Effect of dietary supplementation of *Ulva rigida* C. Agardh extract on several of physiological parameters of grey mullet, *Mugil cephalus* (Linnaeus)

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Abstract

The present research was aimed to assess the effect methanol extract from *Ulva rigida* on biochemical response and digestive enzyme activities in *Mugil cephalus*. A control diet without and three other experimental diets were supplemented by *U. rigida* extract (UE diet) at the inclusion levels of 5, 10 and 15 g kg^{-1} diet, respectively. One hundred and twenty grey mullets with the mean weight of 14.95 ± 2.01 g were randomized into 12 tanks and fed twice per day (09:00 and 17:00) for 60 days. After 60 days of the feeding trial, serum total protein and globulin levels of the fish receiving UE diet had incremental trend and better performance, except for samples fed UE5. The UE10 diet resulted in the highest amylase, lipase and protease activities. Significantly different was observed in survival rate, glucose and triglyceride levels between control and UE supplemented groups ($p< 0.05$). There was significant reduction in glucose and triglyceride levels in the fish received UE diet compared to the control ($p<0.05$). According to the present results, *U. rigida* extract as a new introduced dietary supplement is suitable for improving metabolism of carbohydrate and enhancing digestive enzyme activities in grey mullet.

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Keywords: *Ulva rigida*, *Mugil cephalus*, Biochemical response, Digestive enzymes, Dietary supplement.

Introduction

There are currently a variety of chemical compounds, including hormones, antibiotics and vitamins, in mariculture as factors enhancing growth or with antibacterial activity and other purposes (Nwabueze 2012). Despite the reported

positive effects of different chemicals on fish and shrimp, their residual impacts in the muscle of aquaculture species make them inappropriate commercially. The resistant microbial strains have been emerged following the indiscriminate use of antibiotics as prophylactic therapy in marine fish hatcheries, increasing the need for alternative antibiotics (Sahu, Das, Mishra, Pradhan & Sarangi 2007). Moreover, the larval growth and defense mechanisms of the fish might be reportedly suppressed because of taking antibiotics.

Many sensitivities and adverse complications have been observed due to antibiotics and other chemicals (Shalaby, Khattab & Abdel rahman 2006).

Plants and related derivates contain safer and inexpensive natural compounds varied effective properties in aquaculture industries, including anti-stress activity, enhancement of growth, induction of appetite and stimulation of immune system (Ju, Forster & Dominy 2009; Kakoolaki, Akbary, Zorriezahra, Salehi, Sepahdari, Afsharnasab, Mehrabi & Jadgal 2016). The natural alternative appetizers have attracted recently further attention of researchers due to the knowledge of consumers and concerns raised for unsafe foods. Some of natural plant products reported as growth promoters, immunostimulants and appetizers are microalgae (Ju *et al.* 2009), seaweeds (Yeh, Lee & Chen 2006.) and herbal extract (Sankar, Elavarasi, Sakkaravarthi & Ramamoorthy 2011). Accordingly, feed consumption and digestion can be promisingly improved in the aquaculture industry (Supamattaya, Kiriratnikom, Boonyaratpalin & Borowitzka 2005). There is effective information available for promoting fish growth and immunity regarding dietary seaweeds, including *Porphyra purpurea* (Roth) in the thick-lipped grey mullet ,*Chelon labrosus* (A.Risso), (Davies, Brown & Camilleri 1997), *Hizikia fusiformis* (Harvey) in the olive flounder, *Paralichthys olivaceus* (Choi, Kim, Han, Nam & Lee 2014), *Pyropia yezoensis* in the olive flounder (Choi, Lee & Nam 2015) and *Sargassum ilicifolium* (Turner) in the rainbow trout, *Oncorhynchus mykiss* (Walbaum), (Zamannejad, Emadi & Hafezieh 2016). Interestingly, dietary seaweed species and its concentrations can affect the role of dietary seaweed supplementation (Yeh *et al.* 2006., Zamannejad *et al.* 2016). Although reports are available about beneficial effect of seaweed on growth, immunity and biochemical responses as a dietary supplementation in aquaculture species, there is no data in line with the dietary role of *U. rigida* (C. Agardh) extract on biochemical responses and digestive enzyme activities in grey mullet. The mullet is one of the widely consumed species in aquaculture industries among countries grown in polyculture with shrimp, carps, tilapia and milkfish have been recognized as very desirable for pond culture in Iran, China, Egypt, Hawai, Italy, Japan, Philippine, Taiwan and other regions throughout the world (El-Dahhar, Salama, Moustafa & Zahran 2000). The feed intake and adequate nutrients provided to the fish are leading parameters to promote the quality of fish.

