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SESBANIA GRANDIFLORA LEAF EXTRACT ENHANCES GROWTH AND NON-SPECIFIC IMMUNE RESPONSES OF SHRIMP (*Penaeus monodon*)

Halima Tus Sadia, Alokesh Kumar Ghosh*, Sheikh Shaon Ahmmed, Joya Biswas, Abul Farah Md. Hasanuzzaman and Ghausiatur Reza Banu

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

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Abstract

This study determined the effects of *Sesbania grandiflora* (SG) leaf extract on the growth, feed utilization efficiency and non-specific immunological responses in black tiger shrimp (*Penaeus monodon*). Three diets were prepared by mixing the methanol extract of SG onto a pellet feed (34% protein) at concentrations of 0% (control), 0.05% (T1) and 0.1% (T2), respectively. Ten shrimps (mean weight 2.1g), were reared in each tank of 80 L, and the weight and immunological reaction of shrimp were received after a feeding trail of 28 days. The dietary extract treated shrimp had better growth performance (WG, SGR), and feed utilization efficiency (FCR, PER) compared to the control group. The treated shrimp exhibited enhanced immunological responses; the total haemocyte count, prophenoloxidase activity, superoxide dismutase activity, and shorter haemolymph clotting time compared to the control shrimp. In conclusion, a lower level of SG (0.05%) was found to be most effective in terms of immune response enhancement; hence, this plant could be a good source for feed additives for enhancing sustainable shrimp production.

Keyword: *Sesbania grandiflora***,** Shrimp, Growth, Immune response, Antibiotics

Introduction

Aquaculture is widely acknowledged as a prominent industry with the capacity to enhance global nutrition and food safety. Projections indicate that by 2030, aquaculture is estimated to contribute to above 60% of the worldwide production of aquatic organisms (Kumar *et al*., 2021; Yu *et al*., 2022). Due to its high value and comparatively short production time, shrimp culture is undergoing rapid expansion among the different aquaculture industries. It contributes to the protein needs of a growing global population and makes a substantial contribution to the economic and social progress of coastal populations in many emerging nations (Asche *et al*., 2021; Yu *et al*., 2022). With the on-going enlargement of shrimp farming, this industry has been plagued by different bacterial and viral infections that cause considerable losses in shrimp supply and global trade and, as a result, hinder the economic and social development of coastal regions (Citarasu *et al*., 2022; Yin *et al*., 2023).To achieve higher levels of production, antibiotics are currently employed frequently to control bacterial infections in shrimp farms. Probiotics and chemical treatment (disinfection) have been the focus of another strategy to control this disease (González- Gómez *et al*., 2022; Orozco-Ochoa *et al*., 2022).

Nevertheless, the widespread utilization of antibiotics has evolved to the appearance of multidrug-resistant bacteria, indicating a remarkably high resistance rate to several antibiotics, including those now employed in aquaculture, but also in the treatment of humans (Narayanan *et al*., 2020; Orozco-Ochoa *et al*., 2022).

Medicinal plants are utilized as immunostimulants for several thousand years, and can be safe and natural alternatives to antibiotics, chemicals and immunoprophylactics (Vijayaram *et al*., 2022; Mariappan*et al*., 2023). Medicinal plant products are easy to prepare, inexpensive, typically have a low risk of causing adverse effects; their bioactive compounds possess antibacterial activity, the ability to increase growth and enhance immune function, are able to promote appetite and have anti-stress capabilities in shrimp (Van Hai, 2015; Ghosh *et al*., 2021; Mariappan*et al*., 2023). *Sesbania grandiflora* (L.) Poir. (Fabaceae family), commonly referred to as "*Agathi*" in India. This plant

*Corresponding author :< alokesh_ku@yahoo.com>

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species is indigenous to Sri Lanka, Malaysia, Myanmar, India, Indonesia, and the Philippines (Munde-Wagh *et al*., 2012; Thissera *et al*., 2020). The utilization of the entire plant of *S. grandiflora* is a common practice in traditional medicinal systems for the management of a variety of conditions including bacterial disease, fever, inflammation, swellings, diarrhoea, dyspepsia, rheumatic ulcers, febrifuge, gastralgia, nasal catarrh, and scabies remediation (Roy *et al*., 2014; Thissera *et al*., 2020). Earlier studies have documented the presence of anxiolytic, anticonvulsive, antiurolithiatic, and antioxidant activities in different components derived from *S. grandiflora* (Parasa *et al*., 2018). Considering the medicinal value, the objective of the research was to explore the latent medicinal properties of the plant in relation to its effects on the growth and immunological response of shrimp following oral administration with feed.

