

Short Communication

Effects of walnut (*Juglans regia*) green peel extract on growth performance and challenge to enteric redmouth disease (*Yersinia ruckeri*) in rainbow trout (*Oncorhynchus mykiss*)

Filiz Karadaş¹, Nejdet Gültepe^{2a}¹ Department of Aquacultural Engineering, Atatürk University, ² Department of Fundamental Fisheries Sciences, Atatürk University

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This study aimed to investigate the effects of walnut (*Juglans regia*) green peel extract on growth performance parameters growth performance and challenge to enteric redmouth disease (*Yersinia ruckeri*) in rainbow trout (*Oncorhynchus mykiss*). The triplicate experimental study was performed for 105 days to evaluate the effect of 1, 2, and 4 mg kg⁻¹ of walnut green peel extract (WPE). Although there was no significant difference in growth performance between the groups, the growth performance and survival rate of the extract-added groups were slightly better than the control group. Some blood parameters such as mid-range cells (MID), platelet (PLT), platelet distribution width (PDW), procalcitonin (PCT), platelet large cell ratio (P-LCR), and cholesterol (CHOL) values significantly improved in walnut green peel extract-added groups. Phagocytic activity (PA), lysozyme activity (LA), and myeloperoxidase activity (MPO) were significantly increased between all groups compared with the untreated group. Although respiratory burst activity (RBA) increased, no significant difference existed between groups. As a result, it was determined that although walnut green peel extract does not directly affect growth performance, it can be used as a feed additive with its immunomodulatory aspect.

INTRODUCTION

With the increasing global population, people have focused more on healthy nutrition. Increasing aquaculture due to people's demand for food naturally increases the incidence and potential of some fish diseases. Antibiotics, prebiotics, probiotics, and other immune-supportive additives are used for prevention and treatment in combating diseases. However, due to the adverse effects of these substances on ecology and human health, the use of herbal products is increasing. Additives used to enhance productivity in aquaculture can lead to environmental damage and resistance development against pathogens. To mitigate these negative effects, herbal products, and extracts are used to improve the growth and reproductive performance of fish and to strengthen their immune system. In rainbow trout, antibiotics are being successfully replaced with herbal oils, plant extracts, and probiotics.¹⁻³ In recent years, the use of plant extracts with antimicrobial properties such as phenols has become widespread; it has also been known that the antioxidants found in these plants provide positive contributions against various pathogens in fish and have an immunomodulatory effect.

Common bacterial pathogens in rainbow trout farms worldwide include *Lactococcus garvieae*, *Listonella anguillarum*, and *Aeromonas hydrophila*, while the main diseases in Turkey are Vibriosis and Yersiniosis.⁴ *Yersinia ruckeri*, the main causative agent of enteric redmouth disease, has caused significant losses in salmonid fish in various countries.^{3,5,6}

The green walnut shell, a source of natural components with antimicrobial and antioxidant properties that emerge during walnut harvesting, is considered agricultural forestry waste rich in bioactive compounds. It is also referred to as a by-product of producing dried fruits.⁷ The green walnut shell contains 13 different phenolic components, including juglone, caffeic acid, chlorogenic acid, gallic acid, sinapic acid, ferulic acid, ellagic acid, protocatechuic acid, vannilic acid, syringic acid, catechin, myricetin, and epicatechin. Particularly, juglone has been identified to exhibit antimicrobial properties.^{8,9} Therefore, the present study aims to assess the effects of walnut (*Juglans regia*) green peel extract (WPE) on growth performance parameters growth performance and challenge to enteric redmouth disease (*Yersinia ruckeri*) in rainbow trout (*Oncorhynchus mykiss*).

a Corresponding author. Nejdet Gültepe, Tel.: +905326334769, e-mail: nejdetgultepe@yahoo.com

