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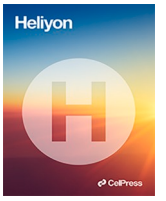

Research article

Dietary impact of *Ocimum tenuiflorum* leaf extract on the growth metrics and immune responses of shrimp (*Penaeus monodon*) against white spot syndrome virus (WSSV)

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ABSTRACT

The present study was conducted to evaluate the efficacy of *Ocimum tenuiflorum* extract against the white spot syndrome virus (WSSV) in black tiger shrimp (*Penaeus monodon*) following oral administration *in vivo*. The methanol extract derived from the extraction was sprayed into feed at a concentration of 0.0 %, 0.05 % and 0.1 %. The feeding trial was continued for four weeks, following which the shrimps were injected with WSSV and mortality was checked two weeks after the trial. The findings revealed that shrimp fed with extract had significantly improved ($p < 0.05$) growth parameters (weight gain, specific growth rate) and feed utilization efficacy (less feed conversion ratio, high protein efficiency ratio) compared to the control group. The extract improved the immunity of shrimp significantly, as demonstrated by increased immunological parameters including total haemocyte count, prophenoloxidase activity, superoxide dismutase activity and up regulation of immune-related genes, which ultimately increased the disease resistance capability in *Penaeus monodon* against WSSV. Based on the experiment, 0.1 % extract had the highest growth and immune response against WSSV followed by Treatment 1 and control. Therefore, the extract of *Ocimum tenuiflorum* could be used as an immunostimulant with feed to boost the growth and resistance capacity of shrimp against WSSV as well as for the sustainable production of shrimp.

1. Introduction

Shrimp aquaculture has undergone tremendous global expansion during the last two decades. The expansion of shrimp aquaculture is propelled by the rising global demand resulting from population and economic development, while the availability of wild shrimp remains stagnant [1]. Shrimp constitutes over 16.4 % of the worldwide traded fish and fisheries products with respect of value, positioning it as the second most important commercial food item after tuna. *Penaeus monodon*, referred as black tiger shrimp, is a highly precious species in the trading industry and its production exceeded 717.1 thousand tons, accounting for 6.4 % of the entire production of crustaceans [2]. Besides export earnings, shrimp farming generate employment opportunities, food security and earnings for the rural populations in coastal regions which lack the other sources of livelihood possibilities [3,4]. However, the quick increase of intensive shrimp farming system results in an occurrence and blowout of viral and bacterial diseases that pose the greatest significant hazard to the shrimp farming sector throughout the world [5,6]. White spot disease (WSD) is the most prevalent and

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infectious disease for global shrimp production caused by white spot syndrome virus (WSSV) resulting in significant economic loss and host death in a limited time period (3–10 days) after showing clinical signs [6,7]. WSSV is the sole known representative of the genus *Whispovirus* and Nimaviridae family, is a double-stranded, rod-shaped and circular DNA virus [8]. Due to having a wide range of existing host and frequent replication, this virus seems difficult to be prevented and spread in the farming system [9,10]. The worldwide economic losses caused by WSD diseases have surpassed $3 billion annually, significantly affecting a global sector valued at US$19 billion per year [11]. The first action used to avert the spread of WSSV in farms is the implementation of biosecurity protocols, which involve rigorous quarantine procedures, equipment disinfecting, and the introduction of shrimp stock that is free from the disease [12]. To enhance the shrimp production, the application of antibiotics, veterinary drugs and disinfectant are on the rise [13,14] in which the misuse of these compounds has led to shrimp resistance and heightened environmental contamination, potentially jeopardizing the safety of humans and the sustainable development of shrimp culture system [15–17] (see Fig. 3).

For these reasons, there is an immediate need to create novel therapies for both the avoidance and management of WSSV infection. Currently, there are no authorized antiviral medications available for the prophylaxis of viral outbreaks in shrimp farming, thus it is very necessary to develop antiviral agents that can control the WSSV infection in shrimp farming [10]. Because of having several advantages such as, natural safety, normally available, non-toxicity and plenty of bioactive compounds, herbal remedy could be the important solution for eco-friendly and pollution free agents along with other benefits including growth promotion, disease resistance, appetite stimulant and immunostimulation in shrimp [18,19]. Research showed that extracts from several plants such as, *Cynodon dactylon* [20,21], *Gracilaria corticata* [22], *Gynura bicolor* [23], *Ceriops tagal* [24], *Zingiber officinale* [25] showed significant activity against the infection of WSSV in shrimp.

