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RESEARCH ARTICLE

Effect of *Tilia tomentosa* methanolic extract on growth performance, digestive enzyme activity, immune response and haematological indices of common carp (*Cyprinus carpio*).

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ABSTRACT

This study was conducted to determine the effect of dietary supplementation with *Tilia tomentosa* on the growth performance, digestive enzyme activity, haematological indices and nonspecific immune indices of juvenile common carp (*Cyprinus carpio*). Fish with an average weight of 4.35 ± 0.16 g were fed a diet supplemented with an aqueous methanolic extract of *T. tomentosa* at a dose of 0% (control), 0.01%, 0.05% or 0.1% for 45 days. The final weight, weight gain and specific growth rate were observed to be significantly higher for the 00.1% and 0.1% groups compared with the control group ($P < 0.05$). The feed conversion ratio was significantly decreased in the 0.05% and 0.1% groups compared with the control ($P < 0.05$). The activities of various digestive enzymes (amylase, lipase and trypsin) were also measured and no significant differences were observed compared to the control ($P > 0.05$). The mean cell volume of the 0.01% group was significantly increased compared to the control ($P < 0.05$) and increased lysozyme activity was observed in the 0.05% and 0.1% groups. Respiratory burst activity was significantly increased ($P < 0.05$) on days 15 and 30 for the 0.1% and 0.05% groups, respectively. No differences were observed for myeloperoxidase activity among the four groups. These results suggest that aqueous methanolic extract of *T. tomentosa* has a growth-promoting and immunostimulatory effect on common carp.

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Introduction

Aquaculture is the most important sector in the world. Also it considered as the most growing food produce industry with an average growth rate more than 7.7% per year through the last decades, the majority of aquaculture production are come from Asia (Gjedrem et al., 2012). Approximately 600 aquatic species are raised in captivity in around 190 countries for produce in fish culture system of varying input intensity

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and technological sophistication (Aklakur et al., 2015).

During last decades there has been a continuous growth of aquaculture industries all over the world and such intensive production would experience disease problems. Infectious diseases that occur as sporadic events in wild fish populations may cause high mortalities when appearing in intensive fish farming (Gudding et al., 1999). Enhancing the immune system of cultured fish appears to be the most promising method for preventing disease. Fish typically depend on nonspecific immune mechanisms to a much greater extent than most animals (Chakrabarti and Vasudeva, 2006).

Medicinal plants contain phytochemicals, which exhibit biological activities such as the prevention of chronic degenerative diseases (Fukumoto and Mazza, 2000). Herbal medicines have been used in aquaculture to promote growth, improve the immune system, and stimulate the appetite and fight against microbes and other stressors, due to the presence of various active compounds like flavanoids, alkaloids, phenolics, pigments, steroids, terpenoids and essential oils (Citarasu, 2010). Recently, immunostimulants of herbal origin have been shown to boost the disease resistance of fish towards a number of diseases by improving their nonspecific and specific defence mechanisms (Harikrishnan et al. 2011). Indeed, many herbs have been reported to enhance the immune response of fish (Khondoker et al, 2016).

The *Tilia tomentosa* extracts contained flavonols (quercetin, kaempferol, apigenin derivatives) as principal components with the exception of a single commercial extract with hydroxycinnamic acids as the most abundant metabolites (İleri et al., 2015). *T. tomentosa* is used as a medicinal plant in Turkish folk medicine (Baser et al., 2005). The present study was carried out to determine whether the *T. tomentosa* extract influences the digestive enzyme activity, growth, haematological parameters, or activity of the nonspecific immune response of the common carp (*Cyprinus carpio*).

