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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Immunity-Related Enzyme Gene Interactions in *Archocentrus centrarchus* Infected with *Lactococcus garvieae*

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Abstract: Unsuitable environmental conditions and low host resistance in the aquatic environment are the most important factors in fish diseases. Bacterial diseases in fish can be treated with different preparations. The aim of this study is to determine the disease symptoms, expression levels of TNF and IL genes and some biochemical parameter changes in *Archocentrus centrarchus* by using herbal extract for the treatment of the disease caused by *Lactococcus garvieae* bacteria. Gene expression was performed by Real-Time PCR and enzyme activities were determined by spectrophotometric analysis method. It was observed that the expression level of the TNF gene was 2.5 times more effective in the antibiotic (oxytetracycline) group and 14 times more effectively in the extract application. It was found that the antibiotic group had a close expression level compared to the control at the IL gene expression level, while the extract group expressed 5.5 times more. Glutathione s-transferase activity in the extract group increased significantly compared to the control and antibiotic groups. Superoxide dismutase activity was significantly higher in the extract group compared to the antibiotic and bacterial groups. It can be said that oxidative stress caused by bacterial infection is tried to be eliminated more in the extract group. It was determined that the andiz root extract at the patent stage, which was used for the first time in the treatment of *A. centrarchus* fish infected with *L. garvieae*, one of the most important bacterial agents in the field of aquaculture, is applicable.

Keywords: Cichlid fish, fish diseases, gene expression, IL, TNF.

Lactococcus garvieae ile Enfekte *Archocentrus centrarchus*'ta Bağışıklıkla İlişkili Enzim Gen Etkileşimleri

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Öz Akuatik ortamda olumsuz çevre koşulları ve düşük konakçı direnci, balık hastalıklarda en önemli etkenlerdir. Balıklardaki bakteriyel hastalıklar farklı preparatlarla tedavi edilebilir. Bu çalışmanın amacı, *Archocentrus centrarchus* balığında *Lactococcus garvieae* bakterisinin neden olduğu hastalığın tedavisi için bitkisel öz kullanarak hastalık semptomlarını, TNF ve IL genlerinin ekspresyon seviyelerini ve bazı biyokimyasal parametre değişikliklerini belirlemektir. Gen ekspresyonu Real-Time PCR ile gerçekleştirilmiştir. Enzim aktiviteleri spektrofotometrik analiz yöntemi ile belirlenmiştir. TNF geninin ekspresyon seviyesinin antibiyotik (oksitetrasiklin) grubunda 2,5 kat, ekstrakt uygulamasında ise 14 kat daha etkili olduğu gözlenmiştir. Antibiyotik grubunun IL gen ifadesi düzeyinde kontrole yakın bir ifade düzeyine sahip olduğu, ekstrakt grubunun ise 5,5 kat daha fazla ifade ettiği tespit edilmiştir. Ekstrakt grubundaki glutatyon s-transferaz aktivitesi, kontrol ve antibiyotik gruplarına kıyasla önemli ölçüde artmıştır. Süperoksit dismutaz aktivitesi, antibiyotik ve bakteri gruplarına göre ekstrakt grubunda önemli düzeyde yükselmiştir. Bakteriyel enfeksiyonun neden olduğu oksidatif stresin ekstrakt grubunda daha fazla giderilmeye çalışıldığı söylenebilir. Su ürünleri yetiştiriciliği alanında en önemli bakteriyel etkenlerden biri olan *L. garvieae* ile enfekte olmuş *A. centrarchus* balığının tedavisinde ilk kez kullanılan patent aşamasındaki andız kökü özünün uygulanabilir olduğu tespit edilmiştir.

Anahtar kelimeler: Balık hastalıkları, çiklit balığı, gen ifadesi, IL, TNF.