Ocimum tenuiflorum, is widely recognized as the “Icon of herbs” due to its extensive medical properties and significant historical significance. Due to its spiritual significance on the Indian subcontinent, this plant is popularly known as “Holy basil,” “tulasi,” or “tulsi.” The plant is a member of the family Lamiaceae and is a fragrant perennial. It is often found in many regions of Southeast Asia [26]. *Ocimum tenuiflorum* is utilized to treat a variety of remedial ailments such as the cold, headaches, gastrointestinal complications, inflammatory conditions, cardiovascular disorders, poisoning, and malaria as well as to mitigate psycho-physical distress, conjunctivitis, and asthma because of its curative medicinal characteristics [27,28]. *Ocimum tenuiflorum* reduces free radicals, safeguards against oxidative damage, and maintains the equilibrium of antioxidant enzymes. The constituents of *O. tenuiflorum* leaf extract consist of oleanolic acid, ursolic acid, eugenol, linalool, and other compounds [29]. *Ocimum tenuiflorum* has been shown to have potential in inhibiting the transmission of lethal viruses including HSV, IHNV, Adenovirus, Enterovirus 71, H9N2, hepatitis B, Hepatitis C virus, and SARS-CoV-2 among others [30]. Previously, it has been found that combining *O. tenuiflorum* plants with other plants can decrease the mortality rate of shrimp when subjected to WSSV [31]. Although *O. tenuiflorum* exhibits potent antiviral properties, its efficacy against the WSSV in shrimp has not been evaluated by oral administration. Therefore, this study was undertaken to examine the impact of *O. tenuiflorum* on shrimp growth, immunological response, and disease resistance against WSSV.

2. Materials and methods

2.1. Preparation of O. tenuiflorum extract through large scale extraction

The fresh leaf of *O. tenuiflorum* was collected from Khulna, Bangladesh and was taxonomically confirmed by the Bangladesh National Herbarium (BNH), Mirpur, Dhaka where the samples were deposited in the library of BNH (DACB 51327). The leaves were washed, evaporated in the oven at 40 °C and pulverized using a mechanical blender to form powdery. Approximately, 150 g of dried powder was mixed with 1.5 L of ~100 % methanol (as methanol was the most potent solvent for extracts against WSSV, that was reviewed by Ref. [32]) in a 2.5 L glass bottle, sonicated (30 min) four times at 6 h interval and passed through muslin cloth following filter paper. The chemical solvent was subsequently evaporated using a rotating vacuum vaporizer (Hahnvapor, Hahnshin, Korea) and the whole process was repeated until the yield became insignificant. The yield of the extract was 7 %. The residues were stored at −20 °C until used.

2.2. Preparation of diet

The necessary amount of extract from the large scale extraction of *O. tenuiflorum* was dissolved in ethanol and comprehensively applied to the pellet feed which comprise protein (32 %), crude fat (7 %) and moisture (12 %) (AOAC method) with a sprayer and was homogenized [33]. Two tests diets were formulated according to the extract dosage such as 0.05 % (Treatment 1, OTT1) and 0.1 % (Treatment 2, OTT2) with the shrimp feed. Subsequent to the application of the spray on the feed, the moist pellet was initially air-dried and subsequently subjected to drying in an oven at 40 °C. The preparation of the control feed consisted of spraying an identical quantity of the ethanol without adding extract. The stability of feed in water was assessed following the methodology outlined by Mohamad et al. [34]. Approximately 2 g of pellet were soaked for a particular period of time in a flask (250 mL), comprising 100 mL of water. Following the specified leaking duration, the submerged pellet was strained using filter paper. The retrieved feed sample was dehydrated in the oven at 100 °C for 24 h. The solid that was obtained was examined to determine its pellet stability in relation to the retention of dry material, by applying the formula, [Feed remaining (g)/initial feed (g)] × 100]. The binding gel was applied to the diet to limit the amount of extract that was lost in the water [33].

2.3. Shrimp collection and husbandry management

Healthy, about similarly sized (2.1 g) and disease-free shrimps were procured from a shrimp farm and transported to the research facility with aeration. The shrimps were assessed for exterior indicators of WSSV, and were validated using PCR analysis. Salinity was maintained at 10 ppt by adding the fresh water with collected brine (salinity = 150–200 ppt). Water was subjected to a potable UV treatment prior to being transferred into the research tanks (60L). To keep the required water temperature, a mini submersible water heater was installed in each tank. Before conducting the research, 10 juvenile shrimp were stocked in every tank and acclimatized for ten days. During the acclimatization period, extra shrimps were reserved to substitute any deceased shrimp in the trial tanks. The shrimps were fed at 5 % body weight of shrimp twice a day and the amount of feed was checked regularly after feeding. Waste was removed twice per day before feeding via siphoning. To ensure that the water quality parameters were maintained at their best, UV-treated water was kept and used to replenish 25 % of the water in the tank on a regular basis. An individual air-stone ensured continual oxygenation in each tank to sustain adequate DO levels during the experiment. Daily measurements and recordings were conducted across multiple water quality measures, with the subsequent values upheld: Temperature: 28–30 °C, pH: 8–8.5, Salinity: 10–12 ppt, Dissolved Oxygen: >6 mg/L, Ammonia: <0.1 mg/L [35].

2.4. Formulation of WSSV samples and challenge protocol

The WSSV cultures were created using the protocols established by Tsai et al. [36] and Kang et al. [37]. In brief, 0.5 g of infected *P. monodon* gills were crushed and homogenized with 4.5 mL of PBS. After that, the sample was centrifuged at 400×*g* for 10 min at 4 °C. The resultant liquid was obtained, filtered by a 0.45-μm pore filter, and subsequently diluted tenfold to infest the test shrimp at a dosage of 5 μL/g of shrimp weight. The injection had been given in the dorsal lateral section of the 4th abdominal section of the shrimp.