Materials and Methods

Formulation of extract and experimental diet

A large-scale extraction of *Sesbania grandiflora* (SG) leaf was developed using the procedure outlined by Kouakou *et al.* (2019). In brief, the leaves were washed, placed in an oven set to 40°C, and dried before being pulverized into a fine powder by a grinder. About 100 gram of dry powder was extracted in one liter of approximately 100% methanol, and the resulting extract was subsequently strained through muslin cloth and then filtered using filter paper. The solvent was subsequently evaporated using a revolving evaporator. The remnants were stored at -20 °C until they were utilized.. To make the diet, the dried extract was first dissolved in ethanol, and sprinkled onto pellet feed and mixed (Ghosh *et al*., 2023). The shrimp feed was treated with the extract at varying concentrations of 0%, 0.05%, and 0.1%, respectively. The pellets were air-dried and heated in a 40°C oven to ensure they were completely dry. The pellets were then coated with a binding gel (Nutriegel, Sanzyme Biologics) to keep the extract from leaking out when they were put in water (Balasubramanian *et al*., 2008). An equivalent amount of solvent (ethanol), but none of the extract, was used to treat pellets for the diet that served as the control.

Experimental shrimp collection and rearing regime

The shrimp were procured from a farm located in Dumuria, Khulna and subsequently transported to the laboratory while ensuring aeration. The water was mixed with brine (150-200 ppt)) to achieve a salinity of 10 ppt. Water was poured into the experimental tanks (60L) after passing it through a potable UV device (UV Ballast, 6W) to inhibit microorganisms. Prior to commencing the experiment, a total of 10 juvenile shrimp were introduced into each tank, and were allowed a period of ten days to become acclimated to their environment. During the period of acclimatization, an additional 100 shrimp were stored so that they could be used to replace any of the test shrimp that perished. The shrimps were provided with feed twice a day, amounting to 10-5% of their body weight, with the feeding amount adjusted gradually to their body weight. To keep the appropriate water quality standards, waste products were eliminated twice daily prior to feeding by siphoning, while 25% of the tank water was changed regularly with stored UV-treated water. Throughout the entire experiment, a single air stone served as the constant source of aeration for each tank, thereby ensuring that the dissolved oxygen (DO) remained at the appropriate level. Different water quality parameters were measured and recorded daily; these values were maintained within the specified ranges: temperature 29-31°C, salinity 10-11 ppt, DO > 6 mg/L, pH 7.8-8.5, and NH3< 0.1 mg/L (Pholdaeng*et al*., 2010).

Experimental design

Depending on the amount of extract in their diet, shrimp were placed into three experimental categories (one control, two treatments), each of which was kept in a 80 L plastic tank with 60 L of water. There were 10 juvenile shrimp (2.1 g) in each tank, and the experiment was done in triplicate. The shrimps were provided with a diet that consisted of 0%, 0.05%, and 0.1% of *S. grandiflora* extract for the control (C), T1 (treatment 1), and T2 (treatment 2) groups, respectively. Growth performance data were gathered after the trial, and immune response parameters including THC, HCT, proPO, and SOD activity were assessed.

Growth performance indices

The shrimp from each tank were gathered, counted, and weighed. The following indices of shrimp growth and feed utilization were evaluated (Abdel-Tawwab*et al*., 2022):
Average weight gain (AWG, g) = Wt – W0;
Individual Growth rate (GR, g/day): (Wt – W0)/28;

Specific growth rate (SGR; %/day) = 100 [(LnWt – LnW0)/28];
Where, Wt denotes the final weight (g) and W0 is the initial weight (g);
Feed conversion ratio (FCR) = feed intake (g)/weight gain (g);
Protein efficiency ratio (PER) = weight gain (g)/protein intake (g);
Shrimp survival (%) = 100 (final shrimp count/beginning shrimp count).

