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Research Article

Effect of sea cucumber, *Holothuria parva*, methanol extract on survival rate and immunity factors of white leg shrimp (*Penaeus vannamei*) exposed to *Vibrio harveyi*

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Abstract

This study aimed to investigate the effect of sea cucumber methanol extract on *Vibrio harveyi* and also to investigate the measurement of changes of factors in farmed shrimp, *Penaeus vannamei*. *Holothuria parva* with an average length of 15-10 cm were collected from tidal coasts of Dayyer city, Bushehr province. Different concentrations of *H. parva* extract (50, 100, and 150 µg/mL) were used to prepare the food of the experiment. *Vibrio harveyi* bacterial strain (PTCC: 1755) was obtained from the Collection Center of Industrial Microorganisms of Iran. Thousand shrimp with an average weight of 10±1 g were collected from culture ponds and distributed among 15 aquariums containing 100 liters of water with a salinity of 40 ppt. The mean comparison of survival rates showed that there was no significant difference among treatments on the first day (p>0.05). Also, there was significant difference among survival rates in other treatments with negative and positive control treatments (p<0.05). The results also showed that there was significant difference among the groups in PO (phenol oxidase), POD (peroxidase), THC (total haemocyte count), TPP (total plasma protein), and SOD (superoxide dismutase) with the amounts of negative and positive control treatments during the experimental days (p<0.05). The highest amounts of measured factors were 538.18±14.45 U min^{-1} mL^{-1} (Treatment 3) for PO on the fifth day, 7.19±0.3 nmol min^{-1} mL^{-1} (Treatment 2) for POD on the fifth day, 37.98±6.49×10^5 cell mL^{-1} (Treatment 3) for THC on the third day, 93.67±14.8 mg mL^{-1} (Treatment 3) for TPP on the third day, and 1663.21±37.07 activity U mL^{-1} (Treatment 3) for SOD on the third day. According to the results, survival was directly related to the concentration of methanolic extract. The highest increase in safety factors was observed in treatment 3, with concentration of 150 µg / mL methanolic extract of sea cucumber.

Keywords: *Holothuria parva*, Immunity factors, *Penaeus vannamei*, Methanol extract, Survival rate, *Vibrio harveyi*

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Introduction

Aquaculture is one of the food production activities whose development directly reduces pressure on marine and ocean aquatic stocks (Supamattaya *et al*., 2005). However, due to lack of animal protein to meet human consumption needs, aquatic products play a very important role in providing healthy and inexpensive source of animal protein (Lebel *et al*., 2010; Xiong *et al*., 2016). White leg shrimp, scientifically known as *Penaeus vannamei* is native to western coasts of Latin America in the Pacific Ocean, from Peru in the south to Mexico in the north. Since late 1990s this species has grown successfully on a commercial scale in Asia (Bachère, 2000). White leg shrimp culture is possible in very high densities up to 150 shrimp per square meter and in closed and controlled condition the density can increase up to 400 shrimp per square meter. It requires less protein food compared to other commonly cultured species (20 to 35%). The highest average production of *P. vannamei* shrimp with a high hygienic-viral control and in a super-dense system is reported to be up to 63 tons per hectare (Bachère, 2000; Rebouças *et al*., 2011).

Aquatic diseases and health problems are some of the main challenges in aquaculture production, especially in shrimp farming industry. Shrimp farmers lose millions of dollars in disease damage each year as one of the main problems in development of this industry. So far, about 20 viral diseases, 4 bacterial diseases, 3 fungal diseases, and several parasitic diseases have been reported in crustaceans, especially shrimp, which cause damage to the shrimp breeding industry. Understanding the relationships among shrimp, environment, and pathogens have a very important role in the study of shrimp diseases and are the basis of proper management in reproduction and breeding of this crustacean (Bachère, 2000; Xiong *et al*., 2016). There is no specific treatment for some shrimp diseases (mainly viral diseases) and several strategies are developed to increase the growth and survival of shrimp and simultaneously reduce the incidence of diseases and destructive effects on the environment. These strategies include improving environmental conditions, specific pathogen-free larvae (SPF), and increasing shrimp resistance to pathogens by immune stimulants (Takahashi *et al*., 2000; Chotigeat *et al*., 2004; Supamattaya *et al*., 2005).

