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# Effects of ribwort plantain (*Plantago lanceolata*) extract on blood parameters, immune response, antioxidant enzyme activities, and growth performance in rainbow trout (*Oncorhynchus mykiss*)

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**Abstract** In this study, we examined changes occurred in blood parameters, immune responses, antioxidant enzyme activities, and growth performance of rainbow trout (*Oncorhynchus mykiss*) administered with ribwort plantain (RP) through feed. Fish (mean weight 36.56 ± 1.99 g) were fed a diet supplemented with an aqueous methanolic extract of RP at variable doses, 0 (control), 1 (RP1), 2 (RP2), and 3 g $kg^{-1}$ (RP3) for 90 days. The final weight, weight gain, and specific growth rate were significantly increased in RP1, RP2, and RP3 treatment groups compared to that of the control. Among examined blood parameters, hemoglobin value in RP1 group (9.77 ± 0.10 g $dl^{-1}$) only was significantly high on the 30th day of the study. When immune response parameters were evaluated, we observed that oxidative radical production and lysozyme activities were affected positively in experimental groups ($P < 0.05$). The highest oxidative radical production was determined in fish of RP3 group. Glutathione peroxidase and glucose 6 phosphate dehydrogenase were increased in RP3 group compared to control and other treatment groups. Based on these results, it is concluded that ribwort plantain promotes growth, enhances immune responses and antioxidant enzyme activities in rainbow trout, and therefore, may be used in aquaculture.

**Keywords** Antioxidant · Immunostimulant · Growth promoter · Medicinal plant

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## Introduction

Aquatic animals, especially fish, play an important role in providing animal protein requirement that increases day by day (Aydın et al., 2018). Total global production of fish, crustaceans, mollusks, and other aquatic animals has reached 170.9 million tons in 2016. According to FAO (2019), aquaculture is the fastest growing food-producing sector accounting nearly 50% of the total aquatic production now.

Fish are exposed to stress due to several reasons such as transportation, high stocking density (Bilen et al. 2015; Bilen et al. 2013a), poor water quality, or handling for various purposes. Elevating antioxidant status of fish to prevent adverse effects of stress is also beneficial to producers. Concordantly alongside fighting against diseases, plant extracts are used in strengthening antioxidant status of fish, getting higher price by inducing growth performance, and thus producing healthier products. Several scientific studies endorse the use of medicinal plants in aquaculture (Almabrok et al. 2018;

Altunoglu et al. 2017; Bilen et al. 2018; Bilen et al. 2014; Mohamed et al. 2018).

Medicinal plants and their bioactive compounds including alkaloids, phenolic compounds, and steroids have been successfully applied to aquatic organisms to promote appetite, growth performance, stress responses, and immunity (Altunoglu et al. 2017; Amhamed et al. 2018; Bilen et al. 2019; Bilen et al. 2019; Laith et al. 2017). Ribwort plantain (*Plantago lanceolata*) is a medicinal plant used in traditional medicine all over the world due to its bioactive compounds (Nizioł-Łukaszewska et al. 2019). It has antioxidant properties (Hausmann et al. 2007) and positive effects on wound healing (Kurt et al. 2018) in mice, growth-promoting effects in quails (Temür and Uslu 2019), and antimicrobial effects in human (Ferrazzano et al. 2015). Ribwort plantain is used internally to suppress cough associated bronchitis and upper respiratory inflammation, to reduce skin inflammation, for treatment of wounds and as a laxative (Baytop 1999).

For this purpose, we used aqueous methanolic extract of ribwort plantain as an additive in rainbow trout feed and evaluated changes in blood parameters, growth performance, antioxidant enzyme activities, and immunological parameters caused by this extract.

## Material and methods

Study protocol was approved in advance by the Kastamonu University local ethics committee of animal trials with the approval number of 2016.21.

### Fish and experimental design

This experiment was conducted in triplicate in 12 tanks (400 L) in Fisheries Faculty, Kastamonu University for a period of 90 days. Experimental rainbow trout, with an average body weight of 36.56 ± 1.99 g, were obtained from Kastamonu University Inland and Marine Fish Research and Application Center, Turkey. A total of 40 fish were stocked in each tank with three replicates per treatment. Aqueous methanolic extract of ribwort plantain (RP) was added to the basal diet of fish at 0 (control), 1 (RP1), 2 (RP2), and 3 (RP3) g $kg^{-1}$ by spraying. Before starting the experiment, fish were acclimatized to the experimental feeding regime using a commercial diet for two weeks (trout commercial pellet, 2 mm). After acclimation, fish of treatment groups were fed with prepared food twice a day ad libitum for 90 days, whereas fish of control group were fed with the commercial trout feed (Table 1). Pooled fish live weight increment was measured at two-week intervals and feed intake was recorded daily throughout the study. At the end, weight and length of individual fish were recorded for determining growth performance parameters. In addition, three fish from each tank (nine fish per treatment) were used for blood and other physiological parameter analyses. Fish tissue samples were kept at − 80 °C until antioxidant enzyme analysis.

