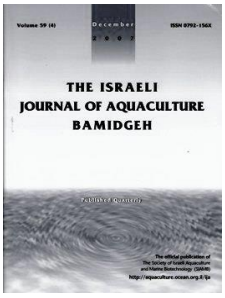

Effects of Dietary Incorporation of Tetra (*Cotinus coggygria*) Extract on Immune Response and Resistance to *Aeromonas hydrophila* in Koi Carp (*Cyprinus carpio*)

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Abstract

In this study, immunostimulant effects of dietary supplementation of tetra (*Cotinus coggygria*) extract on the non-specific immune response, and protection against *Aeromonas hydrophila* infection in Koi carp (*Cyprinus carpio*) were investigated. Koi were fed with tetra extract incorporated diets containing 0 (Control), 0.5 (Te1), 1.0 (Te2) and 1.5 g/kg (Te3), for 30 days. At the end of the study there were no differences in the values of hematological parameters between treatments. However, red blood cell counts were significantly increased ($P<0.05$) in Te2 group. Nitroblue tetrazolium activity was higher in all the treatment groups compared to the control, and highest values were recorded in Te3, Te1 and Te2 groups, respectively. Lysozyme and myeloperoxidase activity of the treatment groups was significantly enhanced compared to control ($P<0.05$), and higher values of lysozyme and myeloperoxidase activity were seen in Te3, Te2 and Te1 groups, respectively.

In the challenge study with *A. hydrophila* (10^8 CFU/ml) administered after 30 days of feeding where the Koi received Te3, Te2, Te1, and control diets, they had 13.3, 20.0, 26.7, and 40.0% mortality, respectively. Tetra extract supplemented diets enhanced the immunological responses and triggered the immune system of Koi carp against *A. hydrophila* infection.

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Introduction

Ornamental fish production is an economically important branch of the aquaculture industry. Koi carp (*Cyprinus carpio*) is a widely cultured ornamental fish species worldwide. Koi can survive and acclimatize to different water conditions and environments enabling this species to be propagated in several new locations. The necessity for improved health and nutrition of the ornamental fish industry has led to increased use of antibiotics and chemotherapeutants. This, in turn has led to the development of drug-resistant strains of pathogenic microorganisms (Amabile-Cuevas *et al.,* 1995). Pathogens such as *Aeromonas hydrophila* cause huge losses in Koi carp. *A. hydrophila* is an opportunistic Gram-negative pathogen causing ulcerative symptoms and abdominal dropsy, hemorrhagic septicemia, and fin and tail erosion in freshwater fish (Austin & Austin, 1993).

Immunostimulants in aquaculture have been reported to provide beneficial effects and their use is an important management tool in fish culture (Sakai, 1999). Immunostimulants increase resistance to infectious diseases by enhancing both specific and nonspecific defense mechanisms of fish and animals (Gopalakannan & Arul, 2006; Gupta *et al.,* 2008).

Tetra or Smoketree (*Cotinus coggygria*) is widely used in Turkish folk medicine and distributed in Kırklareli Province, which is located in the European part of Turkey (Kültür, 2007). Tetra possesses immunostimulants (Bilen *et al.,* 2011), high antioxidants (Niciforovic *et al.,* 2010), antimicrobial and antibacterial qualities (Dulger et al. 2009), and these attributes make it an important herbal medicine.

In this study, we investigated the potential recovery of Koi carp (*C. carpio*) infected with *A. hydrophila* after tetra (*Cotinus coggygria*) treatment and associated immunological and hematological changes under laboratory conditions. The growth promoting effect of tetra on Koi was also checked.

Materials and Methods

Experimental design. Koi fish were obtained from Akdeniz Akvaryum Limited Company and retained for acclimatization for two weeks. A total of 360 fish with average body weight of 4.14 ± 0.28 g were divided into 12 tanks (80 l each. Fish were kept in each of the triplicate aquaria designed for the treatment groups and fed ad libitum twice daily for 30 days with tetra extract supplemented diets.

