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Research Article

Therapeutic advantages of gamma irradiated propolis in mitigating the risk of *Ichthyophthirius multifiliis* infection in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

This study investigated the effects of irradiated-propolis (GI-EEP) supplementation on rainbow trout (*Oncorhynchus mykiss*) during an *Ichthyophthirius multifiliis* (Ich) challenge, aiming to determine if it could enhance the fish immune system and improve its response against Ich. In this experiment, 150 fish (35.80±3.95 g) were divided into five groups and fed diets containing gamma-irradiated or non-irradiated propolis extracts or no treatment (control) for 60 days. The fish were then challenged with 5000 *I. multifilis* theronts per fish. Then, five days post-challenge, blood and skin samples were taken for hematological, biochemical, and gene expression analysis. The results showed that 0.5 g kg^{-1} of gamma irradiated- ethanol extract propolis (GI-EEP) supplementation regulated electrolyte levels, improved blood biochemical parameters, and enhanced the immune system's response in Ich-infected rainbow trout. It increased lysozyme activity, elevated white blood cell counts, and improved red blood cell counts. Supplementation with GI-EEP positively influenced these parameters, including increasing total protein and albumin and decreasing globulin levels. It also elevated alkaline phosphatase activity, indicating improved inflammation control. Gene expression analysis revealed increased levels of inflammatory and immune response markers, including interleukin 8, and immunoglobulin M. No significant changes were observed in C3 complement mRNA gene expression levels. Elevated major histocompatibility complex II mRNA levels indicated an effective immune response. Overall, administering IPE to Ich-infected rainbow trout regulated electrolytes, improved blood parameters, and boosted the immune system's ability to combat the infection. Results showed that the optimal propolis dose for regulating physiological parameters and immune responses in Ich-exposed fish was 30 KGy.

Introduction

Propolis is a natural substance made by honeybees that collect resin from various plants. It is a protective sealant for beehives and has gained recognition for its potential health benefits, commonly used as a dietary supplement or in topical applications. Propolis contains over 500 chemical compounds, including polyphenols, flavonoids, and essential oils, contributing to its antioxidant, anti-inflammatory, and antimicrobial properties (Farag *et al.,* 2021). These compounds have demonstrated antimicrobial, antiviral, antioxidant, and anticancer properties. It is effective against bacterial infections, viral infections, and cancer (Abdelmagid *et al.,* 2022).

Moreover, the consumption of propolis can boost the body's immune response. It can promote the production of immune cells, such as white blood cells, which are crucial for fighting illness. Propolis contains bioactive compounds that help regulate and balance the immune system, enhancing its defense against pathogens. Moreover, its antioxidants help reduce oxidative stress and inflammation, further strengthening the immune system (Fernandes Junior *et al.,* 2005; El-Guendouz *et al.,* 2018; Bouchelaghem, 2022).

Propolis exhibits broad-spectrum antimicrobial activity against bacteria, fungi, and viruses. Studies have shown that propolis can inhibit the growth of harmful bacteria such as *Staphylococcus aureus*, *Streptococcus mutans*, and *Helicobacter pylori* (Almuhayawi, 2020). Propolis contains antioxidants, including flavonoids, phenolic acids, and vitamins, which neutralize free radicals and protect cells from oxidative damage (Almuhayawi, 2020). It also has anti-inflammatory properties, inhibiting the production of pro-inflammatory cytokines and enzymes. Propolis regulates the immune system and enhances the activity of immune cells, strengthening the body's defense against infections. Moreover, it promotes wound healing by accelerating tissue regeneration and reducing inflammation (Fernandes Junior *et al.,* 2005; El-Guendouz *et al.,* 2018; Bouchelaghem, 2022). Numerous studies suggest that propolis may possess potential anti-cancer properties; however, additional research is required to substantiate these findings. Furthermore, propolis demonstrates anti-diabetic effects, regulates glucose metabolism, and enhances insulin sensitivity. Additionally, it contributes to cardiovascular health by reducing blood pressure and lowering cholesterol levels (Abdelmagid *et al.,* 2022). The specific biological properties of propolis depend on the plants used by bees to produce it (Talas and Gulhan, 2009). However, different purification methods can play a crucial role in improving the quality of propolis.

