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Research Article

Effects of aqueous and acetone extracts of Persian walnut (*Juglans regia*) leaves on responses of immune system in farmed western white shrimp (*Litopenaeus vannamei*) infected to *Vibrio harveyi*

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Abstract

The main objective of the present study was to evaluate the effects of aqueous and acetone extracts of Persian walnut (*Juglans regia*) leaves on the responses of the immune system in the farmed western white shrimp (*Litopenaeus vannamei*) infected to *Vibrio harveyi*. Shrimps were randomly divided into 10 groups: 3 groups treated with the aqueous extracts at concentrations of 100, 200, and 300 mg/kg respectively, 3 groups treated with Estonian extracts at concentrations of 100, 200 and 300 mg/kg respectively, 2 groups selected as negative control groups and 2 groups as positive controls. Experimental groups were randomly tested in triplicates over a 40-day period. The parameters of immune responses of shrimp samples were measured at three-time points of baseline (day 0), midterm (day 20), and end line (day 40). The results of this study showed that the administration of acetone extract of Persian walnut leaves at a concentration of 200 mg/kg caused a significant change in total hemocyte count, differential hemocyte count, and total plasma protein levels in the western white shrimp ($p \leq 0.05$). The results indicated that the walnut leaf extract can be used as a supplement to shrimp farming because of its cost-effectiveness and availability, and can significantly increase the productivity of shrimp farms.

Keywords: Western white shrimp (*Litopenaeus vannamei*), Persian walnut leaves (*Juglans regia*), *Vibrio harveyi*

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Introduction

Shrimp is one of the most important farmed seafood around the world, especially in the Asian region. It has high quality and nutritional value and so is very popular. According to the Food and Agriculture Organization (FAO), the Penaeidae family comprises about 110 species of commercial shrimp, accounting for 80% of global shrimp production (FAO, 2018). One of the major problems for the aquaculturists in the early stages of farming is a decrease in the maintenance and survival rate of shrimp larvae, especially when starting active feeding. Enhancing the immune system of shrimp larvae, especially in valuable species, is one of the most important approaches of researchers in this regard (Zuo *et al*., 2018). It has affected the industry economically, in addition to the emergence and spread of diseases along with the development of the aquaculture industry, so that some diseases are difficult to control today (Javanmardi *et al*., 2020). One of the effective ways to improve the ability and resistance of shrimp is to use the stimulants of growth and immune system of fish and other aquatic species (Poonkodi *et al*., 2016). Use of these substances in the aquaculture industry has become more popular recently to improve and stimulate nonspecific immunity and disease resistance. In this regard, some antibiotics (oxytetracycline and chloramphenicol) have been studied and used to stimulate growth and health in commercial species such as shrimp, carp, trout and Nile tilapia (De Silva *et al*., 2018). Use of antibiotics and chemicals has disadvantages, including the risk of pathogen resistance (Sotomayor *et al*., 2019), the accumulation in the body of farmed fish and their contaminant effects on the environment (Albuquerque Costa *et al*., 2015).

Nowadays, immunostimulants are used to solve this problem. The use of herbal compounds as immunostimulants in aquaculture has recently been popularized to enhance the immune system (Bababaalian Amiri *et al*., 2020). Walnut (*Juglans regia* Linn) is a plant of the family Juglandaceae that has 21 species, all of which are deciduous, have edible fruits, and are valuable and medically useful species (Yang *et al*., 2019). Research has shown that the walnut leaves have antibacterial and antifungal properties (Ho *et al*., 2018). Walnut bark contains various chemical constituents such as beta-cytosterol, ascorbic acid, folic acid, gallic acid, juglone, regiolone and quercetin 3-O-Alpha-L-arabinoside (Anjum *et al*., 2017), which have shown reportedly antimicrobial effects (Calcabrini *et al*., 2017). Due to the abundance, availability and affordability of walnut leaves without damaging the tree, they can be a good alternative to synthetic and semi-synthetic drugs (Fiorito *et al*., 2014). The aim of this study was to evaluate the effects of the aqueous and acetone extracts of Persian walnut (*Juglans regia*) leaves on the function of immune system and the prevention of vibriosis in the farmed western white shrimp (*Litopenaeus vannamei*).