Vibrio harveyi is the main pathogen of the genus *Vibrio* which under favorable conditions (stressful conditions in shrimp) can affect the shrimp (Rungrassamee *et al*., 2016). Some bacterial pathogens include extracellular products (cysteine proteinase, phospholipase, and hemolysin), lipopolysaccharide, bacteriophage agents, and bacitracin (Austin and Zhang, 2006). *V. harveyi* pathogenicity is dependent on the bacterial strain and indicates a synergistic interaction between individual and associated factors, namely hydrophobicity, biofilm formation, survival in fish skin mucosa,

serum, proteolytic, hemolytic, and cytotoxicity of ECPs (Won and Park, 2008; Darshanee Ruwandeepika *et al*., 2012).

Sea cucumbers form a large group of aquatic animals whose biological activities include anti-cancer, anti-viral, anti-coagulant, anti-hypertensive, anti-inflammatory, antimicrobial, antioxidant, anti-atherosclerotic, anti-tumor, and accelerates wound healing. The reason for existence of these properties in sea cucumber can be presence of substances such as; compounds with chemical structure of glycoterpenoid, chondroitin sulfate, sulfated polysaccharide, glycoprotein, glycosphingolipid, and essential fatty acids (GAGs) attributed to cucumber glycosaminoglycans (Bordbar *et al*., 2011). Several studies are conducted on the extraction of compounds from sea cucumber and other organisms to improve aquatic diseases, including shrimp (Takahashi *et al*., 2000; Prasetio *et al*., 2013; Bai *et al*., 2014; Farjami *et al*., 2014; Li *et al*., 2016; Shadi and Oujifard, 2019).

Chemicals and antibiotics are commonly used in the traditional treatment of shrimp disease. Shrimp ponds are treated with large amounts of chemicals, and continued use of antibiotics can lead to microbial resistance and entry of antibiotics into shrimp tissue. Application of these methods has many problems, for example microbial resistance in reproduction and culture farms became a global problem and many researchers payed attention to it (Tsoumas *et al*., 1989; Song and Sung, 1993; Mahasneh *et al*., 1995). Biomolecules extracted from aquatic organisms have powerful bioactive properties. Efforts to develop marine biochemicals led to discovery of substances with anticancer, antibiotic, growth regulator, hemolytic, anticoagulant, analgesic, antispasmodic, antihypertensive, and even anti-AIDS agents (Bhakuni and Rawat, 2005). In this study, it is assumed that methanol extract of sea cucumber is effective in preventing bacterial disease caused by *Vibrio harveyi* and changes in safety and survival factors. Therefore, this study aimed to investigate the effect of extracted methanol from sea cucumber on *Vibrio harveyi* and to investigate the changes in the measured factors in farmed shrimps.

Materials and methods

Sea cucumbers collection

Samples of *H. parva* were collected with an average length of 15-10 cm from the tidal area of Dayyer (N: 27° 50' 12.49", E: 51° 53' 33.62"), located in Bushehr province. The collected samples were transferred using ice bags to ecology laboratory of Persian Gulf University. Sea cucumbers were cleaned of foreign matter and necrotic parts were isolated. The specimens were cut from the anus to the mouth and their gastrointestinal tract was cleared. They were then stored at -20°C. After 20 minutes, a white precipitate was collected at the end of the Falcon (Jaeckle and Strathmann, 2013). One drop of the precipitate was spread on the slide using a sampler and matched using a microscope

(magnifications 10 and 40) with identification keys (Kelman *et al*., 2006; Dabbagh *et al*., 2011).

Methanol extraction

The methanol extract of sea cucumbers was made with a freeze dryer and a rotary machine (Farjami *et al*., 2014). After leaving the freezer, sea cucumbers were cut into smaller components. They were then placed in freezer for 24 hours (Tamarit-Pino *et al*., 2020). Then, dried sea cucumbers were powdered by electric mill, and the soaking method was used to obtain sea cucumber tissue extract. Sea cucumber powder (400 mL of solvent per 100 g of tissue) was placed in 94% methanol solvent and then placed at room temperature on a shaker for 72 hours. The extract was then passed through a filter paper (Whatman No. 1) and concentrated under a vacuum using a rotary machine (rotary vacuum distillation) at 45°C (Sinurat *et al*., 2016).

