The efficacy of the red seaweed (*Laurencia snyderiae*) extract on growth performance, survival and disease resistance in white shrimp (*Litopenaeus vannamei*)

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Abstract

Shrimp aquaculture has expanded rapidly in many countries and this rapid development resulted diseases outbreaks and further considerable utilization of antibiotics. Use of natural products as antimicrobial has been reported as a resolution problem. The crude extract of a red seaweed (*Laurencia snyderiae*) collected from the Persian Gulf was evaluated for shrimp growth performance and to determine in vivo efficacy of this seaweed to prevention of shrimp Vibriosis. The ethanol extract of *Laurencia snyderiae* (EELS) was not toxic to the Artemia instar- I when it was fed to them for enrichment. The white shrimp (*Litopenaeus vannamei*) Juveniles fed with these enriched Artemia at 0 mg mL-1 (Control group), and three treatments of 200 , 400 and 600 mg mL-1 for a 30 days period. Results showed an increase in survival rate in treatment groups compared with control group but wasn't significantly ($P<0.05$). Shrimps that fed with enriched Artemia showed a significant in growth parameters comparing to Control group ($P<0.05$). The notably lower mortality was observed when these juvenile shrimps were challenged to Vibrio harveyi (after 30 days) in comparison with Control. The results indicated that EELS has a good potential in growth- promoting and antibacterial activity against *V.harveyi* in which is useful in shrimp aquaculture.

Keywords: red seaweed, *Laurencia snyderiae*, *Litopenaeus vannamei*, Vibrio harveyi.

Introduction

During the last decades, shrimp aquaculture has expanded rapidly in many countries especially in developing countries. Shrimp aquaculture in Iran also has started in 1990's in Bushehr province and rapidly extended to other areas besides Persian Gulf in south of Iran (Kakoolaki 1997). One of the main concerns in shrimp aquaculture is mortality and economic impacts due to diseases outbreaks. Rapid development of shrimp aquaculture and diseases outbreaks has resulted high amount utilization of antibiotics in shrimp aquaculture industry. Antibiotics are frequently used at therapeutic levels to disease treatment or at sub-terapeutic levels as prevention of shrimp disease or to increase of feed efficiency and growth rate improvement (Selvin, Huxley & Lipton 2004).

Sustainability of the industry depends on the shrimp health and efficient shrimp disease prevention (Selvin, Ninawe & Lipton 2009). Thus, control of the disease and health management has been considered as a preference for shrimp aquaculture industry (Roch 1999). The main species causing Vibriosis in shrimp aquaculture are *Vibrio harveyi*, *V. fluvi-*

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alis, *V. parahaemolyticus*, *V. damsella* and *V. vulnificus* (Chythanya & Karunasagar 2002). In most shrimp farms and hatcheries antibiotics and chemicals were being used for vibriosis without proper scientific investigation (Dashtiannasab, Kakoolaki, Sharif Rohani & Yeganeh 2012). The environmental effluence and development of antibiotic-resistant pathogens were being raised (Karunasagar, Pai & Malathi 1994). The antibiotic resistant bacteria in shrimp hatcheries and farms is a universal problem that has investigated by many researchers (Song & Sung 1993; Hektoen, Berge, Hormazabal & Yndeslad 1995; Mahasneh, Jamal, Kashashneh & Zibdeh 1995; Herwing, Gary & Weston 1997; Rahim, Sanyal, Aziz, Huz & Chowdhury 1998). The appear of antibiotic resistance of pathogens in aquaculture needs new efficient antibiotics to treat maricultured species (Braithwaite & McEvoy 2005; Bansemir, Blume, Schröder & Lindequist 2006). Among different alternatives in shrimp aquaculture, use of natural products as antimicrobial has been reported as a resolution problem (Selvin et al. 2009). Some efforts have been done to find new antibiotics from marine organisms such as macroalgae to control bacterial pathogens (Immanuel, Vincybai, Sivaram, Palavesam & Marian 2004; Bansemir et al. 2006; Dashtiannasab et al. 2012). Concerns of human health and ecological safety due to some chemicals and antibiotics have encouraged the increasing interest in more "natural-green" alternatives as antibiotics. Seaweeds are considered as effective source of bioactive compounds that are competent to produce different important secondary metabolites described with great biological activities. The use of bioactive compounds from seaweeds to enhance disease resistance and growth improvement in aquaculture also has been reported (Huang, Zhou & Zhang 2006; Yeh, Lee & Chen 2006; Fu, Hou, Yeh, Li & Chen 2007; Kanjana, Radtanatip, Asuvapongpatana, Withyachumnarnkul & Wongprasert 2011).

