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RESEARCH ARTICLE

CHARACTERIZATION OF ALGINIC ACID EXTRACTED FROM *SARGASSUM WIGHTII* AND DETERMINATION OF ITS ANTIVIRAL ACTIVITY ON SHRIMP *PENAEUS MONODON POSTLARVAE* AGAINST WHITE SPOT SYNDROME VIRUS

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ABSTRACT

The polysaccharide- alginic acid was extracted from brown seaweed *Sargassum wightii* and the yield observed was 3.932%. The purity of alginic acid was determined by preliminary phytochemical analysis and the result indicated that it has only the presence of carbohydrates and its derivative saponins. The physicochemical properties of alginic acid were analyzed. The FT-IR, ^{13}C and ^{1}H NMR analysis indicated the presence of carbons and anomeric protons of guluronic acid and manuronic acid in purified and hydrolyzed alginic acid, respectively. The characterized alginic acid was enriched with instar II stage *Artemia* nauplii at 100, 200, 300 and 400 mg L^{-1} concentrations for 12 h and they were fed to *Penaeus monodon* postlarvae (PL15) for 20 days. After feeding experiment, the *P. monodon* PL35 were challenged with WSSV. The control group of shrimp PL fed with unenriched *Artemia* nauplii showed 100% mortality within 8 days, but the alginic acid (100-400 mg L^{-1}) enriched *Artemia* nauplii fed shrimp PL showed less mortality (79 – 100 %) within 21 days of WSSV post challenge. The reduction in mortality percentage of alginic acid enriched *Artemia* nauplii fed groups over control group was ranged between 17.15 and 49.99 %. The RT-PCR analysis confirmed the considerable reduction of WSSV DNA copy numbers (71757 – 5.73 WSSV DNA copies) in shrimp postlarvae with respect to the concentration of alginic acid. The present result concluded that *P. monodon* PL fed with alginic acid of *S. wightii* enriched *Artemia* nauplii has increased the resistance against WSSV infection.

Key words: Alginic acid; *Sargassum wightii*; *Penaeus monodon*; WSSV; *Artemia franciscana*.

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INTRODUCTION

Alginates are one of the polysaccharides naturally present in the cell walls of brown seaweeds (Kloareg and Quatrano, 1988). These polysaccharides show interesting rheological properties: they enable to enhance aqueous solutions viscosity at low concentration, and to form gels or thin films. They are widely used in various fields of industries such as textile, food, paper, cosmetics, pharmaceuticals, etc. (Perez *et al.,* 1992). Alginate has a combined feature of abundant resources with a linear copolymers of L-guluronic acid and D-mannurunic acid units (Xu *et al.,* 2006). The major structural polysaccharide of brown seaweeds in alginic acid, a linear copolymer of (1→4) linked β-D-mannopyranuronic acid (m) and (1→4) linked α-L-gulopyramuronic acid (G) residues, arranged in heteropolymeric and homo polymeric blocks (Painter, 1983; Larsen *et al.,* 2003). The content of uronic acids with species and tissue types, and partial hydrolysis of alginic acid allows the preparation of fractions enriched in water and homopolymeric blocks (Haug *et al.,* 1974; Craigie *et al.,* 1984). The sulfated polysaccharides (SPs) have been shown to possess antiviral activities. It has been reported that high molecular weight sulfated galactans (SG) from red seaweeds have antiviral properties against herpes simplex virus (HSV), human cytomegalo virus (HCMV), dengue virus (DENV) and respiratory syncytial virus (RSV) (Mazuder *et al.,* 2002; Hidari *et al.,* 2008). Hidari *et al.* (2008) reported that fucoidan from the brown marine alga *Cladosiphon okamuranus* inhibits DEN2 infection. White spot syndrome virus (WSSV) is a pathogen that has devastated the shrimp farming industry (Lightner and Redman, 1998; Jiang *et al.,* 2006); currently it is considered as the most serious shrimp viral pathogen in the

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world (Flegel, 2006; Sanchez-Martinez *et al.,* 2007). WSSV is found in almost all shrimp producing countries and lethal to all commercially cultivated penaeid shrimp species (Wang *et al.,* 2000; Sanchez-Martinez *et al.,* 2007; Escobedo-Bonilla *et al.,* 2008). WSSV is a rod-shaped enveloped dsDNA virus (275×120 nm in size) with a tail-like appendage at one end. WSSV is identified by the presence of white spot on the inner surface of the exoskeleton of shrimp from which the name is derived (Lo *et al.,* 1996). Other clinical signs include anorexia, lethargy and reddish discoloration of the body (Wang *et al.,* 1999). Cumulative mortalities in infected populations may reach 100% within 2-10 days of the onset of clinical signs (Chou *et al.,* 1995; Lightner, 1996; Xu *et al.,* 2006). Previous strategies generally used to control WSSV including immunostimulation, neutralization, vaccination, quarantining, and environmental management (Xiang, 2001). Protective effect of various herbal immunostimulants has been reported against WSSV infection in shrimp *P. monodon* (Rameshthangam and Ramasamy, 2007; Balasubramanian *et al.,* 2007). The antiviral plant extract of *Cynodon dactylon* against WSSV infection and immunostimulatory effect on *P. monodon* through oral administration was studied (Balasubramanian *et al.,* 2008).