Irradiation is a commonly used technique in the food industry to sterilize and improve quality by exposing the food to controlled doses of ionizing radiation, such as gamma rays, X-rays, or electron beams. This process eliminates harmful bacteria, parasites, molds, spoilage organisms, and extends shelf life, while preserving nutritional value (Joshua Ajibola, 2020; Chiozzi *et al.,* 2022).

Besides sterilization, irradiation can reduce harmful chemical and biological pollutants, including pesticides and

contaminants, enhancing food safety (Ekezie *et al.,* 2017). For propolis, gamma radiation eliminates microbial contaminants, including bacteria and fungi, and increases its quality (Heidarieh *et al.,* 2021).

Research has demonstrated that irradiated propolis exhibits superior characteristics compared to non-irradiated propolis. The quality improvement can be attributed to increased bioactive compounds, specifically polyphenols and flavonoids, which significantly enhance antioxidant properties and stimulate the immune system (Kashkooli *et al.,* 2011; Spinelli *et al.,* 2015). Consequently, irradiated propolis enhanced antioxidant and immune-stimulating effects when administered to fish. However, questions remain regarding the effectiveness of irradiated propolis in treating fish infected with different pathogens. Therefore, in the present study, fish were fed irradiated propolis and exposed to the *Ichthyophthirius multifiliis* to address this issue.

Ich (*I. multifiliis*), a ciliated protozoan parasite, is a major pathogen in freshwater aquaculture, causing significant mortality in fish species worldwide. Yang *et al.* (2023) conducted a comprehensive study on the genetic diversity of *I. multifiliis* isolates in China, identifying three distinct genetic groups and noting frequent cross-border interactions with isolates from the U.S. and Turkey, indicating a global distribution and potential for genetic exchange. It has a complex life cycle, with a free-swimming theronts stage that invades the fish and feeds on its skin and mucus. They eventually detach from the fish and enter the encysted stage, forming white cysts that are visible to the naked eye. These cysts fall to the bottom of the water and divide into multiple daughter cells called tomites. Infected fish show white spots on their skin, fins, and gills, irritation, scratching, rapid breathing, and loss of appetite. In response to *I. multifiliis* infection, freshwater fish activate both inflammatory and adaptive immune defenses (Buchmann, 2020; Shiota *et al.,* 2021). Zhou *et al.* (2024) reported that vaccination significantly increased serum IgM levels and C3 content, indicating that koi carp exhibited an immune response to *I. multifiliis* infection. Von Gersdorff Jørgensen (2016) observed a robust innate immune response in zebrafish to *I. multifiliis*, with a 3.4-fold neutrophil increase at infection sites within 24 hours, followed by a decline over 72 hours. Moreover, changes in key immune-related genes, including IgM, IgD, cytokines (IL-1βa, IL-1βb, IFN-γ, and TNF-α), immune cell receptors (CD4, CD8-α, MHC I, MHC II β, TcR-α, and TcR-β), and complement factors after challenge with theront and trophont antigens were reported in different fish species (Xu *et al.,* 2016; Buchmann, 2020; Shiota *et al.,* 2021).

