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RESEARCH ARTICLE

Immune responses and growth performance of the aqueous methanolic extract of *Malva sylvestris* in *Oncorhynchus mykiss*

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ABSTRACT

Growth promoting and immune stimulating effects of common mallow (*Malva sylvestris*) aqueous methanolic extract (AME) in *O. mykiss* were examined. Two different concentrations of common mallow AME [0.1 (CM1) and 0.5 (CM5) g kg^{-1} of feed] commixed a basic diet and a control diet without the common mallow extract were fed to rainbow trout for 30 days to evaluate growth rate and immune responses. At the end of the study, fish growth performance was determined as significantly higher in the group fed with 0.1 g kg^{-1} common mallow AME compared with control diet fed group (P<0.05). In fish fed with CM5 diet, oxidative radical production (ORP) was the highest (P<0.05). Similarly, myeloperoxidase (MPO) activity was increased significantly in CM5 group. Lysozyme (LYS) and phagocytic activities (PA) were not altered in treated fish groups compared to the control group (P<0.05). The pro-inflammatory (IL-1β, TNF-α1, IL-8,), anti-infammatory (IL-10), lymphocyte agonist (TGF-β) and cell-mediated immune regulatory, IL-12 cytokines were generally down-regulated insignificantly compared to control. Although, common mallow AME could not elicit cytokine-mediated immunity and resistance to bacterial pathogen, *A. hydrophila* in rainbow trout, elevation in ORP and MPO activities in treated groups and growth promoting effect were noticed at a low dose. Therefore, application AME of common mallow at 0.1 g kg^{-1} of feed as growth promoter and non-specific immunostimulant is advocated for rainbow trout.

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Introduction

According to FAO (2018), among food producing sectors, aquaculture industry is the fastest rising sector in the world. The occurrence of fish disease and use of chemicals such as antibiotics are the most determining factors in aquaculture. However, it is a hard reality that use of chemicals and antibiotics in aquaculture may result in antibiotic resistance in bacteria and finally leads to environmental hazards (Boran, Terzi, Altinok, Capkin, & Bascinar, 2013; Capkin, Terzi, & Altinok, 2015; Terzi et al., 2020). Furthermore, other unwanted characteristics of antibiotics include residual effects that persist in the environment for a long time (Jones, Voulvoulis, & Lester, 2004; Terzi, 2018) and cultured fish species as well (Cabello, 2006; Corum, Durna Corum, Er, Terzi, & Uney, 2018; Corum et al., 2020). Another important issue is, using new techniques such as oxygen supplementation or floating fish farm unit to obtain maximum production efficiency from the unit area by increasing stocking density, could result some immune deficiency problems and diseases (Bilen, Bilen, & Önal, 2015; Bilen, Kızak, & Bilen, 2013). Therefore, discovering environment friendly products and developing their appropriate application plans are of utmost importance currently. On this direction, studies are being undertaken to find out suitable organic products that are environment friendly, sustainable and cost-effective.

Of late, plant-derived immunostimulants have been tested and several works have been performed related to application of medicinal herbs (Almabrok, Amhamed, Mohamed, Bilen, & Altief, 2018; Arslan, Sönmez, & Yanık, 2018; Dawood, Eweedah, Moustafa, & Farahat, 2019; Mohamed et al., 2018; Moustafa et al., 2020). Several medicinal plants have the potential positive effects on growth performance, survival rate and immune system activation in fishes (Amhamed, Mohamed, Almabrok, Altıef, & Bilen, 2018; Bilen, Altief, et al., 2019; Bilen, Özkan, Alagöz, & Yürüten Özdemir, 2018). Traditional medicines are becoming popular in Turkey because of its rich diversity of medicinal plants. Common mallow (*Malva sylvestris*) is an important medicinal plant cultured in Turkey and it has several beneficial effects (Alan & Padem, 1989; Kaya, İncekara, & Nemli, 2004; Özdestan & Üren, 2008; Tugay, Bağcı, Ulukuş, Özer, & Canbulat, 2012). Recently, positive effects of common mallow were demonstrated in European sea bass, gilthead sea bream and common carp (Bilen, Filogh, Ali, Kenanoğlu, & Zoral, 2019; Bilen, Kenanoglu, Terzi, Ozdemir, & Sonmez, 2019). Therefore, the present experiment was performed to evaluate the effects of dietary supplementation of AME of common mallow on growth, and different cytokine and adaptive immune responses, such as oxidative radical production, phagocytic activity, myeloperoxidase activity and lysozyme activity in rainbow trout which is an important commercially cultured finfish in Europe.