Administration of cidofovir ((S)-1-3-hydroxy-2-phosphonyl methoxy propyl cytosine) (HPMPC) supplemented with a marine blue green algae *Spirulina platensis* in shrimp diet has been shown to delay the mortality of WSSV infected shrimp (Rahman *et al.,* 2006). *In vivo* screening of mangrove plants for antiWSSV activity in *P. monodon* and evaluation of *Cereops tagal* as a potential source of antiviral molecules has been reported by Sudheer *et al.* (2011). Huynh *et al.* (2011) have reported the white shrimp *L. vannamei* immersed in seawater containing *S. hemiphyllum var. chinense* powder and its extract showed increased resistance against WSSV. Immanuel *et al.,* (2012a) and Sivagnanavelmurugan *et al.,* (2012) have reported the effect of fucoidan from brown seaweed *S. wightii* on WSSV resistance and immune activity in shrimp *P. monodon*. Immanuel *et al.,* (2012b) have studied the effect of sodium alginate extracted from *S. wightii* retards mortality in WSSV challenged shrimp *P. monodon*. Ergosan, an algal extract containing alginic acid was also observed to increase the nonspecific defense response of snake head *Channa striata* (Miles *et al.,* 2001), rainbow trout *Oncorhynchus mykiss* (Peddie *et al.,* 2002) and sea bass *Dicentrarchus labrose* (Bagni *et al.,* 2005). Considering the importance of the above, the present study was undertaken to extract and characterize the alginic acid from brown seaweed *S. wightii* and to determine its antiviral effect on *P. monodon* post larvae against WSSV.

MATERIALS AND METHODS

Collection of seaweed

The brown seaweed *S. wightii* was collected from the coastal villages of Kanyakumari District, Tamilnadu, India. The collected seaweed was washed thoroughly and dried under shade at room temperature. The dried seaweed was ground well by using mixer grinder and sieved using nylon sieve (0.45 µm pore size) in order to remove unpowdered materials (Immanuel *et al.,* 2010).

Extraction of alginic acid

The alginic acid was extracted from the brown seaweed *S. wightii* by following the modified method of Torres *et al.* (2007). 100 g of milled seaweed sample was weighed and soaked in 2% formaldehyde in an air tight conical flask for 24 h. After 24 h, the formaldehyde solution was filtered out and the residue was washed with distilled water for 2 to 3 times. Then 0.2 M HCl was added to the residue and kept at room temperature for 24 h. After 24 h, the solution was removed and the residue was washed with distilled water for 2 to 3 times. The residue was extracted with 2 % sodium carbonate for overnight and the extract was filtered through muslin cloth bag. Then 5 % HCl was added to the filtrate for precipitation of alginic acid. The precipitate was separated by centrifugation method (5000 rpm for 15 min). Further the product was dried and made in to powder and calculate the yield.

Determination of purity of alginic acid (phytochemical analysis)

To determine the purity of alginic acid, tests for alkaloids, carbohydrates, flavonoids, steroids, terpins, saponins, tannins and phenols were carried out by the methods proposed by Harborne (1973); Trease & Evans (1989) and Sofowora (1993).

Physicochemical properties of alginic acid

The colour, odour, taste and texture of extracted alginic acid were evaluated by using the methodologies described previously by Kumar *et al.,* (2011). A digital pH meter (Model 2001, Digisum Electronics System) was used to determine the pH of 1% alginic acid solution. Moisture content of the powder was determined using the Indian Standards Institution method (ISI 1984). Protein, carbohydrate, lipid, fucose and sulfate contents of alginic acid were estimated using standard methods (Lowry *et al.,* 1951, Seifter *et al.,* 1950, Folch *et al.,* 1957, Dubois *et al.,* 1956, Dodgson and Price, 1962). Ash content was determined by combusting 1 g of alginic acid powder in a silica crucible in a muffle furnace at 600°C and, once cooled and the weight of ash was determined. The prepared ash was then boiled in 25 ml of 2 N HCl for 5 min, and any insoluble ash was collected on ash-free filter paper and washed with hot water. This insoluble ash was transferred into a silica crucible, combusted and weighed as described above. The procedure was repeated to get an average weight to accurately determine the percentage of acid-insoluble ash. Ash was boiled similarly in 25 ml water for 5 min, and insoluble ash was collected and washed as above, transferred to a silica crucible, combusted for 15 min and weighed. The procedure was repeated to get an average weight of water-insoluble matter that was subtracted from total ash weights to determine the percentage water-soluble ash.

Hydrolysis of alginic acid

In order to reduce the viscosity of the sample as well as to convert the polysaccharide into monosaccharide, the purified alginic acid was subjected for hydrolysis. For this, 20mg of alginic acid was dissolved in 5ml of distilled water and heated at 90^{o} C for 1h. Then 1ml of 0.1 N HCl was added to the sample, it was heated at 90^{o} C for 2h and further the sample was freeze dried (Marais and Joseleau, 2001).

FT-IR analysis

The qualitative analysis of the active principles of hydrolyzed alginic acid was done by Fourier Transmission Infra Red (FTIR) method, described by Kemp (1991).

^{13}C and ^{1}H NMR analysis

After hydrolysis, the alginic acid sample was dissolved in 0.5ml D_2O (Deutrium dioxide), and the proton number and carbon number of alginic acid were identified and confirmed by ^{1}H and ^{13}C NMR experiments using a Bruker Biospin Avance 400 NMR spectrometer (^{1}H frequency = 400.13 MHz, ^{13}C frequency = 100.62 MHz) at 298 K using 5-mm broad band inverse probe head equipped with shielded z-gradient and XWIN-NMR software version 3.5 using TMS as an internal reference. One-dimensional ^{1}H and ^{13}C spectra were obtained using one pulse sequence. One-dimensional ^{13}C spectra using Spin Echo Fourier Transform (SEFT) and Quaternary Carbon Detection (QCD) 42 sequences were also performed to aid the structure identification (Jayaprakash and Kalaiselvi*,* 2007).