### Preparation of aqueous methanolic extract of ribwort plantain (*Plantago lanceolata*)

Ribwort plantain leaves were collected from Kastamonu province and its sub-provinces which are located in Western Black Sea Region of Turkey. RP leaves were brought to laboratory and dried under shade. After drying, plants were powdered using a high speed mill. The powder was used to prepare aqueous methanolic extract according to Bilen et al. (2016). In brief, 50 g RP was percolated with 1 L methanol (40%) for 72 hours and then filtered. The solvent was evaporated and lastly

**Table 1** Proximate composition of the feed

| | Diets[1] | | | |
|---|---|---|---|---|
| | Control | RP1 | RP2 | RP3 |
| Dry matter | 60.65 ± 0.17 | 62.08 ± 0.08 | 62.62 ± 0.22 | 63.82 ± 0.09 |
| Crude protein | 44.31 ± 0.53 | 44.36 ± 0.52 | 44.24 ± 0.24 | 44.55 ± 0.27 |
| Lipid | 15.48 ± 0.58 | 15.11 ± 0.48 | 15.00 ± 0.42 | 15.75 ± 0.52 |
| Ash | 10.92 ± 0.16 | 10.70 ± 0.06 | 10.50 ± 0.05 | 10.32 ± 0.07 |
| Crude cellulose | 1.76 ± 0.01 | 1.60 ± 0.11 | 1.51 ± 0.31 | 1.64 ± 0.30 |

[1] RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g $kg^{-1}$ diet, respectively

concentrate was dissolved in 50 mL distilled water (50 °C). Exact amount of the extract solution was mixed with experimental diets according to the doses. The experimental diets were kept at − 20 °C until use. In order to expose the feed to absorb plant extract more efficiently, prepared feed was vacuum packed using a machine (Lipovak MV-30, Turkey) and kept at 4 °C prior to use.

Component analysis of ribwort plantain (*Plantago lanceolate*) by GC-MS

The contents of the ribwort plantain (*Plantago lanceolata*) aqueous methanolic extract were determined according to Özkan et al. (2017) using Rtx-5MS capillary column (30 m × 0.25 mm; coating thickness 0.25 μm) (Shimadzu GCMS-QP 2010 ULTRA system). Results are given in Table 2.o

Growth parameters

All fish were weighed before start of the experiment and at the end of 90 days to record initial body weight (IBW) and final body weight (FBW) values. Feeding was paused 24 h before weighing. Weight gain (WG), specific growth rate (SGR), and feed conversion ratio (FCR) were calculated according to formulas given below (Ricker, 1979) (Table 2):

$$Weight\ gain\ (\%) = \frac{(Final\ weight - Initial\ weight)}{Initial\ weight}\ x\ 100$$

$$Specific\ growth\ rate\ (SGR) = \frac{(\ln final\ weight - \ln initial\ weight)}{Days}\ x\ 100$$

$$Feed\ conversion\ ratio\ (FCR) = \frac{Feed\ intake}{Weight\ gain}$$

Blood parameters

During the study, three fish from each tank containing forty fish were randomly selected and anesthetized with clove oil. Blood samples were collected from the caudal veins on the 30th, 60th, and 90th days using heparinized syringes. Samples were immediately used for determination of red blood cell (RBC), hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Blood plasma from rest of the samples were separated and stored at − 80 °C until immunological analyses.

**Table 2** Ingredients of extract of ribwort plantain (*Plantago lanceolata* L.) used in the study

| Peak | Area (%) | Name |
|---|---|---|
| 1 | 4.53 | Vanillic acid |
| 2 | 2.33 | 3,4,5-trihydroxybenzoate |
| 3 | 1.14 | Protocatechuic acid |
| 4 | 1.65 | 4-Hydroxybenzoic acid |
| 5 | 0.18 | 0.18 2,5-Dihydroxybenzoic acid |
| 6 | 2.31 | Cinnamic acid |
| 7 | 1.00 | Caffeic acid |
| 8 | 0.97 | *p*-Coumaric acid |
| 9 | 0.63 | Ferulic acid |
| 10 | 0.04 | Sinapic acid |
| 11 | 78.34 | Chlorogenic acid |
| 12 | 0.10 | Aesculetin |
| 13 | 2.03 | Apigenin |
| 14 | 0.12 | Apigenin-7-O-glucoside |
| 15 | 0.04 | Apiin |
| 16 | 0.01 | Vitexin |
| 17 | 1.61 | Luteolin |
| 18 | 1.31 | Luteolin-7-O-glucoside |
| 19 | 0.16 | Chrysoeriol |
| 20 | 0.50 | Quercetin-3-O-rutinosid |
| 21 | 0.41 | Quercetin-3-O-glucoside |
| 22 | 0.07 | Quercetin |
| 23 | 0.01 | Quercitrin |
| 24 | 0.33 | Syringic acid |
| 25 | 0.11 | Amentoflavone |
| 26 | 0.07 | Kaempferol-3-O- glucoside |

*Red blood cell*

RBC count was performed according to Blaxhall and Daisley (1973) using hemocytometer.

*Hemoglobin*

Amount of hemoglobin was measured via colorimetric cyanomethemoglobin method using Biodiagnostic Company kit (Drabkin and Austin 1932).

*Hematocrit*

Hematocrit determination was performed according to Britton (1963) using a microhematocrit centrifuge.

*Other hematological indices*

After RBC, Hb, and Hct determination, other hematological indices, such as MCV, MCH, and MCHC, were calculated using formulas of Lewis et al. (2006).

Non-specific immune parameters

In this study, oxidative radical production was determined using the reduction of nitroblue tetrazolium [(NBT) Sigma–Aldrich St. Louis, MO, USA] on the 30th, 60th, and 90th days, as per the previously described method (Siwicki et al. 1994).