Material and Methods

Design of Experiment

Rainbow trout was acquired from Inland and Marine Fish Research and Application Center, Kastamonu University, and maintained for 14 days at laboratory condition for acclimatization. To start the experiment, nine aquaria (300 L) were stocked with rainbow trout (mean weight: 54.97±0.03 g) at 20 number/aquarium. There were three triplicate aquaria assigned randomly for each treatment group. Experimental diet contained common mallow extract at 0.1 (CM1) and 0.5 g kg^{-1} (CM5), whereas the control diet had no supplementation (0 g kg^{-1}, CM0). During 30 days of the study, fish were fed ad libitum with experimental diets twice a day. At the end of the study, fish was sedated using 0.02 mg L^{-1} of phenoxyethanol. The blood samples were collected from the caudal vein and kept in heparin containing tubes. Head kidney was aggregated and immersed directly into liquid nitrogen then stored at -80°C until further use. During the experimental period, water quality parameters of the fish holding aquaria were as follows: temperature 15±0.8°C, dissolved oxygen 9.01±0.3 mg L^{-1}, pH 8.1±0.1, total ammonium N 0.001±0.001 mg L^{-1}, nitrite-N 0.002±0.002 mgL^{-1}, and nitrate-N 0.01±0.01 mg L^{-1}, conductivity 424±11 µS.

The experimental procedures were permitted by Kastamonu University, Local Ethics Committee for Animal Research Studies (Protocol Number: 03.04.2017-2017.08).

Preparation of Common Mallow Extract

The extraction was performed according to (Bilen, Ünal, & Güvensoy, 2016). Briefly, common mallow (*Malva sylvestris*) was collected from country side in Kastamonu (41°26'33.20"N, 33°47'30.48"E). The herbs were washed with deionized water and shed-dried. One hundred g sample was extracted with 1 L methanol (40%) and filtered after the solvent was dehumidified by a rotary evaporator. Finally, 3.12 g concentrate was dissolved in 25 mL deionised water. This solution was mixed with the feed at a rate of 0, 0.1 and 0.5 g kg^{-1}. The feed were kept at –20°C until further use.

Growth Efficacy

At the end of the 30-day trial, individual fish was weighed. Growth performances (weight gain, WG; specific growth rate, SGR, feed conversion ratio, FCR; protein efficiency ratio, PER) were determined as follows:

$$WG\ (\%) = \frac{Daily\ feed\ intake\ (g)}{Biomass\ (g)} \times 100$$

$$SGR = 100 \times \left[\frac{(\ln final\ weight - \ln initial\ weight)}{Days\ fed\ (g)}\right]$$

$$FCR = \frac{Feed\ intake\ (g)}{Weight\ gain\ (g)} \times 100$$

$$PER = \frac{Wet\ body\ mass\ gain}{Crude\ protein\ intake}$$

Designation of Adaptive Immune Parameters

In the present study, oxidative radical production was measured according to Siwicki and Anderson (1993), lysozyme activity was determined according to Ellis (1990) and myeloperoxidase activity was measured according to Quade and Roth (1997) slightly modified by Sahoo, Kumari, and Mishra (2005). The phagocytic activity (PA) was assayed following Siwicki, Anderson, and Rumsey (1994).

