The effect of ethanol extract of a macroalgae *Laurencia snyderia* on growth parameters and vibriosis resistance in shrimp *Litopenaeus vannamei*

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Abstract

Bacterial diseases have been reported to be the major limiting factor in shrimp production. The use of natural products such as antimicrobials has been reported as a solution to the problem. The crude extract of a red seaweed *Laurencia snyderiae* obtained from the Persian Gulf was evaluated for shrimp growth performance and to determine *in vivo* efficacy of this seaweed in the prevention of shrimp Vibriosis. The ethanol extract from *L. snyderiae* (EELS) that was fed to the *Artemia* instar I for their enrichment was found to be non toxic to them. Shrimp *Litopenaeus vannamei* juveniles were fed with these enriched *Artemia* at 0 mg mL^{-1} (Control group), 200 mg mL^{-1}, 400 mg mL^{-1} and 600 mg mL^{-1} for 30 days. The results obtained showed a significant increase (p<0.05) in survival rate in treatment groups compared with that in the control group. Shrimps fed with enriched *Artemia* showed a significant improvement in growth parameters when compared to those in the control group. When these juvenile shrimps were exposed to *Vibrio harveyi* (after 30 days) they showed notably lower mortality than the control. These results indicate that EELS has a good potential in promoting growth and antibacterial activities against *V. harveyi* that is useful in shrimp aquaculture.

Keywords*:* Red seaweed, *Laurencia snyderiae*, *Litopenaeus vannamei*, *Vibrio harveyi*

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Introduction

Bacterial diseases have been reported to be a major limiting factor in shrimp production. Among the bacteria *Vibrio harveyi* (VH) is one of the most important pathogens (Vezzulli *et al.*, 2010). In the past decades, mass mortalities caused by *V.harveyi* were reported in hatcheries and farms (Karunasagar *et al.*, 1994; Raissy *et al.*, 2011). The use of antibiotics for preventing bacterial diseases, have led to the advent of some problems such as resistant bacterial pathogens (Rahman *et al.*, 2010).

Sustainability of this industry depends on shrimp health and efficient shrimp disease prevention (Selvin *et al.*, 2009). Thus, disease control and health management have been considered as priorities for the shrimp aquaculture industry (Roch, 1999). Among different alternatives in shrimp aquaculture, the use of natural products such as antimicrobials has been reported as the solution to this problem (Selvin *et al.*, 2009). Some efforts have been made to find new antibiotics from marine organisms such as macroalgae to control bacterial pathogens (Immanuel *et al*., 2004; Bansemir *et al*, 2006; Dashtiannasab *et al*., 2012; Dashtiannasab *et al*., 2014). Concerns for human health and ecological safety due to the use of some chemicals and antibiotics have encouraged increasing interest to find more "natural-green" alternatives as antibiotics. Seaweeds are considered as an effective source of bioactive compounds that are competent to produce different important secondary metabolites described with great biological activities (Cruz-Suarez *et al.*, 2008; Chojanca *et al.*, 2012). The use of bioactive compounds from seaweeds to enhance disease resistance and growth improvement in aquaculture have also been reported (Huang *et al.*, 2006; Yeh *et al*., 2006; Fu *et al.*, 2007; Kanjana *et al*., 2011).

It seems that the use of these natural compounds is an excellent strategy to control shrimp disease (Chojanca *et al.*, 2012). But there have been few studies in the development of natural products in the prevention and therapeutic control for shrimp disease. The aims of this study are: to evaluate the efficacy of ethanol extract of a red seaweed, *Laurencia snyderiae*, obtained from the Persian Gulf coastline on shrimp growth performance and to determine *in vivo* efficacy of this seaweed in the prevention of Vibriosis in shrimp.