Total haemocyte count (THC) determination

100 µL of hemolymph was extracted from the ventral sinus situated at the bottom of the first abdominal portion of shrimp. This was accomplished utilizing a 26-gauge needle that was affixed to a 1 mL pipette containing an anticoagulant (Le Moullac*et al*., 1997). The haemolymph was diluted at the ratio of 2:1 (200 µL anticoagulant: 100 µL haemolymph) so that the THC could be counted instantly. A single droplet of Rose Bengal staining reagent was introduced into a 20 µL mixture of the sample. to make the haemocytes easier to see, so they could be counted quickly and accurately. After that, the total amount of haemocytes was calculated by placing a drop of the mixed solution inside a haemocytometer (Precicolor HBG, Germany), capping it off with a cover slip, and analysing it under a microscope (Labomed, USA). The THC and the dilution factor (Dcf) were computed utilizing the subsequent formulas (Japitana *et al*., 2017).
THC= [(A+B+C+D)/4] x 10^4 x Dcf (cells/mL)
A B C and D indicates block of haemocytometer
Dcf= (volume of anticoagulant + volume of haemolymph)/volume of haemolymph

Haemolymph clotting time (HCT) determination

The HCT was measured according to Liu *et al.* (2019). A volume of 100 µL of haemolymph was taken from shrimp as described in the upper section, and placed in a chilled Eppendorf tube (on ice); a 25 µL sample was extracted and placed to a pre-chilled glass capillary tube.. Once this was done, the tube was set vertically so that gravity would pull the haemolymph down from its apex. When the haemolymph approached the bottom end of the tube, it was inverted, and the process was continued till the haemolymph coagulated, which resulted in the determination of the clotting time.

Prophenoloxidase (proPO) and Superoxide dismutase (SOD) activity assay

The proPO activity in the haemolymph of shrimp was evaluated employing spectrophotometry (Peak instruments, C-7200, USA) by recording the synthesis of dopachrome from L- dihydrophenylalanine (L-DOPA). This was done following the procedure outlined by Le Moullac et al (1997). The SOD was determined using the protocol published by Creative BioMart, Inc., USA, as modified by Marklund and Marklund (1974) and Jing and Zhao (1995). In accordance with the directions provided by the manufacturer, two solutions, a TRIS-EDTA buffer (solution A) and a 0.2 mM pyrogallol solution (solution B) were prepared.

Statistical analysis

The data was examined by the SPSS program (Version 16) by the application of one-way ANOVA. The significant difference was examined using the multiple comparisons (Tukey's) test. Prior to analysis, the data underwent a Kolmogorov-Smirnov and Shapiro-Wilk test in order to determine whether or not they followed a normal distribution. When $p < 0.05$, differences were measured to be significant.

Results

Impacts of leaf extract on growth and feed utilization parameters

Following a 28-day trial period, several growth parameters of shrimp were estimated and are presented in Table 1. The inclusion of *S. grandiflora* extract to the shrimp's diet had a significant impact on the shrimp's growth and ability to utilize feed at both doses. The treatment 1 (0.05%) exhibited the highest weight gain, SGR, and PER, while the control group had the lowest values for these parameters (Table 1). Furthermore, it was observed that the treatment 1 and treatment 2 had a reduced FCR ($p < 0.05$), whereas the control group demonstrated the highest value. Regarding extraction-concentration effect, there was no statistically significant difference ($p > 0.05$) observed in growth and feeding efficiency parameters between the two treatment groups. Nevertheless, no significant variation ($p > 0.05$) was perceived in the survival rate between the control group and the treatment groups of shrimp (Table 1).