Lysozyme activity (LA) was determined according to the method used by Ellis (1990).

Total myeloperoxidase (MPO) activity in the serum was measured according to the method used by Sahoo et al. (2005).

Antioxidant enzyme activity

Liver and white muscle tissues were collected from the fish of which blood was taken on the 30th, 60th, and 90th days of the study and stored at − 80 °C prior to use. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glucose 6-phosphate dehydrogenase (G6PDH), and lipid peroxidation (LPO) activities were determined in order to evaluate antioxidant effects of ribwort plantain. SOD, CAT, GPx, and G6PDH activities were determined in liver tissues, whereas LPO was determined in both liver and white muscle tissues. Following commercial kits were used according to the instructions of manufacturers: SOD (Sigma-Aldrich, kit no.: 19160), CAT (Cayman, product no.: 707002), GPx (Cayman, product no.: 703102), G6PDH (Cayman, product no.: 700300), LPO (Cayman, product no.: 10009055).

Statistical analysis

Data are presented as mean ± standard error. Comparison between treatment groups was performed with one-way ANOVA followed by Duncan's multiple range test for different parameters. Statistical analysis was performed using SPSS version 23 for windows.

## Results

Growth performance

At the end of study, growth performances of fish from different experimental groups were presented in Table 3. Final weight, weight gain, and SGR were significantly higher in all the RP extract treated groups compared to the control group ($P < 0.05$). There was no difference on FCR values among different groups ($P > 0.05$).

Hematology

Hematological analyses were performed using blood samples collected at monthly intervals. On the 30th day of study, hemoglobin levels in fish of treatment

**Table 3** Growth performances of rainbow trout fed with ribwort plantain (*Plantago lanceolata* L.) aqueous methanolic extract supplemented feed

| Dietary treatments | | | | |
|---|---|---|---|---|
| | Control | RP1 | RP2 | RP3 |
| IW (g fish$^{-1}$) | 36.25 ± 0.09 | 36.48 ± 0.21 | 36.12 ± 0.15 | 37.39 ± 1.54 |
| FW (g fish$^{-1}$) | 135.45 ± 1.2$^{d}$ | 166.07 ± 0.97$^{b}$ | 169.60 ± 1.23$^{a}$ | 152.36 ± 0.1$^{c}$ |
| WG (%) | 273.70 ± 0.77$^{d}$ | 355.28 ± 0.44$^{b}$ | 369.56 ± 0.74$^{a}$ | 308.19 ± 1.35$^{c}$ |
| SGR | 1.46 ± 0.04$^{d}$ | 1.68 ± 0.02$^{b}$ | 1.72 ± 0.04$^{a}$ | 1.56 ± 0.07$^{c}$ |
| FCR | 1.2 ± 0.01 | 1.09 ± 0.01 | 1.11 ± 0.01 | 1.08 ± 0.1 |

All data are given as mean ± SE ($n = 3$). Means with different superscript letters in a row are significantly different ($P < 0.05$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g kg$^{-1}$ diet, respectively; IW is initial weight, FW is final weight, WG is weight gain, SGR is specific growth rate, and FCR is feed conversion ratio

groups were higher than that of control group. However, this increase was merely significant in RP1 group ($P < 0.05$). On 30th day, RBC, HCT, MCV, MCH, and MCHC values of fish were not affected in any of the treatment groups (Table 4). On the 60th day, there were no differences between groups ($P > 0.05$). Similar observations were recorded for hematological parameters between treatment groups and the control on the 90th day ($P > 0.05$). At the end of study, it indicated that ribwort plantain extract supplementation did not affect hematology of rainbow trout.

## Immune responses

Oxidative radical production (ORP) of fish was examined on the 30th, 60th, and 90th days (Fig. 1). On the 30th day, ORP in fish of RP2 group had no significant difference compared to control group whereas that of RP3 group was higher. On the contrary, ORP in fish of RP1 group decreased significantly. On the 60th day, there was no significant difference between groups. On the 90th day, ORP results were similar to those of the 30th day. Overall, ORP values in fish of RP1 group were the lowest between the treatment groups.

MPO activity did not vary between groups at all sampling times. In fish of the control and all treatment groups, MPO activity increased as the experiment progressed with the highest values on the 90th day (Fig. 2).

On the 30th day, lysozyme activity in fish of all groups was the lowest in comparison to those of the 60th and 90th days. On the 30th day, fish of RP2 and RP3 groups had an increased lysozyme activity compared to the control ($P < 0.05$). However, on the 60th day, fish of RP1 treatment group had the highest lysozyme activity, while values in other treatment groups and control did not differ. On the 90th day, fish in control group exhibited the highest lysozyme activity ($P < 0.05$). Although fish of treatment groups had higher lysozyme activity as the dose increased, these differences were not significant (Fig. 3).

## Antioxidant enzyme activities

SOD activities determined on the 30th, 60th, and 90th days are given in Table 5. The highest SOD activity was recorded in control group ($77.21 \pm 1.06$) on the 60th day, whereas, the lowest was in RP2 group fish ($62.67 \pm 1.59$) on the same day. Although there were differences in SOD values between groups and sampling periods, these differences were not significant ($P < 0.05$).

In terms of CAT, the highest value was in RP1 group fish on the 90th day (Table 6). The lowest value was estimated in fish of the control group on the same day. Similar to SOD results, CAT values did not vary among groups at all sampling days ($P < 0.05$).

