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Expression of IL-8 Gene in Rainbow Trout (*Oncorhynchus mykiss*) Leucocytes Fed with Uryani plum (*Prunus domestica*) Extract

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Abstract

Aim to study: In the present study, interleukin-8 (IL-8) gene expression was determined in rainbow trout (*Oncorhynchus mykiss*) after treatment with uryani plum (*Prunus domestica*) extract.

Material and methods: Fish leucocyte was stimulated with plum extract at the rate of 0 (control), 5 mg/ml, 10 mg/ml and 15 mg/ml. Cells were collected at 1, 4, 12, 24, and 48 hours of the study and IL-8 gene expression was determined in each group.

Results: The result showed an increase in IL-8 expression in all experimental groups compared to the control at the end of the study.

Conclusion: The results suggested that uryani plum (*Prunus domestica*) extract could activate immune responses of rainbow trout head kidney leukocytes in rainbow trout.

Keywords: Fish, gene expression, IL-8, immune response, *Prunus domestica*.

Üryani Eriği (*Prunus domestica*) Ekstraktı ile Beslenen Gökkuşağı Alabalığı (*Oncorhynchus mykiss*) IL-8 Geninin Ekspresyonu

Öz

Çalışmanın amacı: Bu çalışmada, üryani eriği (*Prunus domestica*) ekstraktı ile muameleden sonra gökkuşağı alabalıklarında (*Oncorhynchus mykiss*) interleukin-8 (IL-8) gen ekspresyonu belirlenmiştir.

Materyal ve yöntemler: Balık lökositleri; 0 (kontrol), 5 mg/mL, 10 mg/mL ve 15 mg/L oranında erik özütü ile uyarılmıştır. Hücreler çalışmanın 1, 4, 12, 24 ve 48. saatlerinde toplanmış ve her grupta IL-8 gen ekspresyonu belirlenmiştir.

Bulgular: Çalışma sonunda tüm deney gruplarında IL-8 ekspresyonunun kontrole kıyasla arttığı görülmüştür.

Sonuç: Sonuçlar, ince kabuklu erik (*Prunus domestica*) ekstraktının gökkuşağı alabalığı baş böbreği lökositlerinin immün yanıtlarını aktive edebileceğini göstermiştir.

Anahtar kelimeler: Balık, gen ifadesi, IL-8, immün yanıt, *Prunus domestica*.

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Introduction

Rainbow trout (*Oncorhynchus mykiss*) is one of the most important coldwater fish species in aquaculture and is the 15th most produced species in the world. Global aquaculture production of rainbow trout has been increasing; according to Fishery and Aquaculture Statistics of Food and Agriculture Organization (FAO), global aquaculture production of rainbow trout was 916,540 tonnes in 2019 (FAO, 2022).

However, the emergence of various fish diseases has become one of the limiting factors in intensive aquaculture. Increasing stocking densities to produce more to meet growing demand has led to an increase in organic loading, which degrades water quality in the environment, and an imbalance in water parameters such as dissolved oxygen and pH, which are important for fish health. In addition, temperature (Bilen et al., 2013), poor water quality, and malnutrition trigger and manage the emergence of infectious diseases (Reverter et al., 2020). Chemotherapeutic agents, including antibiotics, provide an effective treatment option for disease outbreaks in aquatic animals; however, resistance has emerged due to their overuse against aquatic pathogens (Thanigaivel et al., 2016). In recent years, medicinal plants have been considered as feed additives for their growth-promoting, antioxidant, and immunostimulant activities. To minimise the use of antibiotics, the use of herbal immunostimulants in aquaculture is considered as one of the safest options. Herbal immunostimulants such as Greek juniper (*Juniperus excelsa*) aqueous methanolic extract (Bilen et al., 2021a), laurel leaf (*Cistus laurifolius*) ethanolic extract (Bilen et al., 2021b), black mustard (*Brassica nigra*) seed oil (Lakwani et al., 2021) and ribwort plantain (*Plantago lanceolata*) (Elbesthi, et al., 2020) were found to be effective against some important fish pathogens in fish.

On the contrary, *in vitro* studies evaluating the immunomodulatory effects of phytoextracts or phytochemicals on fish immune cells are still particularly scarce (Yin et al., 2006; Zanuzzo et al., 2012; Picchietti et al., 2013). Nevertheless, *in vitro* or *in vivo* approaches represent cost-effective pre-tests for subsequent *in vivo* experiments (Galeotti, 1998), in line with the 3Rs principle of replacement, reduction and refinement that should be applied to animal welfare (Midtlyng et al., 2011).