Determination of Cytokine Expression

Total RNA extraction from head kidney sample

Total RNA from 20 µg head kidney sample was isolated using RNeasy Plus Micro RNA isolation kit (Qiagen, Germany) according to manufacturer's protocol. Total RNA was treated with 1 U DNase I (Thermo, Lithuania) and reverse transcribed using Revert Aid RT synthesis kit (Thermo, Lithuania), making 1 µg of template RNA, 15 pmol/µL oligo dT primer, 0 U/µL Revert Aid M-MuLV RT enzyme, 1 mM of dNTP mix, 4 µL 5× reaction buffer and 6 µL of NFW. cDNA was synthesised using thermal cycler (Thermo, Lithuania) for 60 min at 42°C.

qRT-PCR procedure

Rotor-Gene qPCR detection system (Qiagen, Hilden, Germany) and Rotorgene SYBR Green PCR kit (Qiagen, Hilden, Germany) were used for qRT-PCR analysis. Specific primers were used previously determined by Altunoglu, Bilen, Ulu, and Biswas (2017). qRT-PCR mixture contained 0.4 µM of specific forward and reverse primer (β-actin as reference, IL-1β, IL-8, IL-10, IL-12p40, TNF-α1, TGF-β), 0.1 µg template DNA, 12.5 µL of 2× SYBR Green Master Mix and NFW to the final volume of 25 µL. Samples were denatured at 95°C for 10 s, annealed and extended together at 60°C for 40 s. Relative gene expression level was calculated following $2^{-\Delta\Delta CT}$ method as described elsewhere (Altunoglu et al., 2017).

Experimental Challenge Test

All challenge procedure was performed according to Bilen et al. (2016). 14 fish from each tank were injected intraperitoneally with LD_{50} dosage (1×10^8 CFUs mL^{-1}) of *A. hydrophila* (SB-Ah1) (Bilen, Filogh, et al., 2019) and survival was noted during 14 days after injection.

Statistical Analysis

All results were given as mean (±) standard error except survival rates. Differences among groups in terms of growth rate, immune responses and cytokine expressions were tested by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test at $P<0.05$ using SPSS 23.

Results

Results related to effects on growth were summarized in Table 1. FCR and PER did not vary among the experimental groups (P>0.05). All growth parameters such as SGR, weight gain, total feed consumption and final weight were determined significantly advanced in CM1 group (P<0.05) compared to control (CM0). However, there were no differences in these variables between CM5 and CM0 groups (P>0.05).

Table 1. Effects of different doses of methanolic extracts of common mallow on the performances of rainbow trout after a 30-day feeding trial

Measurements	Control (CM0)	CM1	CM5
Initial weight (g)	54.97±0.03	54.97±0.03	54.97±0.03
Final weight (g)	79.13±0.19^b	102.93±0.39^a	84.25±0.67^b
Weight gain (%)	43.96±10.11^b	87.49±11.77^a	53.15±9.78^b
SGR (% day^{-1})	1.21±0.25^b	2.10±0.02^a	1.42±0.1^b
Total feed consumption (g)	739.91±11.51^b	1455.43±21.90^a	912.29±762.32^b
FCR	1.02±0.12	1.01±0.01	1.04±0.03
PER	2.18±0.11	2.20±0.01	2.14±0.09

Note: CM0, CM1 and CM5 indicate common mallow extract doses at 0, 0.1 and 0.5 g kg^{-1} diet, respectively. Values are mean±SD of three replicates (n=3); Different superscript letters in a row indicate significant differences among the experimental groups ($P<0.05$). FCR: feed conversion ratio; SGR: specific growth rate; PER: protein efficiency ratio.

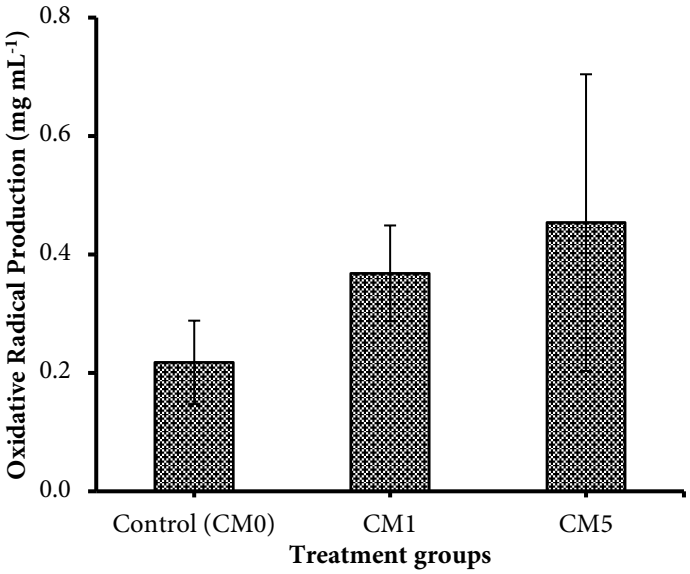

Figure 1. Oxidative radical production in rainbow trout fed with diets supplemented with different doses of common mallow methanolic extract. CM0, CM1 and CM5, common mallow methanolic extract at 0, 0.1 and 0.5 g kg^{-1} diet, respectively. Different letters on bars indicate significant differences among groups (P<0.05). Data are presented as mean± SD (n=3)