Artemia enrichment

To determine the experimental treatment concentrations, the II instar stage of *Artemia franciscana* nauplii (Great Salt Lake, USA) were fed (enriched) with alginic acid. For this, four different concentrations *viz* 100, 200, 300 and 400 mg L^{-1} of alginic acid were prepared individually as enrichment media. The *Artemia* nauplii were stocked at the rate of 20 ml^{-1} in total volume of 5 L seawater in glass containers. Mild aeration was given into the medium in order to maintain the oxygen level and to keep uniform dispersion of the dietary particles of alginic acid in the medium. The enrichment media were delivered into two doses at 6 h intervals to the *Artemia* nauplii, the enrichment duration was 12 h. To ensure the encapsulation of the diets, the enriched *Artemia* nauplii were examined under the microscope to assess 100 % gut loading. After 12 h, the encapsulated *Artemia* nauplii were sieved from the respective containers, washed carefully and kept individually ready for feeding.

Collection and maintenance of experimental animal

The shrimp *P. monodon* postlarvae (PL7) were obtained from Matsyafed. Hatchery (Quilon, Kerala). Immediately after arrival in to the laboratory, the PL were stocked in 1000 L fibre glass tank at room temperature (28 ± 1º C) with the salinity of 32 ± 1 ppt. Natural filtered seawater was used and it was well aerated to maintain the oxygen level above 6 ppm. The PL were kept in the tank for 8 days and fed with live feed (unenriched *Artemia* nauplii) for acclimatization prior to start of the experiment.

Feeding experiment

After measuring the length and weight, uniform (0.0108 ± 0.0036 g) size of *P. monodon* postlarvae at PL15 stage were selected from the acclimatized stock and transferred in to individual experimental tanks (control-unenriched *Artemia* diet; alginic acid with respective concentrations of 100, 200, 300 and 400mg L^{-1} enriched *Artemia* diet), each containing 100 L of filtered sea water at ambient temperature (28 ± 1^{0}C) and salinity (32 ± 1 ppt). The PL were maintained at the stocking density of 5no's L^{-1}.

Mild aeration was given continuously in order to maintain the optimal oxygen level. An *ad libitum* feeding regime was applied to all tanks throughout the experiment, and the food (enriched *Artemia* nauplii) density was adjusted 3 times a day (6:00, 14:00 and 18:00 h) at the rate of 30, 30, and 40 %, respectively. The control group was fed with unenriched *Artemia* nauplii. The uneaten *Artemia* nauplii were collected after the respective hours of feeding and 50% water was exchanged daily during the experimental period. To maintain the nutritional quality of *Artemia,* the remaining enriched *Artemia* nauplii were kept in cold storage at 4 to 10^{0}C with gentle aeration (Leger *et al.,* 1983). The experiment was prolonged for 20 days (PL15–35). Simultaneously, triplicate tanks were maintained in each group.

Preparation of viral inoculum

The WSSV-infected *P. monodon* with prominent white spots on the exoskeleton were collected from local shrimp farms. Head soft tissue from cephalothorax including gills was homogenized and centrifuged at 3000 xg for 20 min at 4^{o}C. The supernatant was recentrifuged at 8000 xg for 30 min at 4^{o}C and the final supernatant was filtered through a 0.4-µm membrane filter. The filtrate was then stored at -20^{o}C for infectivity studies (Yoganandhan *et al.,* 2003). The presence of WSSV in inoculum was checked by nested PCR and this result showed severe infection (912bp) equal to 2000 WSSV DNA copies (IQ 2000^{TM} manual).

WSSV challenge experiment

After feeding experiment, the pathogenesity of WSSV to *P. monodon* PL 35 was carried out separately by immersion (bath) challenge at 2 animals per litre (100 PL 50 L^{-1}) respectively, were reared separately in FRP tanks (75 L) containing 50 L of sterilized, aerated sea water. Air stones and air tubes were sterilized by immersing in 2.6 % sodium hypochlorite and then washed thoroughly with sterilized tap water before use. The tanks were covered to prevent contamination. Aseptic techniques were observed throughout the experiment. The PL were fed on the respective enriched *Artemia* nauplii diet and control group was fed with unenriched *Artemia* nauplii. The WSSV inoculum (stored fluid filtrate) was added to the tank water at a volume equal to 0.1% of the total rearing medium (1 ml L^{-1}) (Chen *et al.,* 2000). Simultaneously, triplicate tanks were maintained in each group. After inoculation of WSSV, the survival of PL was monitored at regular intervals of 8 h until all the animals had succumbed. PL not reacting to gentle mechanical stimulation with a soft paintbrush was considered to be dead. The non-reacting animals were removed from the respective tanks during each observation intervals. The results obtained in every 8 h intervals were pooled and presented as per day interval. The challenge experiment was conducted for 21 days. The cumulative mortality index (CMI) was calculated by the formula described in Immanuel *et al.* (2012b).

Real Time Polymerase chain reaction (RT-PCR) analysis

After challenge experiment, the WSSV infection in *P. monodon* larvae was detected by RT-PCR analysis. The experimentally infected shrimp PL were preserved in 70% ethanol and subsequently were rehydrated in distilled water for 1h before the RT-PCR analysis.

The RT-PCR analysis for WSSV DNA quantification was performed by the methodology described in Immanuel *et al.* (2012a).

Statistical analysis

The data obtained in the present study were expressed as Mean ± SD and were analyzed using one way ANOVA at 5% significant level. Further a multiple comparison by Tukey's test was conducted to compare the significant differences among the parameters using computer software Statistica 6.0 (Statosoft, UK).