The present research aimed to evaluate the effect of dietary inclusion *U. rigida* extract on biochemical response and digestive enzyme activities in grey mullet, *M.cephalus*.

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Materials and Methods

Preparation of *Ulvae. rigida* methanol extract

To this end, 2 kg of *U. rigida* algae was collected from Chabahar coast in Iran, dried in an oven at a temperature of 60°C, crushed by mortar and pestle to powder and finally passed through the sieve. In the next step, 50 g of the prepared powder was placed in 10 L of 99% methanol (10%w/v) at ambient temperature (24 ± 1.2°C) for 48 h to achieve the extract. Afterward, the rotary evaporator (IKA, Germany) was used to concentrate the extract up to 300 ml, resulting in the extract of 6.1 g of powder ml^{-1}. The extract was diluted with 300 ml of distilled water and sprayed on the diet (Choi *et al.* 2015).

Experimental diets and Feeding conditions

Four diets were supplemented with PE at different inclusion levels of 0, 5, 10 and 15 g kg^{-1} diet analyzed proximate compositions are presented in Table 1. For the preparation of four experimental diets, a commercial 1.6-mm extruded pellet (Beyza Feed Mill, Iran) was ground into a powder (0.5 mm particle size), added by 30% distilled water, mixed, pelletized to 1-mm particles using a chopper device. The resulting diets were freeze-dried at -40˚C overnight and stored at -20°C until testing (Choi *et al.* 2015). The feeding test was fulfilled at Fisheries Research Center, Chabahar, Iran. One hundred and twenty grey mullets with the mean weight of 14.95 ± 2.01 g were randomized into 12 tanks (60 L) at a density of 10fish/tank as triplicates per treatment and hand feeding to satiation was performed by twice a day, 09:00 and 17:00, during 60 days and feed intake was recorded on daily basis. The measurements showed dissolved oxygen (DO) concentration of 7.01 ± 0.87 mg L^{-1}, ammonia nitrogen content of 0.11 ± 0.04 mg L^{-1} and pH value of 7.8 ± 0.4. The photoperiod was regulated as a 12:12 h (dark/light) cycle. Biometry was done during the first and last days of the experiment.

Table 1. Ingredients (g kg^{-1}) and chemical composition (%) of the experimental diets

	Diets			
Ingredients (g kg^{-1})	UE0	UE5	UE10	UE15
P.astraulis	0	5	10	15
Fish meal	427	427	427	427
Soybean meal	192.5	192.5	192.5	192.5
Wheat flour	93	93	93	93
Dried yeast	37.5	37.5	37.5	37.5
Fish oil	55	55	55	55
Soy oil	27.5	27.5	27.5	27.5
Choline chloride	2	2	2	2
Bi calcium phosphate	3.7	3.7	3.7	3.7
Lecithin	28.15	28.15	28.15	28.15
Premix[a]	9.4	9.4	9.4	9.4
Proximate composition(%)				
Crude protein	51.6	51	50.6	51.6
Crude lipid	11.9	11	11.4	11.2
Crude ash	12.1	12	11.8	12.6
Dry matter	92.2	92.1	92	92

[a]Premix (mg kg^{-1}) KI, 250; $MnSO_4 \cdot H_2O$, 2800; $ZnSO_4 \cdot H_2O$, 2350; vitamin K, 225; biotin ,3500(%2) ; niacin, 4850; calcium pantothenate, 11,000; folic acid, 2000; vitamin B1, 1500; vitamin B_2, 2000; vitamin B_6, 2000; and vitamin C, 50,000.