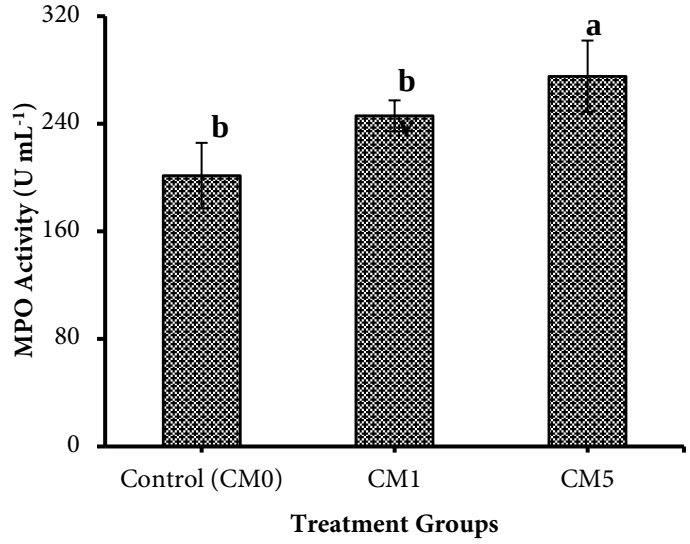

Figure 2. Myeloperoxidase (MPO) activity in rainbow trout fed with diets supplemented with different doses of common mallow methanolic extract. CM0, CM1 and CM5, common mallow methanolic extract at 0, 0.1 and 0.5 g kg^{-1} diet, respectively. Different letters on bars indicate significant differences among groups (P<0.05). Data are presented as mean± SD (n=3)

Oxidative radical production was increased in two experimental groups compared to control. The oxidative radical production was highest in CM5 group (0.454±0.25), followed by in CM1 group (0.368±0.08) (P<0.05) (Figure 1). MPO activity was determined significantly higher in CM5 group compared to that of CM0 and CM1 groups (Figure 2). Lysozyme activity was not affected by common mallow extract administration in rainbow trout (Figure 3). Similar to LYS activity, phagocytic activity was not influenced by common mallow extract administration in rainbow trout of any treatment group as well as the control (Figure 4).

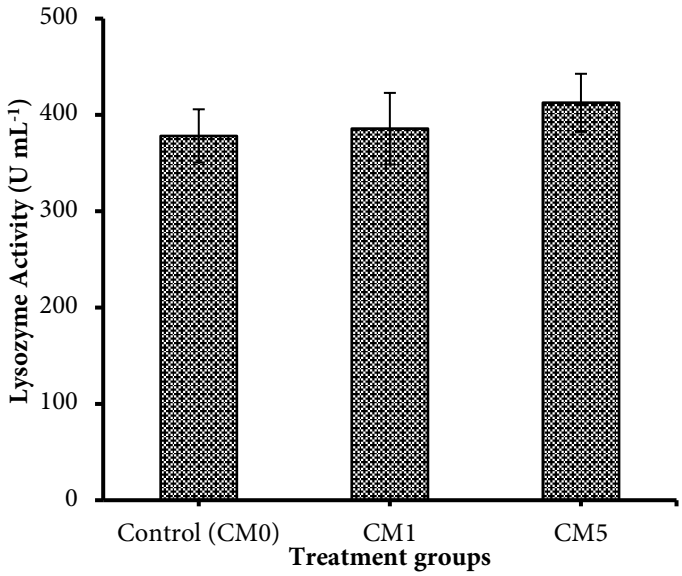

Figure 3. Lysozyme activity in rainbow trout fed with diets supplemented with different doses of common mallow methanolic extract. CM0, CM1 and CM5, common mallow methanolic extract at 0, 0.1 and 0.5 g kg^{-1} diet, respectively. Data are presented as mean± SD (n=3)

