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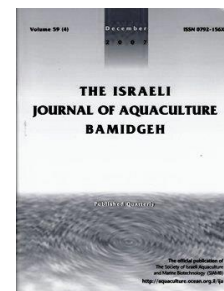
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## Effects of Dietary *Tribulus terrestris* Extract Supplementation on Growth, Feed Utilization, Hematological, Immunological, and Biochemical Variables of Nile Tilapia *Oreochromis niloticus*

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Key words: Nile tilapia, immunostimulant, herb extract, hematology, *Tribulus terrestris*

### Abstract

The present study was conducted for 88 days to evaluate the effect of dietary *Tribulus terrestris* supplementation on growth performance, feed utilization, and hematological, immunological, and biochemical indices of Nile tilapia, *Oreochromis niloticus*. A total of 144 Nile tilapia, average body weight  $2.61 \pm 0.35$  g, were divided into four experimental treatments. There were three replicates of each treatment. Four isonitrogenous (40% CP) and isolipidic (10% CL) experimental diets contained *T. terrestris* extract at levels of 0, 200, 400 and 600 mg/kg respectively. Growth performance and feed utilization of Nile tilapia were significantly higher ( $p < 0.05$ ) in all treatment groups fed with *T. terrestris* extract supplemented diets than those fed the control diet. There were no significant differences between body composition of fish ( $p > 0.05$ ). Hematocrit, hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and myeloperoxidase variables were not significantly affected by herb extract ( $p > 0.05$ ). However, mean corpuscular volume (MCV) value and lysozyme activity increased with increasing *T. terrestris* extract ( $p < 0.05$ ). Serum albumin, total protein, and triglyceride levels were not affected by *T. terrestris* extract. Serum globulin and glucose levels increased and cholesterol levels decreased in Nile tilapia fed with herbal supplemented diets. The results of the present study suggest that Nile tilapia fed diets containing at least 400 mg/kg *T. terrestris* extract enhanced growth performance, feed utilization, hematological, immunological, and biochemical indices.

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## Introduction

Tilapias are important species for fish culture; they are preferred due to their high growth and reproduction rate in captivity. They also feed on low trophic levels (El-Sayed, 2006). Herbal supplements have been tested in aquaculture as an alternative to chemicals as they are efficient and environmentally friendly in disease management, immunity enhancement, and resistance to pathogens (Bhuvaneshwari and Balasundaram 2006; Raa, 1996; Harikrishnan et al., 2010). Many active plant extracts are frequently utilized to treat a wide variety of clinical diseases including liver disease (Chattopadhyay, 2003).

*Tribulus terrestris* (Zygophyllaceae) is a ground-spreading herb widely distributed in India, China, Japan, Korea, western Asia, southern Europe, and Africa; it is traditionally used for treating liver diseases (Rakesh et al., 2009). It contains different substances including saponins, flavonoids, and alkaloids which have anti-inflammatory, anti-tumor, and immunomodulatory activities (Kumar et al., 2006). Flavonoids are one of the most widespread groups of natural compounds reported to have antioxidant and hepatoprotective properties (Allan and Miller, 1996). These compounds have a broad spectrum of chemical and biological activities, including radical scavenging properties (Kavitha et al., 2011). *T. terrestris* is also consumed by people, allegedly for muscle building.

Hematological and biochemical variables are among the most significant physiological indicators of fish health, stress, and welfare (Blaxhall and Daisley, 1973; Campbell, 2004). Previous studies have shown that starvation (Echevarria et al., 1997), seasonal changes (Kavadias et al., 2004), an improper culture environment (Coz-Rakovac et al., 2005), high stocking density, confinement, and harvest (Vazzana et al., 2002; Roncarati et al., 2006; Di Marco et al., 2008) can negatively affect hematological and biochemical variables in fish.

