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THE IMPACT OF VASAKA (*JUSTICIA ADHATODA*) LEAF EXTRACT ON THE GROWTH AND IMMUNE RESPONSE OF SHRIMP (*PENAEUS MONODON*)

Joya Biswas, Alokesh Kumar Ghosh*, Sheikh Shaon Ahmmed, Halima Tus Sadia, and Md. Golam Sarower

Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna-9208, Bangladesh

KUS: 1125: 25062023

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Abstract

Justicia adhatoda, also known as "Vasaka plant," is employed in Ayurvedic medicine to treat a wide range of human diseases. This study aimed to explore the potential application of Vasaka leaf extract (VLE) as a viable option for enhancing the growth and immune response of *Penaeus monodon*. Three diets containing varying amounts of Vasaka leaves (0 g [Control], 0.5 g/kg feed [VLE0.5], and 1 g/kg feed [VLE1.0]) were consumed to replicate groups of shrimp in 60 L aquarium tanks for 28 days. The assessment of growth was conducted by the evaluation of many parameters, including weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio and survival rate. The results of the experiment indicate that there were no statistically significant differences in the growth performance and survival rate of the shrimp across the various experimental groups. The introduction of Vasaka leaf as a dietary supplement resulted in a significant enhancement of shrimp immunity through the upregulation of total haemocyte count, prophenoloxidase activity, and superoxide dismutase activity, accompanied by a concurrent decrease in haemolymph clotting time. These results propose that nutritional supplementation with Vasaka leaf took no negative effect on the growth of *P. monodon* but did increase its non-specific immune response and can be considered as safe in shrimp culture.

Keyword: *Justicia adhatoda, Penaeus monodon*, Extract, Growth, Immunity

Introduction

Shrimp, a type of crustacean, has gained significant global popularity as a seafood product and is recognized as a very profitable species in aquaculture because of high value and demand (Lee *et al*., 2022). Nonetheless, the expeditious progression of the shrimp aquaculture industry has been associated with the appearance of many diseases which have resulted in significant production losses and hindered international trade. Consequently, these challenges have impeded the economic and social progress of coastal regions (Yu *et al*., 2022). Antimicrobials and antibiotics are widely utilized in the context of shrimp farming as a primary method for preventing disease. The inappropriate utilization of antibiotics has accelerated the development of antibiotic-resistant microbes, leading to notable environmental risks and the accumulation of residues from antibiotics in tissues, potentially impacting human well-being (Hossain *et al*., 2022). The growing apprehension about antibiotic resistance and the need for substitutes to synthetic antibiotics has led to a surge fascinating in medicinal plants as possible options for combating diseases. Numerous nations have already attained concerning levels of antibiotic resistance, with certain countries relying solely on their final line of protection against bacterial diseases (Haifa-Haryani *et al*., 2022; Hossain *et al*., 2022). Recently, there has been a noticeable rise in the interest surrounding the utilization of herbal medicines within the field of aquaculture. This heightened attention may be attributed to several factors, including the perceived effectiveness of these medicines, their diminished levels of toxicity and adverse reactions, and the inherent nature of their active constituents as organically derived compounds (Yin *et al*., 2014; Ghosh *et al*., 2023). Numerous research have documented the widespread use of medicinal plant extracts for the purpose of managing shrimp diseases and enhancing shrimp nonspecific immune responses (Abidin *et al*., 2021; Pu and Wu, 2022; Yin *et al*., 2023; Ghosh *et al*., 2023).

*Corresponding author: <alokesh_ku@yahoo.com>

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Justicia adhatoda L., usually referred as Vasaka or Malabar nut, a member of the Acanthaceae family, is a widely distributed shrub found in Southeast Asia and the Indian subcontinent. The leaves, flowers, fruits and roots of this plant are utilized in the therapeutic management of respiratory ailments such as cough, asthma, bronchitis, and tuberculosis. This botanical species holds significance due to its properties as an expectorant, as well as its abilities to act as an anti-spasmodic and anti-helminthic agent. The utilization of plant leaves in the management of various ailments is widespread and serves as a primary basis for the development of pharmaceutical preparations (Das and Ghosh, 2022; Mishra *et al*., 2023). Recent scientific studies have revealed that extracts derived from the leaves of the *J. adhatoda* plant exhibit a wide range of antibacterial and antifungal properties, effectively combating many pathogenic microorganisms (Pa and Mathew, 2012**;** Mishra *et al*., 2023). The immunological modulatory effects of *J. adhatoda* extract have been found to be considerable in both human (Chavan & Chowdhary, 2014) and vertebrate fish (Saha & Bandyopadhyay, 2020), as demonstrated by previous studies. Nevertheless, there is a lack of research investigating the efficacy of *J. adhatoda* extract on the immune functions of shrimp. Therefore, the present study aims to examine the impact of *J. adhatoda* leaf extract on the immunity and growth of shrimp.