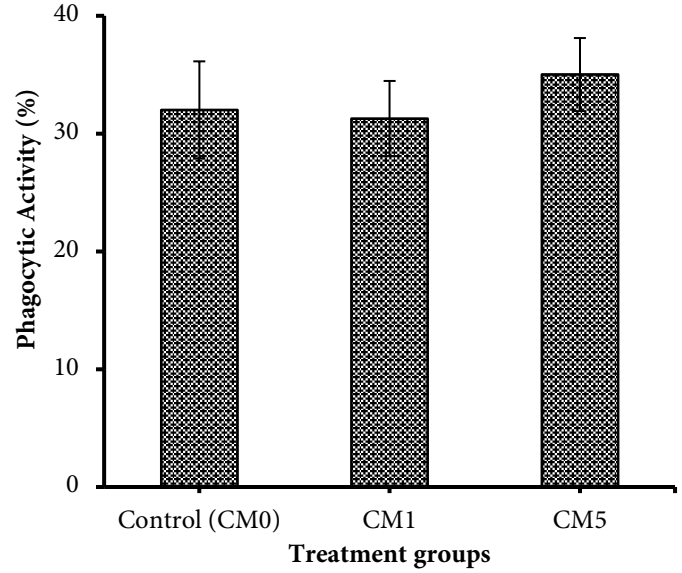

Figure 4. Phagocytic activity in rainbow trout fed with diets supplemented with different doses of common mallow methanolic extract. CM0, CM1 and CM5, common mallow methanolic extract at 0, 0.1 and 0.5 g kg^{-1} diet, respectively. Data are presented as mean± SD (n=3)

Cytokine gene expression such as IL-1β, IL-8, IL10, IL-12, TNF-α1 and TGF-β, was analysed using qRT-PCR (Fig. 5). IL-1β, TGF-β, IL-8, IL-10 and TNF-α expression generally decreased in the CM1 and CM5 groups compared with control but this decrease was not significant (P>0.05).