RESULTS AND DISCUSSION

Yield of alginic acid

The yield of alginic acid obtained from *S. wightii* was 3.932 ± 0.22 %. Generally, the alginate content of various seaweeds is varied much. Torres *et al.* (2007) reported the yield of alginate as 16.90% extracted from *S. vulgare*. Similarly, Davis *et al.,* (2004) found the yield within the range of 21.1 – 24.5 % in *S. fluitans* and 16.3 – 20.5% in *S. oligocystum* with variations being depended on the methodology followed for alginate extraction, as well as species which are used for extraction. In the present study, the yield of alginic acid extracted from brown seaweed *S. wightii* was very low with 3.932 ± 0.22 %. Larsen *et al.* (2003) reported that, a change in yield of alginate in different seaweeds like *S. dentifolium* (3.25 %), *S. asperifolium* (12.4 %) and *S. latifolium* (17.7 %) was due to species dependent variation and also extraction methods followed.

Purity of alginic acid

The purity of alginic acid was determined through phytochemical tests. The result indicated that, the phytochemical constituents such as alkaloids, saponins, tannins, phlobatannins, flavonoids, steroids, terpenoids, cardiac glycosides and phenols were absent.

Only carbohydrate as well as sugar derivative of saponins were found to be present in alginic acid, which confirmed the better purity of the algnic acid (Table 1). Similarly, Kumar *et al.* (2011) have determined the phytochemical characteristics as well as the purity of tamarind seed polysaccharide, which indicated the absence of alkaloids, steroids, flavonoids, saponins, tannins and phenols, however only carbohydrate was found to be present, which confirms the purity of this particular polysaccharide.

Physicochemical characters of alginic acid

The organoleptic characters such as colour, odour, taste and texture of alginic acid were in the order of white to yellowish brown colour, odourless, salty taste and powdery appearance, respectively. The pH of 1% alginic acid solution was 2.35. The moisture content of alginic acid observed was 18 ± 1.07%. Similarly, Cyber colloids Ltd. (E400 Alginic acid) have reported the color, odour, texture, pH, moisture content of alginic acid were white to yellowish brown color, nearly odourless, granular or powder, 2 to 3 and 15%, respectively. The Good Scent Company, have studied the physical parameters of alginic acid of seaweed and they observed the color, odour, taste and texture of alginic acid were white to pale yellow color, odourless and tasteless, respectively.

Chemical Book. (2008) have published the colour of alginic acid of seaweed was off white to light yellow powder. FMC BioPolymer (2008) have reported that the color, odour, texture, taste and pH of alginic acid were white to yellowish color, odourless, tasteless, free flowing powder, 1.5 - 3.5 (in 3% aqueous dispersion), respectively. The major biochemical component of alginic acid was carbohydrate (44.36 ± 1.42%) with little amount of protein (5.82 ± 0.72) and lipid (4.06 ± 0.341%) contents. The fucose content of alginic acid was 28.99 ± 1.09%. The ash values such as total ash, acid insoluble ash and water soluble ash of alginic acid were 1.41 ± 0.032, 0.098 ± 0.0064 and 0.676 ± 0.052%, respectively. The sulphate content of alginic acid was 25.91 ± 0.390 % (Table 2).

Table 1. Determination of purity of alginic acid extracted from brown seaweed *S. wightii* through phytochemical analysis3

Phytochemical tests	Phytochemical characteristics
Alkaloids	-
Saponins	+
Tanins	-
Phlobatannins	-
Flavonoids	-
Steroids	-
Terpenoids	-
Cardiac glycosides	-
Phenols	-
Carbohydrates	+

+ : Present ; - : Absent

Table 2. Physicochemical and organoleptic characters of alginic acid extracted from the brown seaweed *S. wightii*

Characters	Parameters	Alginic acid
Organoleptic characters	Colour	White to yellowish brown colour
	Odour	Odourless
	Taste	Salty taste
Physical characters	Texture	Powder
	pH	2.35
	Moisture content (%)	18.0 ± 1.07
Chemical characters	Protein (%)	5.82 ± 0.72
	Carbohydrate (%)	44.36 ± 1.42
	Lipid (%)	4.06 ± 0.341
	Fucose (%)	28.99 ±1.09
	Ash content (%)	1.41 ± 0.032
	(i) Acid insoluble ash (%)	0.098 ± 0.0064
	(ii) Water soluble ash (%)	0.676 ± 0.052
	Sulphate content (%)	25.91 ± 0.390

Similarly, Torres *et al.* (2007) have studied the biochemical composition of alginate extracted from *S. vulgare*. They reported that the protein values determined were 1.1 and 1.0 for *S. vulgare* low-viscosity alginate (SVLV) and *S. vulgare* high-viscosity alginate (SVHV) samples, respectively. The moisture and ash contents of SVLV and SVHV were 14 & 16% and 2 & 1%, respectively. Larsen *et al.* (2003) have studied the biochemical composition of alginates of algae harvested from the Egyptian Red Sea coast. They observed that total carbohydrate and fucose contents were 74.93 and 11.63%, respectively in *C. trinode,* 57.87 and 5.62%, respectively in *S. dentifolium*; 32.16 and 4.15%, respectively in *S. asperifolium* and 42.26 and 8.24%, respectively in *S. latifolium*.

FT-IR analysis of alginic acid

The FT-IR result indicated that in the region of 3600–1600 cm^{-1}, five bands appeared with a broad band centered at 3414.74 cm^{-1}. It was assigned to hydrogen bond (O–H) stretching vibrations, the weak signal at 2929.30 cm^{-1} due to C–H stretching vibrations, the wavelength at 2360.18 cm^{-1} indicated the presence of $R_2C=N=N$ and the asymmetric stretching of carboxylate O–C–O vibration at 1611.26 cm^{-1}. The band at 1417.64 cm^{-1} may be due to C–OH deformation vibration with contribution of O–C–O symmetric stretching vibration of carboxylate group. The weak bands at 1038.05 cm^{-1} may be assigned to C–O stretching, and C–O and C–C stretching vibrations of pyranose rings. The spectrum showed a band at 887.25 cm^{-1} assigned to the C1–H deformation vibration of β-mannuronic acid residues. The band at 812.91cm^{-1} seems to be characteristic of mannuronic acid residues (R=C=CHR). The band at 618.68cm^{-1} may be due to C≡C-H stretching vibration (Fig. 1). In accordance with these, Leal *et al.* (2008) have reported the FT-IR analysis of alginate in three species of brown seaweeds. They observed a spectral band at 948.5 cm^{-1}, which was assigned to be the C–O stretching vibration of uronic acid residues, and one at 888.3 cm^{-1} assigned to the C1–H deformation vibration of β-mannuronic acid residues and the band at 820.0 cm^{-1} seems to be characteristic of mannuronic acid residues.