Materials and Method

Preparation of Vasaka leaf extract

To obtain the extract, a collection of fresh, mature deep green leaves of Vasaka was obtained from the trees. The leaves were washed with distilled water, cut into tiny pieces, and desiccated in an oven at 40°C for about two days. The dehydrated leaves were smashed into a tiny powder employing a grinder and afterwards stored at 4°C for future utilization. To prepare extract, 100 g of Vasaka leaves powder was placed in a glass bottle and immersed in 1 L of 100% methanol (approximately), sonicated in a water bath at 6-hour intervals, strained using a muslin cloth and filter paper (Whatman No. 1). The methanol was subjected to evaporation using a rotary vacuum evaporator. The remains were stored at 4°C until they were utilized.

Preparation of experimental diet

In the context of diet formulation, the study employed commercial shrimp feed together with varying amounts of Vasaka leaf extract (VLE). First, extract was dissolved in ethanol and employed at varying concentrations on pellet feed (moisture, crude fat and protein were 12, 7 and 31%, respectively) and then combined (Ghosh *et al*., 2023). Three experimental diets control (feed devoid of VLE), VLE0.5 (Basal feed + VLE 0.5 g/kg) and VLE1.0 (Basal feed + VLE 1.0 g/kg) were prepared. Following the combination of the extract and the feed, the resultant wet feed underwent a process of air-drying, subsequently being subjected to 40°C within an oven to finalize the drying procedure. A binding gel was applied to the diet to coat it prior to its introduction into water, so mitigating the potential loss of extract in water body (Balasubramanian *et al*., 2008).

Collection of shrimp and management practices

The shrimp were collected from a shrimp farm (Dumuria, Khulna) and carried to the laboratory with adequate aeration. The shrimp were disease-free and more or less the same size (2 g). Prior to filling the testing tanks (60 L), a UV device was employed to eradicate germs present in the water. The temperature was regulated by employing little submersible heaters (RS electrical, 200 W) that were fitted in each tank. A total of ten shrimps were allocated to each tank and allowed a period of ten days for acclimation prior to the commencement of the experiment. In every tank, a solitary air-stone was employed to ensure continuous aeration, hence sustaining the requisite levels of dissolved oxygen (DO) over the entire duration. The tanks were separated into three groups: one control group (C) and two treatment groups, VLE0.5 and VLE1.0, with multiple replications. The shrimp were administered the experimental diets twice daily, at 09:00 and 16:00, at a dosage of 5-7% of their body weight throughout the duration of the 28-day trial. Prior to each feeding, the waste materials were eliminated by means of siphoning, and a daily replacement of one-third of the water volume was conducted. Daily measurements were taken and recorded for a range of water quality attributes: temperature ranged from 29-30°C, pH 7.8-8.5, salinity 10-11 ppt, DO >6 mg/L, and NH_3 0.1 mg/L.

Data collection and growth performance measurement

At the completion of the experiment, the final weight was determined, and the following equations were used to calculate the weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival rate (Huang, 2020).

WG (g) = (final weight (g) - initial weight (g))

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

FCR = feed intake (FI) / (final weight (g) – initial weight (g))

PER= (final weight (g) – initial weight (g)) / protein intake (g)

Survival (%) = 100 (final shrimp number/beginning shrimp number)

Hematological examination and immunological evaluation

Total Haemocyte count (THC)

A volume of 100 µL of haemolymph was obtained from the ventral sinus, which is situated at the base of the 1st abdominal segment of the shrimp. This was achieved by employing a 1 mL syringe containing an anticoagulant. To enhance the appearance of haemocytes and facilitate their countability, a single droplet of stain solution (Rose Bengal) was introduced into a 20 µL sample of mixed haemolymph. A drop of the mixed sample was applied to a haemocytometer (Precicolor HBG, Germany) to assess the concentration of THC. The specimen is enclosed within a glass casing and observed via the utilization of a microscope (Labomed, USA). The formulas utilized for the calculation of THC (cells/mL) was as follows (Japitana *et al.*, 2017).

