ISSN:2521-0912 (Print)
ISSN: 2521-0513 (Online)

Contents List available at RAZI Publishing

Journal CleanWAS

Journal Homepage: http://www.razipublishing.com/journals/journal-cleanwas/

https://doi.org/10.26480/jcleanwas.01.2017.26.29

Immunomodulatory Effect of Alginic Acid from Brown Seaweed Sargassum Wightii on Disease Resistance in Penaeus Monodon

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ARTICLE DETAILS

Article history:

Received 27 September 2016
Accepted 13 December 2016
Available online 10 January 2017

Keywords:

Alginic acid, Sargassum wightii, Penaeus monnodon, Propheoloxidase, NBT assay, Superoxide dismutase, Vibrio aemolyticus

ABSTRACT

In recent days, bacterial diseases are very common among all the aquatic organisms. Particularly in grow out ponds they cause heavy loss in aquaculture. To overcome this problem, the use of antibiotics become a common practice, thus leads to the development of antibiotic resistant strains. In this regard, a new attempt has been made to study the immunostimulatory effect of alginic acid, a sulphated polysaccharide derived from brown seaweed Sargassum wightii, on the disease resistance in Penaeus monodon. The immunological parameters, such as Total Haemocyte Count (THC), Prophenoloxidase activity, Respiratory burst (NBT assay), Superoxide dismutase activity and Phagocytic activity were monitored after challenging against Vibrio parahaemolyticus. Before the challenge study, the test animals were fed with different concentrations (1,2,3 g/kg as A1, A2, A3) of alginic acid fed diet for 45 days. All the immunological parameters showed a significant increase with an increasing concentration of alginic acid, in the test animals compared to the control throughout the experimental study period.

1. INTRODUCTION

Among aquaculture practices, shrimp farming plays a major role and it fetches high economy in certain countries. However, there was a sharp decline in shrimp production during the 1003 due to disease outbreak, resulting in two third reductions [6]. It has been estimated that various diseases continued to lose shrimp farming industries worldwide about US$ 3 billion per year, bringing down world shrimp production by 40% [9,15]. Vibrio has been implicated as the main bacterial pathogen of shrimp [3,1]. During the past fifteen years, commercial shrimp farming based on local species such as Penaeus monodon, Marsupenaeus japonicus and the exotic species Littopenaeus vannamei have suffered with disease outbreaks associated with bacteria like Vibrio alginolyticus [13], V. parahaemolyticus [19]and V. vulnificus [18].

Vibriosis is found to be deadliest disease in shrimp aquaculture, which causes mass mortality in both hatcheries and grow-out ponds [17]. Several strategies have been used to control these bacterial diseases, the use of antibiotics become the most common practice in India [21]. This resulted in the development of antibiotic resistant strains and loss of efficacy of antibiotic treatments [12].

Recent studies focused on the utilization of marine seaweeds and seaweed polysaccharides as therapeutic agents as well as making antibiotic drugs. Fucoidan, a marine sulphated polysaccharide from seaweed appear to inhibit macrophage function in shrimps [23]. Carrageenan, a polysaccharide abundant in certain red seaweeds, induced an increase in macrophage phagocytic activity and in the resistance against bacterial infections after being injected intra-peritoneally in carp (Cyprinus carpio). Chotigeat et al. [5] reported that the protective effect of fucoidan extracted from Sargassum polycystum on disease resistance of black tiger shrimp P. monodon against WSSV. Sodium alginate extracted from Undaria pinnatifida and Macrosystis pyrifera was found to enhance the non-specific defence system of common carp (C. carpio) and its resistance against Edwardsiella tarda [7]. Sodium alginate extracted from brown algae U. pinnatifida and Lessonia nigrecans have been reported to increase the resistance of L. vannamei against V. alginolyticus [4]. Ergosan, an algal extract containing alginic acid was also observed to increase the non-specific immune response in Channa striata [21], O. mykiss and D. labrose [2]. Considering the importance of the above, the present study was undertaken to study the immunostimulatory effect of alginic acid extracted from brown seaweed S. wightii on P. monodon against Vibrio parahaemolyticus infection.

