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EFFECTS OF DIETARY WALNUT LEAF EXTRACTS ON GROWTH PERFORMANCE, HEMATOLOGICAL PARAMETERS, AND DISEASE RESISTANCE IN RAINBOW TROUT, *Onchorynchus mykiss*

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Abstract
The present study investigated the effects of dietary ethanolic extracts of walnut leaves (Juglans regia) on growth performance, hematological parameters and disease resistance of Rainbow trout (Oncorhynchus mykiss) against Aeromonas salmonicida. Five isonitrogenous and isoenergetic diets were prepared to contain walnut leaf ethanolic extract at rates of 0, 750, 1000, 1500 and 2500 mg/kg diet. Fish were fed with experimental diets for 21 days. Growth performance and hematological parameters were found to be similar in all experimental groups. The dietary incorporation of ethanolic extract of walnut leaf significantly improved disease resistance against Aeromonas salmonicida. In conclusion, findings of the present study indicate that walnut leaf ethanolic extract might be a promising substitute for antibiotic additives against Aeromonas salmonicida pathogen in rainbow trout feeds.
Keywords: Fish disease, herb, Juglans regia, furunculosis

Introduction

The amount of fish produced by aquaculture has reached 54.2 million tons in the World, with a contribution of 314 thousand tons by Turkish fish farming enterprises (FAO, 2020). The continuous increase in production has brought some risks by increased pressure on the marine ecosystem. Stress and disease outbreaks in intensive aquaculture production is most responsible for increased mortalities and eventually economic losses.

Various chemicals, hormones, antibiotics and vitamins have been used for many years to eliminate the conditions that adversely affect fish health (Citarasu, 2010). Antibiotics used in animal husbandry around the world have been restricted or completely banned, however, their unconscious use along with various chemicals increases resistance of harmful bacteria, parasites and other microorganisms. It is undesirable for these resistant microorganisms to affect not only fish but also water, the environment, and human as a result of consumption of fish (Harikrishnan et al., 2011).

For the increase of production volumes with improvement fish resistance, the use of organic products and a variety of nutritional additives have become more attractive, instead of using chemicals and antibiotics in aquaculture. Recently, challenging investigations related to variations in blood parameters, growth performance and nutritional compositions upon supplemented feed additives are in focus by many researchers (Ahmadifar et al., 2020). It seems that herbal extracts need more attention for fish welfare and healthy yields from aquaculture harvest. Hence, more investigations are necessary with focus on increased resistance in fish under captive conditions.

Green fresh peels and leaves of the walnut contain phenolic substances and flavonoid compounds in large quantities (Pulido, 2000), which show sedative, vaso-strengthening, hemostatic, antidiarrheal, hypoglycemic, hypotensive properties in alternative medicine and are applied in several diseases (Silva et al., 2004). Favorable phytochemicals provide effective protection against tissue damage by reducing oxidative stress and their free radical scavenging effects provide anti-carcinogenic properties (Pereira et al., 2007). Extracts obtained from the leaves of the walnut are known to be antiviral, anthelmintic, antimicrobial, acardisal (Wang et al., 2007). Although walnut leaf or walnut leaf extracts are used in various fields, no information is available so far regarding their influences on the health status and disease resistance of rainbow trout when fed with walnut leaf extract incorporated diets. Therefore, the present study assessed the growth performance, haematological indices, and resistance of rainbow trout in the challenge with Aeromonas salmonicida infection.

Material and Method

Walnut Leaf Ethanolic Extract

Walnut leaves were purchased from a local market in Çanakkale, Türkiye, and 10 grams of leaves have been extracted with 350 mL ethyl alcohol (96%) in a soxhlet extractor. Afterwards, the alcohol was removed via evaporation and the crude extract was prepared as 100 mg/mL ethyl alcohol.

Experimental Diets

Diets were prepared according to previous report by Yilmaz and Ergün (2018). Briefly, commercial trout feed (49% protein/5% lipid, 4 mm, HEMYEM, Gaziantep, Türkiye) was used

as the basal diet. Each dosage (750, 1000, 1500 and 2500 mg/kg) of walnut leaf ethanolic extract was mixed with 140 g fish oil. Then, 860 g feed was top-dressed with fish oil containing each level of walnut leaf ethanolic extract. Additionally, control feed was top-dressed with 140 g fish oil without walnut leaf ethanolic extract supplementation.