Material and Methods

Preparation of T. tomentosa Extract

The plants were collected from the Kastamonu province in the north of Turkey and extracted using a methanol extraction method (Pakravan et al., 2012) with some modifications (Bilen et al., 2016) as follows: All ripe parts of the fruit except the seeds were ground in a mechanical grinder to a fine powder. Every 50 g of the ground plant were mixed with 1 L of 40% methanol (Sigma-Aldrich) and the mixture was allowed to stand at room temperature for three days with brief shaking once a day to mix. The extract was then filtered through filter paper (Whatman filter paper No. 1) and the filtrate was collected and evaporated in a rotary evaporator at 55–65 °C to remove the alcohol from the fruit extract. The final crude product was dissolved in distilled water and kept in a flask at 4 °C for later experiments.

Experimental Design

Common carp (*C. carpio*) with an average body weight of 4.35 ± 0.16 g were obtained from a commercial fish farm in Antalya, Turkey. The fish were transported to the Faculty of Fisheries and Aquaculture, Kastamonu University, Turkey. The 480 carp were randomly divided into four groups of three replicates each (i.e. four groups of three aquariums, 12 aquariums in total) with 40 fish per aquarium. The fish were acclimatised for two weeks before the experiment started. During acclimation, the fish were fed with a commercial diet twice a day. During the experimental period, the fish were fed the commercial diet supplemented with *T. tomentosa* extract at the following percentages (w/w): 0%, 0.01%, 0.05%, or 0.1%. The fish were fed by hand with the experimental diet for 45 days to satiation twice a day (at 9 am and 4 pm). The fish were maintained under a natural photoperiod (12-h dark/12-h light). The water quality parameters were checked daily and were within the accepted margins throughout the experiment (dissolved oxygen, 6.8–7.2 mg/L; pH, 7.7–8.5; water temperature, 25–28 °C).

Sample Collection

Every 15 days of the feeding trial (i.e. on days 15, 30 and 45), three fish per aquarium (a total of nine carp from each experimental group) were randomly chosen, anaesthetised by phenoxyethanol at 0.01 mL/L and individually weighed and sampled. The kidney tissues were collected and transferred individually to 1.5 mL RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) for direct assay of the immunological parameters. Blood was collected on day 45 of the feeding trials from the caudal vein using heparinised syringes in EDTA-tubes, and later used for the direct assay of the haematological parameters. Samples of the intestine were also collected on day 45 of the feeding trials. These samples were cleaned to remove waste and all visible fat and then stored at −80 °C for the digestive enzyme assay.

Growth Performance Parameters

The weight of each fish was individually measured at the beginning and end of the study. The growth performance was calculated according to the following equations (Tekinay and Davies, 2001):

$$WG(\%) = 100 \times \left[\frac{(Final\ Fish\ Weight - Initial\ Fish\ Weight)}{Initial\ Fish\ Weight}\right]$$

$$SGR\ (weight\ \%\ d^{-1}) = 100 \times \left[\frac{ln(Final\ Fish\ Weight) - ln(Initial\ Fish\ Weight)}{Experimental\ Days}\right]$$

$$FCR = \frac{Feed\ Intake\ (g)}{WG\ (g)}$$

$$SR(\%) = 100 \times \left[\frac{Final\ Number\ of\ Fish}{Initial\ Number\ of\ Fish}\right]$$

In these formulae, WG indicates weight gain, SGR indicates specific growth rate, FCR indicates feed conversion ratio, and SR indicates survival rate.

Digestive Enzyme Activity

The intestine samples were homogenised with a Potter Elvehjem homogeniser in cold double-distilled water (0.1 g/mL) and centrifuged at 9,000 rpm for 20 min at 4 °C. The

supernatant was removed and stored at −80 °C to test for digestive enzyme activity as follows. The amylase activity was determined using 2% starch (Sigma-Aldrich) as a substrate according to the Worthington (1991) method. The lipase activity was determined using hydrolysis of 4-nitrophenyl myristate (Sigma-Aldrich) according to the method described by Gawlicka et al. (2000). The trypsin activity was determined using the method of Erlanger et al. (1961) and benzoyl-DL-arginine p-nitroanilide (Sigma-Aldrich) as the substrate. The protein concentrations were evaluated with Bradford (1976) method.

