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Influence of Tetra (*Cotinus coggygria*) Extract against *Vibrio Anguillarum* Infection in Koi Carp, *Cyprinus carpio* with Reference to Haematological and Immunological Changes

Soner Bilen[1,*], Sevdan Yılmaz[2], Aslı Müge Bilen[1]

[1] Kastamonu University, Faculty of Fisheries, Department of Basic Sciences, 37200, Kastamonu, Turkey.
[2] Çanakkale Onsekiz Mart University, Faculty of Marine Sciences and Technology, Department of Aquaculture, 17100, Çanakkale, Turkey.

* Corresponding Author: Tel.: +90.507 2444155; Fax: +90.366 2802300;
E-mail: sbilen@kastamonu.edu.tr

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Abstract

In this study, the effect of the methanolic extract of tetra (*Cotinus coggygria*) against *Vibrio anguillarum* in cultured koi carp (*Cyprinus carpio carpio*) was investigated. Three different concentrations of tetra extract (0, 0.5, 1, 1.5 g kg^{-1} of feed) were individually mixed with the basal diet and fed to koi (average body weight of 4,14±0,08 g) for a 4-week period to assess the immunomodulatory characteristics of tetra. At the end of the study, koi was challenged with a strain of *Vibrio anguillarum* (10^8 CFU ml^{-1}). Growth performance was not affected by dietary tetra extract intake (P>0.05). Nitroblue tetrazolium (NBT) activity was found to be the highest in the 1.5 g kg^{-1} containing group. Lysozyme and myeloperoxidase activity of the experimental groups was significantly improved in the tetra groups compared to the control group (P<0.05). Red blood cell and mean corpuscular haemoglobin concentration (MCHC) were higher in 1 g kg^{-1} tetra containing group (P<0.05). This study shows that the koi treated with 0, 0.5, 1 and 1.5 g kg^{-1} tetra extract prior the challenge with live *V. anguillarum* had 37.50%, 31.94 %, 18.06 % and 12.50 % mortality. According to study results, tetra is an effective non-specific immunostimulant for koi.

Keywords: Koi carp, tetra, *Cotinus coggygria*, immunostimulant, *Vibrio anguillarum*, hematology.

Tetra (*Cotinus coggygria*) Özütünün Koi Balıklarında (*Cyprinus carpio*) *Vibrio anguillarum* Enfeksiyonuna Karşı Hematolojik ve Immunolojik Açıdan Etkileri

Özet

Bu çalışmada tetranın (*Cotinus coggygria*) metanolik özütünün koi balıklarında (*Cyprinus carpio carpio*) *Vibrio anguillarum* enfeksiyonuna karşı etkileri incelenmiştir. Koiler (ortalama ağırlıkları 4,14±0,08 g) üç farklı tetra dozu (0, 0,5, 1, 1,5 g kg^{-1}) içeren yemlerle 4 hafta boyunca beslenmişlerdir. Çalışma sonunda balıklar *Vibrio anguillarum* ile enfekte edilerek kontrol testi yapılmıştır. Tetranın balıkların büyüme performansı üzerinde etkisi bulunamamıştır (P>0,05). 1,5 kg^{-1} tetra verilen grupta NBT en yüksek olmuştur. Lizozim ve miyeloperoksidaz aktiviteleri tetra uygulanan gruplarda kontrol grubuna göre kayda değer yüksek bulunmuştur (P<0,05). Akyuvar ve hemoglobin konsantrasyonları 1 kg^{-1} tetra içeren grupta yüksek bulunmuştur (P<0,05). 0, 0,5, 1 ve 1,5 g kg^{-1} tetra ile beslenen gruplarda yapılan kontrol testlerinde ölüm oranları sırasıyla 37,50%, 31,94 %, 18,06 % ve 12,50 % olarak tespit edilmiştir. Çalışma sonuçlarına göre tetra koiler için etkili bir immunostimulanttır.

Anahtar Kelimeler: Koi, tetra, *Cotinus coggygria*, immunostimulant, *Vibrio anguillarum*, hematoloji.