It's seems that these natural compounds are an excellent strategy to shrimp disease control but there has been a few work in the development of natural products to prevention and therapeutic for shrimp disease control. The aim of this study was to evaluate the efficacy of ethanol extract of a red seaweed (*Laurencia snyderiae*) obtained from Persian Gulf coastline on shrimp growth performance and to determine in vivo efficacy of this seaweed to prevention of shrimp Vibriosis.

Materials and Methods

Samples

The red seaweed were collected during low tide from a marine rocks area of the Persian Gulf shores in northern part of Bushehr in south of Iran during November and December 2012.

The postlarvae (PL15) of *Litopenaeus vannamei* were purchased from a shrimp hatchery in Delvar, Bushehr province, and acclimatized in two 500 lit 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, in south of Iran.

The pathogen bacteria (Vibrio harveyi) have been deposited in Iranian Research Organization for Science and Technology under Persian Type Collection Center accession numbers PTCC 1755 were obtained from Microbiology laboratory of Iran Shrimp Research Center, Bushehr, Iran.

Algae Extraction

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

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 L-1 cyst) in sterile filtered (0.45 µm) seawater at 28–30°C with strong aeration, under a constant light regime. Just 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 of 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 24 h of exposure by counting the number of dead nauplii and comparing with control group using a stereomicroscope. The experiments were performed in triplicate. The lethal concentration of the EELS was defined as which caused 50% mortality of the Artemia nauplii (LC50) using probit software.

Enrichment of Artemia nauplii

Artemia nauplii (Instar I -II) are regularly used in shrimp hatcheries as live–feed for postlarvae stages. After of cyst hatching the first instar nauplii appeared; they did not feed because their anus is still closed. After 12 h the larvae molts into the second stage the nauplieus (instar-II) started feeding on small particles (<50 µ) and therefore in this stage, the Artemia nauplii enrichment is done with EELS by the method of Kanjana 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 O2 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 utilization.

Experimental design

Shrimp experimental arrangement and maintenance L. vannamei juveniles (PL-15; 1500 Nos.) acclimatized in two 500 lit plastic tanks with clean seawater in standard conditions: 40±1 ppt, 28±1ºC, and constant aeration for 5 days. After 5 days of nursing phase, shrimps that had no signs of disease were chosen for experiments. In adaptation period the shrimps were fed by a formulated shrimp feed (Havoorash Co.) twice per day. After the initially biometric analysis (1.1± 0.2 cm, 7.4 ± 0.27 mg), shrimps were divided into four groups as 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 Treatments 1 (G1), 2 (G2) & 3 (G3) shrimps fed with enriched Artemia in concentrations 0.5, 1.0 and 2.0 mg mL-1 EELS and fed basal diet, respectively.

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 h to 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 fed with 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. The Control group was fed with unenriched Artemia. The experiment was extended for 30 days (PL20–PL50 stage).

Survival and Growth performance

In the termination of the 30-day culture period, the number of live shrimps were counted in each experimental to measure survival rate (formula 1) (Immanuel, Palavesam & Peter Marian 2001). The growth parameters were estimated by measuring the length and weight of the shrimps . 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 methods (formula 2).

1. Survival (% / day) = 100 × (final live shrimp number) / (initial shrimp number)

2. Specific growth rate (SGR) (%)= 100 × [(lnw2 −lnw1)/(t2 −t1)]

Where: ln= Logarithmic number, W2 = Final weight at time t2 , W1= initial weight at time t1

Vibriosis challenge trial

A strain of V. harveyi (PTCC 1755) isolated from diseased L. vannamei in Bushehr province was used for challenge test. The pathogen was cultured on tryptic soy agar (TSA supplemented with 2% NaCl, Difco) for 24 h at 25 ºC before being transferred to 10 mL tryptic soy broth (TSB supplemented with 2% NaCl, Difco), where it remained for 24 h at 25 ºC as stock culture for tests. The broth cultures were centrifuged at 7155 rpm for 15 min at 4 ºC. The supernatant fluids were removed and the bacterial pellets were re-suspended in saline solution at 1×108 cfu mL-1 as bacterial suspensions for the resistance test. Challenge experiments were performed in triplicate with 20 shrimps per replicate. After completing the dietary experiment, the postlarvae fed for 30 days were immersed at 1×108 for 15 min and transferred to rectangular plastic tanks (30 ×50 × 50 cm) containing 20 L filtered seawater including 1×106 of pathogen V. harveyi. In addition to total four Control and Treatments groups (C, G1,G2 and G3); one group was added as negative control namely no exposure with V. harveyi bacteria. The mortality of each replicate was recorded continuously for 5 days.