Table 1. Formulations of isonitrogenous/isolipidic experimental diets

	Ingredients (g/kg)			
	Control	WPE-1	WPE-2	WPE-4
Fish meal	456	456	456	456
Soy meal	280	280	280	280
Wheat meal	80	80	80	80
Corn starch	40	39	38	36
Fish oil (mL)	104	104	104	104
Vit-Min. Mix ¹	40	40	40	40
WPE	0.00	1	2	4
Chemical composition (% DM)				
Protein	45.07	45.07	45.07	45.07
Lipid	15.20	15.20	15.20	15.20
Ash	8.30	8.30	8.30	8.30
Carbohydrate	31.43	31.43	31.43	31.43

¹ Vit-Min. (Vitamin-Mineral) per kg diet: 4x10⁶ IU vitamin A, 4x10⁵ IU vitamin D3, 4x10⁴ mg vitamin E, 2400 mg vitamin K3, 4000 mg vitamin B1, 6000 mg vitamin B2, 4000 mg vitamin B6, 10 mg vitamin B12, 4000 mg vitamin C, 4000 mg niacin, 4000 mg calcium D-pantothenate, 100 mg D-biotin, 1200 mg folic acid, 6x104 inositol, 270 mg nicotinic acid, 75.3 mg Fe, 12.2 mg Cu, 206 mg Mn, 85 mg Zn, 3 mg I, 0.350 mg Se, 1 mg Co.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

The feeding trial was conducted in a recirculating aquaculture system (RAS) and the bacterial challenge trial was conducted in a free-flowing system at Atatürk University, Faculty of Fisheries, Experimental Research Unit. A total of 240 healthy rainbow trout (*Oncorhynchus mykiss*) juveniles with a mean weight of 4.52±0.05 g were randomly placed in RAS with 12 aquariums, each with a volume of 50 L (20 fish per aquarium) in three replicates. Water parameters were daily measured (mean±SE) throughout the experiment, which are temperature 13.6±0.5 °C, pH 7.7±0.3, dissolved oxygen 7.52±0.08 mg L⁻¹. The fish were fed 3 times (08:00, 12:00, and 17:00) a day, at 3% of the body weight with 12L:12D photoperiod. The experiments were performed in accordance with the guidelines for fish research from the animal ethics committees at Atatürk University, Turkey.

WALNUT GREEN PEEL EXTRACT AND EXPERIMENTAL DIETS

Walnut green peel collected from Tuzluca province of Iğdır of Türkiye during mid-autumn (2023) was cleaned with distilled water and freeze-dried. A dried walnut green shell was ground, methanol (20g/0.8 L) was added, and then kept in the dark for three days. After this period, the mixture was filtered through filter paper with a vacuum pump. The methanol was then evaporated at 35°C using a rotary evaporator to obtain the extract. After the two-week adaptation period for experimental feeding four isonitrogenous/isolipidic (45 protein/15 fat) experimental diets were formulated with WPE at 0 g kg⁻¹ (control), 1 g kg⁻¹ (WPE-1), 2 g kg⁻¹ (WPE-2) and 4 g kg⁻¹ (WPE-4) concentrations (Table 1).^{3,10}

CHALLENGE TEST WITH *YERSINIA RUCKERI*

For the challenge test, the 3 × 10⁶ CFU mL⁻¹ dose of *Yersinia ruckeri* (GenBank KX388238.1) was used. *Y. ruckeri* was produced in Tryptic Soy Broth at 22 °C and then washed twice with phosphate-buffered saline (PBS) to adjust the density to 3 × 10⁶ CFU mL⁻¹. After 105 days feeding trial, fish (10 fish/aquarium) were stocked in 50 L aquarium and maintained at 13.6±0.5 °C throughout the challenge experiment. Bacterial density was measured according to McFarland 1 at λ =610 nm, then inoculated 0.1 mL per fish to each treatment. Triplicate treatments were done to monitor the effect of exposure to *Y. ruckeri* in fish. The mortality of fish was monitored daily for 15 days and dead fish were removed from the aquarium.³ Koch postulates were verified from dead fish.