In vivo assays can be performed using primary cell cultures (e.g. leukocytes purified from lymphatic organs), whereas *in vitro* experiments use cell lines specifically developed for immunological research. These approaches provide reproducible results, allow simultaneous screening of many products at different concentrations and avoid the sacrifice of large numbers of fish (Fierro-Castro et al., 2012). In the case of *in vivo* tests, particular attention should be paid to the selection of healthy donors, as cell reactivity is influenced by the physiological status of the fish. Several promising immunostimulants such as glucans, lipopolysaccharide (LPS) or vitamins have been tested on fish immune cells (Mulero et al., 1998; Abarca et al., 2012; Fierro-Castro et al., 2012).

The main objective of the present study was to evaluate the effects of uryani plum (*Prunus domestica*) extract (TSP) on interleukin-8 (IL-8) expression in leukocytes purified from the head kidney of rainbow trout.

Material and Methods

Animals

The experiment was carried out on three rainbow trout (*Oncorhynchus mykiss* W., 1792) with an average weight of 30.61±1.09 g in Germeçtepe Inland and Marine Fish Production, Application and Research Center. Fish were adapted in

recirculating aquaculture systems in the Aquatic Toxicology unit of the Faculty of Fisheries for one week. For the present experiment, dried fruits were purchased from an herbal shop located in Kastamonu, Türkiye. The plum aqueous methanolic-extracts were prepared according to the method of Bilen et al. (2020).

Kidney Primary Cell Culture Medium

For primary culture, head kidney tissues of three rainbow trout were dissected under aseptic or sterile conditions, purified and maintained in a six-well plate and grown at 18 °C for at least 72 hours. Cell and tissue growth medium was Leibovitz's 15 (Gibco cat no. 11415064) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin-streptomycin (P/S) (Gibco, Thermo Fisher, Waltham, MA, USA) (Schnell et al., 2009).

***İn vitro* Immunostimulation**

24 h after seeding the explant for a primary kidney cell culture, it was stimulated with 0 (control), 5 mg/ml (TSP5), 10 mg/ml (TSP10) and 15 mg/ml (TSP15) concentrations of uryani plum. Primary cell cultures in six well plates were exposed to the immunostimulants for 1, 4, 12, 24 and 48 hours at 18 °C. At the end of these periods, the working cells were collected from each experimental well and harvested. Control plates contained the same volume of medium without immunostimulant. All experiments were performed in triplicate and independently repeated twice.

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the samples using Direct-zol RNA MiniPrep extraction kits (Zymo, Cat. No. R2051) according to the manufacturer's instructions. Quantity and quality of RNA samples was checked using a Thermo Scientific Multiskan GO instrument at wavelengths of 260 and 280 nm. After qualitative measurements, RNA samples were synthesized into cDNA using an Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit. cDNA reaction mixture contained 15 ng template RNA, 100 mM 25X dNTP mix, 10X RT random primers, 1 µL MultiScribe™ Reverse Transcriptase, 2 µL 10X RT buffer and 4.2 µL nuclease free water. The reaction mixture was incubated in a thermal cycler (ThermoFisher Scientific) for 10 minutes at 25 °C, 120 minutes at 37 °C and 5 minutes at 85 °C for cDNA synthesis and then stored at -20 °C.

Analyses of IL-8 Gene Expression

Primer sequences and references of the target genes are as indicated in Table.

Table. Gene specific primers with their sequences and references used for qRT-PCR in the study.

Gene	Primer sequence	References
β-actin	F: 5′-ATGGAAGGTGAAATCGCC-3′ R: 5′- TGCCAGATCTTCTCCATG- 3′	Sigh et.al. 2004
IL-8	F: 5′- CACAGACAGAGAAGGAAGGAAAG- 3′ R: 5′- TGCTCATCTTGGGGTTACAGA- 3′	Awad et. al. 2011

Expression levels of the genes were determined using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) with the Wizpure qPCR Master SYBR Kit (Wizbio solutions, USA). The qRT-PCR mix contained 10 µL of 2× SYBR Green Master Mix, 1 µL template DNA (15 ng), 1 µL of each IL-8 forward and reverse primer, and distilled water to a final volume of 20 µL. The qRT-PCR protocol included 35 cycles of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 40 seconds, and extension steps. Fluorescence signals were captured at 530 nm wavelength from 60 °C to 95 °C, with a temperature increment of 0.5 °C per second, for melting curve analysis. Gene expression of the IL-8 was determined according to comparative threshold cycle (C_T) method ($2^{-\Delta\Delta CT}$ method) (Schmittgen and Livak, 2008). IL-8 gene expression in each sample was finally determined after correction with β-actin.