Introduction

The ornamental fish sector is a well-known and global part of international trade, fisheries, and aquaculture. Koi carp (*Cyprinus carpio*) is also one of the important ornamental fish species cultured, imported and exported worldwide. Although the huge importance of these fish, some limited factors such as diseases and stress factors frustrate the production rate. In aquaculture industry, to prevent the fish from diseases many prophylactic studies have been developed and used. Most important prophylactic applications are use of vaccines, antibiotic practice (Nelis *et al.*, 1991) and immunostimulant implementation (Anderson, 1992).

Vibrio anguillarum is a virulent Gram negative fish pathogen which appears from spring to autumn, particularly when temperature is rising or falling (Akaylı and Timur, 2002), and is one of the most

terrifying bacteria in aquaculture (Toranzo and Barja, 1990). The bacteria are also reported in many fish species as a pathogen such as turbot (Grisez *et al.*, 1996), red sea bream (Muroga and Tatani 1982), sea bream (Akaylı and Timur, 2002), cod (Larsen *et al.*, 1994), Atlantic halibut (Bergh *et al.*, 1992), rainbow trout (Akşit and Kum, 2008) and sea bass (Tanrıkul *et al.*, 2004).

Nowadays, medicinal plants have gained potential usage and many infectious diseases are known to be treated with herbal remedies (Sokmen *et al.*, 1999; Yılmaz *et al.*, 2012a; Yılmaz *et al.*, 2012b; Yılmaz *et al.*, 2013). Turkey is a country that has one of the most extensive floras in continental Europe (Schnick *et al.*, 1997) with more than 9000 flowering plant species (Davis, 1965-1984). The accumulation of the knowledge of traditional medicine enabled this region to have a rich tradition in terms of the uses of medicinal plants (Gözler, 1993). Tetra (*Cotinus coggygria*) is an important medicinal plant which is spread throughout the north-west of Turkey (Kültür, 2007) and is found to be an effective immunostimulant for fish (Bilen *et al.*, 2011).

In the present study, the effect of tetra (*Cotinus coggyria*) on nitroblue tetrazolium (NBT) activities, myeloperoxidase activities, lysozyme activities, and some hematological parameters, as well as disease resistance against a pathogenic bacteria *Vibrio anguillarum* in koi carp was investigated.

Materials and Method

Experimental Design and Fish

Koi carp were obtained from Akdeniz Akvaryum Limited Company and kept for acclimatization for 3 weeks. 408 fish (average body weight 4.14±0.08 g) were distributed into 12 aquariums (80 L for each aquarium) for the tetra extract trial. All fish were kept in each of the triplicate glass aquariums designed for every treatment group. Tetra extract was added to the feed at 0.5 g kg^{-1}, 1 g kg^{-1} or 1.5 g kg^{-1}. The control diet contained no supplementation (0%). Experimental diets were fed to the fish ad libitum twice daily for four weeks. Throughout the experiment, water quality was temperature 27±0.1°C, pH 7.3±0.2, dissolved oxygen 7.10±0.2 mg/L, conductivity 502±12 uS, total NH_3 0.10±0.01 mg/L, nitrite 0.07±0.02 mg/L, and nitrate 1.0±0.1 mg/L.

Preparation of Tetra Extract

Tetra (*Cotinus coggyria*) was collected from Kırklareli province North-West of Turkey. The leaves were washed carefully with distilled water and dried under natural conditions and 1 kg sample was extracted by percolation with 6 L methanol (40%) and then filtered. The solvent was evaporated using a rotary vacuum evaporator and then freeze-dried. Lastly, 6 g concrete was dissolved in 100 ml absolute ethanol (Pakravan *et al.*, 2012). After that, it was added to the feed at a rate of 0, 0.5, 1.0, and 1.5 g kg^{-1} for diets T1, T2, and T3, respectively. The feed ingredients of the diets are presented in Table 1. The ingredients were mixed in a mixer. The feed was pressed through a 2 mm die in a pelleting machine, and the pellets were dried in a drying cabinet (40°C) until moisture dropped to around 10% with instant moisture analyzer (IR-35 - Denver Instrument). The pellets were crushed into desirable particle sizes and stored at –20°C until use.