Materials and methods

Samples

The red seaweed *Laurencia snyderiae* was collected during low tide from a marine rocky area of the Persian Gulf coastal line named Rishahr in the north of Bushehr port in the south of Iran during November and December 2012. The post larvae (PL_{15}) of *Litopenaeus vannamei* were purchased from a shrimp hatchery in Delvar, Bushehr Province, in the south of Iran and acclimatized in two 500 liter plastic tanks with clean seawater (40±1 ppt) for 5 days. The brine shrimp *Artemia* cysts (Inve, Belgium) were obtained

from a local hatchery in Delvar, Bushehr Province. The pathogen bacteria (*Vibrio harveyi*) deposited in Iranian Research Organization for Science and Technology under Persian Type Collection Center accession numbers PTCC 1755 were obtained from microbiology lab. of Iran Shrimp Research Center, Bushehr, Iran.

Algae extraction

Algal samples were cleaned of epiphytes, debris and extraneous matter and then the necrotic parts were removed. The surface of algal samples was washed carefully with seawater and in fresh water. Seaweeds were dried under shade for 8 days and cut into small pieces, and made into powder in a mixer grinder (Immanuel *et al.,* 2004). 30 g of dried and powdered seaweed *L. snyderiae* samples were suspended in 500 mL ethanol for 72 h at room temperature. The extraction was repeated twice and the total extracts (1L) obtained were pooled, filtered through Whatman filter paper No. 1 and concentrated under vacuum in a rotary (Heizbad HB digital, Heidolph) evaporate at 45°C to get ethanolic extract of *L. snyderiae* (EELS) and stored at -20°C until use.

In vitro cytotoxicity assay

The toxicity against *Artemia* nauplii (Brine shrimp) was tested according to the method of Caldwell *et al*. (2003) with minor changes. Dried cysts were hatched (5 g cyst per liter) in sterile filtered (0.45 μm) seawater at 28–30°C with strong aeration, under a constant light regime. About 12 h after hatching, the phototrophic nauplii were collected with a pipette and concentrated in a small vial (4.5 mL filtered seawater and 0.5 mL EELS). Each test included exposing groups of 10 nauplii to various concentrations (0, 0.05, 0.1, 0.5, 1, 2, 4, and 8 mg mL^{-1}) of the EELS. The toxicity was determined after 24h of exposure by counting the number of dead nauplii and comparing with the control group using a stereomicroscope. Experiments were performed in triplicate. The lethal concentration of the EELS was defined as that which caused 50% mortality of the artemia nauplii (LC_{50}) using probit software (version 6.7) (Caldwell *et al.*, 2003).

Enrichment of Artemia nauplii

Artemia nauplii (Instar I -II) are regularly used in shrimp hatcheries as live–feed for postlarvae stages. After hatching of cyst, the first instar nauplii appeared; they did not feed because their anus was still closed. After 12 h the larvae molts into the second stage nauplius (instar-II) and starts feeding on small particles (<50 μ). At this stage *Artemia* nauplii were enriched with EELS by the method of Kenjana *et al*. (2011). In brief, the second-instar *Artemia* nauplii were detached from the hatching container and transferred into plastic bottles for enrichment at a density of 100 nauplii mL^{-1} of one liter filtered sea water at the temperature of 27±1°C and salinity 35 ppt. They were enriched for 6 hrs with EELS at the concentrations of 0 (the control), 0.5, 1, and 2 mg mL^{-1} of sea water. Strong

aeration was carried out to keep the O_2 level at 5 ppm. Each experiment was performed in triplicate. The *Artemia* nauplii were collected from the enriched media after 6 hrs. They were washed carefully with tap water and stored at -20°C for further use (Immanuel *et al.,* 2001).

