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Research Article

Antimicrobial Activities and Immune Responses of Methanolic Extract of *Najas indica* on Shrimp (*Penaeus monodon*)

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Keywords: *Najas indica*; Shrimp health management; Antibacterial activity; Immune parameters; Wssv; Aquaculture; Natural medicine

Abstract

The aim of this study was to evaluate the influence of *Najas indica* on the immune system and health of shrimp. Antibacterial activity, immune parameters, and a challenge experiment against White Spot Syndrome Virus (WSSV) were assessed. The results revealed that the methanolic extract of *Najas indica* exhibited significant antibacterial activity with 15±2 mm inhibition zone, indicating its potential as an antimicrobial agent. Furthermore, shrimp fed with a 1 mg/kg methanol extract of *Najas indica* displayed significantly increased levels of Total Hemocyte Count (51.73 ± 2.25×10^5) cells/ml and Superoxide Dismutase (SOD) activity (4.3 ± 0.72 U/mg) compared to other treatments, suggesting enhanced immune response and oxidative stress defense. Significant differences were observed in the clotting times of shrimp hemolymph collected from individuals fed with 1 mg/kg *Najas indica* extract compared to other treatments. Notably, the clotting time was the shortest (3.67 s) in the 1 mg/kg treatment, indicating improved coagulation response. Conversely, the control group (without *Najas indica* extract) exhibited the longest clotting time (10.67 s). In the challenge experiment against WSSV, shrimp fed with *Najas indica* extract (1 mg/kg) showed reduced mortality compared to the control group. Probit analysis determined the LC_{50}, and the control group exhibited significantly faster mortality rates than the treatment group. The cumulative hazard probability of shrimp mortality was lower in the treatment group than in the control group. In conclusion, this study demonstrated that extracts from *Najas indica* possess therapeutic effects and may serve as a potential natural medicine for aquatic animals, specifically in shrimp health management.

Introduction

Aquatic organisms are commonly confronted with a range of infectious agents, including bacteria, viruses, and fungi, which can cause severe health problems and economic losses in aquaculture [1]. The use of antimicrobial agents to control these pathogens is a common practice in the aquaculture industry [2]. However, the emergence of antibiotic resistance in pathogens has become a major concern worldwide, leading to the search for alternative treatments. One of the promising alternatives is the use of natural products, such as plant extracts, which have been reported to have antimicrobial properties and are generally safe for the environment [3-5]. Secondary metabolites in plants are primarily accountable for their antimicrobial activity [6]. *Najas indica* is an aquatic, submerged, invasive plant that grows in fresh water (calm waters such as rivers, slow-flowing aquatic environments, ponds and lakes) and is prevalent in Japan, China, and southeast Asia [7]. In Asia, the aquatic plants *Najas* spp. are typically prevalent at salinities between 10 and 0 ppt. It is typically found in depths ranging from 0.6 to 4.5 meters, loves moderately mild water (8 °C at its coldest), but can also survive in hot summer water [8,9]. Stems are up to 40 cm long and branched [7]. These plants are known for their ability to absorb

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nutrients and filter water, making them a valuable addition to aquaculture systems, such as shrimp farming. In shrimp farming, *Najas indica* offers several benefits such as improved water quality, enhanced dissolved oxygen levels, and reducing the growth of harmful algae. It can also provide important habitat for shrimp and other aquatic organisms, providing both shelter and nutritional support. However, antimicrobial properties of *Najas indica* are still unexplored. Shrimp (*Penaeus monodon*) is one of the most important aquaculture species, but it is susceptible to bacterial infections, particularly those caused by *Vibrio* sp. [10]. Therefore, the evaluation of the antimicrobial activity of *N. indica* and its effect on the immune responses of shrimp is of great interest. *Najas* species can be a valuable tool for shrimp farmers for the health management of shrimp in their aquaculture system. However, almost no research has been conducted on the use of the *Najas indica* plant for shrimp health management. Therefore, the objective of this study was to determine how extract of *Najas indica* affects shrimp immunity and health management.

Materials and methods

Collection of *Najas indica*

All parts of the *Najas indica* plant except the roots were collected from the shrimp farming pond of the Shrimp Research Station located in Bagerhat-9300, Bangladesh. The plants were identified as *Najas indica* from the Bangladesh National Herbarium (Accession number: DACB 90649). Fresh samples were washed well with clean water to get rid of any other impurities that were still there. The specimens were then dried at room temperature, labeled, and transported to the laboratory for further analysis.