Materials and methods

The current research was conducted at the Microbiology and Chemistry Laboratories (ISO/IEC 17025) as well as at the Shrimp Research Center in Bushehr during the summer 1997. The shrimps weighing 11 g were collected from the farming and breeding centers of Bushehr for a 60-day period. The water needed for the study was supplied from the Persian Gulf, which was used after chlorination (25 ppm) in rectangular concrete ponds at Shoghab Research Station in Bushehr city, affiliated to Shrimp Research Center.

Water quality parameters including dissolved oxygen (DO), temperature and pH were measured daily. During the farming period, total weight of shrimp was recorded by a digital balance (0.01 grams), as well as total length and carapace length were measured by a caliper. At each sampling time, physicochemical factors were measured and recorded for the water source of shrimp farming, including temperature, salinity and DO by a portable multimeter device (HACH, Model HQ 40d).

The extraction process was performed in 7 steps as follows. In the pretreatment stage, the fresh leaves of Persian walnut tree were collected from Shiraz city, and then separated from the stem, washed with water, spread on wide and clean plastic strips under the natural conditions and in the shaded place at laboratory temperature, and finally air-dried (Javanmardi *et al*., 2020). The dried leaves were powdered after grinding the plant with a mill machine and then passed through a 400-micron mesh sieve. Since particle size is one of the factors affecting the balance between solvent and solute, the grinding operation was repeated several times to obtain the smallest size of leaf powder. Next, the plant extract was prepared from 100 g samples of dried Persian walnut leaf powder in 500 ml of solvents (water and acetone) using a rotary evaporator (Ho *et al*., 2018).

Preparation of bacterial strain

In this study, *Vibrio harveyi (IS 01 PTTC 1755*) with accession number at GeneBank (GU 974342, 1), which was previously isolated and purified by the Shrimp Research Center, was used as the dominant bacterial strain.

Experiment on live shrimp

Some 480 shrimps used in this study (*Litopenaeus vannamei*) were obtained from the farming and breeding centers of Bushehr (16 shrimp per each replicate), whose non-contamination with warning viral infections was confirmed by Nested PCR, weighted 11±1.32 g, were transferred from shrimp farms to the indoor saloon at Shrimp Research Center in 300-liter tanks containing 200 liters of seawater. Prior to treatment, the shrimps were adapted for 7 days and de-stressed (Osińska *et al*., 2020).

Grouping

Shrimps were randomly assigned to 10 groups of 20 individuals, in triplicate, including 6 treatment groups infected by *V. harveyi* (0.5 McFarland) treated with different concentrations of the aqueous and acetone extracts of Persian walnut leaves, and 4 control groups that received no treatment, including 2

negative control groups with no treatment and 2 positive control groups infected by *V. harveyi*. Treatments were run in triplicate over a 40-day period (Pott *et al*., 2018).

Preparation of diets

The steps of preparing the shrimp diet were performed on the basis of the protocol as described in the pellet processing sections of feed processing plants (Gomes *et al*., 2018).

The concentrated feed No. 4004 (Havoorash Co., Bushehr, Iran) containing squid powder, fishpowder, shrimp shell powder, soy powder, cereal flour, fish oil, vitamin supplements, and minerals (Table 1) was used in this study.

Table 1: Food composition and the percentage of each element.