However, Li *et al.* (2022) and Qin *et al.* (2024) reported *I. multifiliis* infection in *Schizothorax macropogon*, *Schizopygopsis selincuoensis*, *Triplophysa tibetana*, *T. brevicauda*, and *S. thermalis*, causing significant mortality. According to Pasqualetti *et al.* (2021) established a link between *I. multifiliis* and the suspected causative agent of Red Mark Syndrome (RMS-MLO) in farmed rainbow trout (*Oncorhynchus mykiss*), indicating

potential co-infections that could complicate treatment strategies. Nguyen *et al.* (2020) demonstrated a synergistic effect between *I. multifiliis* and the bacterium *Francisella noatunensis subsp. orientalis* (Fno), which increased mortality in hybrid red tilapia (*Oreochromis* sp.), suggesting that understanding the interactions between various pathogens is essential for comprehensive management strategies in aquaculture. Infection due to *I. multifiliis* can negatively influence the osmotic regulation system in fish. Ich spreads through infected fish, water, or contaminated equipment. Treatment includes raising the water temperature and using medication to target its life stages. Improving water quality, disinfecting water, and boosting the fish's immune system can be taken to avoid fish getting infected by the parasite. Preventive measures include quarantining new fish, maintaining water quality, and disinfecting equipment to prevent re-infection (Jafar *et al.,* 2020; Buchmann *et al.,* 2022). Although potassium permanganate, formalin, and copper sulfate efficiently treat *I. multifiliis*, their use can threaten aquatic animals and compromise food safety due to their potential to accumulate in the environment and edible tissues (Garcia *et al.,* 2007; Rahanandeh, 2020).

This study aimed to evaluate some biochemical and immunological parameters of rainbow trout fed with irradiated propolis in the challenge with *I. multifiliis*. This study hypothesizes feeding fish with irradiated propolis can boost the immune system. Moreover, irradiated propolis could be an effective treatment for fish infected with *I. multifiliis*, as it has stronger antioxidant and immune-stimulating properties than non-irradiated propolis. Therefore, after a challenge with *I. multifiliis*, it may respond more appropriately to its pathogenicity.

Materials and methods

Fish

Healthy rainbow trout (*Oncorhynchus mykiss*) were obtained from a local fish farm, Karaj, Iran, and transferred to the Nuclear Science and Technology Research Institute, Karaj, Iran. Before testing, fish health was assessed through visual inspection for physical abnormalities, behavioral observation, disease screening, and even quarantine conditions. Then, fish were temporarily kept in 1000 L fiberglass tanks equipped with aerators (temperature: 15±2°C; pH: 7.2±0.2; dissolved oxygen: 6±1 mg L^{-1}; 80% water exchange rate per day) and fed a formulated diet (Beyza Feed Mill, Shiraz, Iran) for two weeks before the experiment (Wedemeyer, 1996).

Preparation of propolis: Extraction and Irradiation Process

Propolis specimens were sourced from a local apiarist in Marvdasht-farm, Fars, Iran, and subsequently cryogenically preserved utilizing liquid nitrogen (Heidarieh *et al.,* 2021). Then, 100 grams of propolis were ground and mixed with 95% (v/v) ethyl alcohol at a 1:20 ratio in a sealed glass container. The mixture underwent a seven-day infusion at 37°C, incorporating periodic agitation as specified in the study by de Lima *et al.* (2022). Afterward, the solution was filtered, and the residue was re-mixed with 70% ethanol for two additional days. The resulting ethanol

extract was filtered with Whatman filter paper no. 4, and the ethanol was removed by vacuum evaporation. The purified propolis was freeze-dried and stored at -20°C in a sealed container, following the protocol described by Rocha *et al.* (2022).

The ethanol-extracted propolis (EEP) was irradiated using a gamma cell 220 at a radionuclide ^{60}Co source with dose rate of 1.02 Gy sec^{-1} in the presence of O_2 at room temperature in Nuclear Science and Technology Research Institute, Tehran, Iran. Three irradiation doses were applied to the EEP: 10 kGy and 30 kGy. After the irradiation, the samples were stored at 4°C for additional experiments (Heidarieh *et al.,* 2021).

Diet preparation

0.5 grams of GI-EEP (IEP; 0, 10, and 30 KGy) per kilogram of powdered food were combined. The mixture was then blended with 1 milliliter of distilled water per gram to achieve a consistent blend. The dough was then processed through a meat grinder, creating an extruded string, which was dried in an oven at 55°C for 12 hours. Once dried, the strings were broken into pellets approximately 10 millimeters in length. The pellets were deposited into a hermetically sealed container and maintained at a temperature of -20°C for preservation until required for further use. The control diet followed the same process but without any supplements.