Determination of MIC by in vitro method

MIC was performed to evaluate the antibacterial properties using Broth Macro dilution method. *Vibrio harveyi* bacterial strain (PTCC: 1755) was obtained from the Collection Center of Industrial Microorganisms of Iran (in lyophilized form). The initial culture was linearly placed on saline TSA medium and placed at 30°C for 24 to 48 hours (depending on the rate of bacterial growth in the incubator) to use single colonies for the experiment. After the bacterial colonies grew on the pellets, they were removed from the incubator and transferred to the TSB saline medium (2.5% salt) in the test tubes using needles from single colonies. This was repeated until the turbidity of the broth medium was equal to the turbidity of the McFarland medium equivalent to 1.5×108 CFU/ mL of bacteria (0.5 mL of 1% barium dehydrated chloride and 99.5 mL of 1% sulfuric acid). All tubes were closed and incubated for 24 h at 30°C. After 24 hours, the test tubes were removed from the incubator and their turbidity was checked. The control tube did not contain biologically active compounds and became very cloudy because the bacteria had a chance to grow in it. The tubes were compared visually. This experiment was performed with three replications. Then sterile methanol extract of sea cucumber with concentrations of 5, 10, 20, 30, 40, and 50 µg / mL was used to determine the MIC and MBC. Due to the lack of results and inhibition of growth, higher concentrations of 50, 100, and 150 µg/mL were investigated (Farjami *et al*., 2014).

Preparing a diet containing sea cucumber extract

Different concentrations of *H. parva* extract (0, 50, 100, and 150 µg mL^{-1}) with 3 replications were used to prepare the food used in the experiment. At first Food No. 4004 (made by Havoorrash Company) was prepared and powdered. The determined concentrations were added to each kilogram of food. Gelatin and water were added to the food and passed through a meat grinder (with a mesh size of 1.5 mm) at 60°C for 6

hours. After drying, it was carefully crushed by hand and stored in plastic bags in refrigerator until the exposure test (Gholamhosseini *et al.*, 2020).

Preparation of Vibrio harveyi bacteria

Vibrio harveyi with a collection number (PTCC: 1755) and Accession number (NCBI: Gu974342.1) in Gene Bank was isolated and registered from farmed shrimps in Bushehr province. It was selected as a pathogenic species in this experiment. Bacteria were first cultured in a TSA medium (with 2.5% salt for 24 hours at 30°C) to prepare a single colony. The colony was cultured in tubes containing 10 mL of TSB culture medium in shaker incubation. Tubes containing culture media were refrigerated and then centrifuge at 10,000 rpm for 10 minutes to precipitate the bacteria. The supernatant was removed from the culture medium and 10 mL of normal sterile saline was added to each tube and centrifuged again at 10,000 rpm for 10 minutes to remove the present bacterial and metabolic material in the medium. This step was repeated twice a day. Normal saline was added to the remaining sediment in the tubes, and adhering bacteria to the bottom of the tube were dissolved in normal saline by shaker. The obtained bacterial solution was poured into a sterile 100 mL Falcon tube and its concentration was determined by spectrophotometer and McFarland standard diagram. Then disease-causing concentration (3×108 cfu/mL) was determined according to the volume of water in each of the aquariums (Farjami *et al*., 2014).

Preparation of P. vannamei, adaptation, and experiments

One thousand shrimp with an average weight of 10±1 g were collected from culture ponds and were selected after determining quality and health factors. The shrimps were collected by a tank equipped with a diffused aeration system and transferred to the laboratory of the Shrimp Research Institute of Iran. For this experiment, 15 aquariums containing 100 liters of water with a salinity of 40 ppt were used for 5 treatments (positive control, negative control, concentrations 5, 100, and 150 µg mL^{-1}) with 3 replications. The inlet water was disinfected with 10 ppm chlorine after dewatering, storage, passage through a sedimentation pool and sand filter and then used in the experiment. Also, daily water change was done at 10 to 15% rate. The parameters of water, including temperature, pH, and oxygen, were measured and recorded twice a day (morning and evening) and salinity of water once a day (in the morning) by a multi-parameter device. At first the shrimps were adapted to laboratory condition for 3 to 5 days. After the adaptation phase presence of viruses and bacteria was examined by PCR. Shrimps were fed with foods containing methanol extract for 10 days according to specific treatments. The fed shrimps were then exposed to *V. harveyi* at the concentration of 3×108 CFU mL^{-1}. This was repeated for the treatments and the shrimps were kept for 20 days (Osińska *et al*., 2020).