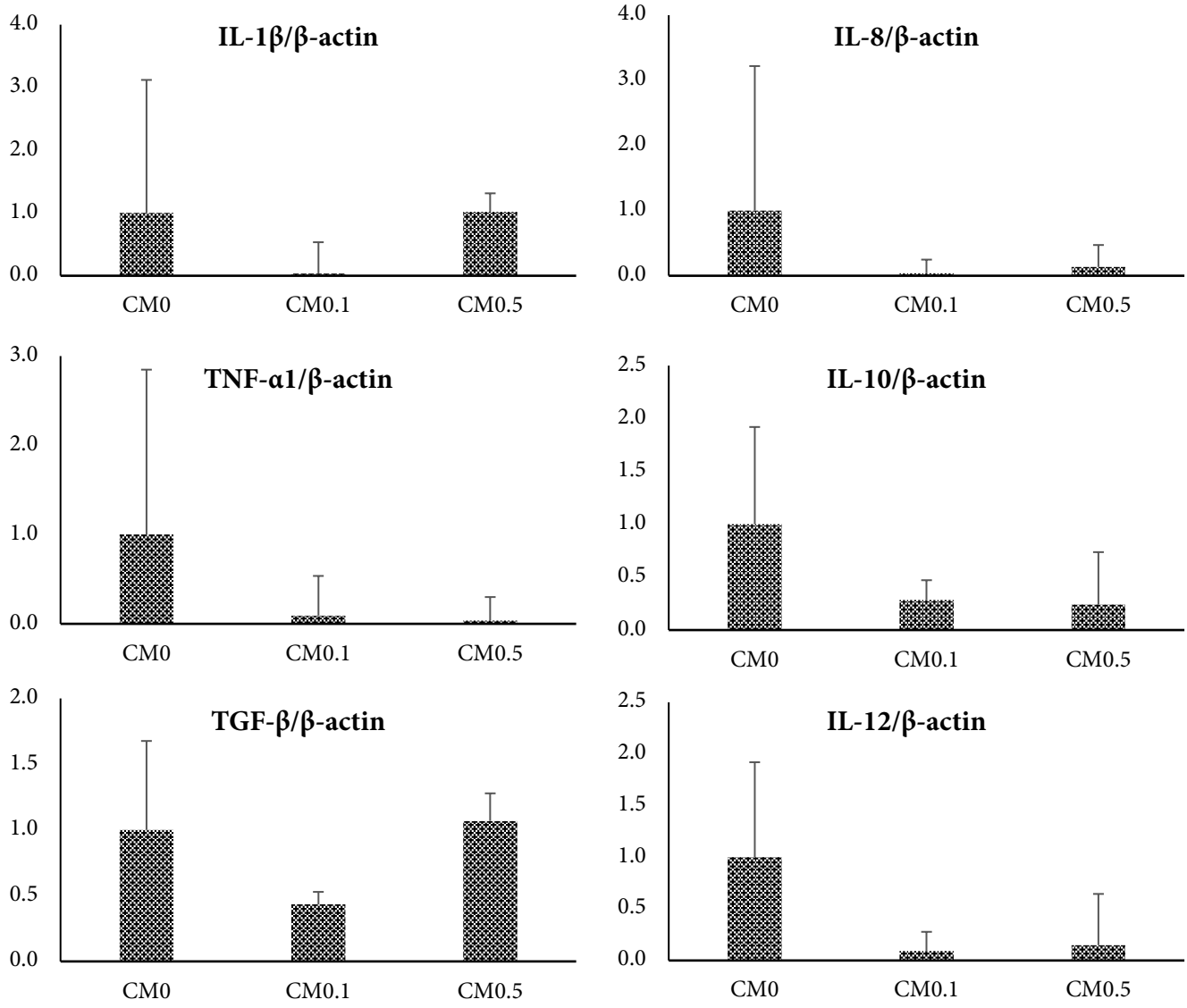

Figure 5. Relative gene expression (mean±SD; n=3) patterns of cytokines in the head kidney cells of rainbow trout fed with diets supplemented with different doses of common mallow methanolic extract. CM0, CM1 and CM5, common mallow methanolic extract at 0, 0.1 and 0.5 g kg^{-1} diet, respectively. Different letters on bars indicate significant differences among groups (P<0.05)

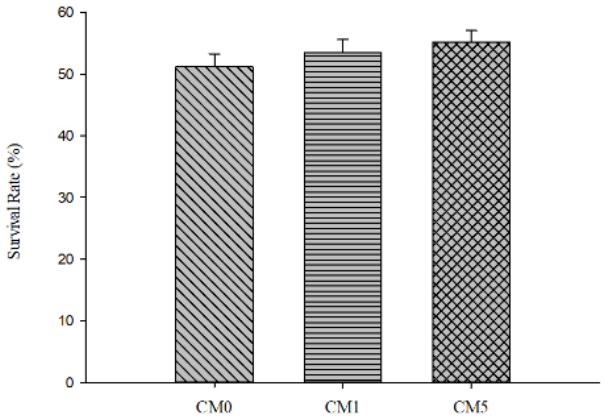

Figure 6. Survival of common mallow extract administered rainbow trout after experimental challenge with *Aeromonas hydrophila*. CM0, CM1 and CM5 indicate black cumin extract doses at 0, 0.1 and 0.5 g kg^{-1} feed, respectively

Survival rate of the fish after challenged with *A. hydrophila* is given in Figure 6. Survival rate was not affected among CM0, CM1 and CM5 groups ($P>0.05$).

Discussion

In this study, we investigated the effect of common mallow extract as a fish feed supplement on growth promotion and immune response in rainbow trout. Previously, beneficial role of common mallow using different application methods was described in fish (Bilen, Filogh, et al., 2019; Bilen, Kenanoglu, et al., 2019; Elbesthi, Yürüten Özdemir, Taştan, Bilen, & Sönmez, 2020). Our results indicated that common mallow extract is effective for adaptive immune responses and growth promotion, but not for innate immune enhancement in

rainbow trout and no protection was determined against *A. hydrophila* (P>0.05).