Description	The final meal	Growing food	Starter food
Protein	33	36	38
Fat	8	8	9
Fiber	4	3	3
Ash	14	14	14
Humidity	10	10	10

In order to perform the micro mill or mill stage, the desired diet was milled and the concentrated feed was obtained with the aid o1f a mill and a 400-micron mesh sieve because the size of the main feed components for the shrimp should be less than 400 microns. Next, the desired concentrations of the aqueous and acetone extracts of Persian walnut leaves (100, 200, 300 mg/kg) were sprayed on powdered concentrated feed to prepare a specific diet for each treatment. Simultaneously, a small electric mixer was used to homogenize and uniformity the extract at all food areas (Mthethwa *et al*., 2019). By adding distilled water to the aqueous extract and liquid oil to the acetone extract, a paste was produced with an approximate moisture content of 30%. To prepare the pellets, the diets were filmed by a meat grinder with a dominant size of 2.5-mm dough, and then placed on stainless steel trays inside an oven at 70°C (Memmert, Model UNB 400) for 8 hours to dry pellets with moisture content less than 10%. Subsequently, the dried pellets were sifted, and the dust removed from the food cycle. Given the biomass weight of each tank (Kaur *et al*., 2019) and the appropriate pellet size for the shrimp studied, the diets were cut into smaller pieces, dried completely, packed in plastic bags, stored in a refrigerator at 4°C with proper ventilation until use (once every 2 weeks), and consumed after the shrimp adaptation to the control diet and tank environment. Feeding was performed with diets containing different concentrations of Persian walnut leaves three times daily (8 am, 1 pm and 8 pm). Feeding rate was based on biomass, water temperature and checking tray method (aquarium floor), so that 5 hours after feeding, the feed contained in the bottom of the aquarium was controlled. Accordingly, if the feed remained in the bottom, the feed volume would be reduced at the next meal, and if there was little or no food left, the feed volume would be increased at the next meal.

Measuring parameters of immune function

The parameters of immune function in the samples of shrimp were measured at three time points of baseline (day 0), midterm (day 20) and end line (day 40) during the 40-day period.

To determine the hemocyte, blood smears were prepared on a slide as a candle flame from hemolymph samples in triplicate, dried completely at room temperature for a minute, fixed in pure methanol, stained with methanol based on May-Grünwald-Giemsa staining protocol (Shen *et al.*, 2010), and observed under a light microscope at a magnification of ×100. Then, 100 hemocytes were counted and divided according to the size and shape of the nucleus relative to the cytoplasm, as three types of hyaline cells (HC), small granular cells (SGC) and large granular cells (LGC). The remaining hemolymph in the Eppendorf, which can be stored in ice for 1 hour after sampling (Ghaednia *et al*., 2011; Rezaei *et al.*, 2020), was centrifuged and the resulting solution was tested by the Biuret method for measuring total plasma protein (Okutucu *et al*., 2007; Akbary *et al*., 2020).

Statistical analysis

Data on survival rate and growth function (total weight, total length and carapace length) and of immune parameters were analyzed by SPSS 21 software between the control and treatment groups using One-way ANOVA and Tukey's post hoc test at 95% significance level, and by SAS9.2 software using general linear model (GLM) to compare two or more variables. In addition, charts were drawn by Excel 2013 software.

Results

Total hemocyte count (THC) at the baseline

According to the findings, the hemolymph THC value in western white shrimp at the baseline indicates that there is no significant difference ($p \geq 0.05$). Further investigations revealed that the highest rate of increased THC was found at day 20 and at the acetone extract concentration of 200 mg/kg as compared to the positive control (containing bacterial suspension of $\times 10^5$ cell ml^{-1} with the THC value of 44.06±3.96 in treatment 50 mg/kg ($\times 10^5$ cell mL^{-1}) with the THC value of 232.29±3.41 which had a significant difference compared to the treatment 10 mg/kg ($\times 10^5$ cell mL^{-1}) with the THC value of 170.11±71.32 ($p \geq 0.05$). In mean values of treatments in the whole treatment period, the treatment of 50 mg/kg was significantly different (Table 2).