Preparation of extracts

The grinder was used to powder the dried samples, thereafter passed through a 40-mesh sieve. Each powdered sample was suspended in methanolic extracts at a 1:10 ratio. Methanol was selected for extraction because it effectively dissolves a wide range of polar and non-polar phytochemicals and it has higher extraction efficiency [11]. Initial phytochemical screening was also conducted of *Najas indica* which demonstrated the presence of phenols, flavonoids, tannin, saponin, anthocyanin and phlobatanin. In order to improve the process of extracting, the sample was placed in a dark environment for a duration of 24 hours, during which it was periodically agitated. Following incubation, the solution was filtered using Whatman No. 11 filter paper (11 µm pore size) under aseptic conditions. After filtration, the residual moist powder was subjected to a further 12-hour extraction in their respective solvents, with intermittent agitation, to maximize the yield of sample powder. The filtrates were concentrated under reduced pressure at 40 °C using a rotary evaporator (RE-300, Sci Labware, UK) until nearly dry. Further concentration was achieved using a Nitrogen evaporator (N-EVP 111, Berlin A 01503, USA). The resulting dried extract was stored at -20 °C [12].

Antibacterial activity assay

Bacterial strains: The assessment of antibacterial properties in *Najas indica* extract was conducted on chosen Gram-negative *Vibrio parahaemolyticus* strains. The bacterial strains utilized in the study were sourced from the Shrimp Health Management Laboratory at the Shrimp Research Station located in Bagerhat.

Antibacterial activity by disc diffusion assay: Disc diffusion method was employed to assess antibacterial activity. Briefly 1 gram of extract was dissolved in 10 ml methanol to prepare stock, resulting in a final concentration of 100 mg/mL. Sterile, blank discs with a diameter of 6 mm were impregnated with 20 µL and 30 µL of the extract and allowed to dry completely. Negative controls, including discs loaded with distilled water, methanol, and acetone, were also utilized. The diameter of the Inhibition Zone (IZ) surrounding the discs was measured in order to assess the antibacterial activity. The discs were placed on the bacterial lawn, and the assay was conducted thrice. The antibacterial activity was measured by determining the average diameter (in millimetres) of the zone of inhibition caused by the plant extract [13].

Shrimp stock and pathogen challenge

An in vivo experimental model was conducted using *Penaeus monodon*, a highly valued crustacean species in aquaculture. Shrimp weighing about 20g were collected and acclimatized for 3 days and then raised in each of 40-L tank with continuous aeration at ambient room temperature. The shrimp were randomly divided into four groups (n = 10 per group) with 3 replications. Where in treatment group methanolic extract (1, 3 and 5 mg/kg) was fed with the commercial feed for 21 days and only commercial feed was fed in control group. After 21 days of feeding, immune parameter was analyzed and on the basis of result of the treatment group, the best one (1 mg/kg) was selected for challenge experiment against the control ones. 1 mg/kg dose of *Najas indica* extract was found to optimally enhance immune responses and disease resistance in shrimp by balancing effective bioactive compound concentration without inducing toxicity or negative side effects. The shrimps were then infected with viral suspension of White Spot Syndrome Virus (WSSV). Briefly, 0.5-g samples of the original frozen infected specimens of *P. monodon* were minced and then mixed properly in 4.5 ml of sterile PBS. After centrifugation at 400 × g for 10 min at 4 °C, the supernatant was filtered through a 0.45-µm membrane and used to infect adult specific-pathogen-free (SPF) *P. monodon* (body weight, 20 g; Pl from Desh Bangla hatchery reared in the pond of Shrimp research Station, Bagerhat) by injection as previously described by Tsai, et al. [14].

Immunological analysis of the shrimp haemolymph

The hemolymph was extracted from both healthy and WSSV-challenged shrimp following the administration of *N. indica* extract. Subsequently, the immunological parameters were assessed according to the following methodology-

Total Hemocyte Count (THC): During the experimental trial, twenty shrimp from each treatment were used for immunological testing. Each shrimp had 0.5 ml of hemolymph extracted from the base of its third walking leg using a syringe containing 1 ml of anticoagulant. After collecting

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the hemolymph, Hemocyte counts were conducted using a hemocytometer. THC (per cubic millimeter) was calculated using the equation established by Song & Hsieh [15]:

$$THC = \frac{Count\ of\ total\ haemolymph \times Dilution\ factor \times 2 \times 10^4}{Number\ of\ square\ counted}$$