Haematological Profiles

White blood cell counts (WBC × 10^7 mm^{-3}), red blood cell counts (RBC × 10^6 mm^{-3}), haemoglobin levels (Hb, g/dL) and haematocrit measurements (Hct, %) were measured according to the methods described by Blaxhall and Daisley (1973). Blood indices included the mean cell volume (MCV, fL), mean cell Hb (MCH, pg) and mean cell Hb concentration (MCHC, %), which were calculated according to the formulae of Lewis et al. (2001).

Nonspecific Immune Parameters

Head kidney cells were isolated from euthanised *C. carpio* according to the method of Kono et al. (2012) with slight modifications as follows. Briefly, the head kidney tissue was carefully removed and gently pushed through a 100-µm nylon mesh (John Stanier & Co., Whitefield, Manchester, UK) into RPMI-1640 medium (Invitrogen) supplemented with 5% foetal bovine serum (Invitrogen) and a 1% solution of 10,000 g/mL streptomycin plus 10,000 U/mL penicillin (Invitrogen). This mixture was then pushed through a 40-µm nylon mesh cell strainer (Becton, Dickinson & Co., Franklin Lakes, NJ, USA). The final homogenate was placed in a 3-mL Falcon tube. Head kidney cell suspensions were pelleted at 1,800 rpm for 3 min at 4 °C. After centrifugation, the supernatant was collected to measure myeloperoxidase activity using 3,3,5,5-tetramethyl benzidine hydrochloride (Sigma-Aldrich) as the substrate (Sahoo et al., 2005). The lysozyme activity was measured using a lyophilised *Micrococcus lysodeikticus* bacterial cell solution (Sigma-Aldrich) as the substrate (Bilen et al., 2014). Each pellet was resuspended in 1 mL of the same medium to directly assay nitroblue tetrazolium (Sigma-Aldrich) reduction, according to the method described by Biswas et al. (2013).

Statistical Analysis

The results were analysed with SPSS software. One-way ANOVA and Duncan's multiple range test were used to determine the significant differences between groups. All results were expressed as the mean ± SE and $P < 0.05$ was considered statistically significant.

Results

Growth

The final weight (FW), FCR, SGR, WG and SR of common carp fed on the experimental diets for 45 days were determined and presented in Table 1.

Table 1. Growth indices of *Cyprinus carpio* supplemented with a methanolic extract of *T. tomentosa* for 45 days.

Groups	IW (g)	FW (g)	WG (%)	FCR	SGR (%/day)
Control	4.09 ± 0.10[a]	6.88 ± 0.08[a]	168.07 ± 5.06[a]	1.68 ± 0.01[a]	1.15 ± 0.04[a]
0.01%	4.06 ± 0.05[a]	7.71 ± 0.09[b]	189.87 ± 6.28[b]	1.78 ± 0.02[a]	1.42 ± 0.02[b]
0.05%	4.23 ± 0.06[a]	7.25 ± 0.07[a]	171.23 ± 4.57[c]	1.24 ± 0.01[b]	1.20 ± 0.02[a]
0.10%	4.12 ± 0.14[a]	7.56 ± 0.21[b]	183.22 ± 6.19[d]	1.56 ± 0.01[c]	1.34 ± 0.03[b]

Note: Values represent the mean ± SE; different superscript letters in a column indicate significant differences between groups ($P < .05$). *IW*: initial weight, *FW*: final weight, *WG*: weight gain, *FCR*: feed conversion ratio, *SGR*: specific growth rate, *SR*: survival rate.

Significant increases ($P < 0.05$) in FW, WG and SGR were observed for fish supplemented with 0.01% and 0.1% extract compared to the control. In addition, the FCR was significantly ($P < 0.05$) lower in the 0.05% and 0.1% supplemented groups compared with the control group. No significant differences were observed between the FW, FCR, SGR and SR of the other treated groups compared with the control group.