Zhang *et al.* (2008) have reported the -OH groups present in alginate are clearly seen at 3400 cm^{-1}. They also suggested that the peaks attributed to the $-CH_2$ groups present at 2931 cm^{-1} and 2926 cm^{-1} in alginate and some distinct peaks such as carboxyl group showed strong absorption bands at 1614 cm^{-1}, 1416 cm^{-1} and 1306 cm^{-1}, due to carboxyl anions. The band at 1648 cm^{-1} is attributed to the absorption band of the carbonyl (-HC=O) stretching. The other band at 1041 cm^{-1} that was assigned to the stretching vibration of (CH-OH) appeared at 1643 cm^{-1} and 1045 cm^{-1} for the composite gel beads.

^{13}C and ^{1}H NMR analysis of alginic acid

^{1}H NMR and ^{13}C spectroscopy is a reliable method for the determination of the composition and also the block structures of alginate molecules (Larsen et al., 2003). The results on ^{13}C and ^{1}H NMR spectral analysis of purified alginic acid are given in the Fig. 2 and 3. The ^{13}C NMR spectrum showed absorptions corresponding to a β-D mannuronic acid at ppm 99.89 (C-1), 66.88 (C-2), 69.79 (C-3), 79.97 (C-4), 75.53 (C-5) and 175.34 (C-6). Similarly, the ^{1}H NMR spectrum showed the correlation of these signals with the ppm of 4.248 (H-1), 3.830 (H-2), 3.693 (H-3), 3.987 (H-4), 3.721 (H-5) and 1.005 (H-6), respectively. Similarly the ^{13}C NMR spectrum showed sharp absorptions corresponding to a guluronic acid at ppm 16.58, 57.11, 64.62, 131.17 and 165.53 respectively for C1, C2, C3, C4, C5 and C6. The ^{1}H NMR spectrum showed the correlation of these signals with the ppm of 0.970, 3.432, 3.450, 3.467 and 3.485 respectively for H1, H2, H3, H4 and H5, respectively. Similarly, Torres et al. (2007) have reported that the NMR analysis of *S. vulgare* alginate and they observed the guluronic acid anomeric proton (G-1) at 5.06 ppm; guluronic acid H-5 (G-5) at 4.4 ppm; and mannuronic acid anomeric proton (M-1) and the C-5 of alternating blocks (GM-5) overlapped at 4.7 ppm. Larsen *et al.* (2003) have studied the NMR spectrum of alginates from algae harvested at the Egyptian Red Sea coast. They reported that the presence of gluronic acid and manuronic acid protons at 3.50 – 3.56 and 3.80 – 4.80ppm, respectively and the carbon at 71.2 – 72.4 and 93.4 – 94.8ppm in *S. asperifolium* alginate.

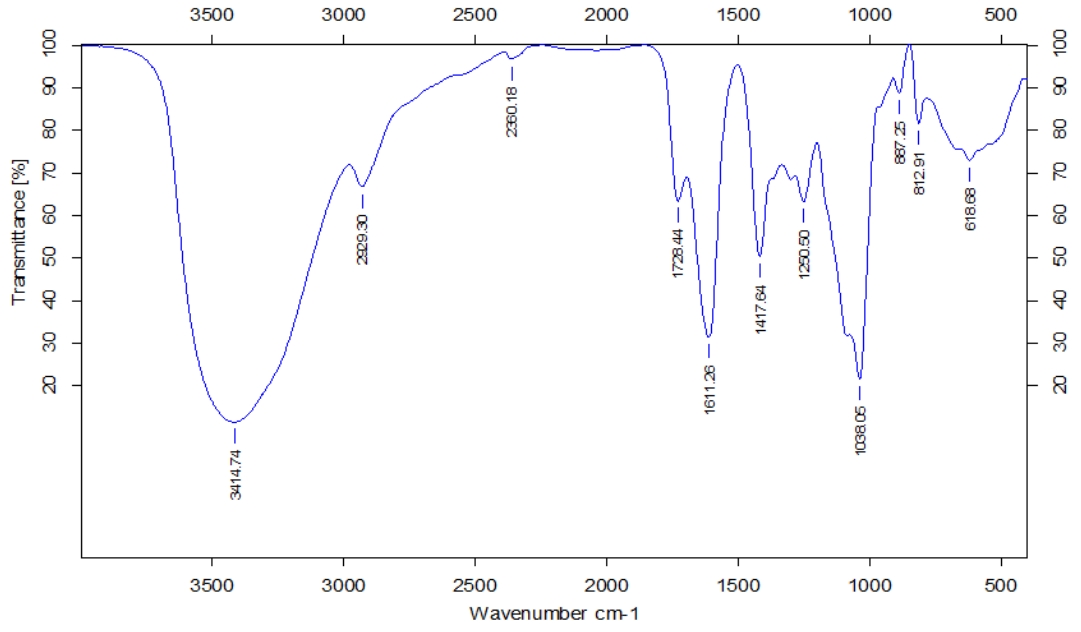

Figure 1. FT-IR analysis of alginic acid extracted from brown seaweed *S. wightii*