Data analysis

All the data are presented as mean ± SD . Mean values were compared between experimental groups and the control using one way analysis of variance (ANOVA) using SPSS for Windows version 18.

Results

Cytotoxicity assay

The seaweed ethanolic extract was estimated for its cytotoxicity at different concentrations at 24 h exposures time. The LC50 of EELS was obtained 1.473 ± 0.241 mg mL-1.

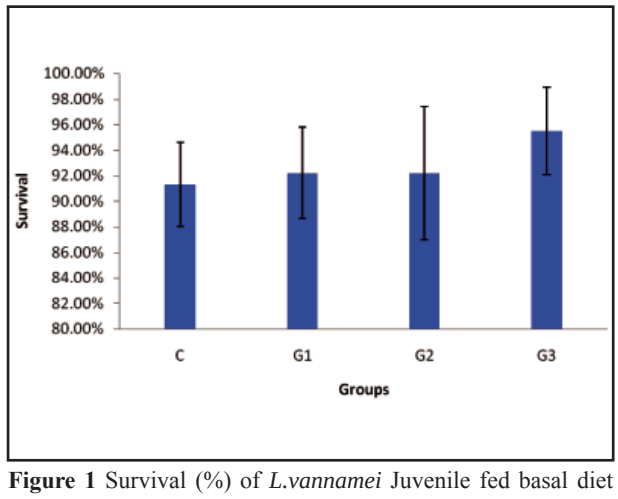

Figure 1 Survival (%) of *L.vannamei* Juvenile fed basal diet (C) or basal diet and EELS enriched Artemia in different concentration (G1-G3) after 30 days culture period.

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 variant and it was maximum (95.5 ± 3.4%) for those shrimps 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, where as the minimum survival of 91.33% was recorded in control shrimps fed with unenriched Artemia (C). But the differences weren't significant (P<0.05) (Fig. 1).

The *L.vannamei* samples obtained maximum weight gain of 333.2 ±3.3 mg in G3 (fed enriched Artemia with 400 mg L-1) group. But the minimum weight gain of 237.4 ± 4.6 mg was displayed in Control group that difference was significant (P<0.05). In treatments (G1-G3) G2, G3, G1 attained further weight gain, respectively.

The specific growth rate (SGR) of the *L.vannamei* juveniles after 30 days culture in different treatment and control were variant, 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 Control and treat-

Table 1 Growth performance of juvenile shrimp *L.vannamei* after feeding with experimental diet 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.

ments were statistically significant (P<0.05) as shown in Table 1.

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

All the unchallenged shrimp (Negative Control) survived. But death began to occur after 24 h 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 that *L.vannamei* Juvenile fed with EELS enriched Artemia had lower mortality (P<0.05) by contrast to positive Control (41.3 ± 3.3%, 30.0 ± 3.3%, 30.0 ± 6.6%, at 5 days in the G1, G2 and G3, respectively) (Fig. 2).

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

Discussion

With attention to toxicity of the ethanol extract, matters are considered stoutly toxic when their LC50 for Artemia instar I larvae are 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, Oliveira, Câmara, Castelar, Carvalho & Lima-Filho 2009). The LC50 of EELS in our study was 1.473 ± 0.241 mg mL-1, that could be considered non-toxic or very weak toxic to Artemia instar I larvae. This study revealed that EELS has a positive effect on survival and growth parameters in *L.vannamei* juvenile for 30 days period under laboratory condition. Also the study suggest that there is an increase in survival and growth parameters when the levels of algae are increased. Cornejo, Curdova, Barajas, Pramo & Clark (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 the subsequent: 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 &Barbosa (2009) also found that the marine algae Hypnea cervicornis and Crypto nemia are feasible for use in the feeding of L. vannamei, with effect on shrimp growth rates. They also found that there is an increase in feed conversion when the levels of algae are increased. Penaflorida &Golez (1996) also, reported that survival was higher in shrimp fed the diet with 3% Kappaphycus alvarezii (red seaweed). Enhancement in growth due to seaweed inclusion was also distinguished by Hashim &Mat Saat (1992). Cruz-Suarez, Tapia Salazar, Nieto Lopez & Rique (2008) found a significant increase in growth rate (53-68%) in white shrimp (*Litopenaeus vannamei*) juvenile (450 mg) fed diets including 2-4% of Mexican kelp (Macrocystis pyrifera) meal by contrast a control diet. The active composite of macroalgae responsible for growth enhancement has not obviously defined, but the benefit has 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 have an anti-VH