*T. terrestris* has demonstrated hepatoprotective and antioxidant activities in *O. mossambicus* (Kavitha et al., 2011). However, there are no reports of studies of the effects of dietary supplementation with *T. terrestris* for *O. niloticus*. This study investigated the optimum dosage of *T. terrestris*, and its effects on the growth performance, biochemical, hematological, and immunological parameters of *O. niloticus*.

## Materials and Methods

**Fish and experimental conditions.** *O. niloticus* fry averaging about  $2.61 \pm 0.35$  g were produced in Muğla Sıtkı Koçman University, Faculty of Fisheries, Muğla, Turkey. Throughout the experiment, the physical qualities (mean  $\pm$  SE) of the fresh water during the experiment were as follows: temperature  $26.1 \pm 0.2$  °C, pH  $7.0 \pm 0.1$ , and dissolved oxygen  $7.0 \pm 0.2$  mg/l.

**Experimental herbs and diets.** Ethanol-water extract of *T. terrestris* L. (40% Saponins, Naturex Inc., Ref No: 333883) obtained from EsleMina LTD., Turkey, was added to the feed at rates of 0, 200, 400, and 600 mg/kg for diets TT-200, TT-400, and TT-600 respectively. The feed components of the diets are presented in Table 1.

Table 1. Formulation and proximate composition of the experimental diets containing supplementation of different *T. terrestris* (TT) extract rate

	Experimental Diets				
	TT-0	TT-200	TT-400	TT-600	
<b>Ingredients (%)</b>					
Fish meal	33.00	33.00	33.00	33.00	<sup>1</sup> Vitamin Mix: Vit. A, 18000 IU; Vit. D3, 2500 IU ; Vit. E, 250 mg/kg; Vit. K3, 12 mg/kg; Vit. B1, 25 mg; Vit. B2, 50 mg; Vit. B3, 270 mg; Vit. B6, 20 mg; Vit. B12, 0.06 mg; Vit. C, 200 mg; Folic acid, 10 mg; Calcium d-pantothenate, 50 mg; Biotin, 1 mg; Inositol, 120 mg; Choline chloride, 2000 mg. <sup>2</sup> Mineral Mix: Fe, 75.3 mg; Cu, 12.2 mg; Mn, 206 mg; Zn, 85 mg; I, 3 mg; Se, 0.350 mg; Co, 1 mg. <sup>3</sup> Nitrogen-free extracts (NFE) = matter – (crude lipid+crude ash+crude protein). <sup>4</sup> Energy calculated according to 23.6 kJ/g protein, 39.5 kJ/g lipid, and 17.0 kJ/g NFE.
Soybean meal	35.00	35.00	35.00	35.00	
Wheat flour	16.00	16.00	16.00	16.00	
Fish oil	6.2	6.2	6.2	6.2	
Vitamin-mineral mix <sup>1,2</sup>	4.0	4.0	4.0	4.0	
Starch	5.8	5.78	5.76	5.74	
TT extract	0	0.02	0.04	0.06	
Total	100	100	100	100	
<b>Chemical analyses (% DM)</b>					
Protein	40.10	40.09	40.11	40.08	
Fat	10.02	10.03	10.05	10.02	
Ash	9.94	9.92	9.95	9.92	
NFE <sup>3</sup>	31.16	31.17	31.18	31.17	
Energy (kJ/g) <sup>4</sup>	18.72	18.72	18.72	18.72	

All the ingredients were mixed in a blender and pelletized in a mincing machine. The pellets (2 mm diameter) were dried in a drying cabinet (40°C) until moisture dropped to around 10%. The pellets were then crushed into desirable particle sizes (150-250 µm) and stored at -20°C until use.

**Experimental design and feeding trials.** The experiment was carried out in triplicate for each diet. Fifteen 60 l aquarium were stocked with 144 fry (12 fish/aquarium). The fish were fed three times daily (09.00, 13.00, and 17.00 h) for 88 days. Each tank was provided with sponge filters connected to an air pump (Resun GF-120). During the experiment, water was exchanged daily at a rate of ~10% of the total volume.