Digestive Enzymes

The digestive enzyme activity levels for each experimental group are shown in Table 2. No significant differences in the activity of amylase, lipase or trypsin were observed for fish supplemented with 0.01%, 0.05%, or 0.1% *T. tomentosa* extract compared with the control group.

Table 2. Digestive enzyme activity in the intestines of *Cyprinus carpio* supplemented with a methanolic extract of *T. tomentosa* for 45 days.

Groups	Enzymes		
	Amylase	*Lipase*	*Trypsin*
Control	1.84 ± 0.54	0.009[a] ± 0.003	0.11 ± 0.01
0.01%	1.58 ± 0.51	0.009 ± 0.001	0.08 ± 0.01
0.05%	3.95 ± 1.26	0.004 ± 0.002	0.12 ± 0.01
0.10%	3.96 ± 1.58	0.006 ± 0.015	0.08 ± 0.05

Note: Values represent the mean ± SE.

Haematological Profiles

Fish supplemented with *T. tomentosa* extract at different concentrations had the same haematological parameters as

the control group throughout the experimental periods (Table 3), with the exception of the MCV of the 0.01% group on day 30 of the experiment ($P < 0.05$) and the MCHC of the control on day 30 of the experiment (highest recorded result compared with the other groups; $P < 0.05$).

Nonspecific Immune Parameters

The immunostimulatory effects of the *T. tomentosa* extract are shown in Figures 1, 2 and 3. The lysozyme activity of fish supplemented with different concentrations of *T. tomentosa* extract was increased on day 30 of the experiment; this increase was found to be significant ($P < 0.05$) only for the 0.1% supplementation compared with the control (Figure 1). On the other hand, the lysozyme activity decreased significantly on day 45 of the experiment for the 0.01% supplementation compared with the control ($P < 0.05$). The lysozyme activity levels of the other supplementation groups did not change significantly over time.

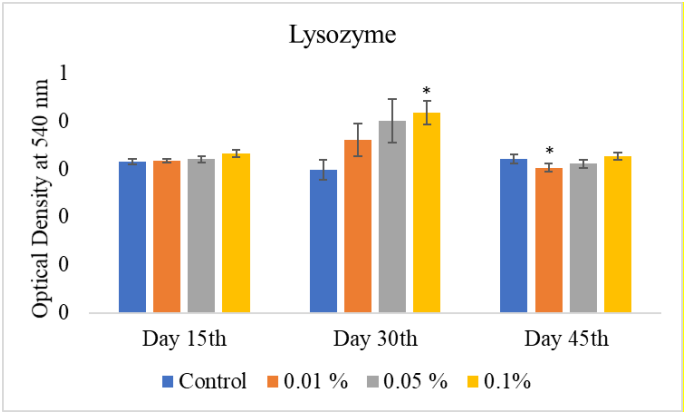

Figure 1. Lysozyme activity in kidney leucocytes of *Cyprinus carpio* fed with different experimental diets for 45 days. Values are expressed as the mean ± SE. Different symbols above the bars indicate significant differences between groups ($P < 0.05$).

The myeloperoxidase activity of the treated fish did not significantly differ from that of the control fish during the study period (Figure 2).

Table 3. Haematological profiles of *Cyprinus carpio* supplemented with a methanolic extract of *T. tomentosa* for 45 days.