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Digestive enzyme activity

Three samples of fish from each tank were randomly selected and sacrificed for preparation of enzyme extracts. Thus, the digestive tracts were dissected carefully, cleaned completely with sterile distilled water, weighed and homogenized separately with cooled buffer phosphate (0.65 %, pH 7, 1: 10 w/v). The supernatant, extracted by centrifugation with 3000 g at 4˚C for 20 min (Centrifuge EBA21, Hettich, Germany), was used for enzyme assays. The 3, 5-dinitrosalicylic acid (DNS) method (King 1965) was applied to assess the amylase activity, so that 0.1 ml of tissue homogenate, 2 ml of phosphate buffer (0.1 M, pH 7) and 0.1 ml of 1 % (w/v) starch solution was mixed and incubated at 30˚C for 35 min. Then, adding 2 ml DNS reagent stopped the reaction. After 5 min in boiling water, the reaction mixture was cooled and diluted with distilled water to record the absorbance at 540 nm. Protease activity was measured by the casein digestion method of king (1965), as 0.1 ml of tissue homogenated, 0.05 M of trisphosphate buffer (pH 7.8), 0.01 N NaOH and 2.5 ml of 1% (w/v) consisted the reaction mixture. The incubation was performed at 30˚C for 10 min and stopped by 2.5 ml, 10% trichloroacetic acid (TCA) and filtered. The reagent blank consisted of only tissue homogenate before stopping the reaction, but with no incubation. The absorbance was read at 320 nm. A unit of amylase activity was defined as the weight (mg) of maltose released for 10 min at 30˚C, and a unit of protease activity was the tyrosine level released within 15 min under the test conditions. Lipase activity was assessed by King (1965) method. Olive oil emulsion, phosphate buffer (pH 7.8, 0.1 M), tissue homogenate and distilled water consisted the reaction mixture. The reaction mixture was incubated at 30˚C for 24 h and added two drops phenolphthalein indicator and 95% alcohol for titration against 0.05 N NaOH upon emerging permanent pink color. A unit of lipase activity was defined as the amount of 0.025 N NaoH essential for neutralizing the fatty acids released within incubation for 18 h in pH of 6.9 at 30˚C. Enzyme unit per gram tissue was calculated for digestive enzymes.

Biochemical analysis

At the end of experiment, 9 fish were anesthetized from each treatment (with clove oil of 5 mg L^{-1}) and the blood samples collected after excising caudal peduncle were poured into un-heparinized sterile tubes 1–1.5 mL to test biochemical factors of the separated serum (Shaluei, Hedayati, Jahanbakhshi & Baghfalaki 2012).

A method proposed by Trinder (1969) was utilized to measure the serum glucose concentration. The concentrations of total protein and albumin in the serum were obtained according to Wootton (1964). Through subtracting albumin level from total protein globulin was calculated. Triglyceride and cholesterol concentrations were measured in accordance with the method described by Sankar *et al.* (2011). Biochemical estimation of blood glucose, protein, cholesterol and triglyceride were determined by means of standard analyses kits (Pars Azmon, Iran) using automatic analyzer (Furuno, CA-270, Japan).

Statistical analysis

Data from all measurements with two replicates were analyzed by SPSS for windows versions 16 using one-way ANOVA at the significance level of $p<0.05$ between the groups. The differences between all groups were analyzed using Duncan multiple comparisons test for significant *F* value for ANOVA. All data are expressed as mean ± standard Error.

Results

Digestive enzyme activities

Significant increase was observed in the specific activity of amylase for fish fed PE diet compared with control. The maximum specific activity of amylase was recorded in those fish fed PE diet at 15 g kg^{-1} of feed. No significant changes were found in the specific activity of protease and lipase observed in those fish fed PE diet at 5 and 10 g kg^{-1} feed over the control except fish received PE diet at 15 g kg^{-1} feed (Table 3).

Table 2. Digestive enzyme activities of *M. cephalus* fed UE diet at different levels for 60 days

Specific activity of enzyme (unit mg^{-1} protein)	UE diet (g kg^{-1} feed)			
	0	5	10	15
Amylase	187 ± 15.58^{d}	205.67 ± 13.38^{c}	243.33 ± 13.52^{a}	225 ± 16.60^{b}
Protease	373.33 ± 22.90^{c}	390.33 ± 17.88^{b}	420 ± 21.15^{a}	393.23 ± 10.29^{b}
Lipase	15.66 ± 0.66^{d}	19.06 ± 1.23^{c}	23.50 ± 1.28^{a}	21.16 ± 2.60^{b}

PE diet, *U.rigida* extract diet. Values (mean ± SE of three replication).In each row not sharing a common superscript are significantly different (P<0.05).