**Growth trial.** Growth performance and feed utilization were calculated according to the following formulae: wt gain (%) =  $100 \times [(\text{final fish wt} - \text{initial fish wt}) / \text{initial fish wt}]$ , specific growth rate (SGR, %/day) =  $100 \times [(\ln \text{ final fish wt}) - (\ln \text{ initial fish wt}) / \text{experimental days}]$ , and feed conversion ratio (FCR) = feed intake/wt gain. Proximate analyses of the diets were performed using standard methods (AOAC 2000). Dry matter was analyzed by drying at 105°C in an oven to a constant weight, crude fat by ether extraction, crude protein by the Kjeldahl method, and crude ash by incineration at 525°C in a muffle furnace for 12 h.

**Blood collection.** After day 88, blood sampling was conducted to assess the effects of dietary *T. terrestris* on hematological variables. In addition, the blood stress indicators, including blood glucose, serum protein, albumin and globulin, and non-specific immune variables of the fish were examined. Nine randomly selected fish from each diet were starved for a period of 24 h and then anesthetized with clove oil. Blood samples of fish were collected from the caudal vein using a syringe after which a sample of blood was added to the tubes containing EDTA (BD Microtainer®, UK). Blood samples were then taken for hematological analysis. Blood serum was separated by centrifugation (4000 x g, 10 min) in plastic biochemistry tubes (Kima-vacutest®, Italy) and stored at -20°C until use for biochemical analysis.

**Hematological analyses.** Hematocrit (Hct, %) and hemoglobin (Hb, g/dl) was determined using the method of Blaxhall and Daisley (1973). Hct was determined using a capillary hematocrit tube. Hb concentration was determined by spectrophotometry (540 nm) using the cyanomethaemoglobin method. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated using the following formula (Bain et al. 2006):

$$\text{MCV}(\mu\text{m}^3) = [(\text{Hct, \%}) \times 10] / (\text{RBC, } \times 10^6 \text{ per mm}^3),$$

$$\text{MCH}(\text{pg}) = [(\text{Hb, g/dl}) \times 10] / (\text{RBC, } \times 10^6 \text{ per mm}^3),$$

and

$$\text{MCHC}(\%) = [(\text{Hb, g/dl}) \times 100] / (\text{Hct, \%}).$$

**Biochemical analyses.** Biochemical indices in serum including glucose (GLU), total protein (TPROT), albumin (ALB), triglycerides (TRI), cholesterol (CHOL), and globulin (GLO) were determined using bioanalytic test kits (Bioanalytic Diagnostic Industry, Co) and measured using a Shimadzu spectrophotometer (PG Instruments, UK). Serum globulin was determined by the following formula: Globulin = total protein - albumin.

### **Immunological Analyses.**

**Lysozyme activity.** Serum lysozyme (Lyso) was assessed using the turbidometric assay (Ellis 1988). A suspension of 875 µl of *Micrococcus lysodeikticus* (Sigma, ATCC 4698) at a concentration of 0.2 mg/ml (in PBS) added to 25 µl of serum samples was measured spectrophotometrically at 530 nm after 0.5 and 4.5 minutes at 25°C, using a spectrophotometer. A unit of lysozyme activity was defined as the amount of serum causing a reduction in absorbance of 0.001/min.

**Myeloperoxidase activity.** Total myeloperoxidase (MPO) content in coagulated blood serum was measured according to Quade and Roth (1997) with minor modifications. 30 µl serum was diluted with 370 µl of HBSS without Ca<sup>2</sup> or Mg<sup>2</sup> 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 a 0.001 increase in absorbance per minute 0.5 ml of reaction mixture ( $\Delta A$  450/min/ml).

**Statistics.** Each value was expressed as mean  $\pm$  SD for each of the measured variables. The data of growth parameters, mortality, and survival rate in the *T. terrestris* groups were compared to the control group with student's t-test using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software.