GROWTH PERFORMANCE

At the end of 105 days feeding trial, fish in each aquarium (n=20) were individually weighed, and then growth performance was calculated according to follows.¹⁰⁻¹⁴

$$\begin{aligned} \text{RGR} &= \frac{W_t - W_i}{W_i} \times 100 \\ \text{SGR} &= \frac{\log(W_t) - \log(W_i)}{t} \times 100 \\ \text{DFI} &= \frac{\text{Initial Feed Consumption}}{t} \\ \text{FCR} &= \frac{\text{Feed Consumption}}{W_t - W_i} \end{aligned}$$

Abbreviations: RGR, relative growth rate; SGR, specific growth rate; DFI, daily feed intake; FCR, feed conversion ratio; W_t, final weight; W_i, initial weight; t, experimental day

BLOOD SAMPLES AND ANALYSES

Random 10 fish from each aquarium were sampled at the end of the trial and anesthetized with tricaine methane-

sulfonate (MS-222, SIGMA, United States) for hematological, biochemical, and immunological analyses. To reduce stress, the head of fish was covered with a wet towel at the blood sampling stage. Blood samples were collected with a sterile plastic syringe from the caudal vein. Red blood cell count (RBC; $\times 10^6$ per mm^3), hematocrit level (Ht; %), hemoglobin concentration (Hb; g/dL), mean corpuscular volume (MCV; femtoliters), mean corpuscular hemoglobin (MCH; pg) and mean corpuscular hemoglobin concentration (MCHC; %), mid-range cells (MID; %), platelet (PLT; $10^3/\text{UI}$), platelet distribution width (PDW; %), procalcitonin (PCT; %), platelet large cell ratio (P-LCR; %), were determined and calculated. Serum glucose (GLU), triglyceride (TRIG), cholesterol (CHOL), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP), albumin (ALB), and globulin (GLO) were determined via the spectrophotometric method in the microplate reader (Thermo Scientific™ Multiskan™ GO, Vantaa, Finland) using the kits (Bio-analytic Diagnostic Industry, Co., İstanbul, Turkey) previously used in the fish. Lysozyme activity (LA), total myeloperoxidase (MPO), and respiratory burst activity (RBA) were assessed using the turbidometric assay. Phagocytic activity (PA) was determined according to the microscopy method.¹⁴⁻¹⁶

STATISTICS

Statistical parameters were examined by ANOVA. Levels of significance were determined using Tukey's HSD test, with critical limits being set at $P < 0.05$. Values are expressed as means $\pm$ standard deviation (SD) for each measured variable.

RESULTS

There were no differences in growth performance between groups in the feeding trial experiment. However, the survival rate of the extract-added groups was slightly better than feeding the unextracted group in the challenge with *Yersinia ruckeri*. Hematological blood parameters such as red blood cell count (RBC), hematocrit level (Ht), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were no difference between groups, however, mid-range cells (MID), platelet (PLT), platelet distribution width (PDW), procalcitonin (PCT), and platelet large cell ratio (P-LCR) values differently found among control and extract-added groups ($p \leq 0.05$). Although cholesterol (CHOL) level found differently between groups ($p \leq 0.05$), glucose (GLU), triglyceride (TRIG), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP), albumin (ALB), and globulin (GLO) were no differently. Immunological parameters in the blood, which are lysozyme activity (LA), total myeloperoxidase (MPO), and phagocytic activity (PA), were different between groups ($p \leq 0.05$) except respiratory burst activity (RBA) (Table 2).