Immunological Parameters

Blood samples of 10 fish randomly selected from each group, anaesthetized by 0.01 mg L^{-1} of phenoxyethanol (Bilen *et al.*, 2011), were collected from the caudal vein using syringe needle at the end of the study. Some of each blood sample was allocated for the hematological assays and the rest of the blood samples were added to heparin containing tubes for the other immunological analyses. Sera were separated by centrifugation at 5000 *g* for 5 min and the immunological analyses were done.

NBT activity was determined as described by Anderson and Siwicki (1994). Briefly, 0.1 ml of heparinized blood was mixed with 0.1 ml of 0.2% NBT solution. The mixture was incubated at 25 °C for 30 minute, 50 µl of the resultant suspension was taken, and 1.0 ml N,N-dimethyl formamide was inserted to the suspension in a glass tube and

Table 1. Basal diet formulation

Ingredients	Concentration (%)
Fish meal	34
Fish oil	5
Corn gluten	14
Wheat meal	12
Wheat gluten	2.5
Soybean cake	18
Starch	9.5
*Vit-Min Premix	5

*Vit-Min Premix (mg kg^{1}, NRC 1977): vitamin A, 5500 IU; vitamin D_3, 1000 IU; vitamin E, 50 IU; vitamin K, 10 mg; choline, 550 mg; niacin, 100mg riboflavin 20mg; pyridoxine, 20 mg; thiamine, 20mg; biotin, 0,1mg; folacin, 5mg; B_{12}, 20µg; inositol, 100 mg; choline chloride, 5000 mg. Mineral premix (mg kg^{-1} diets, H440): NaCl, 1,0; $MgSO_4$, 7; NaH_2PO_4 25; KIO_3 0.0003; $ZnSO_4$ 0.353; $MnSO_4$, 0.162.

centrifuged at 3000 g for 5 min. The optical density (OD) was measured at 540 nm in spectrophotometer.

Lysozyme level in blood serum was determined by a turbidimetric assay (Anderson *et al.*, 1994). Results were presented as Lysozyme units ml^{-1}.

Total myeloperoxidase (MPO) content was measured according to Quade and Roth (1997) and Sahoo *et al.* (2005) with slight modification. 30 µL serum was diluted with 370 ml of HBSS without Ca^{2+} or Mg^{2+} in eppendorf tubes. 100 µL of 0.1 mg/ml 3,3′,5,5′-tetramethylbenzidine dihydrochloride and 0.006% fresh hydrogen peroxide were added. The reaction was followed kinetically by measuring the increase of absorbance. Reaction velocities were determined as IU, defined as the amount of enzyme required to produce an 0.001 increase in absorbance per minute 0.5 ml of reaction mixture (ΔA 450/min/ml).

Hematology

The hemoglobin (Hb) content and hematocrit were measured according to Blaxhall and Daisley (Blaxhall and Daisley, 1973). Hb concentration was determined by spectrophotometry (540 nm) using the cyanomethahaemoglobin method. Hematocrit (Hct) was determined using a capillary hematocrit tube and it was measured by hematocrit scale. Mean corpuscular volume (MCV), mean corpuscular Hb (MCH), and mean corpuscular Hb concentration (MCHC) were calculated by using the standard formulae of Lewis *et al.* (2006).

V. anguillarum and Challenge Test

V. anguillarum was obtained from the Institute of Veterinary Control and Research in Izmir, Turkey. After four weeks of feeding, all fish from each group were injected intraperitoneally (i.p.) with 100 µl PBS containing 1×10^8 *V. anguillarum* strain cells. *V. anguillarum* was re-isolated to confirm the mortality due to the bacterial infection. Mortality was recorded for 7 days which time mortalities had ceased and the observation of surviving fish was extended to 2 weeks (Zhou *et al.*, 2010; Yılmaz *et al.*, 2013b). The pathogenicity was proved by challenge and the bacterium was re-isolated from the dead fish. Relative percent survival (RPS) was calculated according to Ellis (1988) as follows: RPS= 1-(% Mortality T/ % Mortality C) x 100. Where T is the treated group and C is the control group.