Table 2: Results of total hemocyte count (×10^5 cell mL^{-1}) for western white shrimp fed diets containing different concentrations of Persian walnut leaf aqueous and acetone extracts after 0, 20 and 40 days.

Diet containing Persian walnut leaf extract (mg/kg)		Total hemocyte count (×10^5 cell mL^{-1})			
		Day 0	Day 20	Day 40	Mean treatment
Concentration extract					
100	aqueous	79.32± 3.24 ^{a}	93.87± 3.47 ^{cd}	81.54 ±3.72^{c}	84.91± 67.21 ^{d}
	acetone	80.12± 3.18 ^{a}	192.06± 3.47 ^{b}	184.01± 3.91 ^{a}	152.06±49.39 ^{b}
200	aqueous	79.97± 3.16 ^{a}	108.56 ±6.54 ^{c}	145.32± 3.91 ^{b}	111.28± 71.07 ^{c}
	acetone	82.16± 3.79 ^{a}	232.29 ±3.41 ^{a}	195.89± 3.91^{a}	170.11 ±71.32 ^{a}
300	aqueous	80.78± 3.89 ^{a}	100.19± 1.34 ^{cd}	67.76 ±1.27 ^{c}	82.91 ±36.15 ^{d}
	acetone	80.19± 3.48 ^{a}	180.64 ±3.13 ^{b}	172.13± 3.97 ^{a}	144.32±57.82 ^{b}
Negative control		75.29± 2.31 ^{a}	87.90± 3.21 ^{d}	85،15 ±1. 31^{c}	82.78± 2.92 ^{d}
Positive control (containing bacterial suspension)		74.65± 3.98 ^{a}	98.07± 3.19 ^{cd}	71.27± 4.19 ^{c}	81.33 ±18.16 ^{d}

The values in this table are presented as mean±SD. The values labeled with the same letters have no significant difference at the level of 5%.

Differential hemocyte count (DHC)

Hyaline cells (HC)

The empirical data analysis indicated that the HC count in the control treatment at the baseline (day 0) had no significant difference and this value after 20 days in the group treated with 200 mg/kg of acetone Persian walnut leaf extract was 79.77±1.14 (×10^5 cell/mL), which showed a significant difference compared to the positive control, the negative control and other treatment groups (Table 3).

Table 3: Results of hyaline cell count (×10^5 cell mL) for western white shrimp fed diets containing different concentrations of Persian walnut leaf Aqueous and acetone extracts after 0, 20 and 40 days.

Diet containing Persian walnut leaf extract (mg/kg)		hyaline cell count (×10^5 cell/mL)			
		Day 0	Day 20	Day 40	Mean treatment
Concentration extract					
100	aqueous	50.21± 2.91 ^{a}	68.25 ±2.24 ^{b}	68.19± 1.79 ^{b}	62.33 ±3.51 ^{ab}
	acetone	52.17± 3.78 ^{a}	58.65± 3.97 ^{ab}	59.19± 2.77 ^{b}	56.67± 3.23 ^{a}
200	aqueous	53.45± 2.64 ^{a}	68.04± 2.15 ^{b}	60.32 ±1.54 ^{b}	60.60±21.45 ^{ab}
	acetone	51.45± 1.8 9^{a}	79.77± 1.14 ^{c}	64.57± 3.51 ^{b}	64.79± 10.15 ^{b}
300	aqueous	52.09± 2.71 ^{a}	49.21± 4.17 ^{ad}	44.65±1.89 ^{ad}	48.65 ±2.51 ^{a}
	acetone	53.17 ±3.94 ^{a}	40.78 ±2.30 ^{d}	52.14 ±2.64 ^{a}	48.69± 3.15 ^{a}
Negative control		50.16± 1.15 ^{a}	50.41± 1.68 ^{a}	50.75± 1.48 ^{a}	50.44± 3.59 ^{a}
Positive control (containing bacterial suspension)		51.06± 2.98 ^{a}	56.78± 2.91 ^{ab}	50.98 ±3.19 ^{a}	52.94± 3.81 ^{a}

The values in this table are presented as mean±SD. The values labeled with the same letters have no significant difference at the level of 5%.