DISCUSSION

Medicinal plants not only make significant contributions to human health but are also used to make cultivatable species more productive and healthier. Walnut contains effective phytochemicals such as flavonoids, alkaloids, and polyphenolic compounds, and due to these components, walnut has a wide range of uses in the medical field.¹⁷ Due to its green shell content, walnuts can be used for various purposes in modern medicine as well as in traditional medicine, especially in combating diseases such as cancer, bacterial infections, and inflammation, and in relieving pain.¹⁸ Cost-effective, easily accessible, and environmentally friendly, medicinal plants are considered a potential alternative to traditionally used antibiotics, synthetic hormones, and antioxidants in the aquaculture industry.^{3,19} Over the past decade, research on functional feed additives (FuFAs)—supplements designed to deliver growth and health benefits beyond essential nutrients—has demonstrated that plant-based feed additives positively influence fish growth performance and immune system function.²⁰ Examples include *Thymus vulgaris*, *Rosmarinus officinalis*, *Trigonella foenum graecum*, *Tribulus terrestris* and *Pimenta dioica* in tilapia,^{21,22} and *Ocimum basilicum*,²³ *Petroselinum crispum*,²⁴ *Calendula officinalis*²⁵ and *Cyanus depressus*²⁶ in rainbow trout. It is noteworthy that studies generally focus on the effects of plant extracts on immunity rather than on growth performance properties. When the doses of extracts effective on growth performance are examined, it is seen that there is no stability, and the growth performance parameters fluctuate depending on the dose. Similarly, in the present study, growth performance fluctuated, and no difference was found between the groups. To more clearly demonstrate the effect on growth performance, the feeding trial continued for 105 days. Although there was a correlation depending on the dose and no statistical difference, the decrease in FCR value was remarkable. At the same time, an increase in survival rate was observed according to the result of the challenge trial with *Yersinia ruckeri*, but it was insignificant.

Blood parameters in fish provide information about the physiology and health of the fish.¹⁶ In this study mid-range cells (MID), platelet (PLT), platelet distribution width (PDW), procalcitonin (PCT), platelet large cell ratio (P-LCR), and cholesterol (CHOL) Phagocytic activity (PA), lysozyme activity (LA) and myeloperoxidase activity (MPO) were found differently between groups. High MID values are known as monocytosis. High monocytes in the body can be expressed as a blood value that indicates that the body is struggling with abnormal problems such as infection, cancer, and inflammation. It is also possible to say that high MID indicates a disease. The highest MID value was found in the control group, while the lowest was in the 2 g kg^{-1} extract-added group. The fact that the MID value was lower in the other groups besides the control group suggested that the added extract physiologically improved the fish metabolism. In the absence of disease, the first factor affecting the blood parameters of fish is nutrition. Phenolic compounds that enter the body through

Table 2. Growth performance and effects of WPE on fish blood parameters

Parameters	Control	WPE-1	WPE-2	WPE-4
W_i (g)	4.55 ± 0.05	4.53 ± 0.01	4.52 ± 0.06	4.47 ± 0.06
W_f (g)	89.25 ± 6.07	84.13 ± 2.41	85.24 ± 11.21	83.62 ± 6.21
DFI (g)	15.99 ± 1.37	17.24 ± 0.76	18.04 ± 0.80	16.48 ± 2.61
RGR	1848.41 ± 154.48	1738.15 ± 54.35	1827.94 ± 224.51	1138.32 ± 115.22
SGR	1.23 ± 0.03	1.21 ± 0.01	1.21 ± 0.05	1.21 ± 0.03
FCR	1.37 ± 0.21	1.17 ± 0.02	1.17 ± 0.06	1.18 ± 0.14
SR	71.67 ± 12.58	93.33 ± 7.64	96.67 ± 5.77	90.00 ± 17.32
MID	6.40 ± 4.70 ^d	3.83 ± 4.53 ^b	1.94 ± 0.61 ^a	3.66 ± 3.45 ^b
PLT	297.55 ± 73.40 ^a	467.45 ± 164.10 ^b	1272.11 ± 110.89 ^c	1895.50 ± 513.10 ^d
PDW	6.20 ± 0.91 ^{ab}	5.68 ± 0.19 ^a	7.40 ± 1.63 ^c	6.96 ± 1.61 ^{bc}
PCT	0.63 ± 0.15 ^{ab}	0.30 ± 0.10 ^a	0.94 ± 0.10 ^{bc}	1.20 ± 0.15 ^c
P-LCR	0.30 ± 0.01 ^a	0.13 ± 0.05 ^a	0.75 ± 0.08 ^b	0.74 ± 0.10 ^b
CHOL	3.44 ± 1.11 ^{ab}	1.97 ± 0.72 ^a	4.01 ± 0.75 ^{bc}	4.82 ± 0.79 ^c
PA	18.33 ± 0.58 ^a	21.00 ± 1.73 ^{ab}	25.33 ± 5.51 ^{ab}	29.00 ± 3.46 ^b
LA	175.88 ± 49.62 ^a	269.88 ± 51.63 ^a	272.33 ± 99.87 ^a	534.76 ± 151.47 ^b
MPO	130.52 ± 1.86 ^a	157.72 ± 2.63 ^b	161.40 ± 1.67 ^b	168.85 ± 2.68 ^c