Growth Parameters

At the beginning (102 fish per dietary group) and at the end of the study (102 fish per dietary group) each fish was individually weighed. Specific growth rate (SGR) was calculated as: SGR= 100 x [(LN final fish weight)-(LN initial fish weight)]/days fed. Feed conversion ratio (FCR) was calculated as: FCR= feed intake (g)/weight gain (g) x 100.

Statistical Analyses

Data were analyzed statistically by one-way analysis of variance (ANOVA) and Duncan's multiple range tests to determine significant differences using $P<0,05$.

Results

SGR and FCR of koi carp fed with different levels of tetra extract were given in Table 2. Among groups SGR and FCR had no significant difference ($P>0.05$). All diets were accepted by the fish, which avidly consumed the feed during the experiment and survival of fish fed the experimental diets for four weeks was 100%.

Immunstimulatory effects of tetra were given in Table 3. NBT, lysozyme and myeloperoxidase

Table 2. Growth performance of koi fish after feeding the experimental diets

	Control	0.5 g kg^{-1}Tetra	1.0 g kg^{-1}Tetra	1.5 g kg^{-1}Tetra
Initial Wt (g)	4.10±0.64	4.12±0.79	4.07±0.18	4.29±0.87
Final Wt (g)	6.05±1.50	6.12±2.15	6.38±3.44	6.34±2.70
Wt Gain (%)	47.43±3.92	48.59±2.56	56.78±5.54	47.71±2.88
FCR	1.50±0.13	1.65±0.10	1.34±0.15	1.42±0.06
SGR	0.86±0.06	0.88±0.04	1.00±0.08	0.86±0.04

Values are means±SEM (n=3). Different letters in same line indicate significant differences within groups ($P<0.05$).

Table 3. Immune parameters of fish fed with tetra extract for 4-week study period

	NBT	Lysozyme	Myeloperoxidase
Control	$1.96±0.04^{c}$	$158.21±10.11^{d}$	$80.16±2.19^{d}$
T1 (0.5 g kg^{-1})	$2.20±0.07^{a}$	$219.12±11.32^{a}$	$151.14±10.25^{a}$
T2 (1 g kg^{-1})	$2.31±0.05^{a}$	$260.10±12.90^{b}$	$170.11±12.13^{b}$
T3 (1.5 g kg^{-1})	$2.90±0.10^{b}$	$320.11±11.25^{c}$	$199.12±12.23^{c}$

Values are provided as mean ± standard error. Values with different letters are significantly different ($\alpha=0.05$) within the column.

activities of all groups treated with tetra extract were significantly different compared to the control group (P<0.05). NBT activity had no differences between 0.5 and 1 g kg^{-1} groups (P>0.05). The highest level of lysozyme and myeloperoxidase was determined for the groups fed fed with tetra extract at concentrations of 1.5 g kg^{-1}, 1 g kg^{-1} and 0.5 g kg^{-1}, respectively (P<0.05).

In terms of hematology, the RBC count and MCHC increased significantly (P<0.05) in the 1 g kg^{-1} tetra group. All the other hematological parameters determined in the study were remained. The hematological profile of fish fed diets containing different levels of tetra extract was shown in Table 4.

It was observed that groups of koi fed diets supplemented with tetra extract had less mortality following challenge infection with *V. anguillarum* compared with groups of koi fed tetra-free diets (P<0.05). The relative percentage survival (RPS) and survival rate of groups challenged with *V. anguillarum* were given in Table 5.

Discussion

In the present study, the possible immunostimulant effects of tetra (*Cotinus coggyria*) extract and its protection in koi against *V. anguillarum* was investigated. It is known that tetra is a potential immunostimulant for rainbow trout (Bilen *et al.*, 2011). In our study, we observed that the tetra extract showed efficient nonspecific immunostimulant activity and protected the fish against *V. anguilarum*.

In the study growth was not affected by dietary tetra supplementation. Our results are in line with Bilen *et al.* (2011), Pakravan *et al.* (2012), Bilen and Bilen (2012) and Yu *et al.* (2008). However, some herbal immunostimulants affected growth of fish positively and showed promoting effects (Xie *et al.*, 2008; Qui *et al.*, 2002).