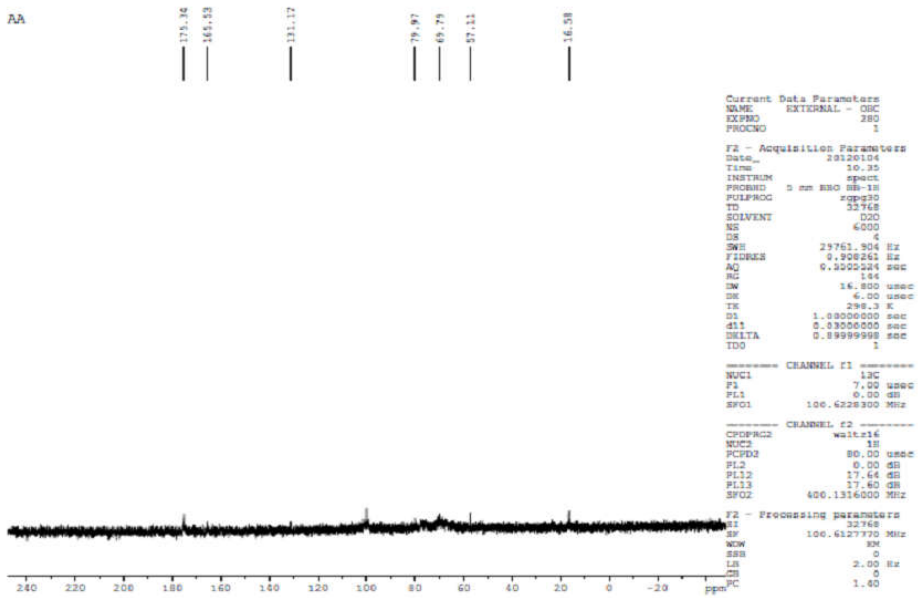

Figure 2. ^{13}C NMR analysis of alginic acid extracted from brown seaweed *S. wightii*

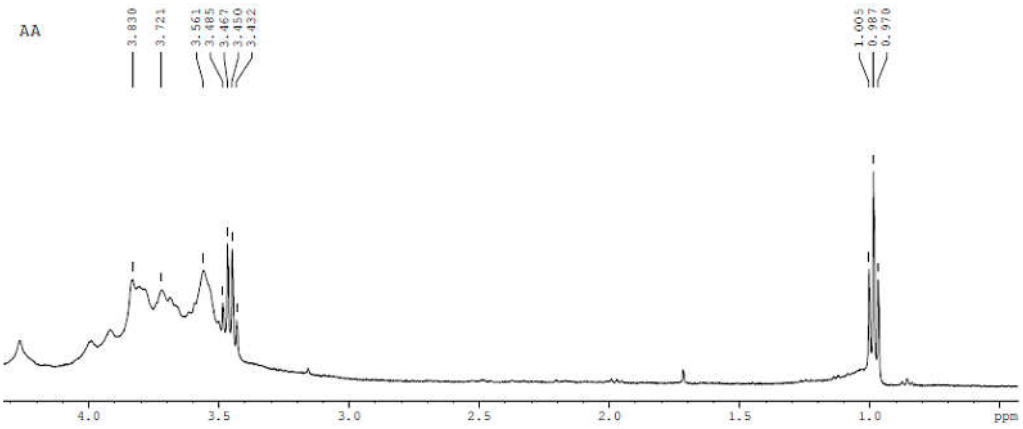

Figure 3. ^{1}H NMR analysis of alginic acid extracted from brown seaweed *S. wightii*

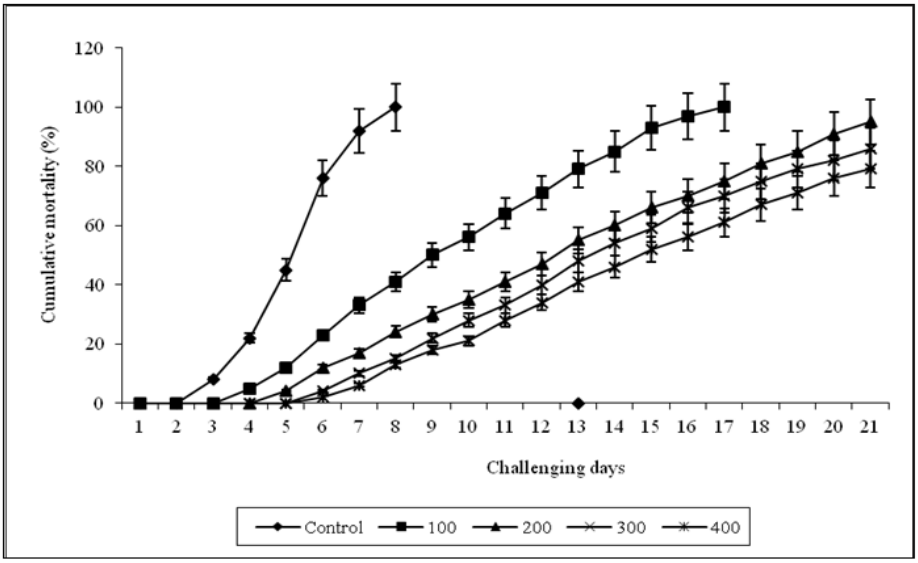

Figure 4. Cumulative mortality percentage of shrimp *P. monodon* PL fed on different concentrations (100 – 400mg L^{-1}) of alginic acid enriched *Artemia* nauplii after bath challenged with WSSV in 21 days interval. Each value is the Mean ± SD of three replicates

Cumulative mortality percentage during WSSV Challenge study

After WSSV challenge test, the *P. monodon* PL were succumbed to death started from 3rd day, on this day 8 % mortality was observed in control group. At the same time, no mortality was observed in experimental groups. The experimental groups with lowest concentrations (100 and 200 mg L^{-1}) of alginic acid enriched *Artemia* nauplii fed PL showed 5 and 4 % mortality during 4th and 5th days of challenge experiment, respectively. But the highest concentrations (300 and 400 mg L^{-1}) of alginic acid enriched *Artemia* nauplii fed groups displayed 4 and 2% mortality respectively on 6th day of challenge experiment. When the duration of the challenge experiment increased, the cumulative mortality was also increased progressively. At last, 100% mortality was observed in control group within 8th day of challenge test. But in the experimental groups, the mortality of PL was decreased with increasing concentrations of alginic acid. Accordingly, the mortality observed was 100, 95, 86 and 79% on 21st day of challenge experiment in 100, 200, 300 and 400mg L^{-1} concentrations of alginic acid enriched *Artemia* nauplii fed PL, respectively (Fig. 4).