## Results

SGR, FCR, and WG% of tilapia fed with different levels of *T. terrestris* extract are given in Table 2.

Table 2. Fish performance and feed utilization for *O. niloticus* fed diets containing different levels of *T. terrestris* (TT) extract for 88 days

	Diets			
	TT-0	TT-200	TT-400	TT-600
Initial weight (g)	2.62 $\pm$ 0.32	2.64 $\pm$ 0.37	2.60 $\pm$ 0.32	2.60 $\pm$ 0.38
Final weight (g)	10.57 $\pm$ 0.41	13.20 $\pm$ 0.46	14.23 $\pm$ 0.49	11.07 $\pm$ 0.45
Weight gain (%)	304.04 $\pm$ 0.16 <sup>a</sup>	398.82 $\pm$ 0.86 <sup>c</sup>	446.35 $\pm$ 1.03 <sup>d</sup>	325.51 $\pm$ 0.20 <sup>b</sup>
Specific growth rate	1.58 $\pm$ 0.00 <sup>a</sup>	1.82 $\pm$ 0.00 <sup>c</sup>	1.93 $\pm$ 0.00 <sup>d</sup>	1.65 $\pm$ 0.00 <sup>b</sup>
Feed conversion ratio	1.73 $\pm$ 0.06 <sup>b</sup>	1.30 $\pm$ 0.04 <sup>a</sup>	1.20 $\pm$ 0.03 <sup>a</sup>	1.64 $\pm$ 0.04 <sup>b</sup>
Survival (%)	100	100	100	100

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

Weight gain (g) = final fish weight – initial fish weight

Weight gain (%) = 100 (final fish weight – initial fish weight) / initial fish weight

Specific growth rate (SGR) = 100 (ln final fish weight) – (ln initial fish weight) / experimental days

Feed conversion ratio (FCR) = feed intake / weight gain

Between the groups there was a significant difference ( $p < 0.05$ ) for SGR, FCR, and WG. The best growth performance and feed evaluation was seen in the TT400 group (Figure 1). The fish accepted all diets and survival rate of fish fed the experimental diets for 88 days was 100%.

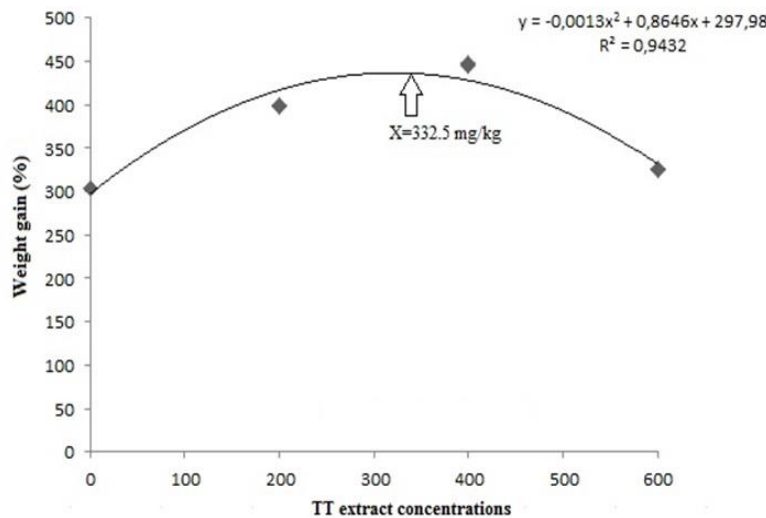


Figure 1. The relationships between dietary TT extract concentrations and weight gain (%) of *O. Niloticus*

Body composition of the fish was not influenced by dietary TT treatment and there were no significant differences between groups ( $p > 0.05$ ) (Table 3)

Table 3. Fillet proximate composition of tilapia fed the experimental diets.

