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Toxicological Evaluation of Dietary Garlic (*Allium sativum*) Powder in European Sea Bass *Dicentrarchuslabrax* Juveniles*

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Abstract

The effects of dietary garlic (*Allium sativum*) powder as a feed additive on hematological and biochemical health characteristics of European Sea bass *Dicentrarchus labrax* juveniles were studied. Experimental fish were fed diets supplemented with garlic powder at 0 (control), 2%, 4%, or 6% levels for a period of 60 days. Results showed that the red blood cell count (RBC), hemoglobin (Hb) concentration, hematocrit (%), and mean corpuscular Hb in fish fed garlic powder diets at dietary inclusion levels of 4% and 6%, were significantly lower than the control values. Serum glucose was significantly lower in Sea bass that were fed garlic powder diets (4% and 6%) compared to the control group. Serum triglyceride and globulin levels in fish fed a 4% garlic powder diet were significantly higher than the control values, whereas these two variables in the 2% and 6% garlic treatments were similar to the control values. The cholesterol levels in the 2% and 6% treatment groups were lower than the value recorded for the control group. As a result, it is suggested that garlic powder supplementation in diets for Sea bass juveniles should not exceed 2%. The present study is the first attempt to examine the effects of dietary garlic powder on the hematological and biochemical status in Sea bass juveniles.

Keywords

Dietary Garlic, *Allium sativum*, European Sea Bass, *Dicentrarchus labrax*, Blood Biochemistry, Toxicological Evaluation

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1. Introduction

There is an increasing need to understand the roles of phyto-additives in aquaculture. To date, a variety of herbs and spices have been successfully used in fish culture as growth promoters and immune-stimulants.

Garlic (*A. sativum*), an herb found virtually throughout the world, has been used in humans to treat rheumatoid arthritis, the common cold, diabetes, malaria, and tuberculosis for over 4000 years [1]. Furthermore, garlic has been reported to have antioxidant, antimicrobial, antifungal, antiviral and antiparasitic effects, and to improve the immune system [2].

Earlier studies have reported that garlic, as a feed additive in fish feed, may stimulate growth, improve antioxidant status, and enhance immunological, hematological and serum biochemical parameters [3]-[8]. The findings of previous studies to determine the optimum dietary inclusion levels varied depending on the dose used (0.05% to 4%), but the efficacy of different doses, particularly at high doses, appeared to be worse based on investigations of toxicology. Overall, the results suggested that high concentrations of phyto-additives may result in negative effects on fish health [9].

Hematological and biochemical variables are known as the most significant physiological indicators of fish health, stress, and welfare [9]-[11]. Previous studies reported that garlic was associated with a reduction in serum GLU in fish [3] [8] [12]. Garlic contains an odorless sulfur-containing compound known as S-allyl-cysteine sulfoxide, which stimulates hypoglycemic activity [13]. Several studies have concentrated on the use of phyto-additives, such as *Thymus vulgaris*, *Rosmarinusofficinalis* and *Trigonellafoenumgraecum* powders [14], garlic and ginger oils [9], and carvacrol [15] to improve immune function, hematological and serum biochemical status, growth and feed utilization or to control bacterial infection in European Sea bass. However, few studies have evaluated the dietary supplementation of garlic powder in terms of its effects on hematological and biochemical blood variables of European Sea bass. Thus, the objective of the present study was to evaluate the toxicological effects of garlic powder in European Sea bass in order to effectively and safely use garlic powder in aqua-diets.

2. Methods

2.1. Fish and Experimental Conditions

A total of 144 European Sea bass juveniles (mean weight ± SD. 10.60 ± 0.18 g) were obtained from a local marine fish farm (Ida Gıda) in Canakkale, Turkey. Fish were kept at the Marine Fish Research and Development Center of the Faculty of Marine Sciences and Technology at Canakkale Onsekiz Mart University (Dardanos-Canakkale, Turkey).