INTRODUCTION

Archocentrus centrarchus (Gill, 1877) is a very popular fish in aquarism. It is also frequently used in studies on diseases seen in aquarium fishes. *Lactococcus garvieae* is one of the most common fish pathogens in the world. It has started to be seen frequently in fish production facilities in our country since the 1990s (Önalan et al., 2020). Rapid identification of pathogens is crucial for the effective control of diseases occurring in aquaculture. Detection of pathogens is important in the environment as well as in infected fish. In the studies on bacterial fish pathogens, genotypic identifications of disease agents are made especially by PCR methods (Crisafi et al., 2011; Fruciano et al., 2019). Many immune-related genes in fish have been characterized recently (Chen et al., 2018, Balta & Karatay, 2021).

In order to determine the oxidative stress caused by the disease, enzyme activity measurements are used in different tissue samples of fish. Bacterial diseases are one of the sources of oxidative stress in fish species. Oxidative stress refers to an imbalance due to excess reactive oxygen species (ROS) or oxidants over the cell's ability to mount an effective antioxidant response. Oxidative stress causes macromolecular damage (Ray et al., 2012). ROS is constantly produced and eliminated by living organisms and is normally kept at certain steady-state levels. However, the overproduction of ROS causes damage to cellular macromolecules (membrane lipids, DNA, carbohydrates and proteins). Organisms have a cellular antioxidant detoxification system consisting of enzymatic antioxidants and non-enzymatic small molecules to deal with the generation of ROS. In particular, the major antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) are most responsible for ROS elimination. Among these enzymes, SOD plays an important role in cellular defense systems against oxidative stress by catalyzing the dismutation of superoxide anions to hydrogen peroxide and molecular oxygen (Kankaya et al., 2015; Kankaya & Kaptaner, 2017; Shi et al., 2022).

A common consequence of oxidative stress is lipid peroxidation (LPO). Malondialdehyde (MDA) is one of the LPO products. The presence of LPO and high MDA levels have been extensively studied in many organs of different fish species exposed to environmental pollutants in both biological monitoring and controlled experiments. MDA levels in different organs of fish and other aquatic organisms exposed to pollutants may decrease or remain unchanged. In general, the MDA response is attributed to its high antioxidant capacity, which counteracts the effects of LPO. However, a similar MDA response was observed without any change or decrease in antioxidant biomarker levels (Kankaya et al., 2015; Garcia et al., 2020). Glutathione s-transferase (GST) is an enzyme belonging to the phase II detoxification enzyme family that can reduce the cellular toxicity of a number of endogenous and environmental chemicals. The primary catalytic activity of GST is the conjugation of electrophilic compounds by facilitating nucleophilic attack with reduced glutathione (GSH). Environmental chemicals that GST detoxifies include carcinogens, pesticides, and reactive intermediates. Expression of the GST isoform is important when considering susceptibility to chemical injury (Trute et al., 2007; Rudneva et al., 2010; Kaptaner et al., 2016). Although there are studies on virulence and pathogenicity tests of bacteria, no study has been found with gene clusters. This study was carried out to determine the disease symptoms, expression levels of TNF and IL genes and enzymatic activity changes in *A. centrarchus* fish infected with *L. garvieae*.

MATERIAL AND METHOD

Trial design: Individuals of *A. centrarchus* fish with similar weights were selected in the experimental groups and 6 of them were used in each group. Four experimental groups were formed, consisting of control (*L. garvieae*), antibiotic (*L. garvieae* + antibiotic - oxytetracycline), extract (*L. garvieae* + extract - andiz root) and negative control (*L. garvieae* + PBS). The fish were kept in 60 L aquariums for 3 periods to adapt to their environment and maintained and fed. The study was conducted using dechlorinated and aerated tap water under the natural photoperiod. Symptoms and mortality rates were monitored in the 7-day period following the *L. garvieae*, antibiotic, extract and PBS injection (Drakopulos & Poe, 2023). The study was carried out with the permission of the local ethics committee for animal experiments dated January 28, 2021, and numbered 2021/01-10.