THC= ((A+B+C+D)/4) x 10^4 x Dcf

A, B, C, D=Block of haemocytometer

Dcf= (volume of anticoagulant + volume of hemolymph)/volume of hemolymph

Haemolymph clotting time (HCT) determination

The capillary method outlined by Jussila *et al.* (2001) was used to measure the haemolymph clotting time of shrimps. A total of 100 µL of hemolymph was taken and kept on ice in an Eppendorf tube. The HCT was efficiently calculated by measuring the flow of 25 µL of undiluted hemolymph through a capillary tube using a specific approach. The tube was oriented in a vertical posture, using the hemolymph sample placed in the uppermost section subsequent to insertion. The tube was maintained in a vertical position until the hemolymph column was compelled to descend towards the bottom end of the tube, following which it was then rotated by 180 degrees once more. This process was performed repeatedly before the hemolymph became coagulated. The clotting duration was recorded when hemolymph flow ceased, indicating complete clotting.

Determination of prophenoloxidase (proPO) activity and superoxide dismutase (SOD) activity

The spectrophotometric measurement of proPO activity was conducted by Huang *et al.* (2020) using the conversion of L-dihydroxyphenylalanine (L-DOPA) to dopachrome, as stated in their method. The SOD activity (E.C. 1.15.1.1) was assessed following the methodology outlined by Marklund and Marklund (1974), with some adjustments as described by Jing and Zhao (1995). The experimental procedure utilizes the pyrogallol autoxidation process, which is a refined version of the Marklund method, in order to assess the enzyme activity. The process of autoxidation of pyrogallol, when conducted in the presence of EDTA at a pH of 8.2, resulted in a 50% reduction.

Data analysis

Initially, the experimental data underwent processing using the software MS Excel. Subsequently, the raw data was imported into IBM SPSS Statistics software for the purpose of conducting a One-way ANOVA analysis (In case of normally distributed data) followed by Post Hoc test (Tukey LSD). The statistical significance was established using a significance level of $p < 0.05$.

Results

Effect of Vasaka leaf extract on growth performance of shrimp

In this study, the final weight, WG, SGR, FCR and PER of shrimp were used to measure the growth performance. The average weight of the control group, VLE0.5 group, and VLE1.0 group was found to be 4.3 g, 4.6 g, and 4.6 g, respectively after 28 days trial, as shown in Table 1. Nevertheless, the statistical analysis indicated that there was no significant disparity between these groups ($p > 0.05$). Similarly, no statistically significant differences were perceived in the WG, SGR, FCR, PER, and survival rate of shrimp across the groups ($p > 0.05$) (Table 1).

Table 1. Summary of growth parameters for *P. monodon* after 28 days of feeding with VLE

Parameters	Control (Mean±SD)	VLE0.5 (Mean±SD)	VLE1.0 (Mean±SD)
Final Weight (g)	4.3±0.8	4.6±1	4.6±0.9
Weight Gain (g)	2.2±0.17	2.5±0.21	2.6±0.27
SGR (%)	2.58±0.14	2.85±0.16	2.86±0.21
FCR	3.2±0.52	2.7±0.64	2.6±0.14
PER	1.01±0.16	1.24±0.30	1.23±0.06
Survival (%)	85±7.1	90±14.1	90±14.1

Effect of extract on total haemocyte count (THC) of shrimp

The THC levels of the shrimps were assessed and presented in Figure 1 following a 28-day feeding period. The average THC levels for the control group, VLE0.5 group and VLE1.0 group were 6.0×10^5 cells/mL, 12.6×10^5 cells/mL and 13.2×10^5 cells/mL respectively. A significant variance ($p < 0.05$) in the level of THC was detected between the shrimp subjected to extract treatment and the control shrimp. The THC content in the VLE1.0 group was found to be significantly higher, exceeding double the amount, compared to the control group. Nevertheless, no significant disparity was noted among the treatments (Figure 1).