Measurement of survival and growth rate of the shrimps

Shrimp survival percentage was calculated based on method of Zhou *et al*. (2009):

Mortality percentage= (Number of dead shrimp /Total number of shrimp)×100

Survival percentage=100 - Mortality percentage

Measurement of shrimp immunity factors

Clinical signs and mortality were recorded from the first day. Water parameters were recorded within 20 days and hemolymph and safety factors including PO (phenol oxidase), POD (peroxidase), THC (total haemocyte count), TPP (total plasma protein), and SOD (superoxide dismutase) were sampled on days 1, 3, 5, 9, 15, and 25 (Huang *et al*., 2012; Afsharnasab *et al*., 2016).

Data analysis

Normality of data was determined by the Kolmogorov-Smirnov test. Significant differences among treatments were tested by one-way analysis of variance (One-way ANOVA). Duncan's multiple range tests were used at a significant level of 0.05 to compare means. Statistical analysis of data was performed using SPSS version 21.0.

Results

Survival

Survival was 100% throughout the period in negative control treatment with optimal culture conditions. But mortality was observed from the third day of exposure in other treatments. The highest mortality was observed on the third day of exposure in positive control treatment with 33.56±1.81 and the lowest mortality on this day was observed in treatment 3 with 18.52±2.1. Mortality in treatments 2 and 3 stopped from the ninth day, but in treatment 1 continued until the 15th day, while mortality in the positive control treatment continued until the 25th day of the experiment (the last day of exposure). The highest survival until the last day of exposure was observed in treatment 3, which was significantly different from other treatments (p<0.05) (Table 1).

Phenol oxidase

Amounts of phenol oxidase (PO) in treatments 2 and 3 were significantly higher than that of other treatments from the first day of exposure (p<0.05). During the exposure period, the amount of PO in treatment 3 was significantly higher than that of other treatments (p<0.05). The amount of PO decreased from the ninth day of exposure to the end of the period in treatments 1, 2, and 3, so that there was no significant difference in the first and twenty-five days of exposure (p>0.05) (Table 2).

Table 1: Survival percentage of *P. vannamei* shrimp fed with methanol extract in different treatments, numbers after ± are standard deviation.

Survival rate	First day	Third day	Fifth day	Ninth day	fifteenth day	Twenty-fifth day
Negative contro	100±00 ^{a}	100±00 ^{a}	100±00 ^{a}	100±00 ^{a}	100±00 ^{a}	100±00 ^{a}
Positive control	100±00 ^{a}	66.44±1.81 ^{d}	48.89±1.81 ^{d}	37.04±2.1 ^{d}	35.56±1.81 ^{d}	34.07±2.1 ^{d}
Treatment 1	100±00 ^{a}	73.33±3.14 ^{c}	51.11±1.81 ^{c}	49.63±2.77 ^{c}	47.41±3.78 ^{c}	47.41±3.78 ^{c}
Treatment 2	100±00 ^{a}	79.26±2.77 ^{b}	56.3±4.57 ^{c}	51.85±3.78 ^{c}	51.85±3.78 ^{c}	51.85±3.78 ^{c}
Treatment 3	100±00 ^{a}	81.48±2.1 ^{b}	73.33±1.81 ^{b}	68.89±3.14 ^{b}	68.89±3.14 ^{b}	68.89±3.14 ^{b}

Different letters in columns show significant difference at 5% level among experimental treatments (p<0.05).

Table 2: PO results (U min^{-1}mL^{-1}) in *P. vannamei* shrimp fed with methanol extract in different treatments, numbers after ± are standard deviation.