Superoxide dismutase activity: The measurement of shrimp's superoxide dismutase activity was conducted using the methodology provided by Creative BioMart, Inc., USA (EC 1.15.1.1). This protocol was adapted from the methods described by Marklund and Marklund [16] and Jing and Zhao [17]. The test is designed based on a conflict between the process of pyrogallol autoxidation by O_2^- and the process of radical dismutation by SOD. A single unit of SOD activity is defined as the ability to reduce the autoxidation of pyrogallol by 50%. A TRIS-EDTA buffer solution (solution A) and a 0.2 mM pyrogallol solution (solution B) were prepared following the instructions provided by the manufacturer (EC 1.15.1.1). The spectrophotometer (Peak instruments, C-7200, USA) was used to measure the absorbance at 325 nm. The SOD activity was estimated using a formula-

$$SOD\ activity\ (U/mg) = \frac{\frac{\Delta A_{325\,blank} - \Delta A_{325\,sample}}{\Delta A_{325\,blank}} \times 100\%}{50\%} \times 4.5 \times \frac{D}{V} \times \frac{V_1}{m}$$

Where, U/img is the SOD activity unit;

$\Delta A_{325\ blank}$ is the auto-oxidation rate determined in the blank test;

$\Delta A_{325\ sample}$ is the auto-oxidation rate determined using a sample;

V is the volume of sample used for testing in mL;

D is the dilution factor of the sample;

V_1 is the total volume of the sample solution in mL;

m is the weight of the solid sample in gram and

4.5 is the total volume of the reaction mixture in mL.

Hemolymph clotting time: The determination of hemolymph clotting time followed the methodology suggested by Jussila, et al. [18]. Hemolymph samples (20 µL) from each of the ten shrimp organisms (n = 10) were introduced into a capillary microtube with a hematocrit of 1.55 mm inner diameter and a length of 75 mm (Mucaps, India). The coagulation time for each sample was assessed through repeated inversion counts. The time measurement commenced from the instance of needle insertion into the shrimp ventral sinus and continued until the hemolymph flow on the microtube ceased.

Based on the best immunity parameter result, shrimp that had been fed 1 mg/kg of *Najas* extract were chosen for the challenge experiment against shrimp that had not been fed any *Najas* extract. Mortality and survival rate were then analyzed.

Statistical analysis

Analysis of variance (ANOVA) was used to determine total Hemocyte counts and the SOD activity of shrimp given extracts from *Najas indica*. One-way ANOVA was used to find differences in means between different sets of factor levels. All of the tests were done three times, and SPSS version 25 was used to do statistical analysis. (IBM, Armonk, NY, USA) [19]. The survival analysis was conducted using the Independent-Samples Kruskal-Wallis Test. The hazard functions were derived using the Kaplan-Meier survival analysis.

Results

Antibacterial activities of *N. indica* extract

The present study investigated the antibacterial activity of *N. indica* extracts obtained using methanol extract against *Vibrio parahaemolyticus*. It showed antibacterial activity, as evidenced by the formation of inhibition zones on agar plates (Table 1).

Immune parameters

Total Hemocyte Count (THC): Shrimp fed with 1 mg/kg methanol extract of *N. indica* had a significantly higher (p < 0.05) THC value than other treatments (Figure 1).

Superoxide Dismutase (SOD) Activity: SOD activity was measured in the shrimp fed with *N. indica* extract (methanol) in different concentrations. Results showed that shrimp fed with 1 mg/kg methanol extract of *Najas indica* had significantly higher (p < 0.05) Superoxide dismutase (SOD) activity than other treatments (Figure 2).

Hemolymph clotting time: In this study, significant differences were found (p < 0.05) between clotting times of the shrimp hemolymph collected from the shrimp fed with 1 mg/kg *N. indica* extract and other treatments. Except for the treatment

Table 1: The antibacterial activities of the *N. indica* extract.

Solvent	Zone of Inhibition (mm)	
	20 µl (Amount of extract/ solvent)	30 µl (Amount of extract/ solvent)
Methanolic extract	15±2	20 ± 1
Only Methanol	No inhibition	No inhibition
Distilled water	No inhibition	No inhibition

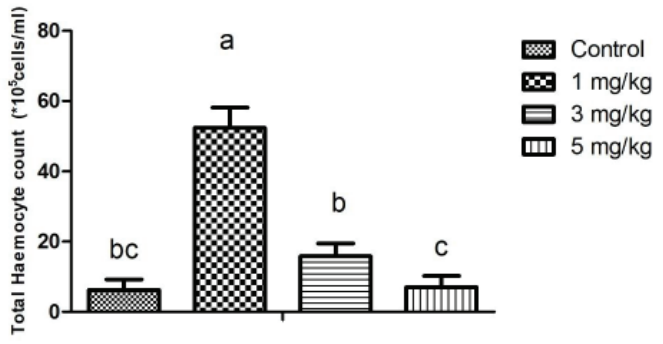

Figure 1: Total hemocyte count (THC) of shrimp of different treatments. Different superscript letters indicate significant difference (p < 0.05) between the treatment and control groups.