Experimental design

Shrimp experimental arrangement and maintenance of *L. vannamei* juveniles (PL-15; 1500 Nos.) were acclimatized in two 500 liter plastic tanks with clean seawater in standard conditions: 40±1 ppt, 28±1°C, and constant aeration for 5 days. After 5 days of nursing phase, shrimps that had no signs of disease were chosen for experiments. In the adaptation period the shrimps were fed on a formulated shrimp feed (Havoorash Co.) twice per day. After the initial biometric analysis (1.1±0.2 cm, 7.4±0.27 mg), shrimps were divided into four groups in a completely randomized plan at a density of 100 shrimps in each plastic rectangular 60 L capacity tanks. Each treatment was in triplicate. Groups were as follows: control group (C): shrimps fed with normal *Artemia* instar II larvae and basal diet (commercial feed). Group 1 (G1): The shrimps fed with enriched *Artemia* at concentration of 0.5 mg mL^{-1} EELS and fed basal diet. Group 2 (G2) the shrimps fed with enriched *Artemia* at concentration of 1 mg mL^{-1} EELS and fed basal diet. Group 3 (G3) fed with enriched *Artemia* at concentration of 2 mg mL^{-1} EELS and fed basal diet.

Feeding program

An ad labium feeding management was applied to all tanks during the experiment, and the feeding schedule was three times a day at 8:00, 14:00, 24:00 hours at rates of 20, 50, 30% of diets, respectively. In the first and third time they received enriched *Artemia* (in experiment groups) or unenriched *Artemia* in control group and in second time they were fed a commercial diet (Havorash Co.). To maintain the nutritional quality of *Artemia*, the remaining enriched *Artemia* were kept in cold storage at 2–5ºC for further use (Immanuel *et al.,* 2004). The control group was fed with unenriched *Artemia.* The experiment was extended for 30 days (PL_{20}–PL_{50} stage).

Survival and growth performance

After a 30-day culture, the number of live shrimp was counted in each experimental group to measure survival rate (formula 1) Immanuel *et al.* (2001). The growth parameters were estimated by measuring the length and weight of the shrimp's total body in each group. The weight gain was calculated by deducting the initial weight from the final weight. The specific growth rate (SGR) was also calculated based on Immanuel *et al.* (2001) as below (formula 2).

1. Survival (% / day) = 100 × (final live shrimp number) / (initial shrimp number)
2. Specific growth rate (SGR)(%)= $100\times [(\ln w_2 - \ln w_1)/(t_2 - t_1)]$

Where: ln=Logarithmic number, W_2=Final weight at time t_2, W_1=initial weight at time t_1

Vibriosis challenge trial

A strain of *V. harveyi* (PTCC 1755) isolated from diseased *L. vannamei* in Bushehr Province was used for the challenge test. The pathogen was cultured on tryptic soy agar (TSA supplemented with 2% NaCl, Difco) for 24h at 25ºC before being transferred to 10 mL tryptic soy broth (TSB supplemented with 2% NaCl, Difco), where it remained for 24h at 25ºC as stock culture for tests (Chythanya *et al.*, 2002). The broth cultures were centrifuged at 7155 rpm for 15 min at 4ºC (Fu *et al.,* 2007). The supernatant fluids were removed and the bacterial pellets were re-suspended in saline solution at 1×10^8 cfu mL^{-1} as bacterial suspensions for the resistance test (Dashtiannasab *et al.*, 2014).

Challenge experiments were performed in triplicate with 20 shrimps per replicate. After completing the dietary experiment, shrimp post larvae fed for 30 days were immersed at 1×10^8 for 15 min and transferred to rectangular plastic tanks (30 ×50 × 50 cm) containing 20 L filtered seawater including 1×10^6 of pathogen *V. harveyi*. Addition four groups (C, G1, G2 and G3) one group was added as negative control where there was no exposure to bacteria *V. harveyi* (Yeh *et al.,* 2009). The mortality of each replicate was recorded continuously for 5 days.

Data analysis

All the data are presented as mean±standard deviation (SD). A multiple-comparison test (Tukey's) test was used to examine significant differences among treatments using the one way analysis of variance (ANOVA) using SPSS for Windows version 18. Before analysis, percent data (resistance study) were normalized using an arcsine transformation. Statistical significance of the difference required that $p<0.05$.

Results

Cytotoxicity assay

The seaweed ethanolic extract was estimated (by probit software) for its cytotoxicity at different concentrations (0, 0.05, 0.1, 0.5, 1, 2, 4, and 8 mg mL^{-1}) at 24h exposure time. The LC50 of EELS obtained was 1.473±0.241 mg mL^{-1}.