2.5. Assessment of antiviral activity with oral dietary passage

As soon as the shrimp had been acclimated, they were separated into three different experimental groups, each with ten juveniles of shrimp with three replications. Group I was administered the control diet, which contained no extract throughout the end of experiment which was regarded as control group (C). Shrimps in group II (treatment 1, T1) and group III (treatment 2, T2), shrimp were fed extract added diet at the rates of 0.05 % and 0.1 % accordingly for 4 weeks. Subsequent to the feeding period, each group of shrimps was challenged with WSSV through injecting and monitored for two weeks after infection. The same feed that was used before the challenge was also used after the challenge and continued until the completion of the study.

2.6. Growth metrics and feed utilization

Prior to the shrimps being subjected to a challenge, data on growth performance was gathered and several variables were determined, including weight gain (WG), and specific growth rate (SGR). The utilization of feed was assessed utilizing metrics including feed conversion ratio (FCR) and protein efficiency ratio (PER) [38]. The formulae were as follows:

$$WG = Wt - W0$$

$$SGR = ((\ln(Wt) - \ln(W0) / t \times 100)$$

$$FCR = TFG / TWG$$

$$PER = WG\ (g) / \text{protein intake}\ (g)$$

where TFG denotes Total feed given and TWG denotes Total weight gained of shrimp

$$\text{Survival}\ (\%) = (Nt / N0) \times 100\%$$

Where Nt represents the ultimate quantity of shrimp and N0 denotes the beginning quantity of shrimp.

2.7. Hematological and immunological analysis

Haematological parameters, including the total haemocyte count (THC), haemolymph clotting time (HCT), and immunological assays such as prophenoloxidase activity (proPO), superoxide dismutase activity (SOD) were assessed both prior to and following the challenge.

2.7.1. Haemolymph collection and total haemocyte count (THC) determination

Haemolymph (100 μL) was collected from the shrimp's abdominal portion employing a 26-gauge needle within a 1 mL syringe having anticoagulant [39]. To count THC immediately, haemolymph was collected and mixed in a tube at a 2:1 ratio of anticoagulant and haemolymph. Twenty μL of collected mixed haemolymph was combined with one drop of solution (Rose Bengal stain) to enhance the visibility of the hemoglobin for counting. A droplet of the amalgamated sample was positioned on a haemocytometer (Precicolor

HBG, Germany), encased in glass and positioned beneath a microscope (Labomed, USA) to conduct THC. The THC and dilution adjustment factor were determined employing the specified formulas, with THC expressed as cell/mL [40].

$$THC = ((A + B + C + D) / 4) \times 10^4 \times Dcf$$

A B C D = Block of haemocytometer

$$Dcf = (\text{anticoagulant volume} + \text{hemolymph volume}) / \text{hemolymph volume}$$

2.7.2. Determination of haemolymph clotting time (HCT)

The HCT was assessed by Liu et al. [41]. One hundred μL of shrimp haemolymph was collected and put in a cold Eppendorf tube prior to putting a 25 μL sample into a pre-chilled glass capillary tube which was positioned vertically so that gravity forces could cause haemolymph to flow from the top and bottom ends. When the haemolymph touched the bottom end, the tube was inverted once again, and the procedure was reiterated till the haemolymph coagulated.

2.7.3. Prophenoloxidase (proPO) and superoxide dismutase (SOD) activity determination

The activity of prophenoloxidase (proPO) in haemolymph was determined by a spectrophotometer (Peak instruments, C-7200, USA) and the method of Le Moullac et al. [39] by keeping the establishment of dopachrome from L-dihydrophenylalanine (L-DOPA). Zymosan (0.1 %) was synthesized in a cacodylate buffer and subsequently subjected to centrifugation at 2000*g* for a duration of 10 min. The resultant supernatant was utilized as an agent for determining the proPO. Haemolymph taken was centrifuged for 10 min at 1000 *g* at 50 °C. After the incubation period, the mixture was subjected to centrifugation for 5 min at a speed of 700 *g*. Then, 180 μL of the resulting liquid were moved into an Eppendorf tube and a 75 μl portion of L-DOPA was introduced. After 10 min, an additional volume of 900 μL of cacodylate buffer was added, and the OD was measured at 490 nm using a spectrophotometer.

The SOD of shrimp muscle was determined using the protocol of Creative BioMart, Inc., USA (EC 1.15.1.1), adapted from Marklund and Marklund [42] and Jing and Zhao [43]. The test evaluates the relationship between pyrogallol autoxidation by O_2^- and radical transformation by SOD, with 50 % autoxidation inhibition of pyrogallol referred to as one unit of SOD activity. The rate of oxidation for the samples was ascertained by introducing 20 μL of the test solution into 2.35 mL of solution A, accompanied by 1.80 mL of purified water, and subsequently incorporating 0.15 mL of solution B. The absorbance at 325 nm was assessed utilizing a spectrophotometer (C-7200, USA) to evaluate the rate of pyrogallol autoxidation by analyzing the variation in absorbance between the blank and the examined sample after a duration of 1 min. The SOD activity was calculated using the formula.