PO	First day	Third day	Fifth day	Ninth day	fifteenth day	Twenty-fifth day
Negative control	330.45±12.13 c	335.65±16.52 c	342.85±11.44 d	334.39±13.99 c	340.95±12.57 b	333.61±12.5 c
Positive control	352.95±7.59 c	365.75±17.65 c	351.76±11.66 d	343.89±13.66 c	360.07±18.37 b	361.59±11.54 c
Treatment 1	353.43±12.98 c	434.49±15.26 b	437.29±15.42 c	435.31±10.25 a	363.62±14.01 b	351.08±18.3 c
Treatment 2	401.25±19.5 b	464.14±15.15 ab	457.37±14.03 b	397.4±14.03 b	407.4±17.56 b	389.67±18.55 b
Treatment 3	447.48±19.5 a	499.05±18.3 a	538.18±14.45 a	440.27±10.95 a	433.46±15.6 a	443.15±19.29 a

Different letters in columns show significant difference at 5% level among experimental treatments (p<0.05).

Peroxidase

The amounts of peroxidase (POD) enzyme on the first day of exposure in all three treatments 1, 2, and 3 were significantly higher than that of positive and negative controls (p<0.05). During the study period, the amount of this enzyme in treatment 3 was higher than that of other treatments. The lowest rate was observed in the positive control treatment on the third day until the end of the exposure period. The level of this enzyme had a decreasing trend from the ninth day of exposure in three treatments 1, 2, and 3 until the end of the period (Table 3).

Table 3: POD results (nmol min^{-1}mL^{-1}) in *P. vannamei* shrimp fed with methanol extract in different treatments, numbers after ± are standard deviation.

POD	First day	Third day	Fifth day	Ninth day	fifteenth day	Twenty-fifth day
Negative control	5.81±0.14 ^{c}	5.73±0.18 ^{b}	5.81±0.13 ^{c}	5.75±0.23 ^{c}	5.84±0.2 ^{bc}	5.88±0.21 ^{c}
Positive control	5.9±0.2 ^{bc}	5.36±0.1 ^{c}	5.17±0.26 ^{d}	5.09±0.26 ^{c}	5.52±0.29 ^{c}	5.75±0.14 ^{c}
Treatment 1	6.05±0.16 ^{a}	6.77±0.18 ^{a}	6.81±0.13 ^{b}	6.53±0.13 ^{b}	6.11±0.13 ^{b}	6.14±0.07 ^{b}
Treatment 2	6.09±0.1 ^{a}	6.91±0.05 ^{a}	7.19±0.3 ^{a}	6.93±0.16 ^{a}	6.11±0.3 ^{c}	6.14±0.17 ^{b}
Treatment 3	6.08±0.09 ^{a}	6.97±0.19 ^{a}	6.88±0.15 ^{b}	6.92±0.14 ^{a}	6.73±0.06 ^{ca}	6.66±0.07 ^{a}

Different letters in columns show significant difference at 5% level among experimental treatments (p<0.05).

Total haemocyte count

The results showed that the amounts of total haemocyte count (THC) in treatments 1, 2, and 3 from the first day of exposure were significantly higher than that of negative control and positive control treatments (p<0.05). THC level in treatment 3 had a significant difference with that in treatments 1 and 2 (p<0.05). During the exposure period,

the amount of THC in treatment 3 was significantly higher than that of other treatments except treatment 2 on the fifth, ninth and twenty-fifth days (p<0.05) (Table 4).

Table 4: THC results (×10^5 cell ml^{-1}) in *P. vannamei* shrimp fed with methanol extract in different treatments, numbers after ± are standard deviation.

THC	First day	Third day	Fifth day	Ninth day	fifteenth day	Twenty-fifth day
Negative control	21.07±0.87 ^{c}	20.96±1.01 ^{bc}	19.1±0.62 ^{c}	21.81±0.42 ^{b}	21.17±1.24 ^{b}	21.1±0.83 ^{b}
Positive control	21.64±1.27 ^{c}	14.31±0.79 ^{c}	13.1±0.65 ^{c}	11.25±0.65 ^{c}	11.81±0.46 ^{a}	15.25±1.03 ^{c}
Treatment 1	25.29±4.74 ^{b}	28.85±4.68 ^{ab}	33.1±6.38 ^{b}	25.59±4.81 ^{b}	26.65±5.79 ^{b}	24.55±4.57 ^{ab}
Treatment 2	27.4±5.89 ^{b}	33.34±5.69 ^{b}	36.59±5.32 ^{a}	34.28±5.32 ^{a}	27.66±6.27 ^{b}	28.43±6.22 ^{ab}
Treatment 3	30.86±5.09 ^{a}	37.98±6.49 ^{a}	37.34±6.32 ^{a}	36.11±7.14 ^{a}	31.48±5.8 ^{a}	31.04±7.31 ^{a}

Different letters in columns show significant difference at 5% level among experimental treatments (p<0.05).