Biochemical indices of serum

Fish fed UE diet showed significant increase in the serum total protein content ($p<0.05$) compared to the control, except for received UE diet at 5 g kg^{-1} feed. The highest total protein content was recorded in those fish group that received UE diet at 10 g kg^{-1} of feed. No significant difference ($p>0.05$) was reported in serum Cholesterol in those fish group fed UE diet compared to the control group. There was significant ($p<0.05$) decline in the levels of glucose and triglycerides in the group fed UE diet compared to the control group (Table 1). Significantly higher serum albumin and globulin levels were only ($p<0.05$) found in fish receiving UE diets with 10 and 15 g kg^{-1} feed compare to the group receiving UE diets with 0 and 5 g kg^{-1} feed (Table2).

Table 3. Serum biochemical parameters of *M.cephalus* fed UE diet at different levels for 60 days

Parameter	UE diet (g kg^{-1} feed)			
	0	5	10	15
Total protein (mg ml^{-1})	4.42 ± 0.4^{c}	4.42 ± 0.38^{c}	5.45 ± 0.10^{a}	4.97 ± 0.4^{b}
Albumin (mg ml^{-1})	2.30 ± 0.5^{b}	2.30 ± 0.7^{b}	2.60 ± 0.5^{a}	2.37 ± 0.11^{b}
Globulin (mg ml^{-1})	2.12 ± 0.8^{b}	2.12 ± 0.9^{b}	2.85 ± 0.21^{a}	2.67 ± 0.14^{a}
Glucose (mg dl^{-1})	55 ± 1.57^{a}	45.66 ± 1.88^{b}	30 ± 2.57^{d}	33.33 ± 2.88^{c}
Triglycerides (mg dl^{-1})	227.33 ± 11.76^{a}	204.11 ± 10.48^{b}	167.33 ± 11.45^{d}	188.26 ± 12.18^{c}
Cholesterol (mg dl^{-1})	69.99 ± 34.19^{a}	104.33 ± 11.20^{a}	75.33 ± 23.71^{a}	82.66 ± 13.71^{a}

PE diet, *U. rigida* extract diet. Values (mean ± SE of three replication). In each row not sharing a common superscript are significantly different (P<0.05)

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Discussion

The purpose of the present research was to evaluate the effect of dietary supplementation of *U. rigida* extract on biochemical response and activities in digestive enzyme of grey mullet (*M. cephalus*). The activities of digestive enzymes in fish are affected by the dietary macronutrients, such as carbohydrate and protein (Lara-Flores, Olvera-Novoa, Guzmán-Méndez & López-Madrid 2003). The synthesize of digestive enzymes are induced by the presence of macroalgae and microalgae in the gut, even at low concentrations (Mian, Hussain, Siddiqui & Immink 2014, Reitan, Rainuzzo, Oie & Olsen 1993). The higher specific activity of digestive enzyme, including amylase, protease and lipase, in UE diet at 10 g kg^{-1} feed might improve the digestion of protein, starch and lipid. In line with this finding, the amounts of digestive enzymes were increased in fishes (Lara-Flores *et al.* 2003) receiving *Zingiber officinalis* as an herbal appetizer (Venkatramalingam, Godwin Christopher & Citarasu 2007) supplemented diets as comparison with the control group. This suggests that dietary UE diet at 10 g kg^{-1} feed like all spices may reduce feed transit time. The digestive enzymes were positively affected by shortened transit time, probably accelerating the total digestive process (Venkatramalingam *et al.* 2007).

Immunostimulant diet is thought to potentate the action of insulin through the increase of insulin binding, insulin receptor number and function through lowering glucose and lipids, thereby regulating the uptake of glucose in to cells (Ahmed & Sharma 1997). The results of present study demonstrate that serum glucose level reduced in fish fed dietary UE. The present physiological finding is in agreement with olive flounder (*Paralichthys olivaceus*) fed *Pyropia yezoensis* extract which also significantly decreased serum glucose (Talpur 2014). Following the immunostimulant diet, a decrease in the blood glucose increased the serum insulin level (Kaleeswaran, Ilavenil & Ravikumar 2012). A possible explanation of the beneficial effect of UE diet attributed that it has stimulated the insulin activity, accordingly reduced the glucose level. Moreover, the present study revealed that feeding of UE diets to *M. cephalus* led to reduce serum triglycerides content in fish. According to the current study, decreased level of triglycerides might enhance the cardiovascular activity of fish (Kaleeswaran *et al.* 2012). Also, feeding of *P. yezoensis* extract in *P. olivaceus* and ethanolic extract of *Cynodon dactylon* in *Catla catla* have reduced serum triglycerides content (Kaleeswaran *et al*. 2012; Choi *et al*., 2015), which were in contrast to our results as it depends on variably dietary seaweed species and different concentrations (Choi *et al*. 2015). Total protein, globulin and albumin contents are recognized as prime factors for retaining a healthy immune system and functions in the blood and are well known to trigger potent innate immune system (Wu, Yuan, Shen, Tang, Gong, Li, Sun, Huang & Han 2007, Kaleeswaran *et al.* 2012 , Talpur & Ikhwanuddin 2012). In the present study, significant positive effects of dietary UE diet at