Bacteria inoculation and injection administration: *L. garvieae* isolates were added to the Tryptic Soy Agar (TSA) medium and incubated at 22°C for 24 h. The freshly cultured bacteria obtained were adjusted to a bacterial concentration of OD_{600} = 0.6 ($1x10^7$ CFU) using a spectrophotometer (Crisafi *et al*. 2011). The extract injection was administered intraperitoneally at a dose of 0.01 mL g^{-1} to the fish. Antibiotic group fish were injected with oxytetracycline prepared at the same dose as the extract. The duration of symptoms and death were observed after injection periods (Pérez-Sánchez et al., 2011, Balta & Dengiz Balta, 2019).

RNA isolation: Muscle tissue samples were taken between the fish's dorsal fin and tail fin, and RNA was isolated with the RNeasy Plus Mini Kit (Qiagen) in the

QIAcube device. The protocol of the QIAcube instrument has been optimized in the elution step for RNA isolation. The modified version of the protocol is given below.

350 µL of RTL buffer was added to 25 mg of tissue and homogenized in Tissue Lyser LT (Qiagen). The homogenate was taken into a 2 mL microcentrifuge tube, 600 µL of 70% ethanol was added and centrifuged at 8000 x *g* (10.000 rpm) for 15 sec. Then, 700 µL of RW1 buffer was added and centrifuged at 8000 x *g* for 15 sec. 500 µL of RPE buffer was added to the spin column and centrifuged at 8000 x *g* for 15 sec. After that, 500 µL of buffer was added to the RPE spin columns and centrifuged at 8000 x *g* for 2 min. Finally, 30 µL of RNase Free Water was added and centrifuged at 8000 x *g* for 1 min and RNA elutions were obtained (Önalan, 2019).

Primer synthesis and cDNA synthesis: The GeneBank accession numbers used in gene expression analysis and the primers and properties of ACTB (housekeeping), IL and TNF genes are given in Table 1. Isolated RNAs were diluted to a concentration of 1 ng μL^{-1} per sample by Real-Time PCR. Then, cDNAs were synthesized with the RT2 First Strand Kit (Qiagen). 2 µL of GE buffer was added to 5 µL of RNA and incubated at 42°C for 5 min. After adding 4 µL of 5X Reaction Buffer, 1 µL of Primer (Primer Array System, UK) and 2 µL of Reverse Transcriptase Mix, the total volume was made up to 20 µL with ultrapure water. The final concentration obtained was incubated at 42°C for 15 min, followed by PCR for 5 min at 95°C (Schwartz et al., 2020).

Table 1: Primers used in this study and some of their properties.

Gene	Sequence (5'-3')	Amplicon (bp)	Binding Temperature	Gen ID
ACTB-F	ATGTTGGTGATGAGGCCCAG	140	60	115784034
ACTB-R	AGGGAAATCGTGCGTGACAT			
IL-F	GAAGGTGGGCAGAAAGACGA	70	60	115789632
IL-R	TTTGAGTTTGACAGCGTGCG			
TNF-F	AGATGAGGATGGGAGCTGGT	216	60	115784139
TNF-R	TTCCACTGCAGCACCATCAT			

Real-time PCR analysis: To determine the gene expression differences, primer design was made for TNF and IL genes. Real-Time PCR analysis protocol using expression primers for TNF and IL genes is given below.

The total volume was optimized to 25 µL by adding 12.5 µL of SybrGreen qPCR Master Mix, 1 µL of Forward and Reverse Assay Primer, 6.5 µL of H_2O and 5 µL of cDNA. In the PCR protocol, preliminary denaturation was applied at 95°C for 10 min, followed by a cycle of 15 sec at 94°C and 30 sec at 60°C 40 times (Önalan & Çevik, 2020).

Tissue homogenization: Liver samples stored at -80°C for analysis were homogenized with tissue lysis (Tissuelyser Qiagen) in a microcentrifuge tube for 3 min in 50 mM KH_2PO_4 buffer (4°C, 1:5 w/v). The homogenate was centrifuged at 9500 rpm for 30 min at 4°C with a refrigerated centrifuge (Inovia, INO-HR/T16M) (Marklund, 1990). The obtained supernatant was used for MDA, GST, SOD and CAT measurements.