Dietary supplementation of medicinal plant products proved to provide positive effects on fish growth (Sönmez, Bilen, Alak, et al., 2015; Sönmez, Bilen, Albayrak, et al., 2015; Bilen, Altief, et al., 2019; Bilen, Filogh, et al., 2019). In the current study, growth was influenced by administration of common mallow extract. Although, FCR remained unchanged, total feed consumption increased significantly. Similarly, growth promoting effect of common mallow extract was demonstrated in carp (Bilen, Filogh, et al., 2019) and European sea bass (*Dicentrarchus labrax*) (Bilen, Kenanoglu, et al., 2019) and rainbow trout fed with aqueous extract of *Malva sylvestris* (Rashidian, Kajbaf, Prokić, & Faggio, 2019). Moreover, fenugreek seeds at 1% and 2% doses caused an enhancement in feed utilization and SGR in Nile tilapia (*Oreochromis niloticus*) (Abdelhamid & Soliman, 2012). On the other hand, Bilen and Bilen (2012) evidenced that *Cotinus coggygria* and *Laurus nobilis* had no effects on growth in rainbow trout. Mohamed et al. (2018) found growth improvement in carp fed with *Apium graveolens* and Almabrok et al. (2018) determined a dose depended growth promoting effect in carp fed with *Tilia tomentosa*.

ORP, MPO, LYS and PA activities were evaluated in rainbow trout fed diet containing common mallow extract. An elevated ORP was detected in both the experimental groups when compared to control. In the fish of CM5 group, ORP reduction level was the highest. Similarly, ORP was elevated in rainbow trout treated with nettle (Bilen, Soydaş, & Bilen, 2014) and black cumin (Altunoglu et al., 2017) extracts. MPO activity was induced by common mallow extract treatment. Bilen, Altunoglu, Ulu, and Biswas (2016) found that caper extracts caused elevation of MPO in rainbow trout. However, lysozyme, which provides non-specific immune responses to pathogens during infection, was not varied by common mallow extract administration in rainbow trout. Moreover, phagocytic activity was not influenced by dietary common mallow extract administration. A similar result was reported in rainbow trout received changed doses of *Nigella sativa* seed extract (Altunoglu et al., 2017).

In this experiment, we examined transcription of six cytokines belonging to different functional groups. The proinflammatory, IL-1β, TNF-α1, IL-8, anti-inflammatory, IL-10, cell-mediated immune regulatory, IL-12 cytokines and lymphocyte agonist, TGF-β were either down-regulated or remained unchanged in their expression in common mallow treated rainbow trout compared to that of untreated control. Similar to our results, no elevation in IL-1β, IL-8, TNF-α1, IL-10 and IL-12 cytokine gene responses was caused by the administration of *Nigella sativa* in rainbow trout (Altunoglu et al., 2017). Contrary to this, Bilen and Elbeshti (2019) observed elevated expression of related genes with our study in rainbow trout treated with *Cotinus coggygria*, and *Usena barbata* (Bilen, Sirtiyah, & Terzi, 2019). Similar to our study, A. Sönmez, Yürüten Özdemir, & Bilen (2018) determined induced pro-inflammatory cytokine gene expression in rainbow trout leucocytes. Therefore, current results suggest that common mallow extract is not effective in induction of cytokine-mediated immunity in rainbow trout.

No differences in survival rate were observed in common mallow treated and control fish groups tested with *A. hydrophila*. Similarly, there was no effect of oyster mushroom in survival of rainbow trout after exposed to the same pathogen (Bilen, Ünal, et al., 2016). However, Bilen, Filogh, et al. (2019) found a decreasing survival rate in common carp when challenged with *A. hydrophila*. Consequently, our results suggest that administration of dietary common mallow extract to rainbow trout could not enhance fish defiance to this bacterial pathogen.

Conclusion

From this study, it was evident that the methanolic extract of common mallow was unable to persuade innate immunity in rainbow trout. However, growth promoting effect was noticed at a low dose. However, non-specific immune responses (ORP, MPO) were elevated. Thus, use of methanolic extract of common mallow at 0.1 g kg^{-1} of feed as growth promoter is suggested for rainbow trout.

Compliance with Ethical Standards

Authors' Contributions

All authors contributed equally.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The experimental procedures were permitted by Kastamonu University, Local Ethics Committee for Animal Research Studies (Protocol Number: 03.04.2017-2017.08).

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