2.8. WSSV detection by PCR

The PCR analysis that was performed after the challenge experiment revealed the presence of WSSV infection in shrimp. The DNA was isolated from the gills and pleopods of shrimp, which were combined, employing the DNA Purification Kit (Monarch® Genomic, Cat no: T3010S, USA) in accordance with the procedure provided by the manufacturer. The concentration and purity of DNA were measured through determining the A260/280 nm ratio using a Nanodrop (Nabi, Korea). The first and second rounds of PCR (Nested) were conducted on every single DNA extract with the OIE-recommended WSSV primer sets [44]. The total volume of the mixture was 25 μL (HotStarTaq® Master Mix Kit, Germany) comprising of primers (0.5 μL, forward and reverse), RNase/DNase-free water (9.5 μL), DNA template (2 μL). A Thermal Cycler (Bio-Rad T100 PCR) was used and the conditions were as to 94 °C (3 min), 94 °C (20 s, 40 cycles), 62 °C (20 s), and 72 °C (30 s), 72 °C (3 min) and maintained at 4 °C. Positive samples are expected to produce a 1447 bp output in the first PCR cycle and a 941 bp output in the nested PCR [45]. Subsequently, the products were segregated in 2 % agarose gels, colored with gel loading dye, and observed by a UV illuminator.

2.9. Immune genes expression by quantitative PCR (qPCR)

The expression of several immune genes was assessed using qPCR. The hepatopancreas of shrimp was utilized to extract total RNA utilizing Trizol in accordance with the manufacturer's instructions. Subsequently, first-strand cDNA was generated utilizing the cDNA Synthesis Kit (ProtoScript® II, Cat No: E6560S, USA) in accordance with the manufacturer's guidelines and preserved at −80 °C for future real-time RT-PCR. Quantitative PCR was conducted with the HYRIS bCUBE™ (UK), including a reaction mixture made up of 2x Luna® Universal qPCR Master Mix, forward and reverse primers, 6.5 μL of water (deionized), and 2 μL template (cDNA), culminating of 20 μL. As a housekeeping gene, 16S rRNA was used [46] and the relative gene expression was determined using the equation below [47].

$$\text{Relative expression of candidate genes} = 2^{-[\Delta Ct \text{ sample} - \Delta Ct \text{ control}]}$$

2.10. Statistical analysis

The data were examined employing one-way ANOVA in SPSS and displayed as mean ± SD (Version 16). A multiple comparisons (Tukey's) test was implemented to ascertain whether there were significant differences among experimental groups. Before analysis, the percentage of survival data was normalized in Excel using an arcsine-transformation. The mean values pre- and post-challenge were evaluated using the Student's t-test. A p-value of less than 0.05 was utilized to ascertain the significance of the difference.

3. Results

3.1. Effect of O. tenuiflorum extract on growth performance and feed utilization of shrimp

The effect of *O. tenuiflorum* extract on the growth, feed utilization of shrimp after four weeks of rearing (prior to challenge) is displayed in Table 1. A significant difference ($p < 0.05$) was found in growth and feed utilization of shrimp among the experimental groups. The weight gain and SGR of the extract-treated shrimps were significantly greater ($p < 0.05$) than the shrimp of the control group. No significant difference ($p > 0.05$) was seen in the initial weight of shrimp among the groups; however, after 4 weeks of rearing, shrimp in treatment 2 (OTT2) exhibited the highest average weight gain (3.0 g) and SGR (3.18 %), while control had the lowest. Similarly, shrimp in T2 had the reduced FCR (2.3) and highest PER (1.45) indicating better feed utilization whereas control shrimp possessed the maximum FCR (3.2) and reduced PER (1.01). The survival of stocked shrimps was 85–100 %, which was not statistically significant ($p > 0.05$) prior to shrimp challenge.

3.2. Effect of O. tenuiflorum extract on total haemocyte count (THC) of shrimp

The dietary extract of *O. tenuiflorum* had a significant impact on THC ($p < 0.05$) in both the conditions (pre and post challenge) of shrimp with WSSV (Fig. 1). The THC levels in shrimp that were served the control feed were significantly less ($p < 0.05$) compared to the THC levels in shrimp in both treatments. Shrimp in T2 (0.1 % extract) possessed the maximum concentration of THC ($p < 0.05$), followed by T1 (0.05 %) and control (0 %). THC levels decreased after shrimp challenge in entirely groups, which was statistically significant ($p < 0.05$) compare to prior challenge (see Fig. 2).

3.3. Impact of O. tenuiflorum extract on haemolymph clotting time (HCT) of shrimp

The HCT of *P. monodon* was significantly varied ($p < 0.05$) by the dietary extract of *O. tenuiflorum* in both situations (pre- and post-challenge). The control shrimp had the longest clotting time (115 s in before challenge and 167 s in after challenge) ($p < 0.05$), while the shrimp nourished with a diet supplemented with 0.1 % extract (T2) exhibited the shortest (41 and 57 s in pre- and post-challenge respectively) clotting time. In comparison to pre-challenged, the HCT was significantly more ($p < 0.05$) in post-challenged shrimp. HCT decreased with increasing the concentration (in T1 and T2) of extract and was always lower than the control group.