Small granular cells (SGC)

The results indicated that the mean SGC count in the negative control group was 31.85±3.15 (×10^5 cell/mL) and this value was not significantly different at day 0 compared to other treatments. The results of Table 4 show that the use of aqueous and acetone extracts of Persian walnut leaf extract had no significant

effect on the SGC at the significance level of 5%.

Table 4: Results of small granular cell count (×10^5 cell ml) for western white shrimp fed diets containing different concentrations of Persian walnut leaf Aqueous and acetone extracts after 0, 20 and 40 days.

Diet containing Persian walnut leaf extract (mg/kg)		Small granular cell count (×10^5 cell/ml)			
		Day 0	Day 20	Day 40	Mean treatment
Concentration extract					
100	aqueous	31.54± 3.14 ^{a}	47.32 ±1.41 ^{b}	42.12 ±3.23 ^{b}	40.32± 3.14 ^{b}
	acetone	32.01± 1.52 ^{a}	47.92± 1.85 ^{b}	49.78 ±3.54 ^{c}	43.23± 1.64 ^{b}
200	aqueous	32.31± 1.45 ^{a}	33.76 ±4.61 ^{a}	37.23 ±2.71 ^{ab}	34.43 ±10.59 ^{a}
	acetone	31.23± 1.65 ^{a}	34.21 ±3.01 ^{a}	37.19± 3.67 ^{ab}	34.54± 13.09 ^{a}
300	aqueous	33.78± 1.48 ^{a}	33.16 ±4.01 ^{a}	34.98 ±2.31 ^{ab}	33.97± 7.49 ^{a}
	acetone	34.12± 3.71 ^{a}	27.15± 2.38 ^{a}	42.17 ±1.17 ^{b}	34.48± 1.98 ^{a}
Negative control		32.43 ±1.34 ^{a}	32 ±5.14 ^{a}	31.12 ±5.6 ^{a}	31.85 ±3.15 ^{a}
Positive control (containing bacterial suspension)		32.89± 1.32 ^{a}	37.67± 2.13 ^{ab}	47.12 ±4.70 ^{bc}	39.22 ±6.71 ^{ab}

The values in this table are presented as mean±SD. The values labeled with the same letters have no significant difference at the level of 5%.

Large granular cells (LGC)

The results of LGC analysis (Table 5) revealed that the control treatment at day 0 had no significant difference with the other groups and this treatment showed a significant difference in the LGC count with 20 days after the experiment with 13.66±1.04 (×10^5 cell/mL) compared to the group treated with 200 mg/kg of aqueous Persian walnut leaf extract with the LGC count of 16.87±0.23 (×10^5 cell/mL) (p≤0.05).

Table 5: Results of large granular cell count (×10^5 cell mL) for western white shrimp fed diets containing different concentrations of Persian walnut leaf Aqueous and acetone extracts after 0, 20 and 40 days.

Diet containing Persian walnut leaf extract (mg/kg)		Large granular cell count (×10^5 cell/mL)			
		Day 0	Day 20	Day 40	Mean treatment
Concentration extract					
100	aqueous	12.09± 3.15 ^{a}	9.25± 1.19 ^{b}	7.25± 1.25 ^{bd}	9.8± 1.05 ^{b}
	acetone	12.12 ±2.15 ^{a}	13.16± 1.71 ^{a}	12.25± 2.65^{a}	12.51± 1.31^{a}
200	aqueous	11.89± 2.13 ^{a}	16.87 ±0.23 ^{c}	11.67± 1.08^{a}	13.47± 2.75^{a}
	acetone	12.14 ±2.12 ^{a}	13.01± 1 ^{a}	13.24 ±2.11^{a}	12.79± 1.04^{a}
300	aqueous	13.23± 0.13 ^{a}	6.98± 1.23 ^{db}	3.53± 2.16 ^{e}	7.91 ±3.19 ^{d}
	acetone	12.65± 1.71 ^{a}	7± 1 ^{db}	3.33± 1.75 ^{e}	7.22 ±4.76 ^{d}
Negative control		12.07± 4.19 ^{a}	12.87 ±2.12 ^{a}	11.69± 1.94^{a}	12.21± 0.42^{a}
Positive control (containing bacterial suspension)		12.22 ±2.81 ^{a}	13.66± 1.04 ^{a}	5.6± 1.08 ^{de}	10.49± 3.81^{b}