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10 g kg^{-1} feed on serum total protein and albumin were detected compared with the other levels of PE or the control group. Also, the fish group receiving the UE diets with 10 and 15 g kg^{-1} feed showed the highest serum globulin levels. Generally, it could be stated that PE diet at 10 g kg^{-1} feed is safe and advantageous to the health of *M. cephalus*. This is in agreement with the finding of Kaleeswaran *et al*. (2012) who found that using of 5% ethanolic extract of *Cynodon dactylon* had significant increased the serum levels of total protein, albumin and globulin concentrations as well as albumin/globulin ratio of *C.catla*. The data are also comparable with the significantly higher serum total protein determined in *P. olivaceus* fed with *P. yezoensis* extract (Choi *et al*. 2015).

In conclusion, the results presented in present study show that diet containing 10 g kg^{-1} UE enhanced total protein, albumin and globulin and declined triglyceride and glucose. Supplementation of dietary UE had marked effect on digestive enzyme activities of grey mullet. These factors play a key role in providing the high quality of grey mullet. According to the present findings, *U. rigida* can be considered as a new feed additive successfully applied in grey mullet feed. It can be one of the effective ways to stimulate appetite in grey mullet.

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Conflict of interests

The authors declare that there is no conflict of interest.

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اثر مکمل غذایی عصاره جلبک الوآ (*Ulva rigida*) و بر برخی پارامترهای فیزیولوژیکی ماهی کفال خاکستری (*Mugil cephalus*)

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چکیده

هدف از این تحقیق، بررسی اثر عصاره الوا بر پاسخ بیوشیمیایی و فعالیت آنزیم‌های گوارشی ماهی کفال خاکستری بود. گروه شاهد (بدون مکمل غذایی) و تیمارهای آزمایشی حاوی سطوح ۵، ۱۰ و ۱۵ گرم عصاره الوا در هرکیلوگرم غذا بود. ۱۲۰ قطعه ماهی کفال خاکستری با میانگین وزنی۲/۰۱ ± ۱۴/۹۵ گرم به صورت تصادفی در ۱۲ تانک تقسیم شده و روزانه دو بار (۹ صبح و ۱۷ عصر) به‌مدت ۶۰ روز تغذیه شدند. بعد از ۶۰ روز، سطوح گلوبولین و پروتئین تام سرم در ماهیان تغذیه شده با عصاره الوا روند افزایشی و عملکرد بهتری را به استثنای ماهیان تغذیه شده با ۵ گرم عصاره جلبک بر کیلوگرم غذا نشان داد. بیشترین فعالیت آنزیم آمیلاز، لیپاز و پروتئآز در ماهیان تغذیه شده با ۱۰ گرم عصاره جلبک بر کیلوگرم غذا مشاهده شد. اختلاف معنی‌داری در میزان بقاء، سطوح گلوکز و تری‌گلیسیرید بین گروه شاهد و گروه‌های حاوی مکمل جلبک مشاهده شد. سطوح گلوکز و تری‌گلیسرید در ماهی‌های تغذیه شده با عصاره جلبک کاهش معنی‌داری را در مقایسه با شاهد نشان دادند ($p<۰/۰۵$). مطالعه حاضر نشان داد که استفاده از عصاره الوا در جیره غذایی ماهی کفال خاکستری به منظور بهبود متابولیسم کربوهیدرات و افزایش فعالیت آنزیم‌های گوارشی توصیه می‌گردد.

کلمات کلیدی: جلبک الوا، ماهی کفال خاکستری، پاسخ بیوشیمیایی، آنزیم‌های گوارشی، مکمل غذایی

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