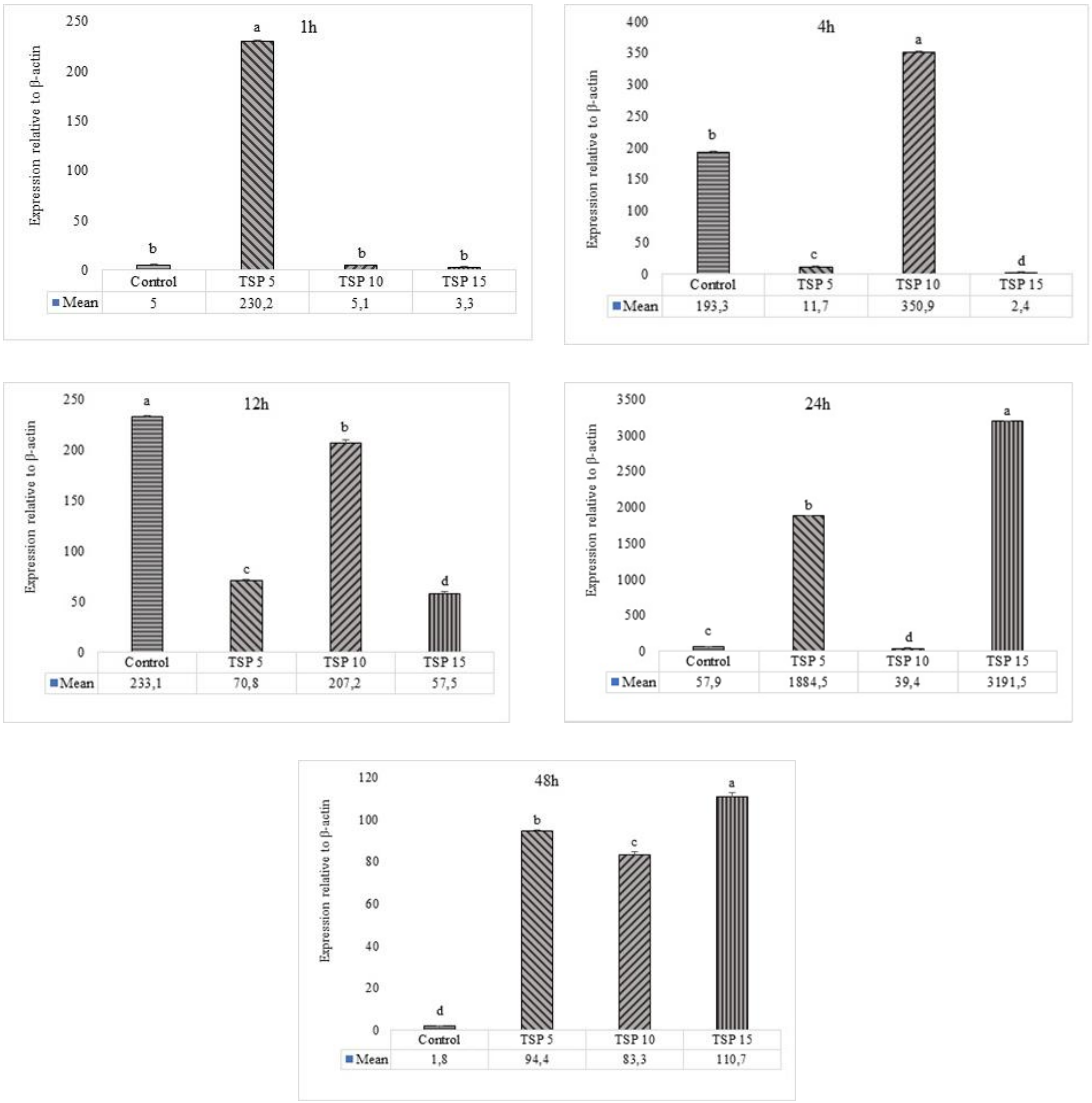

Figure. Comparison of relative gene expression levels (mean ± SD; n=3) of cytokines in head kidney cells of rainbow trout fed diets containing different doses of plum extract at the end of the 1st, 4th, 12th, 24th and 48th hours of feeding. Control, TSP5, TSP10 and TSP15 indicate uryani plum extract doses at 0, 0.1 and 0.5 g kg^{-1} feed, respectively. Different letters on bars denote significant differences among groups ($P<0.05$).