Abbreviations: W_i , initial weight; W_f , final weight; DFI, daily feed intake; IGR, instantaneous growth rate; RGR, relative growth rate; SGR, specific growth rate; FCR, feed conversion ratio; SR, survival rate; MID, mid-range cells; PLT, platelet; PDW, platelet distribution width; PCT, procalcitonin; P-LCR, platelet large cell ratio; CHOL, cholesterol; PA, phagocytic activity; LA, lysozyme activity; MPO, total myeloperoxidase. The values are expressed as means ± SD (n=20 for growth parameters and n=10 for blood analysis). Means with different superscripts in rows are significantly different ($P < 0.05$).

nutrition have biological effects, especially in the blood, apart from their antimicrobial, anti-inflammatory, and antioxidant effects. The main effect on the blood is its effect on platelet aggregation.²⁷ Due to the phenolic compounds contained in the walnut green peel, it was effective on platelet (PLT), platelet distribution width (PDW), procalcitonin (PCT), and platelet large cell ratio (P-LCR) values in the groups to which extract was added. Although cholesterol (CHOL) levels of 2 g kg⁻¹ and 4 g kg⁻¹ extract-added group were increased, the 1 g kg⁻¹ extract-added group decreased. At the same time, the increase in phagocytic activity (PA), lysozyme activity (LA), and myeloperoxidase activity (MPO) values is also associated with the rise in cholesterol in immune system cells. Low cholesterol levels impair the immune system in fish and reduce the inflammatory response.²⁸ The increase in phagocytic activity (PA), lysozyme activity (LA), and myeloperoxidase activity (MPO) values due to the rise in cholesterol levels in the extract-added groups shows that walnut green shell also improves the immune system.

In conclusion, walnut (*Juglans regia*) green peel extract did not result a statistically significant difference in the growth performance of rainbow trout, but it increased the survival rate against *Yersinia ruckeri*. When the decrease in FCR and the increase in SR are examined, it is seen that the use of walnut green shells provides a positive trend for rainbow trout. In addition, the increase in immune parameters showed its positive effects on immune regulation. Therefore, walnut green peel, a waste material, can be used as a sustainable and economical immunomodulatory feed additive in rainbow trout.

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AUTHORS' CONTRIBUTION

Methodology: Filiz Karadaş (Equal), Nejdet Gültepe (Equal). Formal Analysis: Filiz Karadaş (Equal), Nejdet Gültepe (Equal). Investigation: Filiz Karadaş (Equal), Nejdet Gültepe (Equal). Project administration: Filiz Karadaş (Equal), Nejdet Gültepe (Equal). Writing – original draft: Filiz Karadaş (Equal), Nejdet Gültepe (Equal). Conceptualization: Nejdet Gültepe (Lead). Supervision: Nejdet Gültepe (Lead). Writing – review & editing: Nejdet Gültepe (Lead).

COMPETING OF INTEREST – COPE

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICAL CONDUCT APPROVAL – IACUC

The experiments were performed in accordance with the guidelines for fish research from the animal ethics committees at Atatürk University, Turkey (E-75296309-050.01.04-2200366129).

INFORMED CONSENT STATEMENT

All authors and institutions have confirmed this manuscript for publication.

DATA AVAILABILITY STATEMENT

All are available upon reasonable request.

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