Biochemical analyses: MDA content was determined according to Jain et al., (1989) by measuring at 532 nm; GST activity was measured at 340 nm using the method of Habig et al., (1974). In addition to these, SOD activity was determined at 505 nm using the commercial kit manufacturer's procedure (Ransod, Randox Lab., UK) and CAT activity was measured at 240 nm by spectrophotometric method according to the method given by Aebi, (1984).

Statistical analysis: By using the ct values obtained after Real-Time PCR analysis, gene expression levels were determined with the $2^{\Delta\Delta ct}$-log formula and binomial data were obtained. Data were evaluated using one-way analysis of variance (ANOVA), Duncan multiple comparison test, and T-test. Values of $P < 0.05$ in all analyzes were considered statistically significant. Values are given as mean ± standard deviation (Xie et al., 2023).

RESULTS

Symptoms observed after the trial: The mean weight of the fish was 7.1 ± 0.4 g and the average length was 6.5 ± 0.3 cm. The quality criteria of the water used in the study are given below. Temperature: 20.5 ± 0.3°C, pH: 8.43 ± 0.05, dissolved oxygen: 6.9 ± 0.2 mg L^{-1}, oxygen saturation: 98.3 ± 1.4%, electrical conductivity: 705 ± 17 µS cm^{-1}, salinity: 0.31 ± 0.02‰, total hardness: 347 ± 18 $CaCO_3$ mg L^{-1} and total alkalinity: 538 ± 14 $CaCO_3$ mg L^{-1}. By applying clove oil in an amount of 0.4 mL L^{-1}, the time to enter anesthesia for fish was 90 sec and the time to exit anesthesia was 195 sec. During the experiment, Lactococcosis symptoms were observed especially in the control (*L. garvieae*) group of fish that were contaminated with bacteria and not treated. Darkening and exophthalmos were observed as typical symptoms in this group.

Gene expression analysis: TNF gene expression level, which is known to be effective in wound healing and the immune system, was observed at the highest expression level in the extract group compared to the control group. While the expression level of the extract group was 14 times higher than the control group and 2.5 times higher than the antibiotic group, it was observed that it was two times more effective than the PBS group.

In the level of IL, another gene directly related to the immune system, whose expression level was determined in the study, it was observed that the extract group samples were expressed 5.5 times more than the control group samples. While a low expression rate was observed in the antibiotic group, close to the control group, it was determined that the gene associated with the immune system of the body system was expressed two times more

without external intervention. Expression levels of TNF and IL genes are given in Figures 1 and 2.

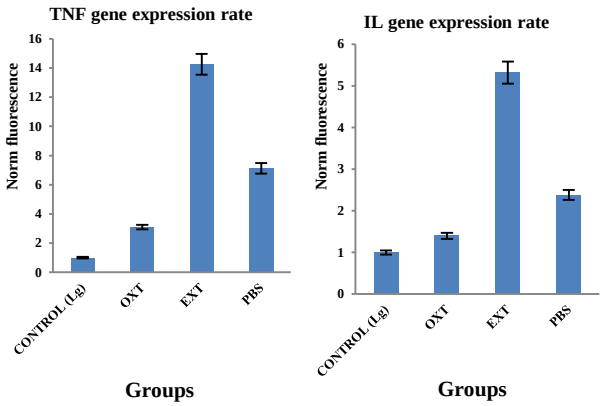

Figure 1: TNF and IL gene expression levels.

When the expression levels of both genes were evaluated together, the expression level of the TNF gene in the antibiotic group was higher than that of the IL gene. It was observed that antibiotics activated the TNF gene at a level two times higher than the control group samples. However, the TNF gene expression level in the extract group was found to be more active than the IL gene expression level in the same group. No significant expression level changes were detected between the two genes in the control group. For this reason, it is thought that the applied substance has an effect on the changes in the expression levels of two genes in the same group. It was observed that the IL gene expression level was expressed more than the TNF gene in the control groups, but it was not at a significant level (Figure 2). In the other groups, the expression level of the TNF gene was overexpressed compared to the IL gene.