Experimental design

To conduct the experiment, 150 fish (average weight: 35.80 ± 3.95 g; average length: 15.27 ± 1.80 cm) were divided into five separate groups, each with three sets (30 fish per treatment in three replicates of ten fish per tank). Before the trial, the fish were given unrestricted access to a commercial rainbow trout diet for at least two weeks.

After the initial feeding period, the fish were fed diets containing different levels of gamma-irradiated and non-gamma-irradiated propolis extract for 60 days. A control group was also included that received no treatment. Group 1 served as the positive control group, where the fish remained untreated throughout the experiment, representing healthy fish. Group 2 served as the negative control group, where the fish were fed a commercial diet and challenged only with *Ichthyophthirius multifiliis* (*I. multifilis*) after 60 days. Group 3 consisted of fish nourished with non-irradiated propolis extract and then challenged only with *I. multifilis* after 60 days. Group 4 included fish exposed to irradiated propolis extract treated with a gamma-ray dose of 10 kGy, and then challenged only with *I. multifilis* after 60 days. In Group 5, the fish were fed propolis extract that had undergone gamma-ray irradiation at a dosage of 30 kGy, and then challenged only with *I. multifilis* after 60 days.

Exposure fish to I. multifilis infection

An *I. multifilis*-infected goldfish were maintained in a 50-L glass aquarium equipped with an air stone under static conditions. Ten healthy fish were introduced to the aquarium to facilitate disease transmission. Deceased infected fish were then placed in a separate aquarium for 4 hours to allow the trophonts to develop into tomonts and detach. The

dead fish were then removed, and the tomonts were left overnight to develop into tomocysts and subsequently transform into infective theronts (Jorgensen *et al.,* 2018). The next day, the water containing theronts was centrifuged at 1500 rpm for 5 minutes, and the theronts were counted using a dissection microscope. The theronts density was expressed as the number per mL (Fu *et al.,* 2017).

Based on McCallum's 1986 study, the theront concentration of 5000 per fish was required to achieve 100 % infection prevalence. To simulate this infestation, a dose of 5000 theronts per fish was added to each tank. To enhance parasite exposure, the water in each tank was replaced with aerated tap water on the second day, and the fish were fed with treatment diets for 60 days. On day 60, groups 2-5 were exposed to live theronts.

Sampling

On the fifth day after the challenge, fish were euthanized using clove oil (50 µL L^{-1}), and blood was sampled from the caudal vein using 2.5 mL syringes. The blood from six fish specimens in each tank was pooled together for analysis. Half of each blood sample was immediately transferred to heparin-coated Eppendorf tubes for hematological examination, while the other half was centrifuged at 1500 g for 15 minutes after being kept on ice for two hours to separate the serum. The serum samples were stored at -80°C for future analysis (Banaee *et al.,* 2022). Furthermore, a 1.5 cm^2 section of fish skin from the right dorsal side was dissected and preserved in liquid nitrogen for real-time PCR analysis. This analysis aimed to investigate the effects of parasitic infection on hematological indices and immune-relevant gene expression.

Hematology indices analysis

After blood sampling, hematocrit values (Ht) were determined by placing fresh blood in capillary glass tubes, which were then centrifuged for 5 minutes in a microhematocrit centrifuge. The blood samples were diluted with Riss fluid to count the red blood cells (RBCs) and white blood cells (WBCs). The diluted samples were then observed under a light microscope (M2015T-KE, 1600X, China) using a Neubauer hemocytometer to count the cells accurately (Gholamhosseini *et al.,* 2020).