Growth and survival data

The mean survival of shrimps *L. vannamei* juvenile after 30 days of culture in control (C) and experimental groups (G1-G3) showed marked variance and it was maximum (95.5±3.4%) for those shrimps that received enriched *Artemia* at 0.6 mg mL^{-1} concentration (G3). The survival shrimps fed enriched *Artemia* at 0.2 (G1) and 0.4 mg mL^{-1} concentration (G2) of EELS were 92.22±3.2% and 92.22±5.2% respectively, whereas the minimum survival of 91.33% was recorded in control shrimps fed with unenriched *Artemia* (C). However the

differences weren not significant (p<0.05) (Fig. 1).

Shrimp *L. vannamei* obtained maximum weight gain of 333.2 ±3.3 mg in G3 (fed enriched artemia with 400 mg mL^{-1}) group. But the minimum weight gain of 237.4 ± 4.6 mg was displayed in control group that was significantly different (p<0.05). In treatment groups (G1-G3) G2 attained further weight gain followed by G3 and G1, respectively.

The specific growth rate (SGR) of the *L. vannamei* juveniles after 30 days culture in different treatments and control varied, G3 group was more than the others and control group was less than the others significantly (p<0.05).

Data summary on multiple comparisons of mean length gain, weight gain and Specific growth rate of *L. vannamei* juveniles between controls and treatments were statistically significant (p<0.05) as shown in Table 1.

Effect of EELS on the resistance of L. vannamei to V. harveyi

Results obtained indicated that all the unchallenged shrimp (control negative) survived, but death began to occur after 24h in the challenged groups. Cumulative mortality for shrimp *L. vannamei* juvenile in the positive control group was 86.6±3.3% within 5 days after VH challenge while *L. vannamei* juveniles fed with EELS enriched *Artemia* had lower mortality (p<0.05) of 41.3±3.3%, 30±3.3%, 30±6.6%, after 5 days in the G1, G2 and G3, respectively as compared to positive control (Fig. 2).

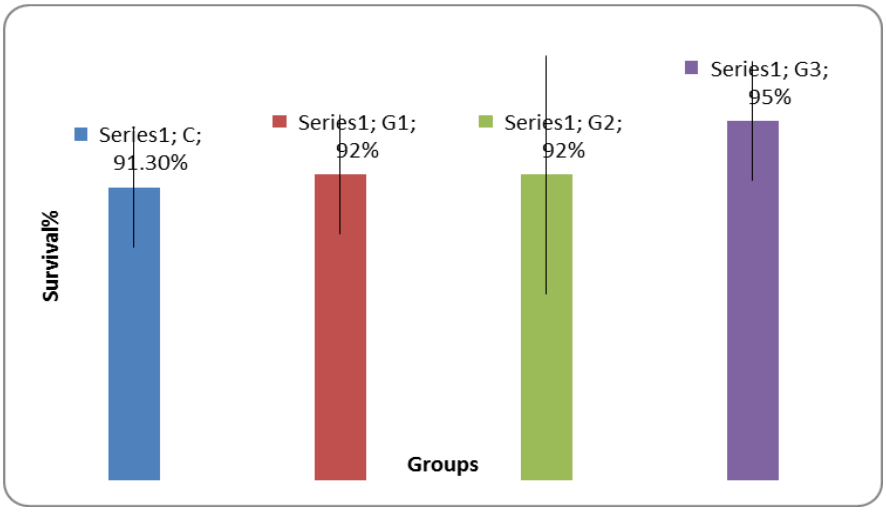

Figure 1: Survival (%) of *Litopenaeus vannamei* juveniles fed basal diet (C) or basal diet and EELS enriched *Artemia* in different concentrations (G1-G3) after 30 days culture period.

Table 1: Growth performance of juvenile shrimp *Litopenaeus vannamei* after feeding on experimental diets for 30 days.