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of 1 mg/kg *N. indica* extract, no significant differences ($p > 0.05$) were observed among the other treatments. Their lowest and highest values were 3.67 and 10.67 Seconds (s), determined in the treatment of feeding 1 mg/kg *N. indica* extract and in the control (No *N. indica* extract), respectively (Table 2).

Effect of the *Najas indica* extract on the shrimps Infected by WSSV

Survival function: The survival function analysis revealed that the probability of cumulative survival in the treatment group was 100% within the first 30 hrs of the challenge test with WSSV. Two consecutive little falls of cumulative survival happened between 30-40 hrs, which then continued (≥ 90%) over 80 hrs, until a sudden fall just below 80% survival of *P. monodon* challenged larvae (Figure 3).

On the other hand, animals without treatment with *Najas*'s extract after WSSV infection might start to die from the 15th h in the challenge test experiment, and with a continuous mortality towards the end of the challenge test. Three sharp falls after 65 hrs reduced the survival from 80% to < 40% at the end of 90 hrs.

Hazard function: The probability of hazard function in the treatment group was less than 10% after 40 hrs of the experiment and continued up till the 85th h of the challenge test, with a sudden increase in hazard for over 20% mortality, which indicated a probability of high protection against WSSV post infection (Figure 4).

On the other hand, the cumulative hazard in the control group started at 15th h, gradually decreased the survival up till 65th hrs, sharp mortality occurred with a higher hazard without *Najas indica* extract treatment (Figure 5).

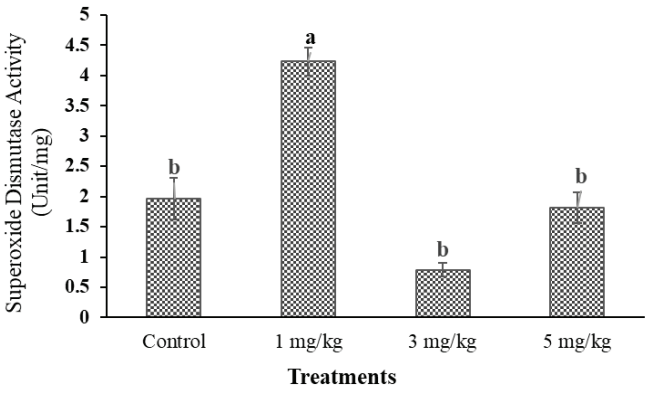

Figure 2: Superoxide dismutase activity in shrimp. Different superscript letters indicate significant differences between treatment groups ($p < 0.05$).

Table 2: Hemolymph clotting time of shrimp in different treatments.

Treatments	Hemolymph clotting time (seconds)
Control	10.67 ± 0.47^{a}
1 mg/kg	3.67 ± 0.94^{b}
3 mg/kg	7.67 ± 2.05^{a}
5 mg/kg	8 ± 1.63^{a}

Means with different superscript letter (a, b) are significantly differenft ($p < 0.05$)

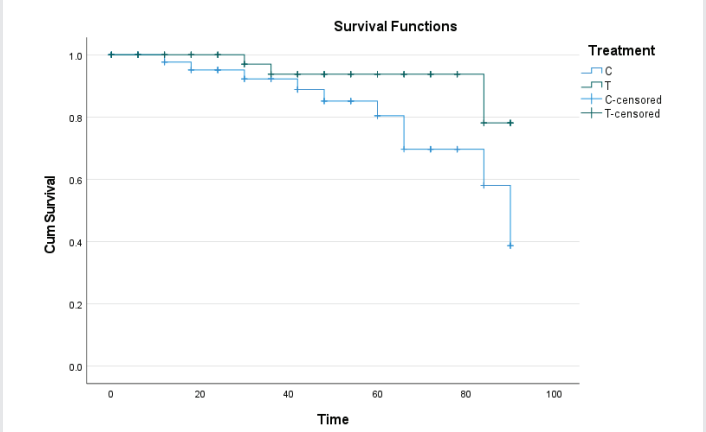

Figure 3: Kaplan Meier survival function graph of shrimp (*P. monodon*) over a challenge test against *Vibrio parahaemolyticus*. Crosses (+) indicate censoring.