Water quality characteristics (mean ± SD) during the experiment were as follows: temperature was 24˚C ± 1˚C, dissolved oxygen concentration was 7.2 ± 0.2 mg/L, pH was 8.3 ± 0.2, salinity was 27.3 ± 0.1‰, and conductivity was 48.1 ± 0.5 mS/cm. The experiments were performed in accordance with the fish research guidelines from the animal ethics committees at Canakkale Onsekiz Mart University, Canakkale-Turkey.

2.2. Experimental Herbs and Diets

Garlic powder (Kotanyi, GmbH, Wolkersdorf-Austria) was added to the feed at increasing levels of 2%, 4% and 6% (g garlic powder/100g diet). Additionally, a control group was fed a diet without garlic powder supplementation. Four isoenergetic (gross energy, 18.8 ± 0.38 kJ/g diet) and isonitrogenous (protein content, 43.2% ± 0.8%) diets were formulated using commercial ingredients (**Table 1**). All ingredients were mixed in a mixer and pressed through a 2-mm die in a pelleting machine. The pellets were dried in a drying cabinet (40˚C) until moisture dropped to approximately 10%, and stored in bags in a freezer at −20˚C until use.

2.3. Experimental Design and Feeding Trials

A total of 144 fish were randomly distributed into recirculating aquariums each with a volume of 84-L (12 fish/aquarium). Experimental fish were acclimatized to the new conditions for 15 days. The fish were fed a commercial diet twice a day during the adaptation period. The experiment was designed in triplicate for each diet. During the feeding trial, fish were fed each experimental diet twice a day to satiation. The total amount of feed consumption was recorded daily. The recirculating system used in the experiment consisted of a sump, filters, a protein skimmer, and a biofilter containing bio-balls. During the experiment, water was exchanged daily at a rate

Table 1. Percentage ingredients (g/100g) of the experimental control and garlic powder supplementation diets fed to sea bass.

Ingredients	Control	GP2	GP4	GP6
Fish Meal	57.5	57.3	57.1	56.9
SBM	20.0	20.0	20.0	20.0
Fish Oil	10.6	10.6	10.6	10.6
b-Corn Starch	8.9	7.1	5.3	3.5
Vit-Min Premix	3.0	3.0	3.0	3.0
Garlic powder	0.0	2.0	4.0	6.0

C = controldiet, GP2 = 2% garlicpowderdiet, GP4 = 4% garlicpowderdiet, GP6 = 6% garlicpowderdiet; Fish Meal: Anchovy, Black Sea origin; SBM: Defatted soybean meal; Fish Oil: Anchovy, Black Sea origin; b-Corn starch: KENTON Corn Starch, Istanbul-Turkey; Garlic powder: KOTANYI GmbH, Wolkersdorf-Austria (Granulated garlic; Energy: 1.498 kJ·100g^{-1}, Protein 15.9 g·100g^{-1}; Carbohydrate 71.1 g·100g^{-1}, Lipid 0.3 g·100g^{-1}).

of approximately 10% of the total volume.

2.4. Blood Collection

At the end of the experiment (60 days), 12 fish per treatment group (4 fish/aquarium) were randomly selected and used for the hematological and serum biochemical analysis. Fish were anesthetized with clove oil at 20 mg/L [16]. Blood samples from fish were collected from the caudal vein by using a syringe, and added to tubes containing EDTA (BD Microtainer, UK). Blood samples were then subjected to hematological analysis. Blood serum was separated by centrifugation (2142 × g for 10 min) in plastic biochemistry tubes (Kima-vacutest, Italy) and stored at −20˚C until use in serum biochemical analysis [17].