	Groups	WBC ($\times 10^7$cells mm^{-3})	RBC ($\times 10^6$cells mm^{-3})	Hb (g dl^{-1})	Hct (%)	MCV (fl)	MCH (pg)	MCHC (%)
15th day	Control	24.47 ± 0.18	1.98 ± 0.04	8.22 ± 0.07	27.82 ± 0.84	141.11 ± 7.12	41.57 ± 1.05	296.47 ± 7.63
	0.01%	25.63 ± 1.26	1.86 ± 0.09	7.77 ± 0.25	27.68 ± 0.55	148.92 ± 3.73	41.76 ± 0.78	281.13 ± 4.32
	0.05%	22.57 ± 1.48	1.97 ± 0.06	8.07 ± 0.10	27.88 ± 0.53	142.44 ± 6.20	41.14 ± 0.89	289.85 ± 8.25
	0.10%	26.03 ± 0.97	1.98 ± 0.05	8.27 ± 0.11	28.09 ± 0.87	143.29 ± 8.22	42.02 ± 1.08	295.31 ± 10.34
30th day	Control	22.61 ± 1.67	1.97± 0.08	8.37 ± 0.17	25.99 ± 0.47	132.72 ± 2.71	42.69 ± 0.89	321.95 ± 2.56*
	0.01%	26.84 ± 0.91	1.76 ± 0.09	7.72 ± 0.15	26.72 ± 0.33	153.71 ± 6.98*	44.23 ± 1.42	289.10 ± 4.66
	0.05%	26.34 ± 1.00	1.81 ± 0.03	7.68 ± 0.36	25.59 ± 0.73	141.85 ± 2.26	42.51 ± 1.21	300.12 ± 7.38
	0.01%	26.35 ± 0.45	1.93 ± 0.07	8.08 ± 0.10	26.84 ± 0.09	140.89 ± 5.95	42.1 2± 1.32	301.50 ± 4.48
45th day	Control	23.76 ± 1.85	1.90 ± 0.09	7.31 ± 0.33	25.59 ± 0.62	134.97 ± 4.07	38.45 ± 0.55	285.78 ± 7.19
	0.10%	24.16 ± 1.73	2.02 ± 0.05	7.81 ± 0.19	26.35 ± 0.64	131.08 ± 4.68	38.70 ± 1.29	269.27 ± 1.26
	0.50%	26.74 ± 1.13	1.97 ± 0.07	7.80 ± 0.10	26.18 ± 0.27	133.49 ± 5.52	39.65 ± 1.08	298.42 ± 6.54
	0.01%	27.41 ± 0.15	1.92 ± 0.06	7.70 ± 0.23	26.87 ± 0.48	140.42 ± 4.23	40.14 ± 0.30	286.51 ± 7.17

The myeloperoxidase activity of the treated fish did not significantly differ from that of the control fish during the study period (Figure 2).

The respiratory burst level of the 1% group was significantly lower ($P < 0.05$) than that of the control on day 15 of the experiment (Figure 3). No significant differences in the respiratory burst level were observed among the other treated groups on day 15 compared to the control group. On day 30 of the experiment, significantly higher levels of respiratory burst were observed for the 0.05% and 0.1% groups, while a significantly lower level was observed for the 0.01% group compared with the control ($P < 0.05$). The same pattern was exhibited on day 45 of the experiment ($P < 0.05$).

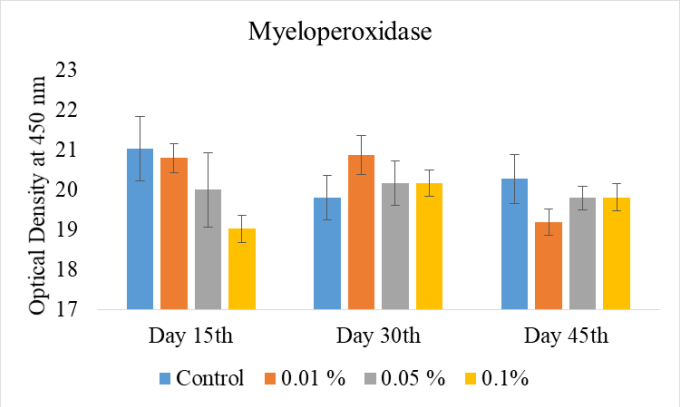

Figure 2. Myeloperoxidase activity in kidney leucocytes of *Cyprinus carpio* fed with different experimental diets for 45 days. Values are expressed as the mean ± SE.