Groups	Weight gain (mg)	Length gain (mm)	SGR%
C (control)	237.4 ± 4.6^{a}	31.6 ±3.4^{ab}	11.71 ±0.43^{a}
G1 (200 mgL^{-1})	282.7 ± 3.8^{b}	34.7 ±2.8^{ab}	12.27 ±0.31^{b}
G2 (400 mgL^{-1})	333.2 ±3.3^{c}	39.6 ±4.2^{bc}	12.81 ±0.27^{c}
G3 (600 mgL^{-1})	302.7 ±5.6^{d}	37.4 ±5.1^{bc}	12.49 ±0.67^{d}

Data in the same column with different letters are significantly different (p<0.05) among different treatments.

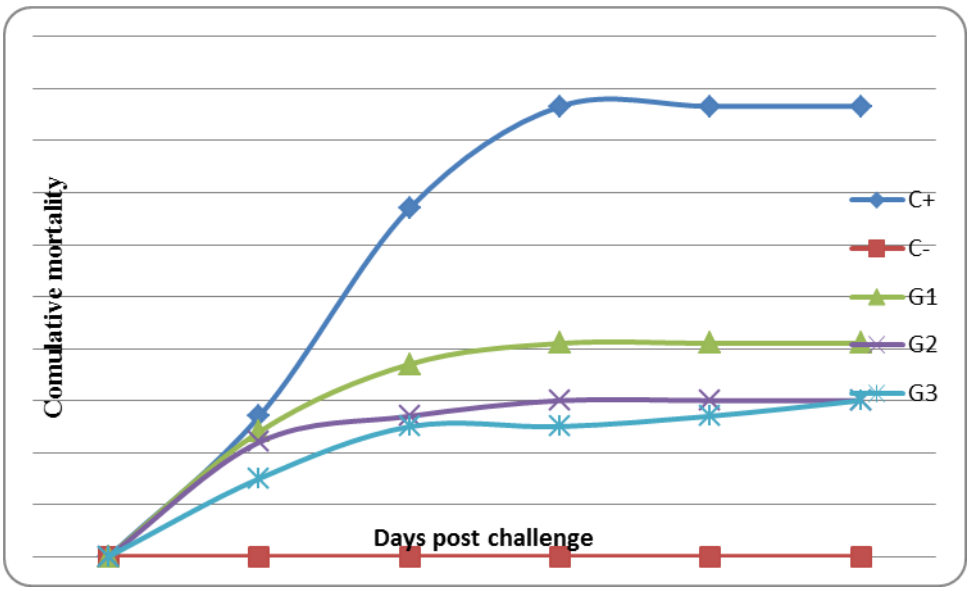

Figure 2: Cumulative mortality (%) of *Litopenaeus vannamei* challenged with VH after being fed basal diet (C+, C-) or basal diet and EELS enriched *Artemia* (G1-G3) for 30 days.

Discussion

The current study assessed the nutritional value of EELS for juvenile *L. vannamei* by measuring survival, growth performance and resistance to vibriosis. The results indicated that use of *Artemia* enriched with EELS is useful for growth and disease resistance under controlled laboratory conditions.

With regard to toxicity of the ethanol extract, it is considered toxic when the LC_{50} for *Artemia* instar I larvae is in the range of 0-80 µg mL^{-1}, moderately toxic at 80-250 µg mL^{-1} and weakly toxic at more than 250 µg mL^{-1} (Ramos *et al.*, 2009). The LC_{50} of EELS in our study was 1.473±0.241 mg mL^{-1}, that could be considered non-toxic or very weakly toxic to *Artemia* instar I larvae.