2.0 Materials and methods

The alginic acid was extracted from the collected brown seaweed (S. wightii) following the methodology of. The extracted alginic acid was added individually and the test diets were prepared at various concentrations such as 0.1, 0.2 and 0.3% respectively. For feeding experiment, uniform size of P. monodon postlarvae at stage PL30 were selected from the acclimatized stock which were maintained before the experiment was conducted.

The PL were transferred into individual experimental tanks (control - unsupplemented pellet feed; alginic acid with respective concentrations of 1,2 and 3g/Kg diet supplemented pellet feed). An ad libitum feeding regime was applied to all tanks throughout the experiment, and the food (pellet feed) amount was adjusted 3 times a day. Control group was fed with unsupplemented pellet feed. The feeding experiment was prolonged for a period of 45 days.

The pathogen V. parahaemolyticus was isolated from the diseased shrimp and it was cultured on TSA plates supplemented with 2.5% NaCl for 24 h at 25˚C. Then the culture was transferred into 10 ml of TSB supplemented with 2% NaCl and the broth was incubated at 25˚C for 24 h as stock culture for the experiment. The broth cultures were centrifuged at 7155 ×g for 15 min at 4˚C. The supernatant fluids were removed and the bacterial pellets were resuspended in saline solution at 1×107 and 2×108 CFU ml-1 as stock bacterial suspension for challenge experiment.

The challenge test was conducted by the injection of 20 µl bacterial suspension (1×107 CFU ml-1) resulting in 2×105 CFU shrimp-1 into the ventral sinus of the cephalothorax. The shrimps that received no alginic acid and then received 2 ×105 CFU shrimp-1 served as challenged control. The shrimp that received no alginic acid and then received saline (20 µl) served as the unchallenged control. Before the injection of V. parahaemolyticus (0 days), the immunological parameters were analysed in each group of shrimps. Subsequently, after the challenge experiment the immunological parameters were again analyzed on 10th and 21st days interval.

The immunological parameters such as Total Haemocyte Count (THC), Prophenoloxidase activity (PO), Superoxide anion activity (NBT assay), Superoxide dismutase activity (SOD) and Phagocytic activity were analyzed by following the standard procedures.

3.0 Results

3.1 Total Haemocyte Count (THC)

The THC count of the control and experimental groups of shrimps after challenged with V. parahaemolyticus is given in Table 1 and Fig. 1. After feeding experiment (45 days), THC in the control group was 51.9×105 cells ml-1. The THC count increased with increasing concentrations of alginic acid. For instance, at the lowest concentration (1g/kg feed), the THC observed was 65.9×105 cells ml-1, whereas it was 73.8 and 83.9 ×105 cells ml-1 in 2 and 3 g/kg alginic acid supplemented diet fed groups respectively. After challenge experiment, the THC was decreased in the control group,

was 48.8×105 cells ml-1, at the same time in the experimental groups, the THC was significantly increased to 67.1, 75.2 and 84.6×105 cells ml-1, respectively in 1,2 and 3g/kg of alginic acid supplemented fed groups. At the end of the challenge study, in both the control as well as the experimental groups, the THC was reduced to 47.3, 66.3, 74.4 and 84.2×105 cells ml-1 respectively.

The two way ANOVA revealed that the THC of P. monodon after challenged with V. parahaemolyticus as a function of variation due to the control and various concentration of alginic acid supplemented diets fed groups was statistically more significant (F = 463.82; P<0.0001), whereas the variation due to different days of intervals of challenge was statistically non-significant (F = 0.6777; P>0.05) (Table 2).