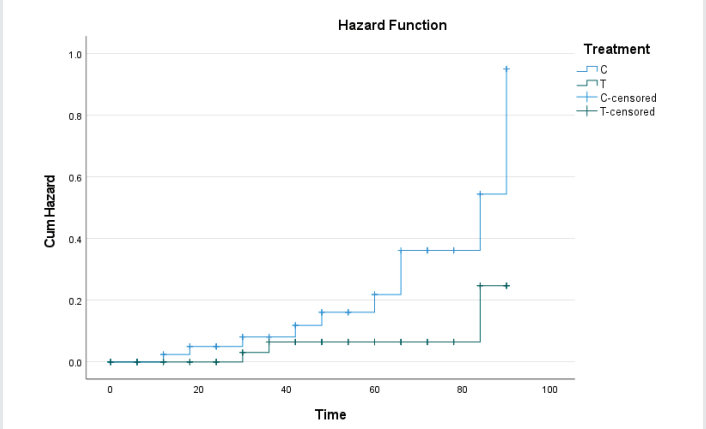

Figure 4: Kapalan Meier hazard function graph of shrimp (*P. monodon*) over a challenge test against *Vibrio parahaemolyticus*. Different horizontal-coloured lines indicate censoring.

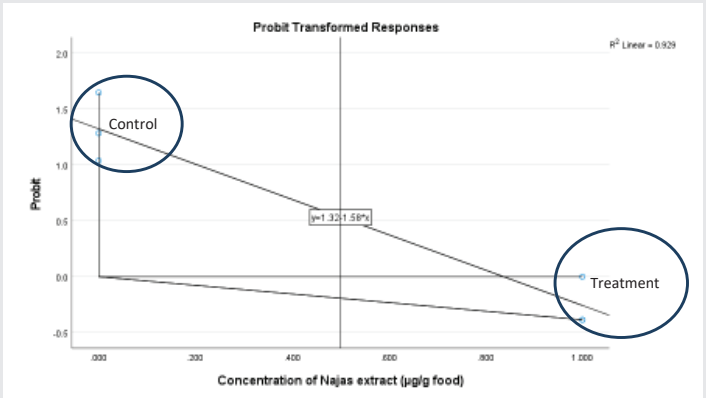

Figure 5: Percentage mortality and probit regression analysis of *P. monodon* juveniles fed with *Najas* extract supplemented diet against WSSV infection.

The probit regression analysis indicated that probit values were higher within the control groups, and 50% of the mortality occurred much quicker in the control group than in the treatment group. Probit analysis indicated that 50% mortality of *P. monodon* juveniles can be happened within the 72 hrs experimental period when the concentration of *N. indica* extract in food is ≤ 0.5 µg/g, which started to reduce the mortality at higher concentrations, which can give a complete protection at the concentration ≥ 1 µg/g.

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Discussion

The bacterial growth inhibition assay revealed that methanolic extracts of *N. indica* inhibited the growth of *Vibrio parahaemolyticus*, which is consistent with earlier results revealing the susceptibility of gram-positive strains to plant extracts of other *Najas* sp and other plant extracts [20,21]. Several plant extracts, including cinnamon, sage, cumin, and other spices, have previously been shown to have significant ($p < 0.05$) antibacterial activity against pathogenic bacteria [22]. Our study revealed that methanolic extract of *N. indica* were efficiently suppressing the growth of *Vibrio parahaemiolyticus* and higher concentration (30 µl) of extract showed higher inhibition zone (20±1 mm). This study supports previous research findings that demonstrate a positive correlation between the concentration of the extract (expressed as a percentage) and the level of antibacterial activity. [23].

In this study, shrimp fed with *Najas* extract improved the immune response of the shrimp specially when it is fed with 1 mg/kg. As shrimp lack immunoglobulin in their bodily fluids, shrimp mostly rely on their non-specific innate immune responses to fend off infections caused by pathogens. All immune parameters like Total Hemocyte Count (THC), Superoxide Dismutase Activity (SOD), Hemocyte clotting time (HCT) was found better in the treatment where 1 mg/kg *Najas* extract was given. Previous studies have demonstrated that the bioactive compounds that are present in some plant extracts have the ability to inhibit the growth of pathogenic bacteria while simultaneously enhancing the resistance of animals to disease [24]. In addition, it was mentioned in a number of distinct areas of research that antinutritional substances of plant origin interfere with biological activities and also display detrimental consequences when administered beyond optimal concentrations [25-27]. This trend was also evident in the current study.