Biochemical parameters analysis

For alkaline phosphatase activity, an equal volume of plasma was mixed with a four mM para-nitrophenyl phosphate solution in a 100 mM ammonium bicarbonate buffer and one mM $MgCl_2$ (pH 7.8 at 30°C). Absorbance was recorded at 405 nm using an ELISA reader over 2 hours. Enzyme activity was quantified as the release of 1 µmol of para-nitrophenyl product per minute, defining one unit of activity (Banaee *et al.,* 2022). Total protein concentration was measured using the biuret method. Samples were diluted, and protein standards were prepared. Biuret reagent was created with copper sulfate and potassium sodium tartrate, then added to standards, samples, and a blank in separate test tubes. After incubation, absorbance was measured at 540 nm using a spectrophotometer. A standard curve was created, allowing protein concentrations to

be determined based on absorbance values. Albumin concentration was measured using the Bromocresol Green (BCG) Method by mixing the sample with a BCG reagent, which forms a green-colored complex with albumin. Absorbance was measured at 540 nm, and albumin concentration was determined by comparing sample absorbance against a standard curve. Globulin concentration was calculated by subtracting the albumin value from the total protein. The serum glucose content was measured using an oxidase-peroxidase reaction at 505 nm using a Parsazmun biochemistry kit. Creatinine levels were assessed according to the Jaffe reaction method using picric acid at 520 nm (Shui *et al.,* 2018; Gholamhosseini *et al.,* 2020).

Calcium levels were determined using the cresolphthalein complexone method at 570 nm. Phosphorous levels were measured using the pyridyl bisphenyl triazine sulphonate method at 560 nm, and magnesium ions were determined by measuring the absorbance of the magnesium-xylidyl blue complex at 510 nm. Sodium (Na^+) and potassium (K^+) levels were assessed using a flame photometer (Jenway PFP 7, England), while chloride (Cl^-) levels were determined by silver nitrate titration following Mohr's method. Lysozyme activity was measured using a turbidimetric assay with a suspension of *Micrococcus lysodeikticus* in a buffer at 450 nm. The decrease in turbidity or optical density is observed over time. To determine the lysozyme activity, the rate of absorbance decrease is analyzed by comparing it to a standard curve generated from known lysozyme concentrations (Shui *et al.,* 2018; Adel *et al.,* 2021).

Gene expression analysis

Total RNA extracted from skin samples using a combination of AccuZol® reagents (Bioneer, South Korea). Samples mechanically disrupted in 1 mL of AccuZol® reagent using a disruption pestle. Then, 200 µL of chloroform were added, and the suspension was centrifuged at 12000 x g for 15 minutes. The clear upper phase was recovered, mixed with an equal volume of 100% ethanol and immediately transferred to RNeasy Mini kit columns. RNA quantity was assessed using a Bio-Rad spectrophotometer (Bio-Rad, CA, USA) at 260 nm, and RNA purity was determined by measuring optical density 260:280 nm which ranged from 1.90 to 2.08. The procedure was continued following the manufacturer's instructions, performing on-column DNase treatment. Finally, the RNA was eluted from the columns in RNase-free water and stored at -80°C until used. One µg of RNA was used to obtain cDNA in each sample using the Bio script reverse transcriptase (Bioline Reagents Ltd) and oligo (dT) 12-18 (0.5 $\mu g.mL^{-1}$) following the manufacturer´s instructions. The resulting cDNA was diluted in a 1:4 proportion with water and stored at -80°C. To evaluate the expression levels of selected immune-relevant genes, a real-time PCR assay was performed in a LightCycler® 480 System instrument (Roche) using SYBR Green PCR core Reagents (Applied Biosystems) and specific primers previously optimized (Table 1). The primers were designed based on the conserved regions of the Goldfish Gene Bank sequences, using Oligo7. QPCR efficiency was also considered when choosing the best qPCR primer pair with specific and correct sizes to validate primers. Gene expressions were standardized using β-actin, which served as a reference gene.

Table 1: List of primers used for assessing the expression of immune-related genes.