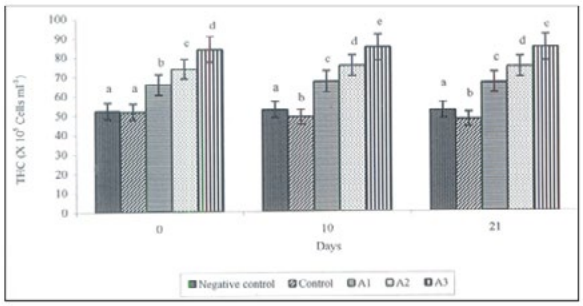

Fig. 1. THC of P. monodon fed on various concentrations of alginic acid supplemented feed after challenged against V. parahaemolyticus in 21 days interval

Table 1. THC (× 105 ml-1) of P. monodon fed on various concentrations of alginic acid supplemented diets during challenge experiment with V. parahaemolyticus in 21 days interval

	Experimental groups	THC (× 10^5 ml^{-1}) 0 days	10 days	21 days
1	NC	52.3±0.32^a	52.6±0.36^a	52.1±0.28^a
2	C	51.9±0.28^a	48.8±0.24^b	47.3±0.22^b
3	A1	65.9±0.42^b	67.1±0.51^c	66.3±0.46^c
4	A2	73.8±0.38^c	75.2±0.46^d	74.4±0.38^d
5	A3	83.9±0.28^d	84.6±0.52^e	84.2±0.42^e

Each value is a Mean ± S.D of three replicate analysis; within each column, means with different superscript letters are statistically significant (t-test, P<0.05 and subsequently post hoc multiple comparison with SNK test)

Table 2. Two way ANOVA for THC of P. monodon during challenge experiment with V. parahaemolyticus as a function of variation between various concentrations of alginic acid as well as variation between different days interval

Sources of variation	SS	df	MS	F-value	P-value
Total variance	2613.616	14	-	-	-
Variance due to control and conc. Of alginic acid	2600.503	4	650.12	463.82	P<0.0001*
Variance due to days intervals	1.9	2	0.95	0.6777	P>0.05**
Error variance	11.2133	8	1.4016	-	-

*P<0.05 - statistically significant; *P>0.05 - statistically non-significant

3.2 Prophenoloxidase activity (PO)

The PO activity of the control and experimental groups is given in Table 3 and Fig. 2. At the beginning of the challenge study (0 days) the PO activity of the control group was 0.1426 (OD-Optical Density), whereas it increased (0.1163 to 0.1745 OD) in the experimental groups fed with various concentrations (1-3g/kg) alginic acid supplemented diets. As the duration of the challenge experiment was increased, the PO activity increased positively in both the control and the test groups. Similar trend was observed at the end of the study.

Two way ANOVA test revealed that the PO activity of P. monodon after challenged with V. parahaemolyticus as a function of variation due to control and various concentrations of alginic acid supplemented diets fed groups was statistically more significant (F = 100.46; P<0.0001), similarly the variation due to various challenge duration was also statistically significant (F = 11.21; P<0.01) (Table 4).

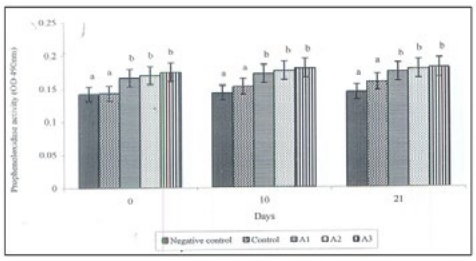

Fig. 2. Prophenoloxidase activity (OD) of *P. monodon* fed on various concentrations of alginic acid supplemented feed after challenged against *V. parahaemolyticus* in 21 days interval

Table 3. Prophenoloxidase activity (490 nm) of *P. monodon* fed on various concentrations of alginic acid supplemented diets during challenge experiment with *V. parahaemolyticus* in 21 days interval

S. No	Experimental groups	Prophenoloxidase (OD 490 nm) 0 days	10 days	21 days
1	NC	0.1420±0.0021^a	0.1428±0.0023^a	0.1433±0.0018^a
2	C	0.1426±0.0016^a	0.1463±0.0020^a	0.1498±0.0026^a
3	A1	0.1663±0.0024^b	0.1715±0.0024^b	0.1739±0.0032^b
4	A2	0.1698±0.0022^b	0.1760±0.0032^b	0.1785±0.0036^b
5	A3	0.1745±0.0032^b	0.1798±0.0028^b	0.1805±0.0028^b