When GPx activity was determined, we observed that the highest activity was in RP3 group fish on the 90th day (Table 7), whereas, the lowest activity was in the control group on the 30th day. On the 30th day, there was no significant difference between groups ($P < 0.05$). On the 60th day, fish in control group had the highest GPx activity together with RP3 group, whereas, fish of RP1 and RP2 groups had low GPx activities ($P < 0.05$). On the last day of trial, RP3 group fish had the highest GPx activity ($78.54 \pm 0.66$), whereas, RP1 group fish had a significantly lower GPx activity compared to that of control ($P < 0.05$).

Among all sampling days, the highest G6PDH activity was recorded in RP3 group fish on the 90th day and it was the lowest in RP2 group on the same day (Table 8). On the 30th day, fish of RP3 group had similar G6PDH values to that of control group. However, G6PDH activity in rainbow trout of RP1 and RP2 groups was significantly lower than that of control ($P < 0.05$). On the 60th day, results revealed that fish in RP2 and RP3 groups had higher activity than that of control group, whereas RP1 group fish had lower value ($P < 0.05$). On the 90th day, G6PDH activity in RP3 group fish was significantly increased once again, whereas RP1 group fish showed no difference with RP3 and RP2 group fish had significantly decreased value compared to that of control ($P < 0.05$).

Lipid peroxidation (LPO) was examined in both white muscle and liver tissues of fish. LPO results determined on the 30th, 60th, and 90th days are presented in Tables 9 and 10. On the 30th day, the highest muscle LPO value was in RP2 group fish. On the other hand, RP1 group fish had the lowest muscle LPO, ($P < 0.05$). On the 60th day, fish of control group had the highest muscle LPO value and it decreased significantly in fish of all the treatment groups ($P < 0.05$). Finally on the 90th day, results displayed no significant difference between groups ($P < 0.05$). In

**Table 4** Hematological profiles of rainbow trout fed with different doses of ribwort plantain (*Plantago lanceolata* L.) aqueous methanolic extract

| | 30th day | | | | 60th day | | |
|---|---|---|---|---|---|---|---|
| | Control | RP1 | RP2 | RP3 | Control | RP1 | RP2 |
| RBC ($\times 10^{-6}$ µl) | 1.13 ± 0.06 | 1.27 ± 0.05 | 1.23 ± 0.04 | 1.23 ± 0.05 | 1.72 ± 0.09 | 1.57 ± 0.05 | 1.10 ± 0.07 |
| Hb (g $dl^{-1}$) | 8.83 ± 0.15[b] | 9.77 ± 0.10[a] | 9.33 ± 0.08[b] | 9.37 ± 0.10[b] | 10.72 ± 0.11 | 10.13 ± 0.12 | 8.04 ± 0.17 |
| HCT (%) | 26.93 ± 0.29 | 29.68 ± 0.23 | 28.70 ± 0.17 | 28.78 ± 0.21 | 35.90 ± 0.36 | 35.23 ± 0.24 | 26.82 ± 0.32 |
| MCV | 238.13 ± 0.38 | 234.73 ± 0.30 | 231.96 ± 0.32 | 234.16 ± 0.25 | 209.77 ± 0.44 | 224.96 ± 0.33 | 223.26 ± 0.27 |
| MCH | 78.27 ± 0.36 | 77.92 ± .32 | 76.12 ± 0.27 | 76.59 ± 0.30 | 62.80 ± 0.46 | 65.20 ± 0.33 | 70.09 ± 0.33 |
| MCHC | 328.83 ± 0.70 | 331.67 ± 0.57 | 328.11 ± 0.46 | 319.22 ± 0.69 | 303.17 ± 0.91 | 288.89 ± 0.60 | 312.22 ± 0.72 |

| | 60th day | 90th day | | | |
|---|---|---|---|---|---|
| | RP3 | Control | RP1 | RP2 | RP3 |
| RBC ($\times 10^{-6}$ µl) | 1.58 ± 0.05 | 1.57 ± 0.06 | 1.60 ± 0.05 | 1.74 ± 0.04 | 1.75 ± 0.04 |
| Hb (g $dl^{-1}$) | 10.89 ± 0.11 | 8.95 ± 0.15 | 10.19 ± 0.17 | 9.80 ± 0.10 | 10.00 ± 0.12 |
| HCT (%) | 36.75 ± 0.19 | 20.82 ± 0.22 | 21.22 ± 0.16 | 22.71 ± 0.15 | 23.21 ± 0.16 |
| MCV | 226.15 ± 0.25 | 133.05 ± 0.32 | 133.34 ± 0.25 | 130.93 ± 0.21 | 132.96 ± 0.23 |
| MCH | 66.32 ± 0.19 | 57.15 ± 0.34 | 63.98 ± 0.42 | 56.32 ± 0.16 | 431.56 ± 0.49 |
| MCHC | 294.89 ± 0.35 | 430.17 ± 0.97 | 480.56 ± 1.14 | 56.99 ± 0.19 | 430.00 ± 0.55 |

All data are given as mean ± SE ($n = 3$). Means with different superscript letters in a row are significantly different ($P < 0.05$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g $kg^{-1}$ diet, respectively; RBC is red blood cells, Hb is hemoglobin, HCT is hematocrit value, MCV is mean corpuscular volume, MCH is mean corpuscular hemoglobin, MCHC is mean corpuscular hemoglobin concentration