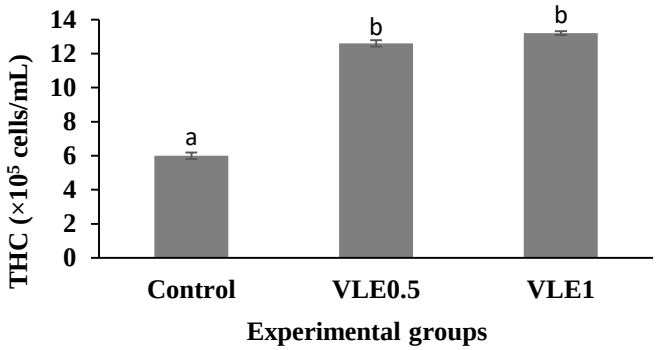

Figure 1. Total haemocyte count of *P. monodon* treated with Vasaka extract. The presence of distinct superscripts within the bar implies significant distinctions among the groupings (5% level of significance).

Effect of extract on haemolymph clotting time (HCT) of shrimp

Following a 28-day period of feeding, the average HCT levels of the experimental shrimp were assessed and shown in Figure 2. The average clotting time for control, VLE0.5 and VLE1.0 were 115 s, 51 s and 54 s respectively. A statistically significant difference ($p < 0.05$) in HCT levels was perceived between the control and treatment groups. The control group of shrimps had the highest HCT, while the VLE1.0 group (treatment 2) displayed the lowest HCT levels. However, no significant difference was established between the treatments.

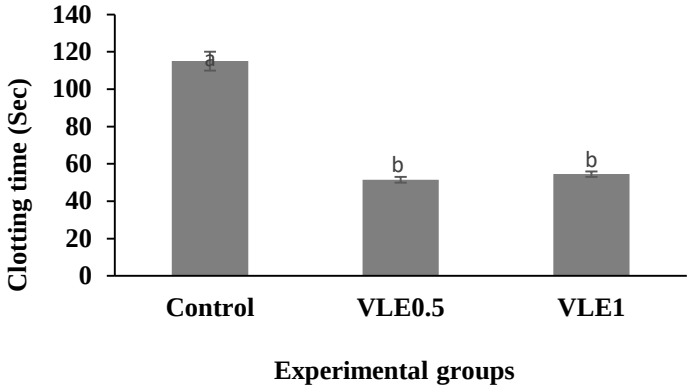

Figure 2. Haemolymph clotting time of *P. monodon* treated with Vasaka extract. Disparities among the groups are represented through numerous superscripts in the bar graph (5% level of significance).

Impact of extract on Prophenoloxidase (proPO) activity of shrimp

The mean phenoloxidase activity (proPO) of shrimps were measured and presented in figure 3. The shrimp proPO was significantly impacted by the dietary inclusion of Vasaka extract. Shrimp in VLE1.0 (1g/kg) had the highest level of proPO ($p < 0.05$), while the control shrimp had the lowest level. No significant variation was found between control group and VLE0.5 group (Treatment 1)

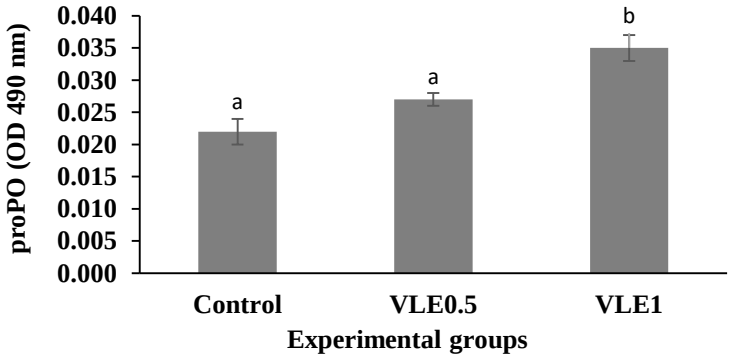

Figure 3. Prophenoloxidase (proPO) activity of *P. monodon* fed extract of *J. adhatoda*. The presence of distinct superscripts in the bar denotes significant disparities among the groups (5% level of significance).