Each value is a Mean ± S.D of three replicate analysis; within each column, means with different superscript letters are statistically significant (t-test, P<0.05 and subsequently post hoc multiple comparison with SNK test)

Table 4. Two way ANOVA for Prophenoloxidase of P. monodon during challenge experiment with V. parahaemolyticus as a function of variation between various concentrations of alginic acid as well as variation between different days interval

Sources of variation	SS	df	MS	F-value	P-value
Total variance	0.0031	14	-	-	-
Variance due to control and conc. Of alginic acid	0.0029	4	0.0007	100.46	P<0.0001*
Variance due to days intervals	0.0001	2	8.21E-05	11.21	P<0.01*
Error variance	5.85E-05	8	7.31E-06	-	-

*P<0.05 - statistically significant

3.3 Respiratory burst activity (NBT assay)

The NBT assay of the control and the experimental groups were given in Table 5 and Fig. 3. At the beginning (0 day), the NBT of the control group was 0.0380, whereas it increased to 0.0439, 0.0546 and 0.0633 OD with increasing concentrations of alginic acid supplemented diet fed groups. As the experiment was prolonged, the respiratory burst activity increased in the test groups, but the trend was dissimilar in the control groups.

For instance, it increased from 0.0466 to 0.0679 OD and 0.0493 to 0.0698 OD during the 10th and 21st days in the experimental groups with the diets of concentration 1-3g/kg respectively. Two way ANOVA test revealed that the NBT assay of P. monodon after challenged with V. parahaemolyticus as a function of variation due to control and various concentrations of alginic acid supplemented diets fed groups was statistically more significant (F = 106.53; P<0.0001), but the variation due to different days intervals was statistically non-significant (F = 1.21; P>0.05) (Table 6).

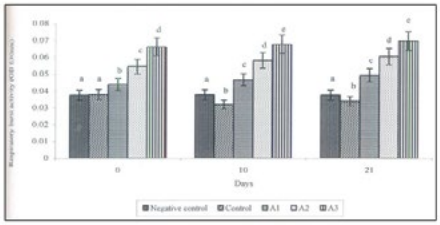

Fig. 3. NBT assay (OD) of *P. monodon* fed on various concentrations of alginic acid supplemented feed after challenged against *V. parahaemolyticus* in 21 days interval

Table 5. NBT assay (490 nm) of *P. monodon* fed on various concentrations of alginic acid supplemented diets during challenge experiment with *V. parahaemolyticus* in 21 days interval

S. No	Experimental groups	NBT assay (OD 630 nm) 0 days	10 days	21 days
1	NC	0.0375±0.0004^a	0.0378±0.0008^a	0.0376±0.0006^a
2	C	0.0380±0.0008^a	0.0320±0.0004^b	0.0340±0.0007^b
3	A1	0.0439±0.0010^b	0.0466±0.0012^c	0.0493±0.0010^c
4	A2	0.0546±0.0008^c	0.0582±0.0010^d	0.0605±0.0008^d
5	A3	0.0663±0.0006^d	0.0679±0.0009^e	0.0698±0.0012^e

Each value is a Mean ± S.D of three replicate analysis; within each column, means with different superscript letters are statistically significant (t-test, P<0.05 and subsequently post hoc multiple comparison with SNK test)

Table 6. Two way ANOVA for NBT assay of P. monodon during challenge experiment with V. parahaemolyticus as a function of variation between various concentrations of alginic acid as well as variation between different days interval *P<0.05 - statistically significant; *P>0.05 - statistically non-significant

Sources of variation	SS	df	MS	F-value	P-value
Total variance	0.0023	14	-	-	-
Variance due to control and conc. Of alginic acid	0.0023	4	0.0005	106.53	P<0.0001*
Variance due to days intervals	1.33E-05	2	6.64E-06	1.212	P>0.05**
Error variance	4.38E-05	8	5.48E-06	-	-

3.4 Superoxide dismutase (SOD) activity The SOD activity of the control and experimental groups of shrimps challenged with V. parahaemolyticus is given in Table 7 and Fig. 4. In the control group, 35.74 Unit/ml of SOD was observed on 0 day, whereas in the experimental groups, the SOD activity increased with increasing concentration of alginic acid. It ranged from 52.14 to 58.97 Unit/ml, respectively in 1-3g/kg of alginic acid supplemented diet fed groups.