Preparation of tetra extract and diets. Tetra (*C. coggygria*) was collected from Kırklareli province in Turkey. The leaves were collected from the plants, washed thoroughly with sterilized distilled water. After washing, they were dried under natural conditions and 1 kg powdered sample was extracted by percolation with 6 l methanol (40%) and then filtered. The solvent was evaporated using a rotary vacuum evaporator and then fridge-dried. Then 6 g concentrate were dissolved in 100 ml absolute ethanol (Pakravan *et al.,* 2012) and added to the feed at a rate of 0 (control), 0.5 (Te1), 1.0 (Te2), and 1.5 g/kg (Te3). The feed ingredients of the diets are presented in Table 1.

Table 1. Formulation of experimental diet used in the study.

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, 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 diets, H440): NaCl, 1,0; $MgSO_4$, 7; NaH_2PO_4 25; KIO_3 0,0003; $ZnSO_4$ 0,353; $MnSO_4$, 0,162.

The ingredients were thoroughly mixed and pressed through a 2 mm die pelleting machine. The pellets were then dried in a drying cabinet (40°C) until moisture dropped to around 10%, crushed into desirable particle sizes, and stored at -20°C until use.

Blood sampling. At the end of the study, nine randomly selected fish from each group were anesthetized with 0.01 mg/l of fenoxyethanol for blood collection. Each blood sample was allocated for hematological assays and immunological analysis. Immunological analyses samples were taken in tubes containing K_3EDTA. Sera were separated by centrifugation at 5000 *g* for 5 min and stored at -80°C for immunological analysis.

Hematology. For hemoglobin (Hb) content determination, 20 µl of blood sample was added to Drabkin's fluid making up a 4 ml sample, and absorbance was measured with a spectrophotometer at 540 nm. The blood sample was taken in hematocrit tubes and centrifuged at 5000 *g* for 5 minutes. The proportion of hematocrit (Ht) was measured using a hematocrit scale. The blood sample was diluted with Dacie's fluid (1/200) and total red blood cell count was measured by Thoma slide. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), were calculated by standard formulae (Bain *et al.,* 2006).

Nitroblue tetrazolium (NBT) activity. NBT activity was determined as described by Siwicki & Anderson (1993) where 0.1 ml of heparinized blood was added in a tube to which 0.1 ml of 0.2% NBT solution was added. The mixture was incubated for 30 min at 25°C. 50 µl of the supernatant was added to 1.0 ml N,N-dimethylformamide in a glass tube and centrifuged at 3000 *g* for 5 min. The optical density (OD) was measured at 540 nm in a spectrophotometer.

Lysozyme activity. Lysozyme level in blood serum was determined by turbidimetric assay according to the method described by Siwicki & Anderson (1993). A unit of lysozyme activity was defined as the amount of serum causing a reduction in absorbance of 0.001/min.

Myeloperoxidase (MPO) activity. Total MPO content was measured according to Sahoo *et al.* (2005) with a slight modification. 30 µl serum was diluted with 370 µl of Hank's Balanced Salt Solution without Ca2+ or Mg2+. 100 µl of 0.1 mg/ml 3, 3', 5, 5' - tetramethylbenzidine dihydrochloride and 0.006% fresh hydrogen peroxide were added to the diluted serum. The reaction was followed kinetically by measuring the increase in absorbance. Reaction velocities were determined as IU, defined as the amount of enzyme required to produce a 0.001 increase in absorbance per minute for 0.5 ml of reaction mixture (ΔA 450/min/ml).

Disease resistance. After 30 days of feeding, 15 fish from each aquarium were challenged with a virulent *A. hydrophila in vivo*, which was obtained from the Institute of Veterinary Control and Research in Izmir, Turkey. In the laboratory, the *A. hydrophila* was grown on nutrient broth for 36 h at 24°C in an incubator, and harvested by centrifuging the culture broth at 5000 *g* for 15 min at 4°C. The cells were then washed three times in phosphate-buffered saline (PBS; pH 7.4), and the final concentration was adjusted to 1×10^8 CFU/ml by serial dilution. The LD_{50} dose was previously determined as 0.1 ml PBS containing 1×10^8 CFU/ml. Fish were challenged with an intraperitoneal injection of 0.1 ml of live *A. hydrophila*. Mortality was recorded for 7 days. Relative percent survival (RPS) was calculated as follows: RPS = 1- (% mortality in treatment/ % mortality in control) x 100.