This study revealed that EELS has a positive effect on survival and growth parameters in shrimp *L. vannamei* juveniles for 30 days under laboratory conditions. Also the study suggests that there is an increase in survival and growth parameters when the levels of dosage of algae are increased. Cornejo *et al.* (1999) also experienced the effect of the seaweed *Caulerpa sertularioides*

on the growth, survival and biomass of the brown shrimp *Penaeus californiensis* for a 10- week period in 150 L tanks with three treatments: Treatment 1- with no seaweed, but commercial feed with 35% crude protein; Treatment 2- indirect presence of seaweed with commercial feed; and Treatment 3- direct presence of seaweed with commercial feed. The results for growth, survival and production were as follows: Treatment 1, 0.46±0.4 g, 68.7±1.2% and 5.6± 1,1 g; Treatment 2, 0.73±0.4 g, 75±1.0% and 7.8±1.2 g; and Treatment 3, 3.98±0.4 g, 100% and 36.24±4.3 g, respectively. The author concludes that the presence of the algae *C. sertularioides* has a direct effect on the growth, survival and biomass of the brown shrimp *P. californiensis* under laboratory conditions. Da Silva and Barbosa (2009) also found that the marine algae *Hypnea cervicornis* and *Crypto nemia* are feasible for use in the feeding of *L. vannamei*, with effects on shrimp growth rates. They also found that there is an increase in feed conversion when the levels of algae are increased. Penaflorida and Golez (1996) also, reported that survival was higher in shrimp fed the diet with 3% *Kappaphycus alvarezii* (a red seaweed). Enhancement in growth due to seaweed inclusion was also distinguished by Hashim and Mat Saat (1992). Cruz-Suarez *et al*. (2008) found a significant increase in growth rate (53-68%) in white shrimp *L. vannamei* juveniles (450 mg) fed diets including 2-4% of Mexican kelp (*Macrocystis pyrifera*) meal by contrast to those fed a control diet.

The active composite of macroalge responsible for growth enhancement has not been obviously defined, but the benefits haves been attributed to their vitamin and mineral content, lipid mobilization and improved absorption and assimilation efficiency ratios (Cruz-Suarez *et al*., 2008).

This work also showed that the EELS has an anti-VH effect. This study revealed that EELS can protect *L. vannamei* juveniles from vibriosis. *L. vannamei* that received EELS enriched *Artemia* at 200 mg mL^{-1}, 400 mg mL^{-1} and 600 mg mL^{-1} showed increased resistance against VH compared to positive control. In another research, the *Sargassum fusiforme* polysaccharide extract (SFPSE) was assessed as a feed additive when supplemented in the diets (0%, 0.5%, 1.0%, and 2.0%) for juvenile shrimp, *Fenneropenaeus chinensis* for 14 days (Huang *et al*., 2006). It was found that the shrimp treated with 1.0% and 0.5% SFPSE displayed significantly lower cumulative mortalities after being injected with *V. harveyi* suspension 24 and 30h later, respectively, compared with that of the control.

The use of algal metabolites for the control of infectious pathogens of *P. monodon* was reported to be most effective by different researchers (Immanuel *et al.* 2004; Jose *et al.,* 2008; Selvin *et al.,* 2009). They showed that herbal and algal extracts may be effectively used as a dietary source to improve the disease resistance

as well as to have better survival and production of *F. indicus* in aquaculture systems.

Banana shrimp *F. merguiensis* fed with *Sargussum platensis* showed resistance against *V. harveyi* infection (Lee *et al*., 2003). *L. vannamei* treated with hot-water extract of *Gracilaria tenuistipitata* via injection exhibited resistance against *V. alginolyticus* (Hou and Chen, 2005).

Seaweeds contain many different polysaccharides, Sulfated polysaccharides inhibit activity of many bacterial species as well as viruses (Leonard *et al.*, 2010). Polysaccharides also can work as prebiotics (substances that stimulate the growth of beneficial bacteria in the digestive track) and provide growth-promoting and health-improving effects (Vidanarachchi *et al.*, 2009).

In conclusion, this study revealed that feed supplementation by bioencapsulation of ethanol extract from the red seaweed *L. snyderiae* has potential of survival, growth-promoting and antibacterial activity against *V. harveyi* in shrimp *L. vannamei* juveniles. The results from this study also may be useful for shrimp hatchery and nursery ponds.

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