3.4. Influence of O. tenuiflorum extract on prophenoloxidase (proPO) activity of shrimp

Dietary extract of *O. tenuiflorum* had a significant impact ($p < 0.05$) on shrimp proPO under both conditions (before and after the challenge with WSSV). Shrimp in T2 (0.01 % extract) had the uppermost level of proPO ($p < 0.05$) followed by T1, whereas the control group exhibited the reduced level of ProPO. The values were greater in post-challenged shrimps compared to pre-challenge shrimp in treatments, and it showed the significant difference ($p < 0.05$) except control group.

3.5. Influence of O. tenuiflorum extract on superoxide dismutase (SOD) activity

Dietary extract of *O. tenuiflorum* had the significant effect on SOD ($p < 0.05$) between treatments and control in the before and after challenge with WSSV in shrimp. Treatment T2 (0.1 % extract) exhibited the highest amount of SOD ($p < 0.05$), whereas the control shrimp showed the lowest level of SOD in both conditions. The SOD value displayed a positive correlation with the content of extract in the diet. The SOD value increased after the challenge with WSSV compared to pre challenge group, excluding the control group, displayed a statistically significant difference ($p < 0.05$) (see Fig. 4).

3.6. Impact of O. tenuiflorum extract on relative expression of immune related genes

The relative expression of various immune genes (Lysozyme, penaeidin, proPO) was analyzed, the results of which are presented in Fig. 5. The dietary extract of *O. tenuiflorum* exerted a significant influence on the shrimp's various genes expressions. In comparison to

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

Parameters	Control	OTT1 (0.05 %)	OTT2 (0.1 %)	*p* value
IW (g)	2.1 ± 0.21^{a}	2.1 ± 0.21^{a}	2.1 ± 0.21^{a}	>0.05
FW (g)	4.3 ± 0.8^{a}	4.3 ± 0.9^{a}	5.1 ± 1.1^{b}	0.04
WG (g)	2.2 ± 0.17^{a}	2.2 ± 0.30^{a}	3.0 ± 0.30^{b}	0.04
SGR (%)	2.58 ± 0.14^{a}	2.59 ± 0.25^{a}	3.18 ± 0.21^{b}	0.03
FCR	3.2 ± 0.52^{a}	2.8 ± 0.38^{a}	2.3 ± 0.59^{b}	0.03
PER	1.01 ± 0.16^{a}	1.17 ± 0.16^{a}	1.45 ± 0.37^{b}	0.02
Survival (%)	85 ± 7.1^{a}	100 ± 0.00^{a}	90 ± 14.1^{a}	>0.05

IW = Initial weight, FW = Final weight, WG = weight gain, SGR = Specific growth rate, FCR = Feed conversion ratio, PER = Protein efficiency ratio. Different superscripts indicate statistically significant changes among the groups ($p < 0.05$), n = 3.

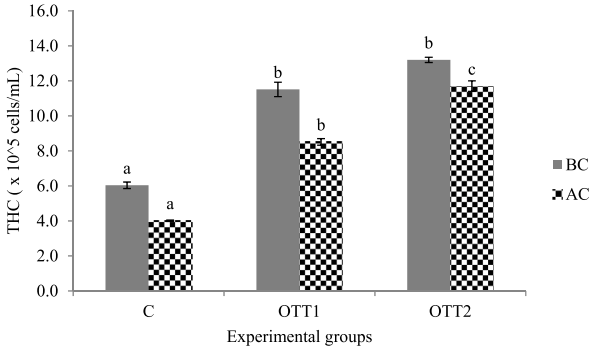

Fig. 1. THC of *P. monodon* treated by extract in both conditions. Dissimilar superscripts in same colour bar designate significant differences between the groups ($p < 0.05$). BC = before challenge of shrimp, AC = after challenge of shrimp by WSSV, C=Control, OTT1 = *O. tenuiflorum* in treatment 1 (0.05 % extract in feed), OTT2 = *O. tenuiflorum* in treatment 2 (0.1 % extract in feed). N = 3.

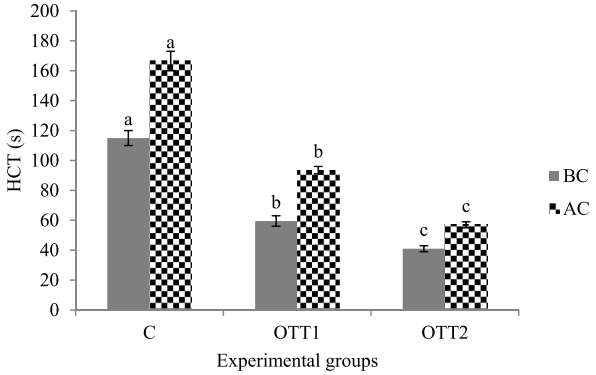

Fig. 2. HCT level of shrimp in extract treated groups challenged and unchallenged with WSSV. Dissimilar superscripts in same colour bar show significant differences between the treatments and control ($p < 0.05$). BC = before challenge of shrimp, AC = after challenge of shrimp by WSSV, C=Control, OTT1 = *O. tenuiflorum* in treatment 1 (0.05 % extract in feed), OTT2 = *O. tenuiflorum* in treatment 2 (0.1 % extract in feed). N = 3.