2.5. Hematological Analysis

Red blood cell (RBC) count (×10^6 per mm^3), hematocrit (Hct, %), and hemoglobin (Hb) concentrations (g/dL) were determined by using the method of Blaxhall and Daisley [10]. The RBC count was obtained with a Thoma hemocytometer using Dacie's diluting fluid. The Hct value was determined using a capillary Hct tube. The Hb concentration was determined by spectrophotometry (540 nm) via the cyanmethemoglobin method. Mean corpuscular volume (MCV), mean corpuscular Hb (MCH), and mean corpuscular Hb concentration (MCHC) were calculated with the following formulae [18]:

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

$$\mathrm{MCH}\left(\mathrm{pg}\right)=\left[\left(\mathrm{Hb},\mathrm{g/dL}\right)\times 10\right]/\left(\mathrm{RBC},\times 10^6\,\mathrm{per\ mm}^3\right),$$

and

$$\mathrm{MCHC}\left(\%\right)=\left[\left(\mathrm{Hb},\mathrm{g/dL}\right)\times 100\right]/\left(\mathrm{Hct},\%\right).$$

2.6. Biochemical Analysis

Biochemical indices in serum, including glucose (GLU), triglyceride (TG), cholesterol (CHOL), total protein (TP), and albumin (ALB) were determined using bioanalytic test kits (Bioanalytic Diagnostic Industry Co.) and were measured with an Optizen POP QX spectrophotometer (Mecasys Co. Ltd., Daejeon, Korea). The serum albumin/globulin ratio (A/G) was also calculated from the biochemical results as the ratio of albumin/(total protein-albumin).

2.7. Statistics

Each value was expressed as mean ± SE for each of the measured variables. The statistical significance (P < 0.05) of growth parameters, hematological and serum biochemical variables were tested using one-way ANOVA followed by a Tukey multi-comparison test with SPSS 17.0 packaged software.

3. Results

3.1. Feed Intake and Garlic Powder Consumption

During the experiment, survival rate was 100% in all treatment groups, showing that all fish were acclimated to experimental conditions and test diets. Overall, the highest feed intake was recorded for fish in the control group fed diets without garlic powder and for fish in the GP4 treatment group which were fed on diets with 4% garlic powder, while the lowest feed consumption was observed for fish in the GP6 treatment group, which received a diet containing 6% garlic powder. Feed intake of fish in the GP4 treatment group was similar to those in the control group, and no significant difference ($p > 0.05$) was recorded between the GP4 and control groups. However, when dietary garlic powder was increased from 4% to 6%, feed consumption of fish in the GP6 treatment group significantly decreased ($p < 0.05$), compared to those in the control, GP2 and the GP4 treatment groups. Garlic powder consumption in the experimental groups significantly increased ($p < 0.05$) with the increase in dietary garlic powder levels from 2% to 6%. The highest consumption of garlic powder was recorded for fish in the GP6 diet group, while the lowest consumption was found in the GP2 treatment group (**Table 2**).

3.2. Hematological Variables

The effects of dietary garlic powder on Sea bass hematological variables are presented in **Table 3**. The RBC count, Hb concentration, Hct, MCH, and MCHC in the GP2 treatment group did not vary significantly from the values observed for the control group ($P > 0.05$). The MCV and MCHC levels in the GP6 treatment groups were lower than the levels recorded in the control group ($P < 0.05$). The RBC count, Hb concentration, Hct, and MCH of fish in the GP4 and GP6 treatments groups were significantly lower than the control values ($P < 0.05$).

3.3. Blood Variables

The effects of dietary garlic powder on Sea bass biochemical variables are given in **Table 4**. Relative to the value

Table 2. Total feed intake (g/fish) and garlic powder consumption (g/fish) of sea bass juveniles during a 60-day feeding period.