Influence of dietary extract on superoxide dismutase (SOD) activity of shrimp

There was a significant impact ($p < 0.05$) of dietary extract of *J. adhatoda* on the SOD level of shrimp. The extract with the highest concentration (VLE1.0) exhibited the greatest level of SOD in comparison to the lowest concentration and the control group which indicates that the SOD level is positively correlated with the concentration of Vasaka dietary extract. The shrimp in the control group had the most minimal level of SOD activity.

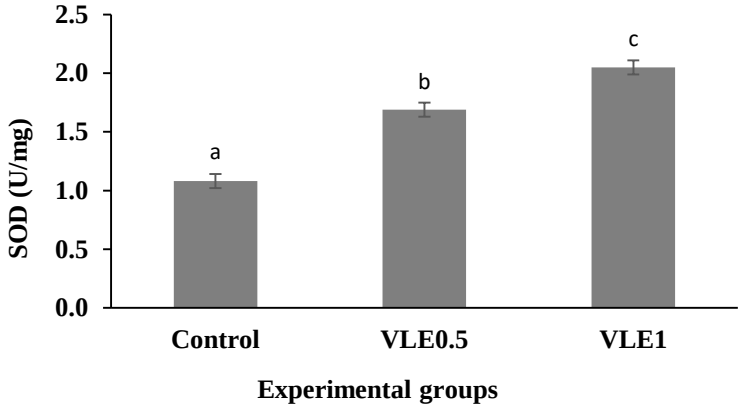

Figure 4. SOD activity of *P. monodon* treated by dietary extract of *J. adhatoda*. Variable superscripts within the bar denote significant distinctions among the groupings (5% level of significance).

Discussion

In contemporary times, the shrimp farming sector has predominantly relied on antibiotics or chemicals to mitigate shrimp diseases by targeting particular pathogens. However, the utilization of medicinal plants in disease prevention is a nascent and evolving field within this industry. Plants harboring bioactive substances have the potential to enhance overall well-being, bolster innate immunity against viral and bacterial pathogens, and contribute to the management and mitigation of many illnesses (Yin *et al*., 2014; Ghosh *et al*., 2023). The herb *Justicia adhatoda* possesses several medical properties; nevertheless, its potential for assessing the immune activity of shrimp had not been previously explored. The current study involved an evaluation of the growth performance and immunological parameters of shrimp by the administration of *J. adhatoda* extract in their diet. The study revealed that the extract did not exhibit detrimental properties on the growth and persistence of shrimp. Nonetheless, it did demonstrate some favorable effects, but these effects were not statistically significant. Despite numerous studies demonstrating the positive effects of plant extracts such as *Andrographis paniculata, Astragalus membranaceus*, or *Moringa oleifera* on the growth of shrimp (Abidin *et al*., 2021; Pu and Wu, 2022; Yin *et al*., 2023), however, some plants, such as *Gracilaria tenuistipitata*, had no effect on growth (Liu *et al*., 2019). The growth is contingent upon the specific characteristics of the plants and extracts utilized, as well as the particular species and size of shrimp involved.

While the growth-enhancing effects of dietary administration of VLE were not found significantly, the other objective of the current study was to investigate the immunological responses connected with VLE consumption. In previous studies, various immunological indicators including THC, HCT, proPO, and SOD activity have been employed to appraise the prospective influence of extracts on decapod immune functions (Huang *et al*., 2020). Shrimp hold an innate or intrinsic immune system which functions as a rapid and efficient means of protection against infections. The immune system of the shrimp is comprised of different components, including haemocytes such as hyalinocytes, granulocytes, and semi-granulocytes. Additionally, it consists of various plasma components, such as antimicrobial peptides, lysosomal enzymes, histones, recognition molecules, and lipopolysaccharide binding proteins. Furthermore, the immune system also involves multimeric systems like the prophenoloxidase system and the clotting protein cascade (Aguirre-Guzman *et al*., 2009). Shrimp haemocytes have a vital function in the immune system, serving as the primary cellular component. These cells are liable for several activities including phagocytosis, encapsulation, nodule development, and the activation of proPO (Aguirre-Guzman *et al*., 2009; Salehpour *et al*., 2021). This study observed a substantial increase in THC levels in shrimps treated with extracts compared to the control group. This finding aligns with previous research that reported elevated THC levels in shrimps fed diets enriched with extracts such as *Psidium guajava* (Yin *et al*., 2014; Dewi *et al*., 2021), *Andrographis paniculata* (Yin *et al*., 2023), and *Astragalus membranaceus* (Pu and Wu, 2022), *Zingiber officinale* and *Aegle marmelos* (Ghosh *et al*., 2023). The obtained phenomena can be ascribed by the expedited maturation of haemocyte precursors inside the haematopoietic tissue. Subsequently,

newly formed cells are released in the circulatory system to uphold the shrimp's haemocyte and ensure its proper functioning (Sirirustananun *et al*., 2011; Huang *et al*., 2020).