Fish and Experimental Design

The study was carried out at Çanakkale Onsekiz Mart University, Faculty of Marine Sciences and Technology. A total of 15 fiberglass tanks, each of 140 L capacity, were used in an experimental recirculated aquaculture system. 450 rainbow trout with an initial weight of 87.10±0.10 g, obtained from Ayazma Keskin Trout Farm in Bayramiç (Çanakkale-Türkiye), were fed with commercial feeds during the 15-days adaptation period. Thereafter, experimental fish were placed into the experimental tanks in triplicate groups, comprising a control group, and four treatment groups incorporated with extracts at levels of 750, 1000, 1500 and 2500 mg/kg diet. Test diets were offered to fish as 3% of the fish body weight as 3 meals a day over a period of 21 days. During the experiment, water parameters were measured daily with YSI 85 probe every morning prior to feeding. For the pH measurements, HANNA C200 (HI 83200) photometer was used. Water parameters were recorded as follows; temperature 13±1ºC, oxygen 8.7±0.2mg/L, conductivity 310±20µs cm^{-1}, pH 7.2±0.2, total ammonia 0.011±0.002mg/L, nitrite 0.01±0.01mg /L and nitrate 0.15±0.05 mg/L.

Growth Performance

Growth performance were evaluated as follows:

Weight Gain (%) = [(Final wet weight(g) - Intial wet weight(g)) / Intial wet weight(g))] x100

SGR (Specific growth rate %/day) = [(Infinal wet weight(g) - In intial wet weight(g))/day] x100

FCR (Feed conversion ratio) = Feed consumed(g) / Weight gain(g)

Haematological Assays

Hematocrit (Hct) levels, hemoglobin (Hb) values and red blood cell (RBC) counts were made using an automatic blood count machine (Mindray/BC 3000 plus), as earlier described by Wu et al. (2016), Yılmaz and Ergün (2018), and Yılmaz (2018). The device was reprogrammed for rainbow trout by comparing it with manual analysis methods before the analysis.

Blood Samples and Assays

Experimental fish were starved for 24 hours after the completion of the 21-days feeding trial. For blood collection, 3 fish were randomly selected from each tank and anesthetized with clove oil in 10 L water-filled containers at a ratio of 20 mg/L clove oil (Inversen et al., 2003). The caudal vein, from which blood was drawn, was cleaned with 70% alcohol to prevent mixing of the mucosa into the blood. With the help of a suitable insulin injector, blood was drawn from the caudal vein of the fish and the blood was put into tubes with K_2EDTA.

Results

GC-MS Analysis of Walnut Leaf Ethanolic Extract

A list of all phenolic compounds were given in our most recent study (Yilmaz et al., 2023). As a result of the GC-MS analysis the most abundant chemical constituents were 13.6% gamma-

sitosterol, 12.4% vitamin E, 8.2% lupeol, 4.6% squalene, 4.3% Octadecatrienoic acid and 4.1% 2,3-dihydro benzofuran.

Growth Performance

Results for growth performance of the experimental fish fed with different levels of walnut leaf ethanolic extract and additive-free (control) feeds are given in Table 1. No statistically significant differences were observed in growth performance data of the fish fed with experimental diets ($p>0.05$).

Haematological Variables

The levels of RBC, Hb and Hct were similar between all treatment groups ($p>0.05$), which are provided in Table 2.

Table 1. Growth performance and feed efficiency of rainbow trout fed with experimental diets

Walnut Leaf Ethanolic Extract Dose	Intial Weight (g)	Final Weight (g)	Weight Gain (%)	FCR	Spesific Growth Rate (%day^{-1})
Control	87.10±0.06	100.33±0.33	15.19±0.32	1.13±0.02	0.67±0.01
750 mg/kg	87.03±0.09	102.00±0.58	17.20±0.75	1.01±0.04	0.76±0.03
1000 mg/kg	87.20±0.17	103.67±1.33	18.88±1.55	0.92±0.08	0.82±0.06
1500 mg/kg	87.17±0.07	102.33±1.45	17.40±1.74	1.01±0.10	0.76±0.07
2500 mg/kg	87.17±0.12	102.33±0.67	17.4±0.74	0.99±0.04	0.76±0.03