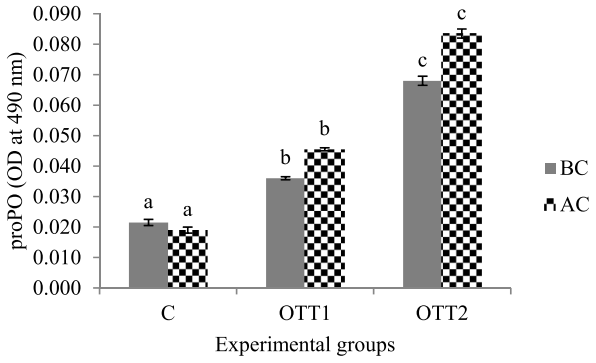

Fig. 3. proPO level of shrimp with extracts of *O. tenuiflorum* in different experimental groups. Dissimilar superscripts in similar colour bar show significant differences between the treatments and control ($p < 0.05$). BC = before challenge, AC = after challenge, C=Control, OTT1 = *O. tenuiflorum* treatment 1, OTT2 = *O. tenuiflorum* treatment 2. N = 3.

T1 and the control group, the expression of the genes was shown to be highest in T2. Unlike the expression of different genes, the proPO gene exhibited higher levels of expression in the treated shrimp.

3.7. Influence of O. tenuiflorum extract on total shrimp mortality in WSSV challenge experiment

Shrimp mortality was noted after WSSV challenge for up to two weeks (14 days) (Fig. 6). Cumulative mortality was differed among the experimental groups. Treatment 2 (OTT2) group had the lowest cumulative mortality (55 %) ($p < 0.05$), followed by Treatment 1

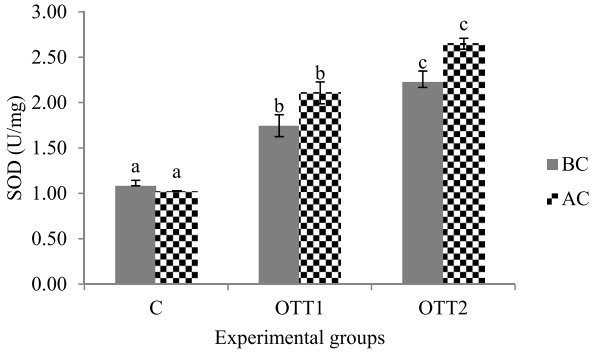

Fig. 4. SOD of shrimp served with extract of *O. tenuiflorum* both in before and after WSSV challenge. Unlike superscripts in identical colour bar show significant differences between the treatments and control ($p < 0.05$). BC = before challenge of shrimp, AC = after challenge of shrimp by WSSV, C=Control, OTT1 = *O. tenuiflorum* in treatment 1 (0.05 % extract in feed), OTT2 = *O. tenuiflorum* in treatment 2 (0.1 % extract in feed). N = 3.

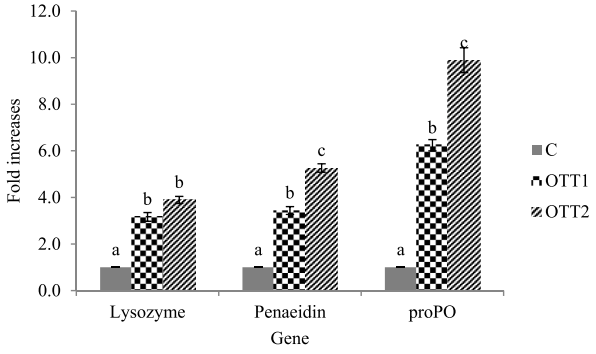

Fig. 5. Comparison of immune gene expression in shrimp under various treatments. The use of distinct superscripts in the similar colour bar implies significant differences among groups for the same gene ($p < 0.05$). C=Control, OTT1 = *O. tenuiflorum* in treatment 1 (0.05 % extract in feed), OTT2 = *O. tenuiflorum* in treatment 2 (0.1 % extract in feed). N = 3.

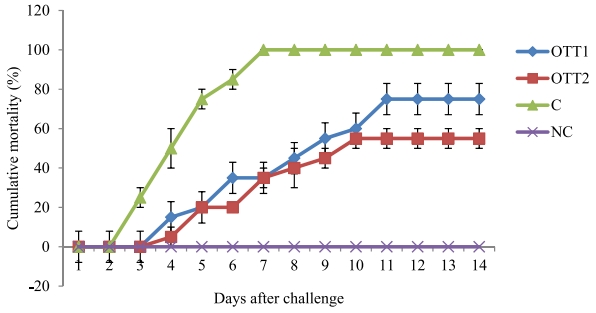

Fig. 6. Cumulative mortality (%) of shrimp fed with *O. tenuiflorum* incorporated diet challenged with WSSV up to 14 days. OTT1 = *O. tenuiflorum* treatment 1, OTT2 = *O. tenuiflorum* treatment 2, C=Control, NC=Negative control.

(75 %), while the control group had the highest (100 %) mortality after two weeks of challenged by WSSV. The total mortality of shrimp in the control group was observed within a span of six days. In the control group, the initial mortality was seen two days following the challenge, but those being treated shrimps exhibited their first mortality four or five days thereafter. However, until the completion of the experiment, there was no death in the unchallenged shrimp (negative control). For the purpose of confirming infection, the PCR analysis was carried out on both unchallenged and WSSV-challenged shrimp. All of the WSSV-challenged shrimps were observed to exhibit the band at 941 bp in nested PCR, but the negative control did not.

