0, P1, and P3, nor among P1, P2, and P3. In weeks 2, 3, and 4, treatment P0 showed significant differences ($p < 0.05$) compared to the treatments (P1, P2, and P3). Additionally, treatment P2 was significantly different ($p < 0.05$) from P0, P1, and P3 during these weeks. However, treatments P1 and P3 did not differ significantly in weeks 2, 3, and 4. The average number of semi-granular cells in each treatment did not show significant differences across all weeks.

For granular cells in the first week, treatment P2 was significantly different ($p < 0.05$) from treatments P0, P1, and P3,

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while P0, P1, and P3 were not significantly different from each other. In weeks 2, 3, and 4, the average granular cell count for treatment P0 was significantly different ($p < 0.05$) from P1, P2, and P3. Similarly, treatment P2 showed significant differences ($p < 0.05$) compared to P0, P1, and P3. However, no significant differences were observed between treatments P1 and P3 during these weeks.

Phagocytic Activity (PA)

The results of Phagocytic activity measurement in white shrimp fed with *P. cruentum* nanoparticles during the study are presented in Table 3.

Table 3. Phagocytic activity of white shrimp fed with *P.cruentum* nanoparticles.

Treatment	Phagocytic Activity (%)				
	Week-				
	0	1	2	3	4
P0	26.75 ± 1.70	28.50 ± 1.29^{a}	30.50 ± 1.29^{a}	32.00 ± 1.41^{a}	35.00 ± 1.63^{a}
P1	26.50 ± 1.29	29.75 ± 1.89^{ab}	34.00 ± 0.81^{b}	38.75 ± 1.70^{b}	43.75 ± 1.25^{b}
P2	26.00 ± 1.82	32.00 ± 1.41^{b}	43.25 ± 1.25^{c}	53.25 ± 1.25^{c}	61.00 ± 1.41^{c}
P3	26.25 ± 1.70	29.25 ± 1.25^{a}	34.50 ± 1.91^{b}	39.75 ± 1.70^{b}	45.00 ± 1.41^{b}

Note: P0 control, without addition of *P. cruentum* nanoparticles in feed, P1 addition of 0.8% *P. cruentum* nanoparticles, P2 addition of 1% *P. cruentum* nanoparticles, P3 addition of 1.2% *P. cruentum* nanoparticles.

Based on the results of observations and measurements of Phagocytic activity in white shrimp, the study showed that shrimp fed with the addition of nanoparticles to the feed (treatments P1, P2, and P3) showed an increasing value each week with a statistically significant difference ($p < 0.05$). The treatment with the addition of 1% *P. cruentum* nanoparticles in the feed showed the highest Phagocytic activity each week and demonstrated significant differences ($p < 0.05$) among the treatments.

Survival Rate

The result of the survival rate measurement in white shrimp fed with the addition of *P. cruentum* nanoparticles during the study is presented in Table 4.

Table 4. Survival rate of white shrimp fed with *P. cruentum* nanoparticles.

Treatment	Survival rate (%)				
	Week				
	0	1	2	3	4
P0	100.00 ± 0.00	100.00 ± 0.00	97.00 ± 0.06^{a}	87.00 ± 0.06^{a}	80.00 ± 0.10^{a}
P1	100.00 ± 0.00	100.00 ± 0.00	97.00 ± 0.06^{a}	97.00 ± 0.06^{a}	97.00 ± 0.06^{b}
P2	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00^{b}	100.00 ± 0.00^{b}	100.00 ± 0.00^{c}
P3	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00^{b}	100.00 ± 0.00^{b}	97.00 ± 0.06^{b}

Note: P0 control, without addition of *P. cruentum* nanoparticles in feed, P1 addition of 0.8% *P. cruentum* nanoparticles, P2 addition of 1% *P. cruentum* nanoparticles, P3 addition of 1.2% *P. cruentum* nanoparticles.

Based on the survival rate results of white shrimp during the study, shrimp fed with feed supplemented with nanoparticles (treatments P1, P2, and P3) showed higher survival rates. In contrast, the treatment without the addition of *P. cruentum* nanoparticles experienced a significant decrease in survival. These results indicated that feeding white shrimp with feed containing *P. cruentum* nanoparticles could enhance the shrimp's immune system.

Nanoparticles are particles measuring between 1 and 100 nm (Ermawati and Ratnawati, 2011). Materials at the

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nanoscale have advantages over their larger (bulk) counterparts. Their extremely small size imparts new properties and functions, allowing control of the material at the atomic level. The preparation of *P. cruentum* nanoparticles is carried out using a top-down method with an HEM tool, which reduces the size of bulk materials to the nanoscale. After the milling process, the nanoparticle size is analyzed using SEM. SEM is a type of electron microscope that takes images of specimens by scanning them with a high-energy electron beam in a raster pattern. The smaller the particle size is, the larger the surface area will be. An increased surface area results in higher reactivity of the material (Mursal, 2018).

According to Setyaningsih *et al*. (2013), this higher reactivity also increases the rate at which bioactive compounds such as extracellular polysaccharides, phycobiliproteins, and fatty acids are absorbed by the shrimp's digestive tract, thus enhancing the effectiveness of the immunostimulant. Risjani *et al*. (2021) also highlighted that bioactive compounds in *P. cruentum* are more efficiently utilized in nanoscale form.