Table 2. Effects of dietary supplementation of walnut leaf ethanolic extract on haematological parameters of rainbow trout

Walnut Leaf Ethanolic Extract Dose	Red Blood Cells (RBC) (10^6mm^3)	Hemoglobin (Hb) (g/dL)	Haematocrit (Hct) (%)
Control	2.19±0.05	8.95±0.32	28.54±0.77
750 mg/kg	2.26±0.06	8.49±0.56	29.66±0.65
1000 mg/kg	2.31±0.05	10.15±0.52	30.61±0.39
1500 mg/kg	2.27±0.05	8.88±0.21	30.01±0.48
2500 mg/kg	2.24±0.05	9.42±0.33	29.75±0.37

Cumulative Survival Rate

All walnut leaf ethanolic extract supplemented groups showed greater resistance to *Aeromonas salmonicida* compared to the control group. The survival of rainbow trout in the control, 750, 1000, 1500 and 2500 mg/kg walnut leaf ethanolic extract incorporated groups were 38.9%, 81.9%, 76.4%, 62.5%, and 48.6%, respectively. Highest survival rate of fish was in the group fed with 750 mg/kg walnut leaf ethanolic extract by the end of day-20 (Figure 1).

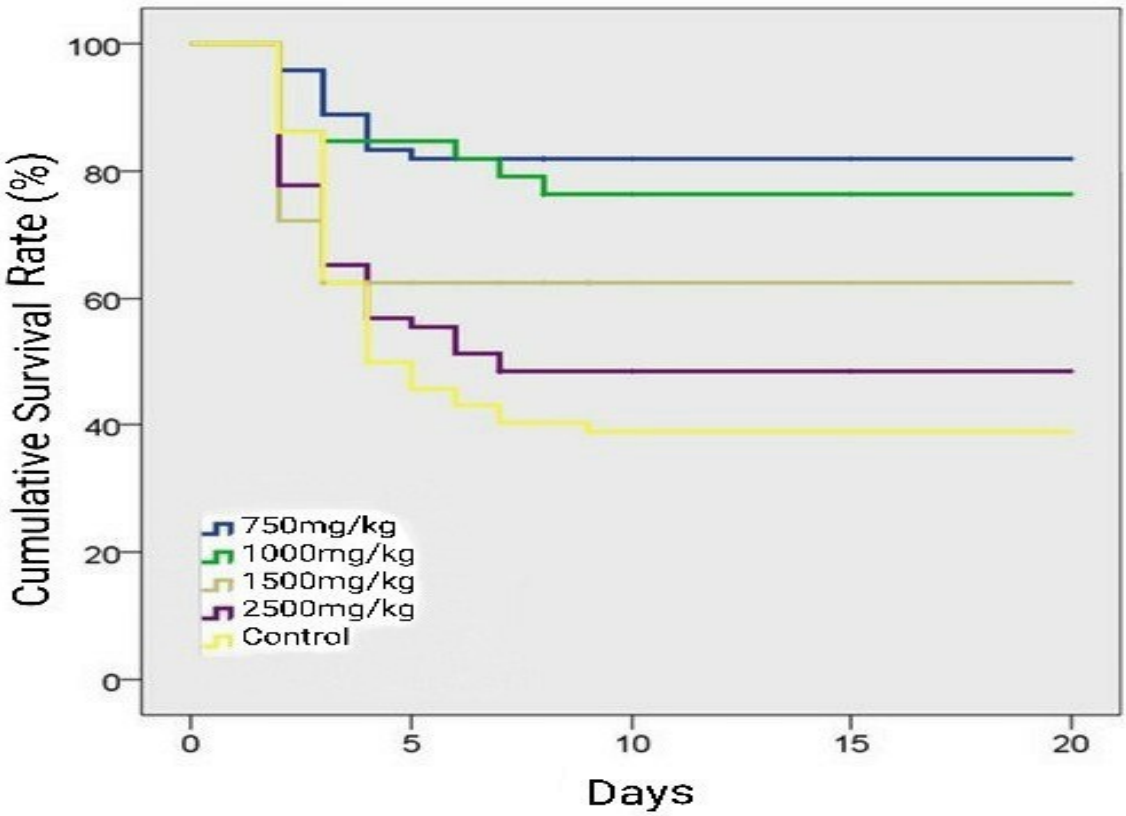

Figure 1. Kaplan–Meier survivorship curves over time for rainbow trout challenged experimentally with *Aeromonas salmonicida*