Cumulative Mortality Index (CMI) and reduction in mortality

The CMI of control group was 21,737, but it reduced considerably to 17.15, 36.21, 43.21 and 49.99 %, respectively in 100, 200, 300 and 400 mg L^{-1} concentrations of alginic acid enriched *Artemia* nauplii fed shrimp (Table 3). The reduction in mortality of all the tested groups increased with increasing concentrations (100 – 400 mg L^{-1}) of alginic acid. The lower concentration of alginic acid exhibited lower inhibitory activity against WSSV. But the higher concentration (400 mg L^{-1}) of alginic acid showed higher inhibitory activity against WSSV. Similarly, Immanuel *et al.* (2012b) have observed that the *P.monodon* (PL35) fed on *Artemia* nauplii enriched with sodium alginate powder (26.5 to 52.4 %) or beads (35.2 to 58.4 %) of *S. wightii* experienced on reduction in mortality rates progressively as alginate concentrations were increased from 100 to 400 mg L^{-1}. Sivagnanavelmurugan *et al.* (2012) have also reported the fucoidan of *S. wightii* enriched *Artemia* nauplii fed groups showed increased resistance against WSSV and they observed the reduction in mortality percentage of experimental groups of shrimp was ranged from 33.71 to 61.65 %, respectively in 100 to 400 mg L^{-1} fucoidan enriched *Artemia* nauplii fed groups, over control group. Immanuel *et al.* (2010) have reported the effect of hot water extract of brown seaweeds *S. duplicatum* and *S. wightii* on WSSV resistance in shrimp, *P. monodon* PL. They pointed out that the mortality percentage of *P. monodon* PL challenged with WSSV reduced to a maximum of 39.35 to 65.83 % in *S. wightii* groups and 16.12 to 47.92 % in *S. duplicatum* groups. In both the seaweed extracts, the reduction in mortality of the tested groups showed increase with the increasing concentrations of seaweed extracts. The lower concentration (250 mg L^{-1}) of both the seaweed extracts showed lower (16.12 and 39.35 %) inhibitory activity against WSSV. But in higher concentration of 750 mg L^{-1}, both the seaweed extracts showed higher (47.92 and 65.83 %) inhibitory activity against WSSV. Chang *et al.* (1999) and Chang *et al.,* (2003) stated that the β-1, 3 glucan has improved the immunity effectively and increased the resistance to WSSV in PL and juveniles of *P. monodon*. In this study, all shrimps in the WSSV challenged, glucon free (control) group died within 5 days. But the mean survival in the WSSV challenged glucon fed group was 12.2 % on 6th day. Chotigeat *et al.* (2004) reported that the oral administration of fucoidan from brown algae *S. polycystum* has reduced the impact of the WSSV infection in the tiger shrimp *P. monodon*. They pointed out that, 4.4, 14 and 44 % of the shrimp survived respectively in 5-8 g shrimp fed on the crude fucoidan of 100, 200 and 400 mg Kg^{-1} of body weight / day before and after challenged with WSSV. The mechanism of inhibition of the virus is that the negative charges of the sulfate group of the alginic acid bind with positive charges of amino acid at V3 loop of viral envelope glycoprotein (gp120). The V3 loop is essential for virus attachment to cell surface heparin sulfate, a primary binding, before more specific binding occurs to the CD4 receptor of CD4+ cell. Therefore, the virus could not invade into the host cells (Witvrouw & De Clerq, 1997).

RT-PCR analysis: WSSV infected *P. monodon* PL from all the experimental and control groups were screened by RT-PCR analysis for the quantification of WSSV DNA (Table 4 and Fig. 5).

Table 3. Cumulative Mortality Index (CMI) and percentage reduction in mortality of shrimp *P. monodon* PL fed on different concentrations of alginic acid enriched *Artemia* nauplii after bath challenged with WSSV against control

Seaweed product	Concentration (mg L^{-1})	CMI	Reduction in mortality (%)
	Control	21737 ± 240.12^{a}	0 ± 0
Alginic acid	100	18007 ± 204.41^{b}	17.15 ± 0.111
	200	13866 ± 183.71^{c}	36.21 ± 0.136
	300	12343 ± 163.29^{d}	43.21 ± 0.130
	400	10870 ± 142.88^{e}	49.99 ± 0.150

Each value is the Mean ± SD of three replicates. Within each column, Means with the different superscript letters are statistically significant (one way ANOVA, $P< 0.05$ and subsequently *post hoc* multiple comparison with Tukey's test).

Table 4. Quantification of WSSV DNA copies by RT-PCR analysis of shrimp *P. monodon* PL fed on different concentrations of alginic acid enriched *Artemia* nauplii after bath challenged with WSSV.