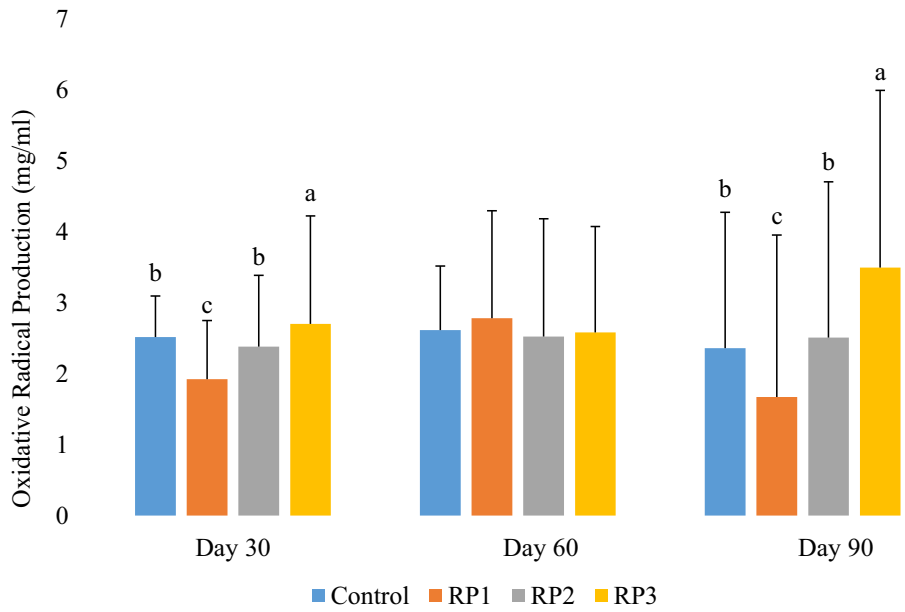


**Fig. 1** Changes in oxidative radical production activity of rainbow trout fed with diet containing aqueous methanolic extract of ribwort plantain for 90 days. All data are given as mean ± SE ($n$ = 3). Different letters above columns indicate significant difference among treatments on the particular sampling day ($P < 0.05$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g $kg^{-1}$ diet, respectively

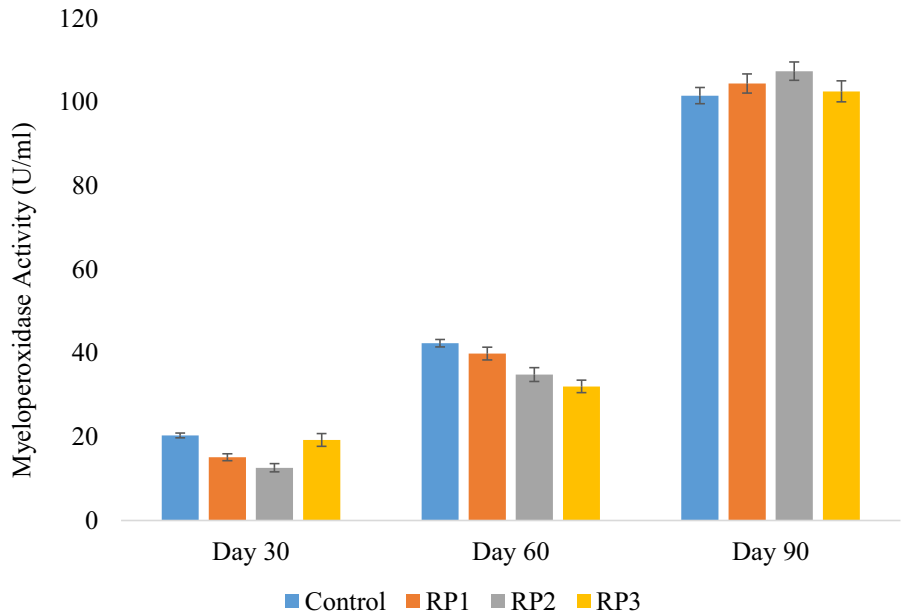


**Fig. 2** Changes in myeloperoxidase (MPO) activity of rainbow trout fed with diet containing aqueous methanolic extract of ribwort plantain for 90 days. All data are given as mean ± SE ($n$ = 3). Different letters above columns indicate significant difference among treatments on the particular sampling day ($P < 0.05$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g $kg^{-1}$ diet, respectively