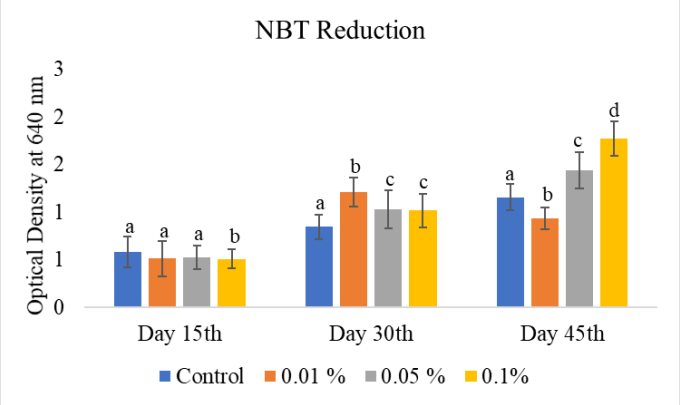

Figure 3. Respiratory burst level in kidney leucocytes of *Cyprinus carpio* fed with different experimental diets for 45 days. Values are expressed as the mean ± SE. Different letters above the bars indicate significant differences between groups ($P < 0.05$).

Discussion

In the present study, *T. tomentosa* was shown to promote the growth of common carp based on observations of increased WG, SGR and efficiency of feed conversion. These results agree with those of previous studies demonstrating that medicinal plants promote the growth of various aquatic animals (Kour et al., 2004; Kaleeswaran et al., 2011; Ojha et al., 2014). The WG was significantly improved when Japanese flounder (*Paralichthys olivaceus*) were supplemented with an herbal mixture to 500 mg/kg (Seung-Cheol et al., 2007). Most probably fat was used for energy, and protein was used for growth in the herbal-supplemented diet (Yılmaz et al., 2012). Nile tilapia fingerlings fed with a basal diet containing 0, 0.5, 1 and 1.5 g/100 g fenugreek (*Trigonella foenum-graecum*) seed meal for three months (Mostafa et al. 2009) and they found that the use of 1 g/100 g fenugreek seed meal improved the fish growth performance. However, although some herbs have positive effects on the fish growth (Xie et al., 2008; Mahdavi et al., 2013), other herbal supplements have not been observed to have any effects. For example, Farahi et al. (2012) found that dietary supplementation with *Aloe vera* did not promote the growth performance of rainbow trout (*Oncorhynchus mykiss*). Yılmaz et al. (2012) reported that the WG, FCR and SGR of sea bass (*Dicentrarchus labrax*) were unaffected by 1000 mg/kg dietary rosemary (*Rosmarinus officinalis*) and fenugreek. Also, the growth rate of koi carp (*Cyprinus carpio*) was unaffected by dietary supplementation with tetra (*Cotinus coggygria*) (Bilen et al., 2013).

Digestive enzymes play a significant role in the hydrolysis of proteins, lipids and carbohydrates, thereby assisting with the assimilation of nutrients. These nutrients are transported into the tissues and incorporated into cellular materials or used as an energy source for growth and reproduction (Furne et al., 2005). The digestion of foods begins with the digestive enzymes in the stomach and continues in the intestine with the digestive enzymes secreted by the pancreas, such as trypsin, chymotrypsin, amylase and lipase (Cockson and Bourne, 1972; Moriarty, 1973; Fang and Chiou, 1989).

Our study did not find any significant differences in the amylase, lipase and trypsin activity levels in any of the experimental groups compared with the control group. This result is in line with a study done by Iqbal et al. (2016), who found no significant differences in the digestive enzyme activity and haematology of juvenile *Labeo rohita* following supplementation with different plant and animal origin feeds (fishmeal). In contrast, a study by Kawai and Ikeda (1973) reported an increase in amylase activity in *O. mykiss* when fed a diet containing increased amounts of plant protein.