	C	TT200	TT400	TT600
Protein	18.19 $\pm$ 0.65 <sup>a</sup>	18.04 $\pm$ 0.96 <sup>a</sup>	17.03 $\pm$ 0.40 <sup>a</sup>	17.08 $\pm$ 0.16 <sup>a</sup>
Lipid	1.73 $\pm$ 0.01 <sup>a</sup>	1.63 $\pm$ 0.02 <sup>a</sup>	1.65 $\pm$ 0.08 <sup>a</sup>	1.79 $\pm$ 0.06 <sup>a</sup>
Moisture	75.25 $\pm$ 0.31 <sup>a</sup>	74.39 $\pm$ 0.42 <sup>a</sup>	74.74 $\pm$ 0.75 <sup>a</sup>	74.06 $\pm$ 0.78 <sup>a</sup>
Ash	2.43 $\pm$ 0.16 <sup>a</sup>	3.37 $\pm$ 0.29 <sup>c</sup>	2.67 $\pm$ 0.25 <sup>ab</sup>	3.27 $\pm$ 0.21 <sup>bc</sup>

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

Immunostimulatory effects and hematological profile of TT are given in Table 4. Lysozyme and myeloperoxidase activities of the fish were influenced by TT extract levels and showed significant differences ( $p < 0.05$ ). The highest level of lysozyme and myeloperoxidase occurred in groups fed TT extract at concentrations of 400 mg/kg ( $p < 0.05$ ). MCV increased significantly with TT extract at concentrations of 400 mg/kg ( $p < 0.05$ ). All the other hematological parameters determined in the study did not differ significantly ( $p > 0.05$ ).

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

	C	TT200	TT400	TT600
Hb (g/dl)	6.24±0.93	7.46±0.98	6.33±1.18	6.14±0.39
Ht (%)	31±1.60	34±3.00	29.2±4.02	31.4±1.70
MCV ( $\mu\text{m}^3$ )	148.09±4.54 <sup>ba</sup>	130.22±18.31 <sup>a</sup>	152.52±11.55 <sup>b</sup>	146.18±4.08 <sup>ba</sup>
MCH (pg)	29.75±3.56	28.50±4.63	33.29±6.11	28.65±2.00
MCHC (%)	20.09±2.32	21.99±2.68	21.75±3.01	19.60±1.10
Lysozyme	3.65±0.93 <sup>ba</sup>	3.59±1.07 <sup>ba</sup>	4.06±0.76 <sup>b</sup>	2.25±0.73 <sup>a</sup>
Myeloperoxidase	137.65±1.11 <sup>a</sup>	138.11±2.20 <sup>a</sup>	138.07±1.18 <sup>a</sup>	130.38±4.50 <sup>b</sup>

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

Hb (g/dl) Hemoglobin

Ht (%) Hematocrit

MCV (Mean corpuscular volume) ( $\mu\text{m}^3$ ) = [(Hct, %) × 10]/(RBC, × 10<sup>6</sup> per mm<sup>3</sup>)

MCH (Mean corpuscular hemoglobin) (pg) = [(Hb, g/dL) × 10]/(RBC, × 10<sup>6</sup> per mm<sup>3</sup>)

MCHC (Mean corpuscular hemoglobin concentration) (%) = [(Hb, g/dL) × 100]/(Hct, %)

Biochemical variables of fish are shown in Table 5. By the end of 88 days the control had significantly lower values of glucose (GLU) ( $p < 0.05$ ). The dietary TT supplementation affected the globulin (GLO) level, the highest values obtained in TT200 groups ( $p < 0.05$ ). Dietary serum cholesterol (CHOL) level decreased when TT levels in the diets increased ( $p < 0.05$ ). Albumin (ALB), total protein (TPROT) and triglyceride (TRI) levels were not affected by TT supplementation ( $p > 0.05$ ).