Statistical analyses

In our study, one-way analysis of variance (ANOVA) was performed to determine whether there was a statistically significant difference between the means of the dependent variables divided into different groups. The analysis was performed using SPSS 23.0 (SPSS Statistics) and the significance level was accepted as 0.05. Duncan's multiple comparison test was used when there was homogeneity of variances; otherwise, a Tamhane post hoc test was applied.

Results

The *in vitro* immunostimulatory effects of plum methanolic extracts at different concentrations were evaluated by changes in the expression levels of the IL-8 gene. At different sampling times, increases in IL-8 gene expression levels were detected in the experimental groups compared to the control group ($P<0.05$). IL-8 expression was found to increase in TSP5 group 1 hour after *in vitro* immunostimulant administration compared to the control group ($P<0.05$). However, at 4 h sampling time, this increase was observed in the TSP10 group ($P<0.05$).

As shown in the figure, IL-8 gene expression levels of the TSP5 and TSP15 groups were lower than the other groups at 12 hours ($P<0.05$). However, in contrast to this situation, as seen in the figure, TSP5 and TSP15 groups were the two groups with the highest gene expression levels compared to the other groups at 24 and 48 hours ($P<0.05$). When the effect of uryani plum extract on head kidney leukocyte of the rainbow trout stimulation *in vitro* was evaluated over 48 hours depending on the level of IL-8 gene expression, the highest effect was found in the TSP15 and TSP5 groups at 24 hours ($P<0.05$). Also, when the results were evaluated independently of the gene expression level, it was found that IL-8 expression in all experimental groups showed a significant increase compared to the control group at the end of 48 hours ($P<0.05$) (Figure).

Discussion

Pharmacological effects and immunomodulatory properties of plant extracts have been an important field of study for fisheries research (Bilen et al., 2016; Altunoglu et al., 2017; Elbesthi et al., 2020). In aquaculture, the immunostimulant effect of plant extracts has been widely investigated with humoral responses (Almabrok et al., 2018; Ali et al., 2022) as well as various cytokine genes and different results have been obtained (Salem et al., 2022; Sönmez et al., 2021; Terzi et al., 2021).

In the present study, IL-8 gene expression was determined at different sampling times in head kidney leucocyties of the rainbow trout. IL-8 is another important pro-inflammatory cytokine produced by a variety of cells including monocytes, macrophages, epithelial cells, endothelial cells, neutrophils, and fibroblasts (Jimenez et al., 2006). Under normal conditions, IL-8 is mainly distributed in the spleen, intestine and gill (Wang et al., 2017, Terzi te al., 2021). At the end of the study all groups IL-8 gene expression was significantly increased ($P<0.05$). IL-8, produced mainly by monocytes, can interact with the G protein-coupled receptors CXCR1 and CXCR2 to recruit neutrophils and induce cytotoxic effects at sites of infection (Kendrick et al., 2014).

Plum extract triggers the immune system and could probably reveal immune responses (Hooshmand et al., 2015). The results of this study clearly showed an increase in IL-8 and this increase was closely related to plum extract. The IL-8 is very important against gram negative bacteria (Wang et al., 2017). The methanolic extract of uryani plum showed an excessive increase in IL-8 gene expression. A sustained up-regulation of IL-8 cytokine was observed in the

kidney after 24 hours. Therefore, plum extract administration may have stimulated neutrophils and phagocytic cells. These results are in agreement with the findings of Salem et al. (2022) and Sönmez et al. (2021) who tested various plant immunostimulants. In contrast to our results, Altunoglu et al. (2017) were not able to detect any effect of black seed on the expression of the IL-8 gene in rainbow trout.

Conclusion

In conclusion, long-term use of uryani plum extract in rainbow trout may be an effective immunostimulant against bacterial diseases in rainbow trout. It is thought that uryani plum methanolic extract may be used not only as an immunostimulant but also as a remady agent.

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Ethical Statement

This study was approved by the Kastamonu University Animal Experiments Local Ethics Committee (KUHADYEK-14.12.2020-2020.35).

Author Contributions

Investigation: S.B., N.C.A. and E.M.Y.; Material and Methodology: K.K. and N.C.A.; Supervision: S.B.; Writing-Original Draft: K.K. and E.M.Y; Writing- review & Editing: S.B.

Conflict of Interest

The authors declared that there is no conflict of interest.

Data Availabilty Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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