In our study, the 1 mg/kg dose of Najas extract was shown to improve immune parameters, including Total Hemocyte Count (THC), Superoxide Dismutase Activity (SOD), and Hemocyte Clotting Time (HCT). However, a reduction in immune responses was observed at higher doses (3 and 5 mg/kg). This decline in immunity can be attributed to the presence of antinutritional factors in the plant extract, which, when administered in excess, may adversely affect shrimp immune responses. Antinutritional factors such as tannins, alkaloids, and saponins, which are often present in plant extracts, can have inhibitory effects on digestive processes and interfere with nutrient absorption [28]. At higher concentrations, these compounds might not only impede nutritional uptake but could also cause oxidative stress, leading to the suppression of immune functions [29].

In challenge experiment, the shrimp fed with *Najas* extract (1 mg/kg) significantly reduced mortality in WSSV-infected shrimp. Shrimp mortality was calculated using probit analysis and an LC_{50}, where 50% mortality occurred in the control group at a significantly faster rate than in the treatment group. A study by Delgado, et al. [30] demonstrated that garlic extract had a notable effect on the immune response in aquatic animals. Similarly, Curcumin has been shown to enhance the immunity of shrimp by increasing antioxidant enzyme activities and protecting against oxidative stress [31]. Both garlic and turmeric exhibit comparable antibacterial and antiviral activities, similar to those observed in our study with *N. indica*. The probability of cumulative hazard to mortality of shrimp in this study was higher in the control group than the treatment group. There is a lack of information about the biological activities of *Najas* species in the relevant literature. However, Topuzovic, et al. [20] reported some antimicrobial activity of *Najas minor as a* phytomedical investigation. The Polysaccharide content present in the plant extract could kill viruses [32]. The immune-enhancing effects and reduced mortality observed in shrimp fed with Najas indica extract are likely due to the presence of polyphenols, antioxidants, alkaloids, and saponins, which help modulate immune responses, enhance antioxidant activity, and exhibit antimicrobial and antiviral properties [33-35]. These compounds may support immune cell function, reduce oxidative stress, and improve resistance to bacterial and viral infections, similar to findings in other plant-based extracts like garlic and turmeric [34,36]. However, excessive doses of antinutritional factors in higher concentrations may counteract these benefits by impairing nutrient absorption and immune function [25,27].

Conclusion

The investigation conducted on *Najas indica*, a frequently encountered botanical species in the coastal regions of Bangladesh, has unveiled its auspicious therapeutic capabilities. The antimicrobial properties of this particular species had not been previously investigated. However, our study has successfully demonstrated its noteworthy antibacterial and antiviral activities. Additionally, our research revealed that *N. indica* possesses the capacity to augment the immune response of shrimp, a trait of particular importance to local shrimp farmers. The antimicrobial and immune-enhancing effects observed in our study suggest that *N. indica* could be integrated into shrimp feed formulations, offering a natural and cost-effective alternative to synthetic antibiotics and immunostimulants. This could improve the health and survival rates of shrimp, thereby boosting farm productivity. Furthermore, with the growing demand for sustainable and eco-friendly aquaculture practices, Najas indica could provide a valuable addition to bio-based feeds. However, its commercial potential will depend on further investigations to determine the optimal dosage, cost-effectiveness, scalability, and potential long-term benefits or side effects associated with its use in aquaculture systems. Besides, there is a need for further field trials and long-term toxicity testing to fully assess its practical application in aquaculture. Field trials will provide valuable insights into the efficacy and practical feasibility of incorporating *Najas indica* into commercial shrimp farming practices under real-world conditions, considering environmental factors and production scales. Additionally, long-term toxicity testing is essential to assess the long-term safety of Najas indica supplementation, ensuring that there are no adverse effects on shrimp health or on the surrounding

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ecosystem. These future studies are crucial to to validate the commercial viability of the plant and long-term benefits.

Ethical statement

The authors confirmed adherence to ethical guidelines for animal research from the relevant authorities. However, the experimental protocol was granted approval by the Bangladesh Fisheries Research Institute (BFRI), and the study was conducted in accordance with the guidelines outlined in the National Parliament of Bangladesh-approved 'Animal Welfare Act 2019'.

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