In crustaceans, haemolymph coagulation is a component of the innate immune functions; it inhibits haemolymph leaking and supports the body's defense against the spread of intruders like bacteria. The process of coagulation in shrimp is triggered with the advent of haemocyte, TGs, which leads to the polymerization of the haemolymph clotting protein, resulting in the formation of a stable gel (Yeh *et al*., 2007). In this study, the HCT in the shrimp that had been treated with extract had a significantly shorter duration than that of the control group which is in accordance with the findings of Balasubramanian *et al.* (2008) for *Cynodon dactylon,* Velmurugan *et al.* (2015) for *Enteromorpha flexuosa*, Ghosh *et al.* (2023) for *Zingiber officinale* and *Aegle marmelos,* in which clotting time was significantly reduced in shrimp fed of extract. Shrimps that were fed the extract treated diet exhibited shortest clotting time, maybe attributable to an increased abundance of haemocytes and/or a higher amount of hemolymph protein in the shrimps subjected to this particular diet (Raja Rajeswari *et al*., 2012). The proPO-activating process is an essential element of the innate immune defenses of invertebrate organisms. When the prophenoloxidase activating enzymes are activated by an invading pathogen, they cleave inactive proPOs to produce active POs, which in turn cause the creation of melanin and the death of the invading pathogens (Amparyup *et al*., 2013; Zhou *et al*., 2021). The present investigation documented a rise in proPO activity in shrimp subjected to the extract, in comparison to the control group which is consistent with previous research. For instance, The proPO function in shrimps was augmented when they were administered a herbal-supplement diet consisting of *Argemone mexicana* (Palanikumar *et al*., 2018), *Gracilaria tenuistipitata* (Sirirustananun *et al*., 2011), *Cystoseira trinodis* (Salehpour *et al*., 2021), *Zingiber officinale* and *Aegle marmelos* (Ghosh *et al*., 2023) against WSSV infection. The extract's immunostimulant compounds potentially engaged with pattern recognition proteins (PRPs), so initiating the activation of the proPO system, afterwards causing in immunomodulation (Citarasu *et al*., 2006; Palanikumar *et al*., 2018).

When haemocytes engage in phagocytosis to devour bacteria, they generate a variety of bactericidal chemicals comprising highly reactive oxygen species that have the potential to be cytotoxic to the host cell (Campa-Córdova *et al*., 2002; Salehpour *et al*., 2021). SOD is a vital antioxidant enzyme for determining the level of oxidative stress in aquatic species, which converts harmful superoxide radicals to less harmful particles of oxygen or hydrogen peroxide (Chang *et al*., 2003; Salehpour *et al*., 2021). The present study revealed that dietary treatment with *J. adhatoda* extract increased SOD activity in shrimp than that of the control shrimp. Similarly, when shrimps were fed with diets containing extracts of *Cynodon dactylon* (Balasubramanian *et al.* 2008), *Gynura bicolor* (Wu *et al*., 2015), *Zingiber officinale* and *Aegle marmelos* (Ghosh *et al*., 2023), there was an rise in the enzymatic activity of SOD. Hence, the inclusion of dietary extracts resulted in a significant elevation in enzymatic activity in shrimp, than that of the control group of shrimp.

Conclusion

The findings of this study demonstrated that the administration of Vasaka extract to shrimp (*P. monodon*) diet resulted in enhanced immunological responses. This was indicated by an elevation in total haemocyte count, prophenoloxidase activity, and superoxide dismutase activity, and a decrease in hemolymph clotting time. Although the extract does not exhibit significant effects on shrimp growth, it possesses the latent to serve as an immunostimulant for shrimp, hence enhancing their resistance against diseases.

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Conflict of interest

The authors declare no conflict of interest.

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