Genes	Primer	Accession no.	Size of amplicon (bp)
MHC-II	Fwd.: 5'-ATGTCGATGCCAATTGCCTTCTA-3' Rev.: 5'-TGTCTTGTCCAGTATGGCGCT-3'	U20943	236
IgM	Fwd.: 5'-TGCGTGTTTGAGAACAAAGC-3' Rev.: 5'-GACGGCTCGATGATCGTAAT-3'	AH014877.2	107
C3	Fwd.: 5'-AGCTTGCTGACTGGCTTTGT-3' Rev.: 5'-TCATAAACGGTGACCCCAAC-3'	AM773828	227
IL-8	Fwd.: 5'-GAATGTCAGCCAGCCTTGTC-3' Rev.: 5'-TCCAGACAAATCTCCTGACCG-3'	AJ279069	162
β-actin	Fwd.: 5'-GTCACCAACTGGGACGACA-3' Rev.: 5'-GTACATGGCAGGGGTGTTGA-3'		174

Each sample was measured under the following conditions: 10 min at 95°C, followed by 45 amplification cycles (15 s at 95°C and 1 min at 60°C). The expression of individual genes was normalized to the relative expression of trout EF-1α. The expression levels calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔCt is determined by subtracting the $EF^{-1}\alpha$ value from the target Ct (Sheikhzadeh *et al.,* 2021; Banaee *et al.,* 2023). Negative controls with no template were included in all the experiments. A melting curve for Each PCR was determined by reading fluorescence at every degree between 60°C and 95°C to ensure that only a single product had been amplified.

Statistical analysis

All experimental data were presented as the mean±SE. After testing the normality of the data and variance homogeneity, the significance of differences was assessed using the one-way analysis of variance (ANOVA) and Tukey post hoc test in the SPSS software version 19.0. The P values of 0.05 or less were considered statistically significant.

Results

Fish fed with irradiated-propolis (GI-EEP) supplement and exposed to *Ichthyophthirius multifiliis*, did not show any instances of death. The study noted that the fish treated with EEP exhibited a significant decrease in harm and skin injuries caused by infection when compared to the infected group that did not receive any treatment.

Fish exposed to parasites and fed with irradiated propolis (10 and 30 KGy) had regulated blood sodium, potassium, and magnesium levels. The findings indicated that propolis irradiated at a dosage of 30 KGy affected chloride levels in fish exposed to Ich. Significant changes were observed in phosphorus levels in fish exposed to Ich and fed irradiated propolis.

Creatinine and glucose levels increased in fish infected with Ich. Results showed that the administration of irradiated propolis did not adjust glucose levels in infected fish. However, feeding fish with irradiated propolis supplement at 30 KGy regulated creatinine levels in fish exposed to Ich.

The highest level of total protein was observed in fish exposed to Ich. The administration of irradiated propolis

decreased the total protein levels in the plasma of infected fish. Nonetheless, the administration of irradiated propolis at a dosage of 30 KGy restored total protein levels in fish that were exposed to Ich. No significant difference was observed in the levels of albumin and globulins in fish exposed to Ich, regardless of their consumption of irradiated propolis.

The activity of alkaline phosphatase (ALP) increased in fish exposed to Ich and consumed irradiated propolis.

There was no significant difference in the red blood cell (RBC) count between the fish exposed to Ich and the control group. However, when fish exposed to Ich were administered irradiated propolis at dosages of 10 and 30 KGy, their RBC counts increased. Moreover, exposure to Ich combined with the consumption of irradiated propolis at various dosages led to an increase in white blood cell (WBC) counts. No significant difference were observed in the hematocrit (Hct) levels between the fish exposed to Ich and the control group, regardless of their consumption of irradiated propolis (Table 2).