Total plasma protein

Total plasma protein (TPP) levels were significantly different from the beginning of exposure (except in positive and negative control treatments) and the highest TPP levels were observed in treatments 3, 2, and 1, respectively. This difference was observed until the third day of exposure, but no significant difference was observed between treatments 2 and 3 from the fifth day (p>0.05). These two treatments were significantly different from other treatments (p<0.05) (Table 5).

Table 5: TPP results (mg/m) in *P. vannamei* shrimp fed with methanol extract in different treatments, numbers after ± are standard deviation.

TPP	First day	Third day	Fifth day	Ninth day	fifteenth day	Twenty-fifth day
Negative control	38.15±2 ^{d}	37.37±2.28 ^{c}	37.39±1.6 ^{c}	37.96±2.99 ^{c}	38.08±0.97 ^{c}	38.62±1.79 ^{d}
Positive control	36.55±2.41 ^{d}	25.69±1.89 ^{c}	22.83±0.94 ^{d}	23.48±0.94 ^{c}	26.55±1.04 ^{c}	27.88±0.72 ^{e}
Treatment 1	58.26±5.85 ^{bc}	65.87±5.55 ^{b}	83.26±3.8 ^{b}	62.93±12.38 ^{b}	63.53±11.73 ^{b}	59.84±2.31 ^{c}
Treatment 2	65.73±11.98 ^{b}	84.95±13.62 ^{a}	91.51±4.45 ^{a}	88.28±4.45 ^{a}	70.1±3.4 ^{a}	70.38±3.76 ^{b}
Treatment 3	73.2±3.61 ^{a}	93.67±14.8 ^{a}	92.16±5.15 ^{a}	88.78±13.47 ^{a}	74.78±4.12 ^{a}	73.65±9.16 ^{a}

Different letters in columns show significant difference at 5% level among experimental treatments (p<0.05).

Superoxide dismutase

There were significant differences in superoxide dismutase (SOD) levels from the first day of exposure among treatments (p<0.05). During the period, the amount of SOD in treatments 2 and 3 was not significantly different on the third day of exposure (p>0.05), but on other days all treatments were significantly different from each other (p<0.05). The amount of SOD was the highest in treatment 3 (Table 6).

Table 6: SOD results (Activity U ml^{-1}) in *P. vannamei* shrimp fed with methanol extract in different treatments, numbers after ± are standard deviation.

SOD	First day	Third day	Fifth day	Ninth day	fifteenth day	Twenty-fifth day
Negative control	539.19±7.45 ^{d}	533.84±5.18 ^{d}	539.93±5.16 ^{c}	534.64±6.06 ^{d}	536.4±8.58 ^{d}	537.16±8.42 ^{d}
Positive control	396.71±13.38 ^{e}	364.47±15.26 ^{e}	353.57±12.98 ^{d}	347.78±12.98 ^{e}	377.9±12.85 ^{e}	395.44±5 ^{e}
Treatment 1	1026.21±16.73 ^{c}	1167.09±21.57 ^{c}	1473.35±21.73 ^{b}	1118.55±35.98 ^{c}	1128.26±25.97 ^{c}	1059.22±17.29 ^{c}
Treatment 2	1158.57±32.98 ^{b}	1493.93±31.74 ^{b}	1605.77±27.87 ^{a}	1554.33±27.78 ^{b}	1241.33±16.5 ^{b}	12.49.7±28.87 ^{b}
Treatment 3	1288.46±33.2 ^{a}	1663.21±37.07 ^{a}	1648.76±33.72 ^{a}	1598.67±26.91 ^{a}	1327.17±38.39 ^{a}	1320.52±38.21 ^{a}

Different letters in columns show significant difference at 5% level among experimental treatments (p<0.05).