The results showed that the average total hemocytes in all treatments increased weekly (Table 1), both in the control group and in the groups fed with the addition of *P. cruentum* nanoparticles. The treatment with the addition of 1% *P. cruentum* nanoparticles in the feed consistently showed the highest total hemocyte count each week and was significantly different ($p < 0.05$) from the other treatments, while the control group showed the lowest total hemocyte count throughout the study. Darwantin *et al*. (2016) stated that this increase in hemocyte count reflects the activation of non-specific immune mechanisms in shrimp, particularly the stimulation of hemocytes to experience degranulation and release immune-related proteins such as lipopolysaccharide-binding protein (LPS-BP), transglutaminase, prophenoloxidase, and antimicrobial peptides like penaeidin and lectin.

Administering the appropriate dose of immunostimulants can enhance the immunity and growth of shrimp; however, excessive doses may suppress growth and act as immunosuppressants (Manoppo, 2014). The increase in total hemocytes indicates that *P. cruentum* nanoparticles added to the feed can enhance the shrimp's immune response. An elevated hemocyte count improves the organism's health status as these cells are likely involved in forming phagocytic cells that play a role in defending against microbial attacks (Syaichudin *et al*., 2019).

In this study, *P. cruentum* was added to the feed in nanoparticle form, with particle sizes ranging from 50.54 to 97.84 nm, which increased feed absorption and utilization efficiency since the particle size affected the organism's ability to absorb nutrients. This, in turn, influenced metabolic and physiological processes, ultimately impacting growth. Hidayat *et al*. (2018) stated that this efficient absorption helps to optimize immune cell function and energy utilization, directly supporting hemocyte proliferation and activity during immune responses.

Hemocyte cells in shrimp are classified into three types: hyaline cells, semi-granular cells, and granular cells. In the treatments supplemented with *P. cruentum* nanoparticles, the number of hyaline cells increased each week and showed significant differences ($p < 0.05$) between treatments. The highest hyaline cell count was observed in the treatment with 1% *P. cruentum* nanoparticles. Semi-granular cells in the nanoparticle treatments showed relatively consistent average values and did not differ significantly ($p > 0.05$) between treatments. Meanwhile, granular cells exhibited a decreasing trend in average counts each week. According to Maftuch *et al*. (2013), the decrease in the percentage of granular cells is compensated by a proportional increase in the hyaline cell population. Similarly, Prastiti *et al*. (2023) reported that increases in certain hemocyte types are due to induced cellular proliferation or rapid differentiation in

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response to antigenic challenges. All hemocyte types are capable of phagocytic activity, but hyaline cells generally play a more active role in this process. During phagocytosis, hyaline cells ingest and destroy pathogens and foreign particles entering the shrimp's body (Jannah *et al*., 2018). Therefore, an increase in hyaline cells enhances phagocytic activity, providing better protection for shrimp against pathogenic bacterial infections.

Phagocytic activity increased each week across all treatments. The control group showed only a slight increase and remained relatively stable, whereas the treatments with the addition of *P. cruentum* nanoparticles consistently showed higher phagocytic activity each week. The group fed with 1% *P. cruentum* nanoparticles exhibited the highest phagocytic activity and showed significant differences ($p < 0.05$) compared to the other treatments. In week 4, the average phagocytic activity values were 35.00% in the control, 43.75% in P1, 61.00% in P2, and 45.00% in P3. According to Zhang *et al*. (2023), total THC is positively correlated with phagocytic activity. This study also demonstrated that as THC increased, phagocytic activity rose accordingly. The increase in phagocytic activity indicated that the addition of nanoparticle-based immunostimulants such as *P. cruentum* could enhance the shrimp's immune system.

The survival rate of white shrimp after one month of feeding with *P. cruentum* nanoparticles reached 100%. In contrast, the control treatment without *P. cruentum* nanoparticles showed a relatively high mortality rate of 20%. This indicates that the addition of *P. cruentum* nanoparticles enhances the shrimp's immune system against pathogenic bacteria. These nanoparticles stimulate increased shrimp resistance by elevating the total hemocyte count, which subsequently raises the number of phagocytic cells (hyaline and semi-granular) responsible for phagocytizing pathogenic bacteria invading the shrimp's body. Rustam *et al.* (2024) stated that the increased survival is not only an indicator of better immune function but also reflects improved resistance to environmental stressors and opportunistic pathogens.

The survival rate of shrimp can be improved by administering immunostimulants. This is supported by Arayamethakorn *et al*. (2023), who reported that bacterial lipopolysaccharide administration increases survival rates in white shrimp. Additionally, Cantelli *et al*. (2019) found that the addition of sulfate polysaccharides enhances phenoloxidase (PO) activity and other nonspecific immune parameters, thereby improving survival rates in white shrimp.

CONCLUSION

From this study, it can be concluded that feeding *P. cruentum* nanoparticles to white shrimp increases total hemocytes, hyaline cells, phagocytic activity, and survival rate.

CONFLICT OF INTEREST

No conflict of interest exists among all authors upon writing and publishing the manuscript.

AUTHOR CONTRIBUTION

Saniya Lailatul Qodriyah and Nova Erika contributed to data collection and fieldwork. Woro Hastuti Satyantini was responsible for the conceptualization and design of the study. Laksmi Sulmartiwi prepared and wrote the manuscript. All authors have read and approved the final version of the manuscript.

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access to the laboratory and field facilities essential for this research.

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