Seaweed product	Concentration (mg L^{-1})	Ct FAM (Cycles)	No. of DNA Copies
Negative control		-	-
Positive Control		18.69	2.23×10^{5}
Alginic acid	100	20.41	71757
	200	21.34	39014
	300	22.18	22442
	400	34.73	5.73

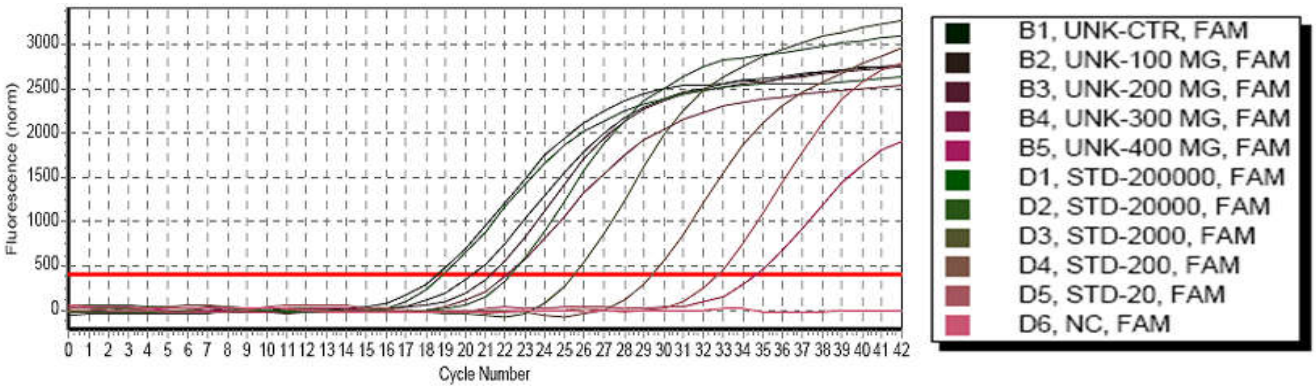

Figure 5. Amplification curve showing 10 fold serial dilutions of standards, control and different concentrations (100 – 400mg L^{-1}) of alginic acid enriched *Artemia* nauplii fed experimental shrimp samples

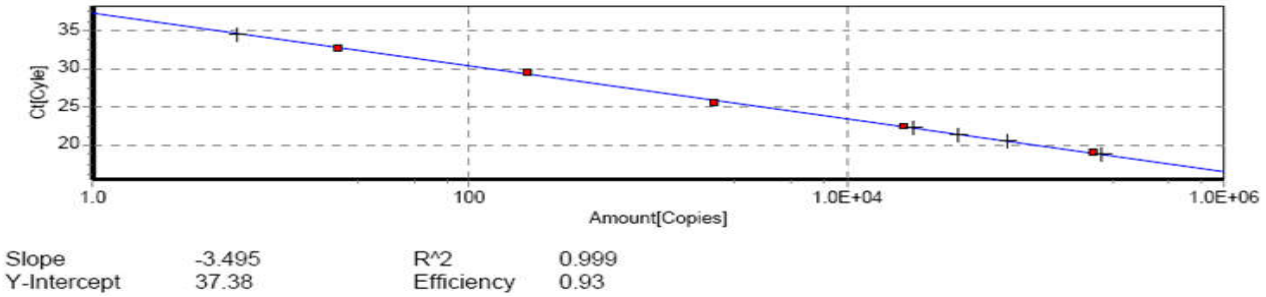

Figure 6. Standard curve of WSSV Taq Man Real Time PCR

The WSSV infection in positive control group showed 2.23 x 10^5 WSSV DNA copies with in 18.69 threshold cycles (Ct FAM). But in the experimental groups, the copy number of WSSV DNA was decreased with increasing concentrations of alginic acid. The experimental group with low concentration (100 mg L^{-1}) of alginic acid enriched *Artemia* nauplii fed shrimp PL displayed 71757 WSSV DNA copies within 20.41 threshold cycles. But in the 200 and 300 mg L^{-1} concentrations of alginic acid enriched *Artemia* nauplii fed groups, 39014 and 22442 WSSV DNA copies were determined with in 21.34 and 22.18 threshold cycles, respectively. Invariably, the shrimp PL fed on the highest concentration (400 mg L^{-1}) of alginic acid enriched *Artemia* nauplii displayed only 5.73 WSSV DNA copies within 34.73 threshold cycles. Negative controls did not show any amplification. Strong linear correlation (R^2= 0.999) was obtained between the threshold cycles (Ct) and the amount of WSSV DNA copies in RT-PCR with reaction efficiency (E= 0.93) and proper slope (M= -3.495) indicating that the assay had a large dynamic range (Fig. 6).

According to the RT-PCR result, the WSSV DNA copy numbers in shrimp PL of *P. monodon* was decreased with increasing concentrations of alginic acid. Similarly, Immanuel *et al.* (2012a) have quantified the WSSV DNA in shrimp *P. monodon* fed on different concentrations (0.1 – 0.3 %) of fucoidan supplemented diets for 45 days and subsequent WSSV challenge test for 21 days. They observed that the positive control group had 1.42 x 10^6 WSSV DNA copies within 16.96 threshold cycles (Ct FAM), whereas in the experimental groups, the copy numbers of WSSV DNA was positively decreased (756 - 11 within 27.23 - 36.26 threshold cycles). Zhu and Zhang (2011) quantified the WSSV DNA copies in WSSV challenged shrimp *Marsupenaeus japonicus* treated with antiviral VP28-siRNA. They recorded significant (P< 0.05) reduction of WSSV DNA copies in treatment groups than that of positive control (no treatment group).

Conclusion

In conclusion, the present findings clearly demonstrated that the brown seaweed *S. wightii* is rich source of gluronic acid and manuronic acid containing polysaccharide-alginic acid. Further it emphasized that the shrimp *P. monodon* postlarvae which received alginic acid of *S. wightii* showed increased resistance against WSSV and also it can be used as a prophylactic agent to improve survival of shrimp against WSSV infection in aquaculture practice.

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