effect. This study revealed that EELS can protect L. vannamei juvenile from vibriosis. L. vannamei received EELS enriched Artemia at 200 , 400 and 600 mg mL-1 showed increase resistance against VH respectively, compared to positive control. In a research, the Sargassum fusiforme polysaccharide extract (SFPSE) was assessed as a feed additive when supplemented in the diet (0, 0.5, 1.0, and 2.0%) for juvenile shrimp, Fenneropenaeus chinensis for 14 days (Huang et al. 2006) they 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 30 h 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, Lipton & Subhash 2008; Selvin et al. 2009) 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 Min-Hsien & Shiau 2003). L. vannamei treated with hot-water extract of Gracilaria tenuistipitata via injection exhibited resistance against V. alginolyticus (Hou & Chen 2005). Seaweeds contain many different polysaccharides, Sulfated polysaccharides inhibit activity of many bacterial species as well as viruses (Leonard, Sweeney, Pierce, Bahar, Lynch & O'Doherty 2010). Polysaccharides also can work as prebiotics (substances that stimulate the growth of beneficial bacteria in the digestive track) and apply growth-promoting and health-improving effects (Vidanarachchi, Iji, Mikkelsen, Sims & Choct 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* j uvenile. The results from this study also may be useful for shrimp hatchery and nursery ponds.

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تأثیر عصاره جلبک دریایی قرمز (*Lurenciae snyderiae*) بر عملکرد رشد، بازماندگی و مقاومت به بیماری در میگوی سفید غربی (*Litopeneus vannamei*)

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چکیده

پرورش میگو در بسیاری از کشورها سریعاً گسترش یافته است و نتیجه این توسعه سریع شیوع بیماری‌ها و استفاده زیاد از آنتی بیوتیک ها بوده است. استفاده از محصولات طبیعی به عنوان یک راه کاهش تلفات گزارش شده است. در این مطالعه عصاره یک جلبک دریایی (*Lurencia snyderiae*) اســتحصالی از خلیج فارس روی عملکرد رشــد میگوها و تعیین تأثیر آن در پیشــگیری از بیماری ویبریوزیس مورد ارزیابی قرار گرفت. عصاره اتانولی *L.snyderiae* (EELS) برای ناپلی آرتمیا ســمی نبود. لذا مرحله اینســتار۱ آرتمیا با این عصاره غنی ســازی شــد و میگوهای جوان *L. vannamei* با آرتمیای غنی سازی شده با دزهای ۰، ۲۰۰، ۴۰۰ و ۶۰۰ میلی گرم در لیتر به مدت ۳۰ روز تغذیه شدند. نتایج نشان داد که میزان بازماندگی در گروههای آزمایش در مقایسه با گروه شاهد افزایش یافت ولی از نظر آماری معنی دار نبود (P>۰/۰۵). پارامترهای رشد در گروه های آزمایش در مقایسه با گروه شاهد بیشتر شده بود (P>۰/۰۵). موقعی که این میگوها پس از ۳۰ روز با باکتری ویبریو هاروی روبرو شدند در مقایسه با گروه شاهد تلفات کمتری داشتند. این نتایج مشخص کرد که عصاره اتانولی snyderiae L. EELS دارای پتانسیل خوبی در تحریک رشد و فعالیت ضدباکتریایی علیه باکتری ویبریو هاروی بوده و احتمالاً در صنعت پرورش میگو مفید خواهد بود.

واژه‌های کلیدی: جلبک دریایی قرمز، *Litopenaeus vannamei*، Lurencia snyderiae، ویبریو هاروی.

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