4. Discussion

In current time, with the continuously expanding scale of shrimp farming, the recurrent incidence of viral infections has become the

most significant concern that threatens the swift advancement of shrimp culture [17,48]. Given the absence of any current treatment approach, it is crucial to know a therapeutic drug that may be created and utilized to effectively engage WSSV in shrimp [17]. Using herbal remedies or their crude extracts as a means of preventing or treating WSSV has been the subject of a large number of research investigations [10,21,22,49].

This study demonstrated that shrimp receiving *O. tenuiflorum* supplemented feed (T2) had superior growth (WG, SGR) and feed efficiency (FCR, PER) relative to the control group. The results of this study are analogous to the ones seen in previous studies examining the effectiveness of diet supplemented with extract such as, *Syzygium cumini* [50], *Forsythia suspensa* [51], *Phyllanthus amarus* [52], *Moringa oleifera* [53], *Zingiber officinale* [25] in fostering the growth of shrimp. In a previous study by Abdel-Tawwab *et* al. [54], it was shown that the growth and feed utilization metrics were not high when Indian shrimp (*Penaeus indicus*) were fed with a different species of basil (*Ocimum basilicum*). However, in the current study, these parameters have been observed to rise. The growth increment and better feed efficiency in the present study might be linked to the better digestion and absorption of nutrients contained in the extract added diet, that prompted the action of digestive enzymes located in the digestive gland of the hepatopancreas [6]. The inclusion of *O. tenuiflorum* extracts in higher amounts (in this study) in feed might promote the release of digestive enzymes, like lipase, amylase, and protease, and worked as an appetizer, enhancing the development and utilization of the diet [53,55].

In shrimp, haemocytes serve as the fundamental component of the immunity and these cells are tangled in the processes of phagocytosis, encapsulation, the development of nodules, the healing of wounds, coagulation, cell-to-cell communication and the activation of proPO [6]. In crustaceans, a lower-than-normal quantity of circulating haemocytes is largely connected with a lowered resistance to infections. This correlation is substantial when the resistance to infections is poor. In this study, the THC levels in extract-treated shrimps were significantly elevated in comparison to the control group in both the challenged-before and challenged-after situations. Parallel results have previously been stated in other research where THC levels increased when shrimps were fed enriched diets including *Cynodon dactylon* [33], *Gynura bicolor* [23] and *Cystoseira trinodis* [6] while conducting experiment with WSSV. While other research have documented an increase in THC levels in shrimp due to different extracts, there is currently no accessible information on the impact of *O. tenuiflorum* on THC levels when exposed to WSSV. The increase in THC levels in treated shrimp was high because possibly the hematopoietic tissue in shrimp rapidly matures haemocyte precursors, and then releases or transfer the cells from tissue to haemolymph, due to shrimps' open blood circulation, where they help keep the haemocyte number and function stable [52,56]. In contrast, the THC levels of post-challenge shrimp were lower than those of pre-challenge shrimp. This outcome aligns with prior research indicating that THC levels were decreased in shrimp subjected to WSSV [33,57,58]. According to the findings of Chang et al. [59], the THC levels dropped dramatically to 60 % following 24 h of challenge with WSSV when compared to pre infection levels with *P. monodon*. The decrease in haemocytes in infested shrimp may be occurred because haemocytes accumulated at the injection site to heal wounds and engulf foreign particles, or because older haemocytes in hematopoietic tissues reached the end of their lifespan. So, it may result in the proliferation of younger and extra functioning haemocytes in treatments than the control [6,33].

The HCT of the shrimp that had been treated with extract was significantly shorter than the control, both pre and post challenged with WSSV. This is consistent with the findings of Balasubramanian et al. [33] for *Cynodon dactylon* and Velmurugan et al. [60] for *Enteromorpha flexuosa*, which demonstrated that the HCT was significantly reduced in shrimp fed extract against WSSV. The shrimp fed with the maximum extent of extract (T2) had the less HCT, perhaps the more quantity of haemocytes and/or greater concentration of haemolymph protein in this diet treated shrimps ([61,62]. The HCT was also raised in the shrimps after they were challenged with WSSV, which is similar to the outcome of Yoganandhan et al. [63] and Hameed et al. [64], who observed that the HCT was elevated in WSSV-injected shrimps in comparison to shrimp that had not been challenged. This could be because infected control shrimp had a higher viral load and a lower haemocyte count in their haemolymph, making it takes longer for their haemolymph to clot than the haemolymph of shrimp fed immunostimulants [57].

In the present investigation, the proPO of shrimp that had been treated with extract was increased in both pre- and post-challenge circumstances relative to the control. Similarly, Balasubramanian et al. [33] discovered that a *Cynodon dactylon*-enriched diet dramatically boosted the proPO activity in WSSV-infected shrimp than the control group. Likewise, proPO was boosted when the shrimp were fed with *Argemone mexicana* [49], *Gracilaria tenuistipitata* [65] as well as *Cystoseira trinodis* [6] herbal enriched diet in order to protect them from WSSV infection. Therefore, in the current study, the extract of *O. tenuiflorum* served as a promoter and assisted in elevating the level of proPO enzyme that was secreted.