	Control	GP2	GP4	GP6
TFI	18.504 ± 1.107^{c}	16.656 ± 0.172^{b}	18.289 ± 0.837^{c}	14.867 ± 0.446^{a}
GPC	0.00 ± 0.00^{a}	0.333 ± 0.003^{b}	0.732 ± 0.033^{c}	0.892 ± 0.027^{d}

Values with different superscripts in the same row are significantly different at $p < 0.05$ (mean ± SD of triplicate groups); (One way ANOVA and Duncan's multiple range test, $P < 0.05$); TFI (total feed intake, g/fish) = (total feed intake (g)/number of fish); GPC (garlic powder consumption, g/fish) = (total feed intake (g) × percent of garlic powder)/number of fish.

Table 3. Hematological variables (mean ± SD; $n = 12$ fish/group) in sea bass fed diets containing different conentrations of garlic powder.

Variable	Control	GP2	GP4	GP6
RBC ($\times 10^6$ per mm^3)	3.20 ± 0.03^{a}	3.15 ± 0.08^{a}	2.84 ± 0.07^{b}	2.78 ± 0.04^{b}
Hb (g 100 ml^{-1})	6.08 ± 0.18^{a}	5.63 ± 0.29^{a}	4.73 ± 0.11^{b}	4.42 ± 0.12^{b}
Hct (%)	32.38 ± 0.38^{a}	32.00 ± 0.73^{a}	28.63 ± 0.78^{b}	27.63 ± 0.46^{b}
MCV (µm^3)	101.25 ± 0.92^{a}	101.65 ± 0.39^{ab}	100.77 ± 0.37^{ab}	99.41 ± 0.31^{b}
MCH (pg)	19.07 ± 0.70^{a}	17.84 ± 0.72^{ab}	16.70 ± 0.52^{b}	15.90 ± 0.42^{b}
MCHC (%)	18.84 ± 0.71^{a}	17.56 ± 0.73^{a}	16.58 ± 0.55^{ab}	16.00 ± 0.43^{b}

Variables are red blood cell count (RBC), hemoglobin (Hb) concentration, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular Hb (MCH), and mean corpuscular Hb concentration (MCHC). Superscript letters indicate inter group statistical differences. Means ± standard deviation of data for triplicate groups with different superscripts in the same row differ significantly (One way ANOVA and Duncan's multiple range test, $P < 0.05$).

Table 4. Serum biochemical variables (mean ± SD; n = 12 fish/group) in sea bass fed diets containing different concentrations of garlic powder.

Variable	Control	GP2	GP4	GP6
GLU (mg 100 ml^{-1})	176.72 ± 3.83^{a}	153.67 ± 6.45^{a}	105.02 ± 8.43^{b}	88.10 ± 5.68^{b}
TG (mg 100 ml^{-1})	94.08 ± 5.85^{a}	112.50 ± 7.45^{a}	64.23 ± 6.43^{b}	95.41 ± 2.16^{a}
CHOL (mg 100 ml^{-1})	138.20 ± 6.05^{a}	72.30 ± 6.43^{b}	121.70 ± 10.78^{a}	81.00 ± 4.70^{b}
TP (g 100 ml^{-1})	9.84 ± 0.30^{a}	9.72 ± 0.41^{a}	9.53 ± 0.40^{a}	11.27 ± 0.74^{a}
ALB (g 100 ml^{-1})	3.13 ± 0.18^{c}	4.15 ± 0.09^{b}	5.18 ± 0.31^{a}	5.84 ± 0.12^{a}
GLO (g 100 ml^{-1})	6.72 ± 0.35^{a}	5.57 ± 0.45^{ab}	4.35 ± 0.26^{b}	5.43 ± 0.72^{ab}
A/G Ratio	0.48 ± 0.05^{b}	0.79 ± 0.08^{a}	1.22 ± 0.10^{a}	1.24 ± 0.19^{a}

Variables are glucose (GLU), triglyceride (TRI), cholesterol (COL), total protein (TP), albumin (ALB), globulin (GLO), and albumin/globulin ratio (A/G). Super script letters indicate inter group statistical differences. Means ± standard deviation of data for triplicate groups with different superscripts in the same row differ significantly (One way ANOVA and Duncan's multiple range test, $P < 0.05$).