Table 5. Changes in biochemical variables in tilapia exposed to different levels of TT extract

	C	TT200	TT400	TT600
GLU (mg/dL)	93.03±15.35 <sup>a</sup>	95.56±4.93 <sup>a</sup>	112.52±23.51 <sup>ab</sup>	142.74±23.00 <sup>b</sup>
ALB (g/dL)	1.23±0.23 <sup>a</sup>	1.82±0.18 <sup>a</sup>	1.58±0.72 <sup>a</sup>	2.00±0.60 <sup>a</sup>
TPROT (g/dL)	5.14±1.26 <sup>a</sup>	7.44±0.85 <sup>a</sup>	6.91±1.34 <sup>a</sup>	7.20±2.56 <sup>a</sup>
GLO (g/dL)	3.91±1.05 <sup>ab</sup>	5.52±0.67 <sup>c</sup>	5.33±0.82 <sup>cb</sup>	3.39±0.65 <sup>a</sup>
TRI (mg/dL)	145.74±40.96 <sup>a</sup>	175.38±25.21 <sup>a</sup>	171.40±44.58 <sup>a</sup>	163.83±25.06 <sup>a</sup>
CHOL (mg/dL)	243.20±64.65 <sup>b</sup>	225.40±22.09 <sup>ba</sup>	222.40±44.24 <sup>ba</sup>	148.80±26.14 <sup>a</sup>

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

## Discussion

In the present study, the possible immunostimulant effects of *T. terrestris* in tilapia diets have been investigated. Dietary *T. terrestris* (TT) significantly affects growth and feed utilization of *O. niloticus*. Second order polynomial regression analyses found that dietary TT level of 332.5 mg/kg produced the most weight gain. Specific information on the effects of TT in fish is scarce but positive effects of other herbal extracts on growth performance of tilapia have been reported (Yilmaz et al., 2013; Pandian and Sheela, 1995; Goda 2008; Francis et al., 2002).

Hematological indices are useful indicators for identification of diseased fish (Harikrishnan et al. 2011). In this study, Hb, MCH, MCHC, and Ht values were not influenced by dietary TT supplement although other supplements such as garlic and ginger oil enhanced MCV values (Yilmaz and Ergün, 2012).



Lysozyme is an antimicrobial peptide that is effective in fighting bacteria (Masschalck and Michiels, 2003) and is an important factor in immune defense (Magnadóttir, 2006). The present study indicated that 400 mg/kg TT increased lysozyme activity when compared to the control group. Other reports also reported lysozyme enhancement in *O. mossambicus* fed *E.alba* leaves (Christybapita et al., 2007).

MPO is an important enzyme for many fish species (Castro et al., 2008) as it enhances neutrophils (Lau et al., 2005) and macrophages (Grattendick et al., 2002) in the blood. Similarly MPO was enhanced in *O. mossambicus* fed with herbal supplements (Alexander et al., 2010).

GLU is one of the stress indicators in fish (Morgan and Iwama, 1997). The present study shows that GLU levels increase with increased amounts of TT extract. The same phenomenon was also reported when a mixed herbal extract of *Azadirachta indica*, *Osmimum sanctum* and *Curcuma longa* was fed to goldfish (Harikrishnan et al. (2010).

Serum TPROT is an important non-specific immune parameter (Magnadóttir, 2006). TPROT enhances the immunity of fish to diseases yet in the present study, serum TPROT was not statistically different from the control group.

TT extract increased the serum ALB and GLO levels in Nile tilapia thought to be associated with a stronger innate immune response of fish (Wiegertjes et al. 1996). TRI levels were not influenced by dietary TT extract treatment. However, CHOL levels decreased when high concentrations of TT extract were given. This may be explained by the cholesterol-lowering effects of herbs (Lin et al., 2007).

In conclusion, *Tribulus terrestris* extract at 400 mg/kg can be beneficial when supplemented to Nile tilapia diet. The present results suggests that supplemented *Tribulus terrestris* extract in feeds for Nile tilapia improves growth performance, nutrient utilization, some immunological and biochemical traits.

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