The values in this table are presented as mean±SD. The values labeled with the same letters have no significant difference at the level of 5%.

Total plasma protein levels

Results of the effects of extracts on total plasma protein in western white shrimp hemolymph indicated that the control treatment at day 0 had no significant difference with other groups fed diets

containing Persian walnut leaf extract ($p \geq 0.05$). Regarding the content of total plasma protein in hemolymph after 40 days, the control group with total plasma protein content of 5.2±0.01 had a significant difference with the treatment containing the aqueous Persian walnut leaf extract of 100 mg/kg (c0.01±2.1) and the treatment containing the aqueous and acetone extracts of Persian walnut leaves of 300 mg/kg. No significant difference ($p>0.05$) between these three treatments was found (Table 6).

Table 6: Results of total plasma protein content (mg/dl) for western white shrimp fed diets containing different concentrations of Persian walnut leaf aqueous and acetone extracts after 0, 20 and 40 days.

Diet containing Persian walnut leaf extract (mg/kg)		Total plasma protein (mg/dl)			
		Day 0	Day 20	Day 40	Mean treatment
Concentration extract					
100	aqueous	2.08±0.02 [a]	6.5±0.01 [a]	3.59±0.02 [a]	9.8± 1.05 [b]
	acetone	2.01±0.02 [a]	2.1±0.01 [a]	2.17±0.02 [a]	12.51± 1.31[a]
200	aqueous	2.4±0.03 [a]	5.01±0.05 [a]	2.93±0.02 [a]	13.47± 2.75[a]
	acetone	2.1±0.02 [a]	1.75±0.01 [a]	2.71±0.01 [a]	12.79± 1.04[a]
300	aqueous	2.89±0.01 [a]	2.375±0.01 [a]	2.821±0.01 [a]	7.91 ±3.19 [d]
	acetone	2.14±0.01 [a]	1.85±0.01 [a]	2.29±0.01 [a]	7.22 ±4.76 [d]
Negative control		4.5±0.03 [a]	4.5±0.03 [a]	5.2±0.01 [a]	4.73±0.03 [a]
Positive control (containing bacterial suspension)		3.2±0.01 [a]	3.31±0.01 [a]	3.9±0.01 [a]	3.47±0.01 [a]

The values in this table are presented as mean±SD. The values labeled with the same letters have no significant difference at the level of 5%.

Discussion

The most important bacterial disease reported in the shrimp farming industry is vibriosis, and one of the most reported species is *V. harveyi* (Reen and Boyd, 2005). The use of alternative agents for antibiotics causing drug resistance in many dangerous bacterial pathogens, which have no complications, have been considered by many researchers (Morris and Nair, 2011). Among these, the use of various extracts from natural materials, such as brown algae (*Sargassum glaucesens*) on Indian white shrimp (*Fenneropenaeus indicus*) (Fan *et al*., 2019) or other medicinal plants as immunostimulants, with production cost lower than other chemicals and disinfectants has been of interest to researchers. The use of various extracts from medicinal plants has been considered as new immunostimulants (Lage *et al*., 2017).