for the control group, serum GLU was significantly lower ($P < 0.05$) in Sea bass in treatment groups GP4 and GP6 (**Table 4**). Serum TG and GLO levels in fish fed the GP4 diet were significantly lower than the control values ($P < 0.05$), whereas these two variables for the GP2 and GP6 treatment groups were the same as the control values ($P > 0.05$). The CHOL levels in the GP2 and GP6 treatment groups were lower than the level recorded in the control group ($P < 0.05$). The TP levels in Sea bass were not significantly affected by feeding with garlic powder diets ($P > 0.05$). Serum ALB and A/G ratios in fish fed the GP2, GP4 and GP6 diets were significantly higher than those of the control group ($P < 0.05$).

3.4. Biochemical Variables

The effects of dietary garlic powder on Sea bass biochemical variables are given in **Table 4**. Relative to the value for the control group, serum GLU was significantly lower ($P < 0.05$) in Sea bass in treatment groups GP4 and GP6 (**Table 4**). Serum TG and GLO levels in fish fed the GP4 diet were significantly lower than the control values ($P < 0.05$), whereas these two variables for the GP2 and GP6 treatment groups were the same as the control values ($P > 0.05$). The CHOL levels in the GP2 and GP6 treatment groups were lower than the level recorded in the control group ($P < 0.05$). The TP levels in Sea bass were not significantly affected by feeding with garlic powder diets ($P > 0.05$). Serum ALB and A/G ratios in fish fed the GP2, GP4 and GP6 diets were significantly higher than those of the control group ($P < 0.05$).

4. Discussion

Physiological stress indicators such as hematological and serum biochemical variables could be useful to evaluate the effects of phyto-additives in fish [9] [14] [19], but for the application of these findings in fish feed preparation, more detailed studies of the dietary effects are necessary to produce safe and effective diets.

Changes in hematological variables are important for the evaluation of the health status of organs [20]. In particular, anemia is an important trigger of increased sensitivity to infectious diseases [21]. The present study demonstrated that hematological variables (RBC, Hct, Hb and MCH) decreased in a dose dependent manner (GP4 and GP6). Earlier studies in which garlic was used as a supplement in fish feed reported that diets containing 0.05% to 3% garlic resulted in hematological improvements [3]-[5] [8]. However, there is no information about the effects of higher levels of dietary garlic (over 4%) on hematological variables in fish. In our study, dietary garlic powder at inclusion levels of 4% and 6% resulted in significant decreases in RBC, Hb, and Hct, leading to anemia in Sea bass. These parameters were also used for the assessment of anemia in fish [22] [23]. The anemia may be due to the inhibition of RBCs (erythropoiesis) and hemesynthesis and an increased rate of erythrocyte destruction in hematopoietic organs [24].

The present study demonstrated that the GLU level decreased with increasing doses of garlic powder. These results suggest that garlic powder will cause hypoglycemia in Sea bass juvenile. Reference [25] reported that persistent hyperglycemia causes hepatomegaly and other health problems, which could negatively affect any growth promoting effects of hyperinsulinemia in fish. This hypothesis about hyperglycemia is also supported by the increased activity of serum AST and ALT enzymes, which are known as indicators of liver injury, in *Oncorhynchus mykiss* fed 40 mg/kg (4%) and 50 mg/kg (5%) garlic diets over a period of 8 weeks [7]. Although the influence of chronic hyperglycemia or hypoglycemia in fish is still unclear, hypoglycemia induced by garlic powder supplementation may be considered as a beneficial effect of garlic powder. Further investigations are needed to address this issue.