The present study indicated that all doses of tetra affected lysozyme activity compared to the control group and the activity was improved with increasing of tetra dosage. Lysozyme is an antimicrobial peptide that is effective against bacteria (Masschalck and Michiels, 2003), and an important parameter in the immune defense of both invertebrates and vertebrates (Magnadottir, 2006). Increase in lysozyme activity is in conformity with the reports of Xie *et al.* (2008), Jian and Wu (2002) and Bilen *et al.* (2011). MPO is an important enzyme in neutrophils of many fish species (Castro *et al.*, 2008) and uses hydrogen peroxide to oxidise several substrates (Hampton and Kettle, 1996). It is also designated with regard to more complex functions of MPO which stimulate neutrophil (Lau *et al.*, 2005), and macrophages (Grattendick *et al.*, 2002) during inflammatory response. In certain studies, increasing activity of MPO in rainbow trout (Siwicki *et al.*, 1994), in *Oreochromis mossambicus* (Alexander *et al.*, 2010) and in sea bream (Sitja-Bobadilla *et al.*, 2005) was reported. These results agree with our findings. The NBT activity was found higher than that of the control group in all groups fed with tetra supplemented diet. The highest dose of tetra extract showed the most elevated NBT activity. Increased NBT values were also reported by Sakai *et al.* (1995) on rainbow trout fed on EF203 and Sarlin and Philip (2011) on *Fenneropenaeus indicus* fed on marine yeasts *Debaryomyces hansenii* (S8) and *Candida tropicalis* (S186). The hematological parameters showed similar results except for RBC and MCHC of 1 g kg^{-1} tetra extract group. The group's RBC and MCHC were found significantly higher than all other groups, which is in agreement with the study of Dada and Ikuerowo (2009).

In this study after challenge with *V. anguillarum*, survival rates were significantly increased in all groups compared to the control group, with the lowest mortality in 1.5 g kg^{-1} group (% 13.3). The *V. anguillarum* is commonly considered halophilic pathogen (Taylor, 1989), but Fujiwara-Nagata and Eguchi (2004) have shown that this pathogen can survive in freshwater conditions and it is also pathogenic for rainbow trout (Ekici *et al.*,

Table 4. Changes in the hematological profile of the experimental groups

	Hb(g dl^{-1})	Ht(%)	RBC (x$10^6$$mm^3$)	MCV(μm^3)	MCH(pg)	MCHC(%)
Control	4.48±0.37	27.33±1.86	0.91±0.08^b	304.97±26.75	49.84±4.22	16.35±0.26
T1 (0.5 g kg^{-1})	5.15±0.28	28.67±1.76	0.98±0.04^b	291.43±11.71	52.31±1.70	17.96±0.14
T2 (1 g kg^{-1})	5.59±0.49	31.00±2.65	1.41±0.19^a	232.71±52.37	41.95±9.51	18.01±0.07*
T3 (1.5 g kg^{-1})	5.26±0.48	29.33±2.67	0.91±0.03^b	324.73±33.92	58.27±6.26	17.94±0.09

Values are provided as mean ± standard error. Values with different letters are significantly different (α=0.05) within the column.

Table 5. Percentage of mortality and survival of the groups of koi fed with tetra extract

Group	No. of challenged fish	No. of fish mortalities	Mortality (%)	Survival (%)	RPS
Control	72	27.00	37.50 ^{a}	62.50^b	
T1 (0.5 g kg^{-1})	72	23.00	31.94^a	68.06^b	14.81
T2 (1 g kg^{-1})	72	13.00	18.06^b	81.94^a	51.85
T3 (1.5 g kg^{-1})	72	9.00	12.50^b	87.50^a	66.67

2005). Alexander *et al.* (2010) reported that *Tinospora cordifolia* leaves gave protection in terms of reduced percent mortality which is reflected in the increased Relative Percent Survival (RPS) values. In contrast, Huttenhuis *et al.* (2006) observed that a challenge with *V. anguillarum* resulted in an initially higher cumulative mortality in the group fed with lipopolysaccharide (LPS).

As a result of this study, oral administration of tetra extract at dose of 1.5 g kg^{-1} increased the immunity of koi carp and protected the fish against *V. anguillarum*. However, the further studies including modern molecular techniques and histological investigations could provide a better understanding of the effects of the tetra extract as an immunostimulant product.

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