SOD is one of the most important antioxidant enzymes and a biomarker that may be used to evaluate the oxidative damage of aquatic species [6]. The current work demonstrated that dietary administration of O. tenuiflorum extract enhanced SOD function in shrimp, leading to a vigorous immune reaction against WSSV. Similarly, shrimps with diets encompassing extracts of *Cynodon dactylon* [33], *Gynura bicolor* [23], *Gracilaria corticata* [22] and *Cystoseira trinodis* [6], *Zingiber officinale* [25], there was a rise in the enzymatic action of SOD against WSSV. In a research conducted by Abdel-Tawwab et al. [54], it was discovered that the SOD and glutathione peroxidase were significantly increased in the *Ocimum basilicum* oil-fed Indian shrimp (*Penaeus indicus*) with the maximum values found in treatments (2.5–5.0 g/kg diet). Consequently, dietary extracts enhanced enzymatic activity levels in shrimp relative to the control group in the current research. The elevated SOD in shrimp after being challenged in this study can be ascribed to the greater expression of enzyme activity produced by the immunostimulant in stressful circumstances, as opposed to normal conditions, as validated by other investigations [50,66].

The current study found that the immune genes were up regulated in the shrimps that were treated with extract, which is reliable with the findings of some previous experiments. Before being subjected to the test, the proPO gene was significantly up regulated in shrimp that had been given *Sargassum wightii* extract compared to control shrimp [66]. Several immune-related genes showed increased expression in shrimp that had been treated with the dietary extract of *Gynura bicolor* [23], *Cystoseira trinodis* [6] while

challenged by WSSV. The combination of the extract and the feed was effective in expressing immune-related genes in shrimp. This occurred either through the enhancement of immune activities or the activation of immune specialized cells, both of which contribute to the shrimp's defensive mechanism.

Ocimum tenuiflorum includes numerous substances including flavonoids and phenolic groups which boost the phagocytic activity and eliminate pathogens as well as neutralize free radicals in the body of shrimp leading lessen oxidative stress which eventually strengthens immune system [67]. *Ocimum tenuiflorum* contains bioactive substances that increase the production of immune-related genes, leading to the synthesis of antimicrobial peptides, prophenoloxidase, and other substance that strengthen the immune system and enables shrimp to effectively defend against infections such as WSSV. The extract of *O. tenuiflorum* can increase the synthesis of antimicrobial peptides (AMPs). These peptides serve as the initial defensive mechanism and counteract microorganisms, including the viral load in shrimp bodies [68,69].

The findings from this study revealed that the cumulative mortality of shrimp treated with *O. tenuiflorum* extract was significantly reduced than the shrimp used as a control, a finding that has been corroborated by a number of other studies conducted during WSSV challenges. Significant lower cumulative mortality was found against WSSV challenged when the shrimps fed with the extract of *Ceriops tagal* [24], *Cynodon dactylon* [33], *Gynura bicolor* [23], *Gracilaria tenuistipitata* [49] which showed better immune response. In this study, the control shrimp succumbed to WSSV within three to four days of being exposed to the virus, whereas the treated shrimps, which displayed improved immunity, were able to live through the ordeal. Therefore, it is commonly observed that some extracts are able to lift the resistance capacity of shrimps against WSSV. This is the ultimate result of the combination of several parameters, and it has been found that this ability can be increased by plant extracts. Antiviral agents inhibit or kill viruses largely by three mechanisms: direct destruction of the virus; stopping from adhering to and invading host cells; and preventing the reproduction of viruses and their biosynthesis or inhibiting the development and discharge of virus. Extracts that target viral components can inhibit further viral transmission and function as an effective method for protecting the host [10,70].

Throughout the experiment, it was not possible to fully investigate the bioavailability and pharmacokinetics of extracts/compounds in the shrimp body. Further study should emphasis on investigating the mechanism by which the extract and discovered compounds work, as well as studying the *in vivo* efficacy of these compounds against WSSV in shrimp aquaculture. To verify the effectiveness of the extract, it is necessary to carry out the entire shrimp culture cycle in the growers' pond or farm.

5. Conclusion

The current research has displayed that the dietary extract of *O. tenuiflorum* enhanced the growth rate and feed utilization efficiency of shrimp. The extract boosted the immunity of shrimp, as demonstrated by improved immunological parameters and up regulation of immune-genes that finally led to the development of disease resistance capacity in *P. monodon* against WSSV with reduced mortality. According to the present experimental settings, the best extract supplementation dosage was 0.1 % of the diet in terms of growth and immunity of shrimp. The findings of this research offer valuable understandings into the impact of *O. tenuiflorum* extract as a shrimp feed additive, namely as a growth enhancer and immunostimulant, in order to promote sustainable shrimp production.

CRediT authorship contribution statement

Md Rejwanul Haque Galib: Writing – original draft, Software, Methodology, Data curation. **Alokesh Kumar Ghosh:** Writing – review & editing, Data curation, Conceptualization. **Wasim Sabbir:** Writing – review & editing, Supervision.

Data availability statement

Data will be made available on request to the corresponding author.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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