Table 1. Effect of *S. grandiflora* leaf extract on the growth and feed utilization of the experimental groups of *P. monodon*

Parameters	Control	Treatment 1 (T1)	Treatment 2 (T2)
FW) (g)	4.3±0.8 [a]	5.2±1.2[b]	5±0.9[b]
WG) (g)	2.2±0.17 [a]	3.1±0.19 [b]	2.9±0.08 [b]
SGR) (%)	2.58±0.14 [a]	3.26±0.13 [b]	3.15±0.06 [b]
FCR	3.2±0.52 [a]	1.9±0.12 [b]	2.1±0.1 [b]
PER	1.01±0.16 [a]	1.67±0.10 [b]	1.50±0.07 [b]
Survival %	85±7.1 [a]	100±0 [a]	95±7.1 [a]

Effect of S. grandiflora extract on total haemocyte count (THC)

After the culture period, the THC level of shrimp was calculated and displayed in Figure 1. The application of *S. grandiflora* extract had a substantial impact on the THC levels of shrimp in comparison to the control group (Figure 1). The haemocyte count in the treatment 1 (0.05%) ($23.0\pm0.37\times10^5$ cell/mL) was significantly greater in comparison to both the control ($6.0\pm0.19\times10^5$ cell/mL) and the treatment 2 (0.1%) ($19.7\pm0.27\times10^5$ cell/mL) (Figure 1). The control group of shrimp, which did not receive any extract in their diet, exhibited the lowest THC levels ($p > 0.05$).

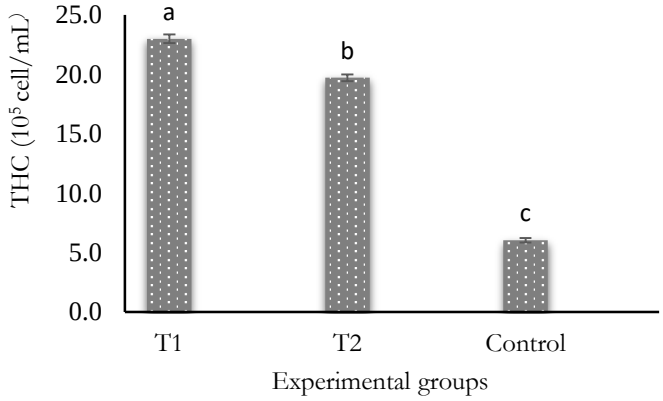

Figure 1. Changes in Total Haemocyte count of shrimp at different doses of *S. grandiflora* extract. The presence of distinct superscripts in the bar denotes statistically significant variations among the groups (5% level of significance).

Effect of leaf extract on haemolymph clotting time (HCT)

The HCT was measured and depicted in Figure 2. The incorporation of dietary extract demonstrated significant impacts on the HCT levels of shrimp (Figure 2). The results indicated that the control group exhibited significantly greater clotting time in comparison to the treatment groups. The treatment 1 had the shortest clotting time than the control and treatment 2 ($p < 0.05$).

Prophenoloxidase (proPO) activity of shrimp

Figure 3 displays the proPO activity of shrimp across several experimental groups. There was a significant difference of proPO activity in shrimp among the groups (Figure 3). The treatment 1 (T1) had significantly greater ($p < 0.05$) levels of proPO activity than the control and treatment 2 while the control possessed the lowest level ($p < 0.05$) of proPO activity.

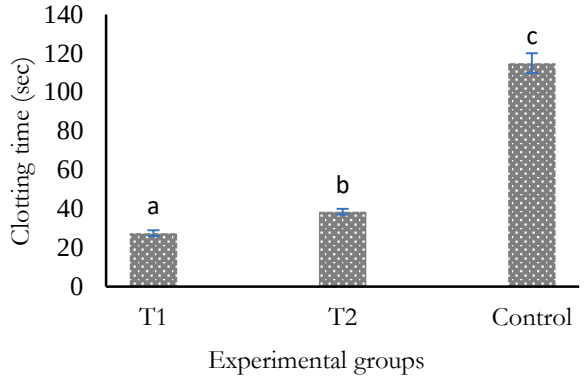

Figure 2. Haemolymph Clotting Time changes of shrimp at different doses of *S. grandiflora* extract. The presence of distinct superscripts within the bar signifies significant distinctions among the groupings (5% level of significance).