As the duration of the challenge experiment was increased, the SOD activity decreased in the control group (34.96 and 33.87 unit/ml in10th and 21st days). It increased in the experimental groups with increasing concentrations (i.e. 54.63 to 58.94 Unit/ml during 10th day and 55.73 to 60.66 Unit/ml during 21st day in 1-3g/kg of alginic acid diet fed groups). Two way ANOVA test revealed that the SOD assay of P. monodon after challenged with V. parahaemolyticus as a function of variation due to control and various concentrations of alginic acid supplemented diets fed groups was statistically more significant (F = 288.85; P<0.0001) but the variation due to the different time intervals was statistically non-significant (F = 1.911; P>0.05) (Table 8).

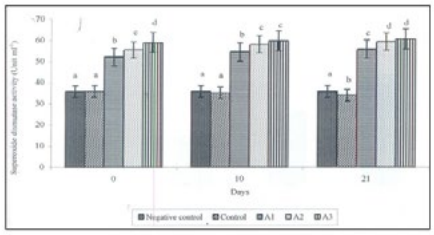

Fig. 4. SOD activity (Unit/ml^{-1}) of *P. monodon* fed on various concentrations of alginic acid supplemented feed after challenged against *V. parahaemolyticus* in 21 days interval

Table 7. SOD activity (Unit ml^{-1}) of *P. monodon* fed on various concentrations of alginic acid supplemented diets during challenge experiment with *V. parahaemolyticus* in 21 days interval

S. No	Experimental groups	SOD activity (Unit ml^{-1})		
		0 days	10 days	21 days
1	NC	35.66±0.32^a	35.70±0.28^a	35.72±0.24^a
2	C	35.74±0.28^a	34.96±0.34^a	33.87±0.18^b
3	A1	52.14±0.34^b	54.63±0.32^b	55.73±0.30^c
4	A2	55.43±0.42^e	58.12±0.42^e	59.42±0.38^d
5	A3	58.97±0.46^d	59.84±0.40^e	60.66±0.44^d

Each value is a Mean ± S.D of three replicate analysis; within each column, means with different superscript letters are statistically significant (t-test, P<0.05 and subsequently *post hoc* multiple comparison with SNK test)

Table 8. Two way ANOVA for SOD activity of *P. monodon* during challenge experiment with *V. parahaemolyticus* as a function of variation between various concentrations of alginic acid as well as variation between different days interval

Sources of variation	SS	df	MS	F-value	P-value
Total variance	1801.12	14	-	-	-
Variance due to control and conc. Of alginic acid	1782.91	4	445.72	288.85	P<0.0001*
Variance due to days intervals	5.8980	2	2.949	1.911	P>0.05**
Error variance	12.34	8	1.5431	-	-

*P<0.05 - statistically significant; *P>0.05 - statistically non-significant

3.5 Phagocytic activity

The phagocytic activity of the control and the experimental groups is given in Table 9 and Fig. 5. At the beginning of the challenge experiment, the phagocytic activity of the experimental group was higher (6.40 to 6.98% in 1-3g/kg diet fed groups) than the control (5.35%). Subsequently, the challenge duration prolonged, the activity decreased in both the control as well as the experimental groups.

For instance, at the 10th day, the activity in the control group was only 4.22%, whereas it was found to be 6.12 to 6.72% in the experimental group with concentrations of 1-3g/kg. Similarly at the end of the experiment (21st day), it decreased to 3.45% in the control and 5.96 to 6.41% in the experimental groups.

Two way ANOVA test revealed that the phagocytic activity of P. monodon after challenged with V. parahaemolyticus as a function of variation due to control and various concentrations of alginic acid supplemented diets fed groups was statistically significant (F = 22.26; P<0.001), similarly the variation due to different days intervals was also statistically significant (F = 4.76; P<0.05) (Table 10).