Discussion

The findings in the present study showed that dietary walnut leaf extract did not affect growth performance of rainbow trout. Similar finding was also reported for tilapia (*Oreochromis niloticus*) with no significant difference in growth performance among treatment groups when fed walnut leaf extract incorporated diets (Yilmaz et al., 2023).

Monitoring of hematological variables is considered as an important biomarker for observation and assessment of the health status of fish (Fazio, 2019). In this study, dietary supplementation with walnut leaf extract did not influence the hematological variables of rainbow trout, which underlines that the dietary supplementation of walnut leaf extract did not negatively affect fish health. However, several previously published studies reported that herbal additives like ginger (*Zingiber officinale*; Haghighi & Rohani 2013), horse mint (*Mentha longifolia*; Heydari et al., 2020) and anthocyanin extract from roselle (*Hibiscus sabdariffa* L calyx; Jomeh et al., 2021) supplements could improve the hematological variables of rainbow trout.

In this study, walnut leaf extract increased the survival rate of rainbow trout after the challenge with *A. salmonicida*. Several similar studies focused on the benefits of Biotronic® Top3 (Menanteau-Ledouble et al., 2017) and curcumin (Yonar et al., 2019) for improving the disease resistance of fish against the challenge of *A. salmonicida*. Moreover, the results in the present study were in agreement with the findings of Yilmaz et al. (2023), who underlined that walnut leaf extract significantly increased the resistance of Nile tilapia challenged with *Plesiomonas shigelloides*.

These findings can be attributed to the antimicrobial, antioxidant and immunomodulatory effects of walnut leaf extract (Sharma et al., 2013). Herein, Yiğit et al. (2009) reported antimicrobial effects of walnut leaf extract against human pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*. Further, Oliveira et al. (2008) evidenced that the ethanolic extract of the leaf parts and green bark were also effective against gram-positive bacteria as *Staphylococcus aureus*, *Basillus cereus*, and *Basillus subtilis*. Moreover, it was found that walnut leaf extract exhibited antifungal activity against the *Candida albicans* and *Candida neoformans* (Pereira et al., 2008) and *C. albicans*, *Candida glabrata*, *Candida tropicalis* and *Candida kefyr* (Yiğit et al., 2009).

Conclusion

In conclusion, in this study, dietary supplementation of walnut leaf extract especially at 750 mg/kg significantly increased disease resistance of rainbow trout after the challenge with *Aeromonas salmonicida* infection without any negative effect on growth performance and hematological parameters. Considering the contradictions among earlier reports on the influence of walnut leaf extracts in fish diets, it might be possible that haematological values in fish can change when treated for a longer period of time or different doses of walnut leaf extract supplementations. Therefore, experiments with longer periods and different incorporation levels of walnut leaf extracts are encouraged in future studies, in order to understand the effect of exposure duration to walnut leaf extract supplemented diets on fish.

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Ethical approval

Fish experiments were performed in accordance with the guidelines for fish research from the animal ethics committee at Canakkale Onsekiz Mart University (Protocol Number: 2018/06-023).

Informed consent

Not available

Data availability statement

The authors declare that data can be provided by corresponding author upon reasonable request.

Conflicts of interest

There is no conflict of interests for publishing this study.
The corresponding author is responsible on behalf of all authors' declaration.

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Contribution of authors
Çağatay BAYİZİT: Conceptualization, Investigation, Methodology, Data source and analysis, Writing original draft
Sebahattin ERGÜN: Conceptualization, Methodology, Resources, Supervision, Validation, Review, Editing
Ayşenil BAYİZİT: Investigation, Methodology, Data source and analysis, Writing original draft
Sevdan YILMAZ: Conceptualization, Investigation, Methodology, Data source and analysis, Writing original draft
Murat YIGIT: Conceptualization, Methodology, Resources, Validation, Review, Editing
All authors have read and agreed to the published version of the manuscript.

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