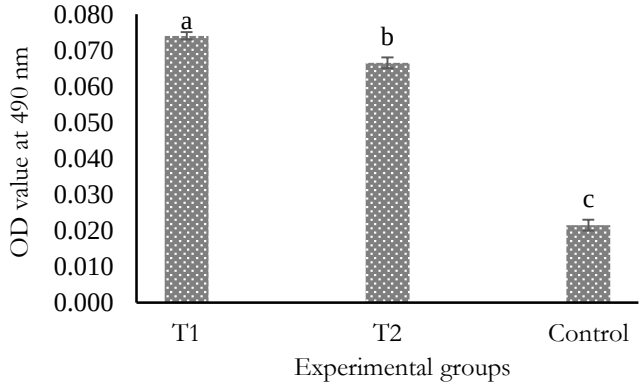

Figure 3. proPO activity of shrimp at different doses of *S. grandiflora* extract. The various superscripts in the bar denote major distinctions among the groups (5% level of significance).

Superoxide dismutase (SOD) activity of shrimp

The extract-treated shrimp demonstrated significant effects of SOD than the control group (Figure 4). The highest SOD value ($p < 0.05$) was perceived in the treatment 1 (T1), while the lowest SOD value was exhibited in the control group. Nevertheless, no significant change was demonstrated between the extract-treatments.

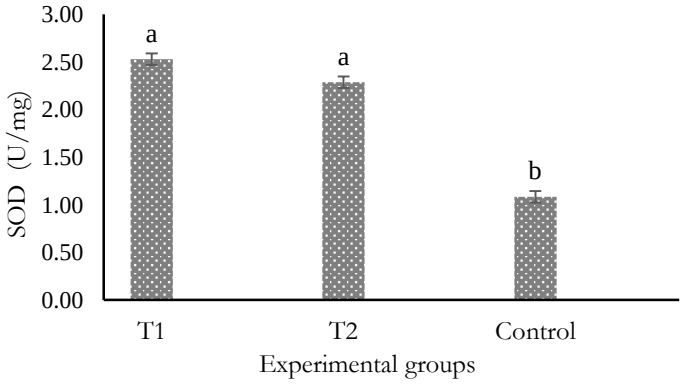

Figure 4. Effect of extract on SOD activity of shrimp. The presence of several superscripts in the bar signifies significant variations among the groups (5% level of significance)

Discussion

The proliferation of shrimp aquaculture has resulted in the introduction of diseases, which have had significant negative impacts on shrimp production and international trade. Consequently, these challenges have impeded the economic and social progress of coastal regions (Citarasu *et al*., 2022; Yin *et al*., 2023). Hence, it is imperative to ascertain the significance of medicinal plants in the context of aquaculture and to formulate alternative strategies for enhancing growth and mitigating diseases. The findings of the study indicated that the incorporation of *S. grandiflora* extract into the diet showed a significant beneficial effect on the growth and feed utilization of *P. monodon*, which was evident in relation to increased weight gain, SGR, PER, and reduced FCR, than the control group. The results of our study are consistent with previous observations made in shrimp that were provided with feed containing plant extracts (Dewi *et al*., 2021; Yin *et al*., 2023; Ghosh *et al*., 2023). For instance, Yin *et al.* (2023) demonstrated that the growth rate and feed utilization of shrimp increased significantly when they were provided with a diet comprising *Andrographis paniculata*. Additionally, Ghosh *et al.* (2023) found that the growth and feeding efficiency of shrimp increased when shrimp were served a mixed diet containing extracts of *Zingiber officinale* or *Aegle marmelos*. The increase in growth could be attributable to bioactive substances in the extract stimulating digestive enzymes, essential for feed digestion or nutrient assimilation, by which feed utilization and growth is promoted (Kaleo *et al*., 2019; Yin *et al*., 2023).