Many fish physiologists have concentrated on haematological studies as this has proved a valuable diagnostic approach for evaluating fish quality (Kori-Siakpre et al., 2005; Oluyemi et al, 2008; Patra et al., 2014). Variations in the haematological parameters of fish are due to environmental stress (Hickey, 1982), malnutrition (Casillas and Smith, 1977), gender (Siddique and Naseem, 1979; Collazos et al., 1998), fish size (Garcia et al., 1992), seasonal differences and breeding efficiency (Cech and Wohlschlang, 1981). The blood characteristics of fish are therefore an effective and sensitive index for monitoring physiological and pathological changes (Iwama et al., 1976; Chakrabarti and Banerjee, 1988; Orun et al., 2003; Patra et al., 2014). The present study indicated no significant effect on the blood parameters of *C. carpio* supplemented with 0.01%, 0.05%, or 0.1% *T. tomentosa* extract over the study period. This result suggested that the extract tested here did not stress the fish physiologically.

Lysozyme is an important enzyme in the humeral nonspecific defence mechanism that provides defence against microbial invasion (Evelyn, 2002). The bactericidal action of this enzyme involves the hydrolysation of the peptidoglycan layers of the bacterial cell wall, which lyses the cells and prevents colonisation by microorganisms (Saurabh and Sahoo, 2008). Lysozyme also induces antibacterial activity in the presence of a complement (Harikrishnan et al., 2011). The present study recorded significantly increased lysozyme activity in the 1% supplemented group compared with the control. Increasing lysozyme activity is in agreement with several reports on herbal immunostimulants (Rao et al., 2006;

Choi et al., 2008; Bilen et al., 2011). Similar results were observed for common carp when fed a diet supplemented with methanolic extracts of *Cotinus coggygria* (Bilen et al., 2014) or various Chinese herbal extracts (Jian and Wu, 2004).

Neutrophils contain myeloperoxidase in their cytoplasmic granules (Rodriguez et al., 2003). Myeloperoxidase is an important enzyme with microbiocidal properties as it utilises a reactive oxygen species (H_2O_2) to produce hypochlorous acid (Dalmo et al., 1997). This process is believed to be key in killing microorganisms (Johnston, 1978). Although some studies have shown increased myeloperoxidase activity in fish following supplementation with, for example, quercetin or black cumin seed oil (Awad et al., 2013; Alexander et al., 2010; Bilen et al. 2013), our study showed no significant effect on myeloperoxidase activity following supplementation with *T. tomentosa* extract at different concentrations.

Phagocytosis and the respiratory burst response by phagocytes in blood and tissues present a major antibacterial defense mechanism in fish (Secombes, 1996). Respiratory burst activity measured by nitroblue tetrazolium (NBT) is one of the most important bactericidal mechanisms in fish (Secombes and Fletcher, 1992). In this study, the respiratory burst levels of common carp were significantly decreased on days 15, 30 and 45 of supplementation with 0.1%, 0.05% and 0.01% *T. tomentosa* extract, respectively, compared with the control group. For the groups supplemented with 0.05% and 0.1% extract, on days 30 and 45 of the experiment we found a significant increase in this parameter compared with the control. This was similar to results observed by Bilen et al. (2011) when *O. mykiss* was supplemented with *Cotinus coggygria* leaves. Harikrishnan et al. (2010) reported a significant increase in respiratory bursts for olive flounder supplemented with three different Korean plants. Haghighi and Rohani (2013) reported a significantly higher respiratory burst level in rainbow trout fed a commercial diet containing *Zingiber officinale*. Our results are also in agreement with several studies on the use of dietary immunostimulants in various fish species (Yin et al., 2009; Bilen and Bulut, 2010).

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Conflict of Interest

The authors declare that there is no conflict of interest.

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