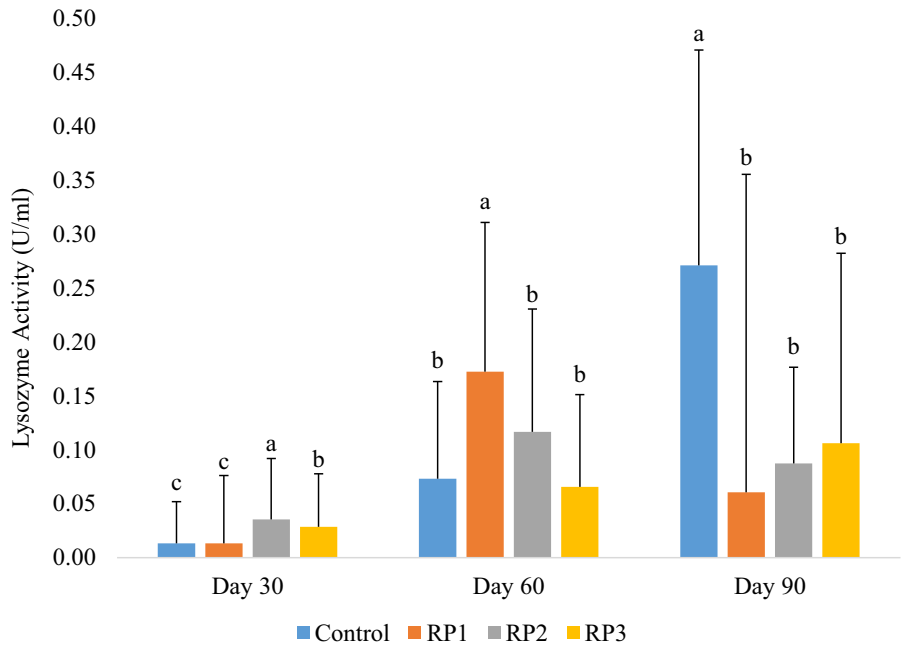


**Fig. 3** Changes in lysozyme activity of rainbow trout fed with diet containing aqueous methanolic extract of ribwort plantain for 90 days. All data are given as mean ± SE ($n = 3$). Different letters above columns indicate significant difference among treatments on the particular sampling day ($P < 0.05$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g kg$^{-1}$ diet, respectively

terms of liver LPO, there was no significant difference between groups at all sampling times.

## Discussion

Results of this study indicate that dietary administration of aqueous methanolic extract of ribwort plantain at the doses of 1, 2, and 3 g kg$^{-1}$ can promote growth in rainbow trout. It was also observed that ribwort plantain extract elevates antioxidant activity and non-specific immune responses. The growth promotion along with stimulated antioxidant and non-specific immune responses could provide a better yield and an early protective immunity in rainbow trout.

In this study, growth performance parameters, such as final weight, weight gain, and SGR levels, were significantly higher in RP administered groups compared to control. Especially, RP2 had a dose-dependent growth promotion. Similar to this study, significantly increased final weight and SGR were reported in rainbow trout fed with capper and nettle extract (Bilen et al. 2016; Bilen et al. 2014). Acar

**Table 5** Liver superoxide dismutase (SOD) activity (U ml$^{-1}$) of rainbow trout fed with ribwort plantain aqueous methanolic extract supplemented feed

| Groups | 30th day | 60th day | 90th day |
|---|---|---|---|
| Control | 73.39 ± 0.72 | 77.21 ± 1.06 | 66.86 ± 0.81 |
| RP1 | 70.62 ± 0.97 | 65.77 ± 1.28 | 68.15 ± 0.94 |
| RP2 | 66.32 ± 0.98 | 62.67 ± 1.59 | 71.78 ± 0.95 |
| RP3 | 73.58 ± 0.78 | 77.14 ± 1.17 | 69.86 ± 0.70 |

All data are given as mean ± SE ($n = 3$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g kg$^{-1}$ diet, respectively

**Table 6** Liver catalase (CAT) activity (nmol min$^{-1}$ ml$^{-1}$) of rainbow trout fed with ribwort plantain aqueous methanolic extract supplemented feed

| Groups | 30th day | 60th day | 90th day |
|---|---|---|---|
| Control | 0.33 ± 0.07 | 0.31 ± 0.06 | 0.27 ± 0.08 |
| RP1 | 0.31 ± 0.05 | 0.28 ± 0.09 | 0.34 ± 0.07 |
| RP2 | 0.28 ± 0.11 | 0.30 ± 0.08 | 0.33 ± 0.08 |
| RP3 | 0.30 ± 0.09 | 0.29 ± 0.09 | 0.29 ± 0.07 |

All data are given as mean ± SE ($n = 3$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g kg$^{-1}$ diet, respectively

**Table 7** Liver glutathione peroxidase (GPx) activity (nmol $min^{-1}$ $ml^{-1}$) of rainbow trout fed with ribwort plantain aqueous methanolic extract supplemented feed

| Groups | 30th day | 60th day | 90th day |
|---|---|---|---|
| Control | 15.32 ± 0.06 | 45.44 ± 0.78$^{a}$ | 59.44 ± 0.65$^{b}$ |
| RP1 | 20.85 ± 0.70 | 20.73 ± 0.21$^{b}$ | 35.35 ± 0.63$^{c}$ |
| RP2 | 19.41 ± 0.39 | 23.28 ± 0.93$^{b}$ | 61.33 ± 0.39$^{b}$ |
| RP3 | 27.53 ± 1.08 | 37.38 ± 0.65$^{a}$ | 78.54 ± 0.66$^{a}$ |

All data are given as mean ± SE ($n = 3$). Different superscript letters indicate significant differences between groups in the same column ($P < 0.05$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g $kg^{-1}$ diet, respectively

**Table 9** White muscle lipid peroxidation (muscle LPO) (U $ml^{-1}$) of rainbow trout fed with ribwort plantain aqueous methanolic extract supplemented feed

| Groups | 30th day | 60th day | 90th day |
|---|---|---|---|
| Control | 2.63 ± 0.16$^{b}$ | 3.18 ± 0.14$^{a}$ | 2.54 ± 0.17 |
| RP1 | 2.29 ± 0.14$^{c}$ | 2.35 ± 0.12$^{b}$ | 2.45 ± 0.14 |
| RP2 | 3.25 ± 0.16$^{a}$ | 2.45 ± 0.18$^{c}$ | 2.41 ± 0.04 |
| RP3 | 2.86 ± 0.17$^{b}$ | 2.72 ± 0.14$^{c}$ | 2.38 ± 0.14 |