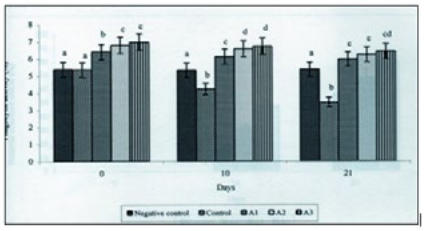

Fig. 5. Phagocytic activity (%) of *P. monodon* fed on various concentrations of alginic acid supplemented feed after challenged against *V. parahaemolyticus* in 21 days interval

Table 9. Phagocytic activity (Unit ml^{-1}) of *P. monodon* fed on various concentrations of alginic acid supplemented diets during challenge experiment with *V. parahaemolyticus* in 21 days interval

S. No	Experimental groups	Phagocytic activity (%)		
		0 days	10 days	21 days
1	NC	5.38±0.018^a	5.32±0.020^a	5.36±0.026^a
2	C	5.35±0.012^a	4.22±0.018^b	3.45±0.016^b
3	A1	6.40±0.022^b	6.12±0.026^c	5.96±0.032^c
4	A2	6.79±0.026^e	6.57±0.032^d	6.23±0.036^e
5	A3	6.98±0.030^c	6.72±0.024^d	6.41±0.028^{cd}

Each value is a Mean ± S.D of three replicate analysis; within each column, means with different superscript letters are statistically significant (t-test, P<0.05 and subsequently post hoc multiple comparison with SNK test)

Table 10. Two way ANOVA for phagocytic activity of P. monodon during challenge experiment with V. parahaemolyticus as a function of variation between various concentrations of alginic acid as well as variation between different days interval

Sources of variation	SS	df	MS	F-value	P-value
Total variance	13.6740	14	-	-	-
Variance due to control and conc. Of alginic acid	11.4243	4	2.8560	22.26	P<0.001*
Variance due to days intervals	1.2236	2	0.6118	4.76	P<0.05*
Error variance	1.0261	8	0.1282	-	-

*P<0.05 - statistically significant

4.0 Discussion

Bacterial and viral are considered to be a major threat to shrimp larviculture [22] and fish hatcheries [8] worldwide. It is responsible for serious losses, which consequently result in severe economic loss [16]. The infection in shrimp may occur via contaminated water and by ingestion of pathogens infected shrimp meat [20]. To solve this problem, several studies were carried out to treat such diseases with marine natural products which are having immunomodulatory effect. Among the products, seaweed based alginates; fucoidans and alginic acid are found to be excellent sources. In the present study, the immonumodulatory effect of alginic acid derived from S. wightii on P. monodon against shrimp bacterial pathogen V. parahaemolyticus was tested.

In the present study, the immunological parameters were analysed after challenge experiment with V. parahaemolyticus. The THC of the experimental groups significantly increased than the control after the challenge experiment. THC in the control group was 51.9×105 cells ml-1. But the haemocyte content increased with increasing concentrations of alginic acid. Similarly, Hou and Chen [10] pointed out that the effect of hot water extract from Gracillaria tenuistipitata on THC of white shrimp Litopenaeus vannamei challenged with V. alginolyticus. They postulated that the THC of L. vannamei that received hot water extract of G. tenuistipitata at 4 and 6 µg/g was significantly higher than that of the shrimps that received saline. Huang et al. [11] reported that the effect of Sargassum fusiform polysaccharide extract on the THC of Fenneropenaeus chinensis after challenged with V. harveyii. They pointed out that the THC of the experimental group was progressively elevated with the dietary supplementation of SFPSE increasing from 0.1% to 2%. The THC of the 2% treatment group was significantly higher than that of the control (P<0.01).