Discussion

In this study, the antibacterial effect of methanol extracts of sea cucumber, *H. parva*, on *V. harveyi* in *P. vannamei* shrimp was investigated. Limited research is done on the antibacterial properties of sea cucumber on this bacterium and most of them confirmed the antibacterial properties of sea cucumber extracts. According to Abraham *et al*. (2002), different levels of *Actinopyga miliaris*, *Holothuria (Halodeima) atra*, and *H. (Metrialtyla) scabra* extracts have an inhibitory effect on *Escherichia coli*, *Enterococcus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Staphylococcus aureus*, but these extracts were not effective on *Bacillus*. In the study of Mokhlesi *et al*. (2011), no antimicrobial effect was observed of ethyl acetate, methanol, and water-methanol extracted from internal organs, salivary fluid, and cell wall of *Bohadschia marmorata*. Farjami *et al*. (2014) showed that methanol extract of *Holothuria (Mertensiothuria) leucospilota* did not affect *Escherichia coli*, but chloroform and hexane extracts prevent bacterial growth at concentrations of 2 and 5 mg/mL. Shadi and Oujifard (2019) showed that ethanolic and methanol extracts of *H. parva* have inhibitory effect against *Escherichia coli*, *Vibrio alginolyticus*, and *Staphylococcus aureus*. Sea cucumber has an innate immune system that is a potential source for discovery of antimicrobial peptides. As a result, sea cucumber is considered a rich source of compounds with antimicrobial properties, which is a good option for the synthesis of medical, pharmaceutical, and antibiotic compounds (Shakouri *et al*., 2017). The antibacterial effect of sea cucumber extracts may be due to accumulation of several bioactive compounds. Based on effect of the extracts, inhibitory action of the membrane and the bacterial wall is weakened and causes the extract to penetrate the bacterial cell and disintegrate (Farjami *et al*., 2014).

Phenol oxidase (PO) is one of the enzymes evaluated in this study. Methanol extract of sea cucumber activated the pro PO system and the amount of PO in T3 increased in shrimp hemolymph during the fifth day. The propene oxidase system (pro PO) is an important component of shrimp defense system (Ayiku *et al*., 2020). This system plays a very important role in identifying non-indigenous components (Liu *et al*.,

2020). Phenol oxidase (PO) is one of the most important enzymes that is created during activation of the pro PO system and plays a key role in the process of melanization due to exposure to particles or foreign pathogens (Hernández-López *et al*., 1996; Liu *et al*., 2020). Activation of this enzyme is done by a number of immune stimulants including peptides and glycans (Cerenius and Söderhäll, 2004), beta-glucans (Cerenius and Söderhäll, 2004; Bai *et al*., 2014) and lipopolysaccharides (Lorenzo *et al*., 1999; Takahashi *et al*., 2000). These results are not consistent with those of the study of Chen *et al*. (2015). They found that using *Agarophyton tenuistipitatum* extract activated some PO-like immune factors in white leg shrimp, but their levels are decreased under stress.

Another effective factor is peroxidase enzyme (POD) in plasma, which is strongly bound to oxygen-free radical molecules (ROS) and is one of the most important defense reactions against invasion of foreign particles during phagocytosis (Le Moullac and Haffner, 2000). POD had the highest amount in T2 during the fifth day. POD plays a very important role in oxygen poisoning and immune defense in shrimp (Liu *et al*., 2016). Yan *et al*. (2010) reported that peroxidase is specifically present in certain organs such as liver, heart, and gills. POD is also present in lipids around the liver. Researchers found that infected shrimp also had POD levels in liver, heart, and gills, but it was decreased in adipose tissue around the liver. In the present study, the amount of POD increased in the main cells of this enzyme with the use of methanol extract, which is consistent with the results of Yan *et al*. (2010) and Wang *et al*. (2012). Plasma peroxidase activity is closely related to ROS in phagocytosis that eliminates pathogens (Le Moullac *et al*., 1998; Le Moullac and Haffner, 2000). This enzyme also has a very sensitive role in shrimp body defense and shrimp immune system and is very effective in disinfection (Li *et al*., 2016). Peroxidase-containing organelles contain large numbers of oxidase, POD, and catalase molecules that can degrade cells from harmful compounds such as H_2O_2 by oxidation reactions to water and oxygen cells (Wang *et al*., 2012; Laguerre *et al*., 2020).