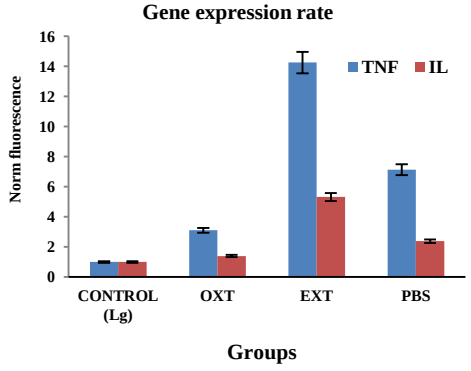

Figure 2: Comparative graph of expression levels of TNF and IL genes compared to the control group.

Biochemical: Enzyme values measured in control, antibiotic, extract and bacterial group fish liver tissue were found as follows; MDA content 15.35 ± 0.37, 7.52 ± 0.4, 15.56 ± 0.28, 7.28 ± 0.17 nmol g^{-1} tissue, GST activity 15.54 ± 0.31, 13.32 ± 0.26, 21.55 ± 0.34, 1.42 ± 0. 28 U g^{-1} tissue, SOD activity 1081.92 ± 11.55, 594.48 ± 6.91, 946.42 ± 8.22, 692.93 ± 7.71 U g^{-1} tissue and CAT activity 1869.97 ± 17.54, 775.4 ± 7.92, 2445.36 ± 10.07, 75.46 ± 1.2 U g^{-1} tissue. When Figure 3 is examined, it can be seen that MDA content decreased significantly in antibiotic and bacterial groups compared to control and extract groups. GST, SOD, and CAT activity changed significantly in all groups.

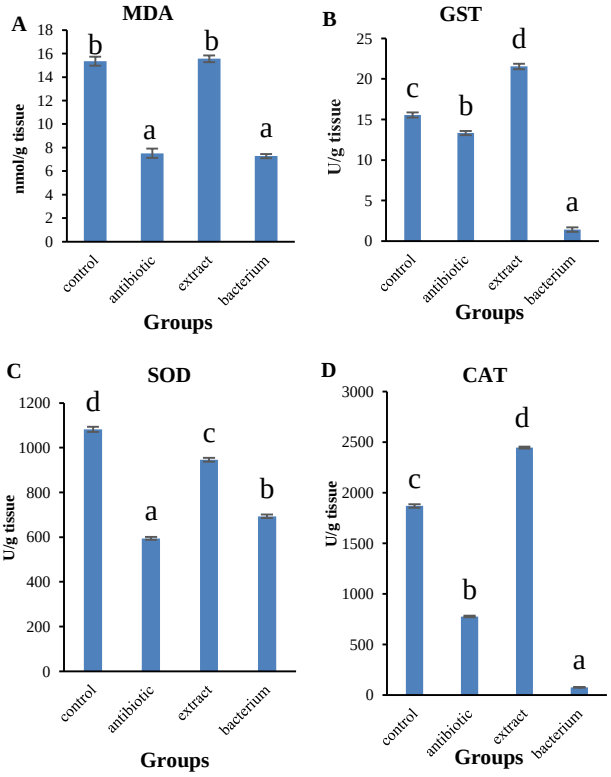

Figure 3: The antioxidant defense system indices in liver tissue of *A. centrarchus* infected with *L. garvieae*: MDA (A), GST (B), SOD (C), and CAT (D). (n = 6)