The present study used Persian walnut leaf extracts, According to previous studies which had antibacterial activity and the ability to control gram-positive and gram-negative bacteria, for bioassay and survival rate of western white shrimp. To the best of our knowledge, no study has been conducted in the world so far in this regard, so similar research was used for comparison (Ghaednia *et al*., 2011). According to the findings of the present study, in the western white shrimp infected with *Vibrio* sp. 90-69B3, mortality rates in the second week had reached 50%. Therefore, the treatments without effective agents and immune boosters will have high mortality rates. This is if the western white shrimp

receiving the Aqueous and acetone extracts of the walnut leaves at different concentrations as immunostimulants should have a higher survival rate (70%) than the positive control treatments at all concentrations of 10, 20 and 50 µg g^{-1}. In a study of Burgents *et al*., who used the yeast V.Xp at different concentrations as immunostimulants, the highest survival rate was found in the second week at concentrations of 1% (70%) and 0.5% (50%). The cases reported showed the effect of immunostimulants on enhancing the function of samples indirectly to control the pathogens (Burgents *et al*., 2004).

A study investigated different levels of dietary astaxanthin as a stimulant to elevate levels of biochemical and nonspecific immune markers in western white shrimp. The astaxanthin was added to the diet at four levels of 0, 50, 100 and 150 mg/kg, then the shrimps were exposed to oxygen deficiency stress at the end of the period. The results indicated that significant differences in growth and immune indices were observed in the treatments that fed with 100 and 150 mg/kg astaxanthin (Jagruthi *et al*., 2014), which is in line with the present study. Suryadi *et al*. (2019) used OMP and LPS to stimulate nonspecific immune system. The results showed that there was a significant decrease in the number of total homocytes in the treated groups compared to the control groups. In a study, different levels of astaxanthin were applied as a stimulant to increase the levels of biochemical and nonspecific immune markers in western white shrimp (Beygi *et al*., 2019). The astaxanthin was added to the diet at four levels of 0, 50, 100 and 150 mg/kg, and then the shrimps were exposed to oxygen deficiency stress at the end of the period (day 40). The results showed significant differences in blood chemical indices and immune function in the treatments that fed with 100 and 150 mg/kg astaxanthin. In addition, the highest THC, HC, SGC, phagocytic activity, and plasma protein were obtained in the treatment of 100 mg/kg, and the highest LGC and glucose content were observed in the treatment of 150 mg/kg ($p \geq 0.05$). In a study conducted by Talpur and Ikhwanuddin, total plasma protein content was decreased in the positive control containing bacterial suspension from 15 mg/mL to 9 mg/mL, due to the energy consumption of shrimp frequently from total plasma protein consistent with the present study (Talpur and Ikhwanuddin, 2012). Contrarily, total plasma protein did not decrease when the shrimp were exposed to different level of *Bohadschia ocellata* (Javanmardi *et al*., 2020).

A study examined the effect of dietary nucleotide at the level of 0.2% on growth, survival and hemolymph markers. The addition of dietary nucleotide to the diet resulted in a significant difference in total plasma protein content in shrimp fed dietary nucleotide compared to the control group (Biswas *et al*., 2012), which is in line with the present study.

On the other hand, a study investigated the effect of fucoidan from brown seaweed *Sargassum wightii*, as immunostimulants, on Indian white shrimp immersed in concentrations of 100, 300 and 500 mg/L of algal extract.

They examined the prevention of White Spot Syndrome Virus (WSSV) and immune activity (Immanuel *et al*., 2012). The results of this study showed that the immersion of Indian white shrimp in concentrations of 300 and 500 mg/L of *Sargassum wightii* in hot water extract for 2 to 3 hours was effective in increasing immune factors and survival rate, which is in line with the present study. The empirical results of ZOI diameter, MIC and MBC indicating the plant antibacterial activity can be attributed to the presence of fatty acids and phenolic compounds, which is in agreement with the findings that plant phenolic compounds are of the main groups in the antioxidant activity and antimicrobial effects (Chraibi *et al*., 2016).

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