In the present study, the level of hemocytes (THC) significantly increased in treatment 3 of shrimps fed with methanol extract in the presence of *V. harveyi* on the third day. This result means that lymph cell-producing tissues are stimulated and amount of lymph cells in the shrimp is increased. Hemocytes play an important role in shrimp safety and the amount of hemocytes in bloodstream is one of the determining factors in shrimp health (Xiong *et al*., 2016; Huang *et al*., 2018). In a study by Cheng *et al*. (2004), glucan-like polysaccharides in the walls of fungi, yeasts, and sodium alginate had positive effects on prevention of vibriosis and white spot in shrimp. Total hemocytes in *P. vannamei* shrimp increased significantly after 10 days of feeding with *Saccharomyces cerevisiae* yeast so that the highest amount of THC

for T1 treatment was recorded on day 25 of the experiment. The results of this study are consistent with the results of Balasubramanian *et al*. (2007), Lin *et al*. (2011), and Wongprasert *et al*. (2014) on *Gracilaria* algae.

Total plasma protein (TPP), similar to total hemolymph (THC), is a determining factor in shrimp health and changes with shrimp health (Yoganandhan *et al*., 2003). In the present study, the amount of TPP in treatment 3 (on the third day) of shrimp fed with methanol extract was higher than that in other treatments. According to Chen and Cheng (1993), changes in TPP levels in treatment depend on size, age, sex, nutritional status, and environmental conditions. Total plasma protein is one of the most important defense parameters in shrimp. Because a large number of receptor recognition patterns are similar to the gram-negative bacteria-binding protein, lipopolysaccharides or beta 1 and 3 glucan protein binders, and fibrinogen-related proteins found in the hemolymph and hepatopancreas and are related to TPP (Wang and Wang, 2013). It seems that the increase in TPP in hemolymph is due to consumption of methanol extract, which increases PRPS in hemolymph. This factor can be effective in protecting shrimp against this bacterial disease. Total plasma protein levels are considered as a determinant of shrimp health (Vogan and Rowley, 2001; Yoganandhan *et al*., 2003). According to Wang and Wang (2013), increase in TPP was due to an increase in pattern recognition receptors (PRPS) such as peptidoglycan recognition proteins (PGRPs), gram-negative binding proteins (GNBPs), and Lipopolysaccharide binding protein (LBP). These proteins can be isolated in the hemolymph or hepatopancreas. Maftuch *et al*. (2013) reported that increase in TPP was due to injected *Vibrio alginolyticus* outer membrane proteins into black tiger prawns (10 to 30 micrograms per body weight). According to a study by Subramanian and Philip (2013), levels of alkaline phosphatase, TPP, and THC enzymes in beta-glucan-fed shrimp increased and these factors led to greater survival in shrimp.

Superoxide dismutase (SOD) is another effective enzyme in shrimp's defense reactions, which occurs extensively in aerobic and anaerobic tissues (Mohankumar and Ramasamy, 2006; Yan *et al*., 2010; Feng *et al*., 2020). This enzyme is also evaluated in health of the shrimp. The amount of SOD in treatment 3 (on the third day) of shrimp fed with methanol extract had the highest amount compared to other treatments. The results of Chang *et al*. (2003) and Pacheco *et al*. (2011) were consistent with the results of the study and showed that use of immune stimulants increased SOD in shrimp. Activated oxygen species including O_2, H_2O_2, OH, and $1O_2$, are formed in shrimp and played an important role in shrimp safety (Wu *et al*., 2015). Rapid and effective removal of reactive oxygen species is necessary for shrimp to survive. This activity is performed in shrimp with an antioxidant system that

includes SOD (Zhang *et al*., 2007; Feng *et al*., 2020).

The results showed that feeding with food containing methanolic extract before exposure to bacteria stimulated and increased the immune factors of shrimp hemolymph. So that, the amounts of PO, POD, THC, and SOD were more than that in negative and positive control treatments from the first day of exposure. Stimulation of the immune system by bacteria caused an increase in irritability, immune factors, and survival rate of shrimps exposed to *Vibrio harveyi*. The amount of methanolic extract was higher in treatments 2 and 3 and mortality rate was stopped from the ninth day. The level of immune factors also decreased due to the effect of vibriosis in these treatments. Mortalities were observed in the positive control treatment until the end of the exposure period, and survival rate of this treatment decreased with increasing the length of the exposure period. According to the results, survival was directly related to the concentration of methanolic extract. The highest increase in safety factors was observed in treatment 3, which had a concentration of 150 µg/mL methanolic extract of sea cucumber.

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