All data are given as mean ± SE ($n = 3$). Different superscript letters indicate significant differences between groups in the same column ($P < 0.05$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g $kg^{-1}$ diet, respectively

(2018) demonstrated that carp fed with diet supplemented with St. John's Wort Oil (*Hypericum perforatum*) at 5 g $kg^{-1}$ attained higher live weight gain and specific growth rate compared to other groups and control. Dissimilar to these our results, use of laurel caused no effects on growth of rainbow trout (Bilen and Bulut 2010). Moreover, Dügenci et al. (2003) reported that mistletoe (*Viscum album*), nettle (*Urtica dioica*), and ginger (*Zingiber officinale*) aqueous extracts did not have any effect, whereas, Sönmez et al. (2015) demonstrated that mint (*Mentha spicata*) oil affected growth performance of rainbow trout negatively.

Hematological parameters are important in determining physiological status and health condition of fish (Fazio 2019). In our study, ribwort plantain did not influence RBC, Hb, HCT, MCV, MCH, and MCHC values at any of the sampling times except RP1 group fish which had an increase in hemoglobin level on the 30th day. Gupta and Mishra (2014) studied the effect of supplementation of false daisy (*Eclipta alba*) leaf extract at different concentrations on blood parameters of African sharp-toothed catfish (*Clarias gariepinus*). They used alcoholic and aqueous extracts and reported that there were significant differences in RBC, Hb, and WBC values of the fish. In another study, Şahan et al. (2016) observed increased RBC and WBC counts in Nile tilapia (*Oreochromis niloticus*) fed with diet containing 5 g $kg^{-1}$ *Spirulina platensis* for 75 days. Similarly, Nobahar et al. (2015) fed beluga (*Huso huso*) with garlic (*Allium sativum*) and nettle (*Urtica dioica*) extract supplemented feed for 60 days. On the 20th and 40th days of the study, they observed increased Hb values in nettle administered groups. On the other hand, on the 60th day, HCT was higher in nettle fed groups compared to control and garlic treated groups. They also reported that MCHC values of garlic treated group increased significantly ($P < 0.05$).

**Table 8** Liver glucose-6-phosphate dehydrogenase (G6PDH) activity (U $ml^{-1}$) of rainbow trout fed with ribwort plantain aqueous methanolic extract supplemented feed

| Groups | 30th day | 60th day | 90th day |
|---|---|---|---|
| Control | 0.85 ± 0.10$^{a}$ | 0.76 ± 0.1$^{b}$ | 0.79 ± 0.11$^{b}$ |
| RP1 | 0.64 ± 0.09$^{b}$ | 0.65 ± 0.09$^{c}$ | 0.72 ± 0.09$^{b}$ |
| RP2 | 0.68 ± 0.07$^{b}$ | 0.85 ± .13$^{a}$ | 0.63 ± 0.09$^{c}$ |
| RP3 | 0.85 ± 0.12$^{a}$ | 0.82 ± 0.14$^{a}$ | 0.87 ± 0.09$^{a}$ |

All data are given as mean ± SE ($n = 3$). Different superscript letters indicate significant differences between groups in the same column ($P < 0.05$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g $kg^{-1}$ diet, respectively

**Table 10** Liver lipid peroxidation (liver LPO) (U $ml^{-1}$) of rainbow trout fed with ribwort plantain aqueous methanolic extract supplemented feed

| Groups | 30th day | 60th day | 90th day |
|---|---|---|---|
| Control | 3.26 ± 0.45 | 2.97 ± 0.34 | 3.01 ± 0.39 |
| RP1 | 2.65 ± 0.16 | 4.89 ± 0.66 | 2.39 ± 0.17 |
| RP2 | 4.43 ± 0.65 | 2.27 ± 0.08 | 2.49 ± 0.18 |
| RP3 | 2.23 ± 0.15 | 2.38 ± 0.09 | 2.19 ± 0.10 |

All data are given as mean ± SE ($n = 3$). RP1, RP2, and RP3 are extracts of ribwort plantain (*Plantago lanceolata* L.) at 1, 2, and 3 g $kg^{-1}$ diet, respectively

Determination of oxidative radical production is very important to assess stimulation of cellular defense in fish. In this process, some immune cells, such as neutrophils and macrophages, consume oxygen to form reactive oxygen species (ROS) which are toxic to pathogenic bacteria (Srivastava and Pandey 2015). These ROS interact with nitroblue tetrazolium (NBT) in a reduction reaction, thus, indicating whether oxidative radical production was increased or not. In our study, ORP was determined on the 30th, 60th, and 90th days. The highest ORP was calculated as 2.7 and 3.5 mg $l^{-1}$ in fish of RP3 group on the 30th and 90th days, respectively. However, it was 2.51 and 2.35 mg $l^{-1}$ in control group ($P < 0.05$). Oxidative radicals are mainly produced and released by phagocytic cells and are very toxic to the bacterial pathogens (Hardie et al. 1996). Our results indicated that phagocytic cells were activated by the dietary administration of plant extract. Dügenci et al. (2003) studied the effects of European mistletoe (*Viscum album*), nettle (*Urtica dioica*), and ginger (*Zingiber officinale*) extracts on nonspecific immune response of rainbow trout (*O. mykiss*). They demonstrated that none of the treatment groups exhibited different ORP values compared to control group ($P < 0.001$). On the contrary, Diler et al. (2015) reported that addition of common mugwort (*Artemisia vulgaris*) extract to feed of rainbow trout (*O. mykiss*) caused an increase in ORP ($P < 0.05$). Therefore, they concluded that common mugwort could stimulate immune response in rainbow trout. Similarly, Bilen et al. (2013b) revealed that ORP level of koi carp (*Cyprinus carpio*) was increased when fed with diet containing European smoketree.