Growth parameters. At the beginning and at the end of the study 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 analysis. Data are presented as means ± S.E. per group. Differences between means were analyzed using one-way analysis variance (ANOVA) followed by Duncan's multiple range test for their comparison at $P<0.05$. Statistical analysis was performed using SPSS for Windows v. 17.0 program (SPSS Inc., Chicago, IL, USA).

Results

Hematological and Immunological Variables. The RBC count increased significantly (P = 0.015) in the Te2 group (Table 2).

Table 2. Hematological parameters of koi carp fed diet containing different levels of tetra extract.

	Hb (g/dl)	*Ht (%)*	*RBC (x10^{6}mm^3)*	*MCV (µm^3)*	*MCH (pg)*	*MCHC (%)*
Control	4.68±0.21	26.67±1.63	0.89±0.05^y	301.37±15.65	53.16±2.82	17.75±0.98
Te1 (0.5 g/kg)	5.27±0.27	29.17±1.64	0.94±0.03^y	310.19±19.35	56.02±3.35	18.08±0.13
Te2 (1 g/kg)	5.49±0.27	30.17±1.47	1.39±0.14^z	230.62±28.89	41.79±5.03	18.19±0.21
Te3 (1.5 g/kg)	5.17±0.23	29.00±1.24	0.89±0.03^y	329.56±18.24	58.76±3.40	17.82±0.09

Values are mean±SE of nine fish; Different superscript letters in a column indicate significant differences between groups (P<0.05).

There were no significant differences in Hb, Ht, MCV, MCH and MCHC. NBT activity was found to be higher in all the treated groups than in the control group (Table 3).

Table 3. Immunological parameters in koi carp fed different levels of tetra extract.

	NBT	*Lysozyme*	*Myeloperoxidase*
Control	1.94±0.04^{y}	147.33±11.51^{y}	76.76±5.69^{y}
Te1 (0.5 g/kg)	2.39±0.08^{z}	203.00±13.61^{z}	140.74±13.65^{z}
Te2 (1 g/kg)	2.29±0.02^{z}	254.17±18.98^{z}	163.19±17.93^{z}
Te3 (1.5 g/kg)	2.78±0.13^{z}	328.17±17.25^{z}	188.10±25.27^{z}

Values are mean±SE of nine fish; Different superscript letters in a column indicate significant differences between groups (P<0.05).

Lysozyme activity was higher in the treatment groups compared to the control. As the tetra extract supplementation level increased, there was a constant, but non-significant increase in lysozyme activity. Similar results were also observed in myeloperoxidase activity. Myeloperoxidase activity was also found to be higher in Te1 (P = 0.026), Te2 (P = 0.032), and Te3 (P = 0.033) groups than control group (Table 3). The highest NBT, lysozyme, and myeloperoxidase activity was recorded in Te3 group.

Challenge test. Fish fed with tetra extract showed significantly higher survival rate, increased RPS, and lower mortality (Table 4).

Table 4. Survival rate of koi carp after challenge with *A. hydrophila.*

Group	*Total no. of challenged fish*	*Total no. of dead fish*	*Mortality (%)*	*Survival (%)*	*RPS*
Control	45	18	40.0^{z}	60.0^{y}	-
Te1 (0.5 g/kg)	45	12	26.7^{y}	73.3^{z}	32.9
Te2 (1 g/kg)	45	9	20.0^{y}	80.0^{z}	49.6
Te3 (1.5 g/kg)	45	6	13.3^{y}	86.7^{z}	66.5

Different superscript letters in a column indicate significant differences between groups (P<0.05).

The highest survival rate was found in Te3 group. All tetra supplemented groups (Te1 to Te2) were significantly different from the control group with P = 0.022, P = 0.020 and P = 0.018, respectively.

Growth parameters. There was no significant difference (Table 5) in SGR and FCR values between treatments. All diets were accepted by the fish and survival of fish fed the experimental diets for 30 days was 100%.

Table 5. Growth performance of koi carp fed experimental diets.