Haemocytes serve as the primary components of shrimp hemolymph, potentially offering insights into the physiological condition and immune system because the quantity of haemocytes in shrimp can be significantly reduced due to disease and prolonged stress (Liu and Chen, 2004; Yin *et al*., 2023).Accordingly, the counting of haemocytes and the assessment of haemolymph clotting time serve as reliable indicators of crustacean well- being, which can be influenced by a multitude of factors (Fotedar *et al*., 2006). In this study, the THC levels of shrimp given an extract-included feed were significantly greater than the THC of the control group. Similar observations (THC level increases) were also reported in shrimp fed diets containing extracts of *Andrographis paniculata* (Yin *et al*., 2023), *Moringa oleifera* (Abidin *et al*., 2022), *Psidium guajava* (Dewi *et al*., 2021), *Zingiber officinale* or *Aegle marmelos* (Ghosh *et al*., 2023).The observed increase in total haemocyte count (THC) in the treated shrimp can be attributed to the extract's potential to stimulate the proliferation of younger and more efficient haemocytes, while inducing death in older and less effective haemocytes inside the shrimp's haematopoietic tissues (Chen *et al*., 2014; Salehpour *et al*., 2021).

An elevation in hemolymph clotting time (HCT) may serve as an indicative measure of stress in shrimp, specifically when they are afflicted with infections (Sarathi *et al*., 2007; Bautista-Covarrubias *et al*., 2022). The current study found that the HCT levels in shrimp treated with the extract were significantly reduced relative to the control group which is similar to the findings of Xie *et al.* (2018) and Ghosh *et al*., (2023), who observed that shrimp given *Forsythia suspense* and *Zingiber officinale* extracts had shorter clotting times compared with higher levels of THC in the control shrimp. The extract-treated diet contributed in the clotting time reduction since it contained immunostimulants which elevated THC, and thus THC might affect clotting time via their TGase as reported by Raja Rajeswari*et al.* (2012). In addition, it has been found that shrimp treated with various extracts have a lower clotting time because they have a higher concentration of a clotting protein (Dewi *et al*., 2021; Abidin *et al*., 2021).

The ProPO system is a crucial component of the humoral immune system in shrimp and crustaceans, serving as an essential defense mechanism (Yildirim-Aksoy *et al*., 2022; Kewcharoen *et al*., 2022). Activation of ProPO results in the generation of PO, which subsequently facilitates the oxidation of tyrosine, leading to the production of reactive quinones and other temporary intermediates that ultimately culminate in the synthesis of melanin from phenol., which adheres to the surface membrane of bacteria and encourages the adhesion of hemoglobin-producing cells to microbes (Munaeni *et al*., 2020; Yildirim-Aksoy *et al*., 2022). The present research observed a rise in proPO activity treated with the extract, relative to shrimp in the control group. This outcome is in line with prior findings that, shrimp proPO activity was boosted by consuming a diet containing specific plant extracts such as *Syzygium cumini* (Prabu *et al*., 2018), *Psidium guajava* (Dewi *et al*., 2021), *Zingiber officinale* (Ghosh *et al*., 2023).The extract's immunostimulant components potentially engaged with the pattern recognition proteins (PRPs) in the shrimp immunity, thereby initiating the proPO activation system (Palanikumar *et al*., 2018).

The SOD is an antioxidant that exhibits a specific ability to scavenge the superoxide anion. Consequently, it enhances the tissue's defensive system by promoting phagocytosis and oxidation (Prabu *et al*., 2018). The current research demonstrated that the implementation of our interventions led to a notable increase in SOD than the control group which coincides with previous research findings in the field. For instance, SOD levels increased in shrimp treated by extracts of *Psidium guajava* (Dewi *et al*., 2021), *Astragalus membranaceus* (Pu *et al*., 2022), *Andrographis*

paniculata (Yin *et al*., 2023), and *Zingiber officinale* (Ghosh *et al*., 2023), which can protect shrimp against oxidative stress. The extract utilized in the shrimp may potentially consist of compounds of antioxidant characteristics that have the ability to induce the formation or enhance the SOD, hence contributing to the body's defense mechanism against oxidative stress.

Conclusion

In conclusion, this study revealed that administration of a *S. grandiflora* extract can promote the growth performance, feed utilization efficiency and immunological response, of shrimp (*P. monodon*). The supplementation of feed with the extract at a concentration of 0.05% resulted in the most beneficial effect of shrimp's growth performance and innate immune responses. Therefore, *S. grandiflora* might potentially act as a nutritional supplement to boost the growth indices and immune functions of shrimp. This would have advantages for promoting sustainable shrimp farming and could potentially replace the need for antibiotics.

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Conflict of Interest

The authors assert that they do not possess any conflict of interest.

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