Myeloperoxidase (MPO) is a principal enzyme that is released by polymorphic nuclear neutrophils and it generates cytotoxic oxidants that impact deleteriously on nitric oxide-dependent signaling cascades within the vasculature (Lau et al., 2005). In the current study, MPO activity did not differ among different fish groups at all the sampling times. Similar to our result, Christybapita et al. (2007) reported that there were no significant differences in MPO activity among treatment groups of Mozambique tilapia (*Oreochromis mossambicus*) fed with diets containing false daisy (*Eclipta alba*) leaf extract for 3 weeks. Bilen et al. (2016), on the other hand, demonstrated that feeding rainbow trout with caper (*Capparis spinosa*) extract for 30 days caused an elevation in MPO activity. Likewise, use of *Tilia tomentosa* increased the MPO activity in rainbow trout (Almabrok et al. 2018).

Lysozyme activity (LA) is an important parameter like phagocytic, neutrophil, and complement activities to understand the immune response of fish (Murray et al., 2003). In the present study, LA was significantly increased in fish of RP2 and RP3 groups on the 30th day ($P < 0.05$). On the 60th day, LA in all fish groups including control increased, although, this increase was merely significant in RP1 group only. On the other hand, results on the 90th day were different from those of the 60th day. Fish of all treatment groups had significant decrease in LA in comparison with control group ($P < 0.05$) on the 90th day. Bilen and Bulut (2010) examined the effects of dietary supplementation of laurel (*Laurus nobilis*) leaf powder on serum lysozyme activity in rainbow trout. They reported that after feeding the fish for 3 weeks, there was not significant differences in LA between the groups ($P < 0.05$). In another study, sage (*Salvia officinalis*) and *Aloe vera* extract supplementation caused a significant increase in lysozyme activity in rainbow trout (Tafi et al. 2018). Similar to this, Bilen et al. (2013b) demonstrated that koi carp (*Cyprinus carpio*) had higher lysozyme activity when fed with diet containing European smoketree methanolic extract.

Antioxidant defense mechanism of fish includes various factors which eliminate or limit the spread of pathogens (Blazer 1992). The first and fundamental antioxidant defense occurs enzymatically (Gökhan 2007). In the present study, we evaluated superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glucose-6-phosphate dehydrogenase (G6PDH), and lipid peroxidation (LPO) activities in fish fed with ribwort plantain additive. Increase in SOD activity could occur due to increase in intracellular superoxide radicals (Cheng et al., 2007). Sönmez et al. (2015) studied dietary effects of sage (*Salvia officinalis*), mint (*Mentha spicata*), and thyme (*Thyme vulgaris*) oils on antioxidant enzymes in rainbow trout. They added 500, 1000, and 1500 mg oil per kg feed and fed the fish for 60 days. According to their results, all the treatment groups had increased SOD, G6PDH, and GPx activities compared to that of control group ($P < 0.05$). In

addition, they reported that CAT, GST, and GR enzyme activities were significantly decreased. In a similar study, Metwally (2009) added different forms of garlic (*Allium sativum*) into the feed of Nile tilapia (*Oreochromis niloticus*), and examined liver and serum SOD, CAT, and GPx activities. They found that garlic addition caused an increase in SOD, CAT, and GPx activities in tilapia ($P < 0.05$). Gabriel et al. (2015) fed GIFT tilapia (genetically improved farmed tilapia, *Oreochromis niloticus*) with *Aloe vera* additive for 8 weeks. They demonstrated that 4% *A. vera* treated group had an increase in liver CAT activity, whereas GPx activities in 0.5% and 1% treated groups were higher than that of control. Also, SOD activities of all treatment groups were significantly increased ($P < 0.05$). In another trial, Manal (2016) investigated the effects of curcuma (*Curcuma longa*) and garlic supplementation in Nile tilapia (*Oreochromis niloticus*). They reported that all the treatment groups had an increase in GSH, CAT, GPx, and GRx activities ($P < 0.05$). We believe in that ribwort plantain is rich in with chlorogenic acid compound. Chlorogenic acid is a polyphenols and has antioxidant property (Sato et al. 2011) and this may explain increasing of some antioxidant parameters.

## Conclusion

In the present study, new information on potential application of ribwort plantain is provided by examining its effects on growth performance, antioxidant activity, and immune responses in rainbow trout. After the 90 days of experiment period, we observed that oxidative radical production and myeloperoxidase activities were affected positively, proving that ribwort plantain (*P. lanceolata*) has an immunostimulatory effect in rainbow trout (*Oncorhynchus mykiss*). In respect to antioxidant defense, GPx and G6PDH activities were also enhanced in treatment groups, especially in fish group that received 3 g $kg^{-1}$ ribwort plantain extract. Based on these findings, we conclude that ribwort plantain can be used as a growth promoter and immunostimulant in rainbow trout culture. However, further studies to determine correct dose and time duration of administration should be conducted in order to maximize the benefit of ribwort plantain.

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