	Control	Te1 0.5 g/kg	Te2 1.0 g/kg	Te3 1.5 g/kg
Initial Total Weight (g)	63.10±0.66	61.77±0.55	60.68±0.17	63.40±0.81
Final Total Weight (g)	100.04±1.58	95.62±0.93	93.13±3.72	95.94±0.89
Weight Gain (%)	58.53±1.06	54.86±2.64	53.50±6.29	51.37±2.28
FCR	1.31±0.02	1.39±0.07	1.47±0.16	1.37±0.04
SGR	1.02±0.01	0.97±0.04	0.95±0.09	0.92±0.04

FCR= Feed conversion ratio; SGR= Specific growth rate; Different superscript letters in a column indicate significant differences between groups (P<0.05).

Discussion

In aquaculture, prophylactic measures such as vaccines, immunostimulants, and antibiotics have been widely used to protect fish against diseases (Selvaraj et al., 2005; Sakai, 1999). Immunostimulants have been used because they have few or no side effects (Kumari & Sahoo, 2006). Organic food production is also relevant, and in this context herbal immunostimulants have proven their effectiveness therefore some are incorporated in commercial diets.

According to our results, tetra extract has beneficial effects on the Koi immune system, and elevates immune system activity. Tetra is known to enhance non-specific immune defense of rainbow trout (Bilen *et al.,* 2011). Tetra also protects the fish against *A. hydrophila* without influencing Koi growth, FCR or SGR. These results regarding Koi growth concur with other findings (Bilen & Bilen, 2012).

Hematological variables have often been suggested as useful indicators of stress in fish. In this study, all hematological indices were similar except for RBC level of Te2 group which was significantly higher than that of other groups. There was a negative RBC effect on goldfish fed with herbal supplemented diets of *Azadirachta indica, Ocimum. sanctum* and *Curcuma longa* in combination (Harikrishnan *et al.,* 2010). Increased RBC of Te2 group suggests that RBC transport more oxygen to the cells and tissues of fish after immunostimulation with tetra.

When compared with the control increased superoxide production was observed in all the tetra supplemented groups with the highest value found in Te3 group. Higher doses of tetra extract (1.5 g/kg) elevated superoxide anion production. These findings are in agreement with previous studies on rainbow trout (Bilen *et al.,* 2011). However, superoxide production was not enhanced in *Oreochromis niloticus* treated with *Lonicera japonica* and *Ganoderma lucidum* (Yin et al., 2008), and *Astragalus radix* and *Scutellaria radix* (Yin e al., 2006).

Myeloperoxidase (MPO) is an important enzyme in neutrophils of many fish species and uses hydrogen peroxide to oxidize several substrates (Hampton & Kettle, 1996). It is also related to more complex functions of MPO which stimulate neutrophil (Lau et al., 2005), and macrophages (Grattendick et al., 2002) during inflammatory response. In the present study, MPO activity was increased in all the tetra supplemented fish, and a trend of increasing MPO activity was recorded with increased tetra doses. Improved activity of MPO is in agreement with the findings of Alexander *et al.* (2010).

Lysozyme, a serum component and an important non-specific immune mediator against parasitic, bacterial, and viral infections, prevents adherence and colonization by micro-organisms, and increases activity in fish blood in response to infection (Kumari & Sahoo, 2006). In the present study, a significant increase in lysozyme activity was observed in the Te1, Te2 and Te3 groups at the end of the experiment. Similar results were observed by Bilen *et al.* (2011) and Xie *et al.* (2003).

In this study, after being challenged with *A. hydrophila*, mortality was significantly reduced in all treatment groups compared to the control, with the lowest mortality in Te3 group (13.3%). Various studies confirmed decrease in fish mortality using different immunostimulants such as triherbal extract treatment on goldfish (Harikrishnan *et al.,* 2009), Astragalus root and Angelica root supplement feeding on Jian common carp (Jian & Wu, 2003), *Achyranthes aspera* seed supplement feeding on *Labeo rohita* (Chakrabarti & Srivastava, 2012), and *Gonoderma lucidum* supplement feeding on *Oreochromis niloticus* (Yin et al., 2008) after being challenged with *A. hydrophila.*

According to our findings, it appears that tetra extract (1.5 g/kg) enhances the nonspecific immunity of Koi carp. Tetra is abundant in northwest Turkey and has great commercial potential for processing and use as an immunostimulant for Koi carp.

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