DISCUSSION AND CONCLUSION

Symptoms, expression levels of TNF and IL genes and changes in enzymatic activity were investigated in *A. centrarchus* fish infected with *L. garvieae*. The herbal extract was used for the first time for therapeutic purposes in infected *A. centrarchus* fish. The effects of bacterial disease agents occurring in aquaculture vary in different temperature and water quality criteria. The virulence and pathogenicity rates of every bacterial disease agent on the same organism also vary. This situation is directly related to the virulence and pathogenicity of the bacteria as well as the immunity of the host. Mortality rates resulting from these differences result in negative situations against different bacteria in the fight against diseases in the field by the producers. Mortality rates in diseases caused by bacteria can vary widely, from 60% to 100%. In the realization of this result, the virulence and pathogenicity characteristics of disease agents originating from different gene clusters or indirectly play a role in the mechanism of action. Popoola et al., (2021) studied the effects of different

carbon sources on immune-related gene expression in *Clarias gariepinus* fish infected with *Aeromonas hydrophila*. In this study, they found that IL-10, TNF-α, TGF-β, and IL-1β immune genes were increased. In other words, they stated that carbon sources increase fish innate immunity and immune-related gene expression. Chen et al., (2018) investigated the immune-related gene expression profile by infecting *Siniperca chuatsi* fish with *A. hydrophila*. They reported that the expression levels of major histocompatibility complex class II (MHC II), T cell receptor α (TCR α), tumor necrosis factor α (TNF α), CC chemokine 3, interleukin 8 (IL-8) and Hepcidin were strongly upregulated in the spleen and anterior kidney tissue of fish. Arslan, (2021) determined that when *Raphanus sativus* is added to the diet against *A. hydrophila* in *Oncorhynchus mykiss* fish, it can improve the immune responses and growth of the fish. Carriero et al., (2020) examined the changes in the expression levels of IL-1β and IL-8 genes in immune-related gene expression analysis in *Piaractus mesopotamicus* fish. They found that these genes could be used in the early response to bacterial infections, particularly the spleen and anterior kidney, under pathogenic conditions. Zou et al., (2020) determined immune gene expression levels in *O. mykiss* fish with IL-1β, IL-8, IL-2A, IL-6A, IL-22 and TGFβ genes. They suggested that gene expression in surviving fish was associated with the resistance conferring trait of several immune genes. In this study according to the expression results, it was determined that the TNF gene expression level was 2.5 times higher in the antibiotic group than in the control group, and it was 14 times more effective in the extract application. The antibiotic group was observed to have a close expression level compared to the control in the IL gene expression level, while the extract group expressed 5.5 times more. Considering the mortality rates, it was observed that the extract reduced the mortality rates against *L. garvieae*, one of fish's most important bacterial disease agents. On the other hand, it can be said that in the antibiotic-treated group, 2.5 times more genes are expressed and work harder than in the control group.

Skuratovskaya et al., (2013) reported an increase in SOD, CAT and GST activities in *Merlangius merlangus euxinus* fish infected with parasites at high density. Adeyemi, (2014) determined LPO level, GST and CAT activity in the liver tissue of *Escherichia coli* and *Vibrio fischeri* infected *C. gariepinus* fish. LPO and GST were found to be significantly higher in infected fish, and CAT was insignificant in infected and uninfected fish. It has been reported that the liver has a higher rate of oxidative stress than the gill and muscle tissue. Nabi et al., (2017) compared the liver tissue enzyme values of *Schizothorax plagiostomus* fish infected and uninfected with the acanthocephalan parasite (*Pomphorhyncus*). They reported that while LPO increased, GST and SOD decreased, and parasitic infections caused oxidative stress. In our study, GST increased significantly in the extract group compared to the control and antibiotic groups. MDA was similar in the extract group to the control group. SOD increased significantly in the extract group compared to the antibiotic and bacterial groups. It is seen that the oxidative stress caused by bacterial infection is tried to be eliminated in the extract group.

It was determined that the root extract at the patent stage, which was used for the first time in the treatment of *A. centrarchus* fish infected with *L. garvieae*, which is one of the important bacterial disease agents in aquaculture, is applicable.

In conclusion in future studies, it is planned to control the applications in different experimental animals and to produce a commercial form of the extract. It has been determined that the extract can be used as an alternative treatment method for bacterial fish diseases. In addition, the extract used in the study was found to activate the immune system. As a result, it can be said that the production of different organic matter derivatives in alternative aquaculture will open new horizons in this field.

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