The prophenoloxidase activity (PO) of the experimental groups

significantly increased than the control after the challenge experiment in the present examination. At the beginning of the challenge experiment (0 days), the PO activity of the control group was 0.1426 (OD), whereas it was increased in the experimental groups fed with various concentrations of alginic acid supplemented diets. At the end of the challenge study (21st day) again the PO activity gradually increased. Similarly, Cheng et al. [4] reported that the effect of sodium alginate on the PO activity of white shrimp L. vannamei after challenged with V. alginolyticus. They reported that the PO activity of the shrimp that received sodium alginate at 20 and 50 µg / g after 1 day was significantly higher than that of the shrimps that received saline after 2 days. However, no significant difference in PO activity was observed among the five treatments after 4 to 6 days. Huang et al. [11] reported that the effect of S. fusiform polysaccharide extract on the PO activity of F. chinensis after challenged with V. harveyii. The author reported that the PO activity of the haemolymph was also affected by the dosage of dietary SFPSE. The PO activity of the 0.5% SFPSE group was significantly higher than that of the control ($P<0.05$). However the PO activity was lower in the higher concentrations.

In the present study, the respiratory burst activity of experimental group significantly increased than the control after the challenge experiment. At the beginning of the experiment (0 day), the respiratory burst activity of the control was 0.0380, whereas it increased in different concentrations of alginic acid supplemented diet fed groups. As the challenge experiment was increased the respiratory burst activity was also increased gradually. Cheng et al. [4] reported that the effect of sodium alginate on the respiratory burst activity of white shrimp L. vannamei after challenged with V. alginolyticus. They reported that the respiratory burst activity of the experimental groups were found to increase gradually along with the time interval. Higher concentrations showed higher activities. Hou and Chen (2005) have reported that the effect of hot water extract from Gracillaria tennuispitata on the respiratory burst activity of L. vannamei challenged against V. alginolyticus. They observed that the respiratory burst activity of shrimps that received hot water extracts at 4 and 6 µg/g was significantly higher than that of the shrimp that received saline as well as the control over 1-6 days.

During the present observation, the superoxide dismutase activity (SOD) of the experimental groups increased significantly than the control. In the control, 35.74 Unit/ml of SOD was observed on 0 day, whereas in the experimental groups it increased with increasing concentrations of alginic acid. As the duration of the experiment was increased, the SOD activity decreased in the control groups and increased in the test groups. Similarly, Huang et al (2006) reported that the effect of S. fusiform polysaccharide extract on SOD activity of F. chinensis challenged with V. harveyi. They pointed out that the SOD activity of shrimp was also slightly enhanced by dietary SFPSE, but there was no significant difference among the treatment groups ($P>0.05$). Similar findings were observed from the studies of Cheng et al. [4] and Hou and Chen [10] on the effect of sodium alginate and hot water extract on the SOD activity of L. vannamei.

Regarding the phagocytic activity, it increased significantly in the experimental groups than the control after the challenge experiment in this study. At the beginning of the challenge experiment, the activity of the test groups increased from 6.40 to 6.98% in A1 to A3 (different concentrations of alginic acid) diets fed groups than in the control (5.35%). As the experiment was prolonged, the activity was reduced in both the test and control groups. Similarly, Cheng et al. [4] observed the effect of sodium alginate on the phagocytic activity of white shrimp L. vannamei after challenged with V. alginolyticus. They found that the activity was significantly higher for test groups that received sodium alginate at 10µg/g than for control, which were maintained for 2 days. They even observed that the activity tends to increase as the concentration was raised to 50µg/g with prolonged time interval (4 days). Comparable remarks were observed by Hou and Chen [10] on the effect of hot water extract from G. tenuistipitata on the phagocytic activity of L. vannamei challenged against
V. alginolyticus.

The present observations on the immunological effect of alginic acid towards the disease resistance in P. monodon clearly indicates that seaweeds can be as an alternate source for biologically active polysaccharides, which may be applied for therapy of shrimp diseases in addition or instead of commercial antibiotics.

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Cite this article as:Kadharsha Kudus, Immanuel Grasian, Sivagnanavelmurugan Madasamy and Akbar John.
Immunomodulatory Effect of Alginic Acid from Brown Seaweed Sargassum Wightii on Disease Resistance in Penaeus Monodon. J. Clean WAS.
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