Major blood lipid components, such as triglycerides, phospholipids, and cholesterol are affected by diet and stress levels in fish [26]. In our study, the levels of serum TG in Sea bass decreased in the GP4 group. On the other hand, the levels of CHOL in theGP2 and GP6 groups were increased. Reference [27] also demonstrated that garlic supplementation resulted in an inhibition of the plasma cholesteryl ester transfer protein (CETP) activity, lowering the concentration of total cholesterol and triglycerides in blood. On the other hand, in this study, serum TG or CHOL levels showed fluctuations in response to garlic diets. The present findings seem to be consistent with other research, which found a similar and regular trend [7]. Serum cholesterol levels are affected by dietary cholesterol (exogenous cholesterol) and synthesized cholesterol in the liver (endogenous cholesterol) [21]. The feed consumption in theGP2 and GP6groups was significantly lower than that in the control and GP4 groups. These findings suggest that lowered serum CHOL would be affected by a decrease in exogenous cholesterol. If the decrease in serum TG observed in GP4 is due to the influence of garlic powder, it is expected that the levels of TG in the GP2 and GP6 groups would show significant decreases compared to the control. However, the levels of TG in GP2 and GP6 were similar to that of the control. In yellowtail, slight starvation caused an elevation of serum TG levels (Maita, unpublished data). It is therefore likely that the levels of TG observed in GP2 and GP6 resulted from both the garlic powder itself (lowered TG) and insufficient feed intake.

The serum TP, ALB and GLO contents are considered to reflect the health status of fish during the herb treatments [9]. In this study, the serum TP and GLO levels were not significantly affected by feeding garlic powder diets. However, serum ALB and A/G ratios in fish fed with all garlic diets were higher than the control and this result was dose-dependent. Therefore, it appears that the increase in serum ALB and A/G ratios were caused by garlic power supplementation. Similarly, [3] conducted an experiment with *Labeo rohita* fingerlings fed a basal diet containing 0%, 0.5% or 1.0% garlic powder for 60 days, and found that 0.5% garlic powder increased serum ALB and A/G ratio values compared to that of the fingerlings fed a control diet. In general, the serum ALB level indicates the status of dietary protein intake. When we consider the significant decrease of feed intake in GP2 and GP6, the dose-dependent increases in ALB observed in the present study seem to be contradictory. Based on the increased levels of ALB and A/G ratios of the fish reported here, the garlic powder seemed to exert a stronger effect than that of the decreased feed intake in this study.

It is well known that GLO concentrations are indisputably essential for a healthy immune system and for maintaining immune functions in fish blood [8]. An increase in serum GLO in fish with the addition of dietary garlic has also been reported in previous studies [4] [8]. In contrast, our study indicated that the blood GLO concentration was decreased in fish fed the experimental diet containing 4% garlic. Similar results have been reported by an earlier study [3], indicating that garlic powder supplementation at 0.5% decreased the serum GLO values in *L. rohita* fingerlings after 20 days of feeding. However, they found that the use of 0.5% garlic powder significantly increased serum GLO values compared to the fingerlings fed the control diet after 60 days of feeding. These fluctuations in GLO values are in agreement with the findings of an earlier study [4], who also reported a decrease and an increase in serum GLO contents in *O. mykiss* fingerlings after 14 days of feeding of oven-dried garlic bulbs at 0.05% and 0.5%, and 0.1% and 1.0%, respectively. Based on the findings in different fish species, it may be concluded that the dose and time of exposure to garlic may alter the effects of garlic on GLO levels in blood.

5. Conclusion

Various studies of dietary herbal supplements in different fish species have demonstrated hematological, serum biochemical or immunological enhancements. In the present study, results showed that the choice of herbs, their dose and time of application is very important for obtaining higher efficiency. High levels of garlic supplementation in diets of Sea bass may have negative hematological and biochemical consequences. Hence, it is suggested not to use more than 2% dietary garlic powder in juvenile European Sea bass diets. The present study is the first attempt to examine the effects of dietary garlic powder on the hematological and biochemical status of Sea bass. Further studies are needed to determine the optimal application frequency for favorable garlic performance and to assess its impacts on infection.

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