Table 2: The effects of irradiated propolis supplements on the biochemical and hematological parameters of fish exposed to *I. multifiliis.*

Biochemical and hematological parameters	Treatments				
	Control	Ich	Ich-P (0 KGy)	Ich-P (10 KGy)	Ich-P (30 KGy)
Sodium (mg dL^{-1})	117.4 ± 4.1^{b}	98.4 ± 3.4^{a}	96.8 ± 3.3^{a}	110.25 ± 3.7^{b}	109.9 ± 4.3^{b}
Chloride (mg dL^{-1})	109.7 ± 2.1^{c}	86.0 ± 3.4^{a}	81.0 ± 3.0^{a}	96.3 ± 2.1^{b}	104.5 ± 2.7^{c}
Potassium (mg dL^{-1})	5.4 ± 3.4^{b}	5.3 ± 0.05^{a}	5.3 ± 0.03^{a}	5.5 ± 0.04^{b}	5.4 ± 0.05^{b}
Calcium (mg dL^{-1})	12.1 ± 0.4^{b}	11.3 ± 0.7^{a}	11.7 ± 0.6^{ab}	11.6 ± 0.6^{ab}	12.2 ± 0.4^{b}
Magnesium (mg dL^{-1})	7.5 ± 1.1^{b}	5.1 ± 1.0^{a}	5.5 ± 1.0^{a}	7 ± 1.5^{b}	7.0 ± 2.2^{b}
Phosphorus (mg dL^{-1})	9.8 ± 1.7^{b}	11.3 ± 2.3^{d}	10.4 ± 2.0^{c}	8.9 ± 1.5^{a}	11.1 ± 2.4^{d}
Glucose (mg L^{-1})	45.3 ± 1.7^{a}	69.8 ± 2.2^{e}	63.2 ± 1.3^{d}	60.8 ± 1.1^{c}	57.1 ± 1.1^{b}
Creatinine (mg L^{-1})	1.29 ± 0.03^{a}	1.59 ± 0.05^{d}	1.46 ± 0.07^{c}	1.38 ± 0.07^{b}	1.28 ± 0.05^{a}
Total protein (g dL^{-1})	4.5 ± 0.2^{a}	5.1 ± 0.2^{c}	4.8 ± 0.2^{b}	4.8 ± 0.2^{b}	4.5 ± 0.2^{a}
Albumin (g dL^{-1})	1.8 ± 1.2^{a}	3.0 ± 1.2^{a}	2.6 ± 1.1^{a}	2.5 ± 1.0^{a}	2.5 ± 1.2^{a}
Globulin (g dL^{-1})	2.7 ± 1^{a}	2.1 ± 1^{a}	2.2 ± 0.9^{a}	2.2 ± 0.9^{a}	2.0 ± 0.9^{a}
Alkaline phosphatase (U L^{-1})	98.3 ± 11.1^{a}	176.3 ± 10.2^{e}	153.4 ± 10.3^{d}	111.5 ± 9.6^{b}	128.8 ± 9.3^{c}
Lysozyme (U L^{-1})	26.7 ± 2.1^{b}	15.9 ± 1.9^{a}	27.1 ± 3.1^{bc}	32.7 ± 2.4^{c}	25.1 ± 1.7^{b}
RBC ($\times 10^{6}$ cell mL^{-1})	1.78 ± 0.5^{ab}	1.73 ± 0.1^{a}	1.82 ± 0.1^{ab}	1.96 ± 0.1^{b}	1.85 ± 0.1^{b}
WBC ($\times 10^{3}$ cell mL^{-1})	26.7 ± 0.2^{a}	42.0 ± 0.1^{c}	35.0 ± 0.1^{b}	33.0 ± 0.1^{b}	40.0 ± 0.1^{c}
Hematocrite (Hct) %	36.5 ± 2.1^{a}	36.3 ± 1.7^{a}	37.3 ± 1.1^{a}	37.5 ± 2.7^{a}	37.0 ± 3.2^{a}

The findings indicated there were no significant changes in IL-8 mRNA levels following the exposure of rainbow trout to Ich. However, fish fed EEP and subsequently challenged with Ich exhibited significantly higher IL-8 levels than the control group. Although no significant changes were detected in the gene expression of C3 complement in fish exposed solely to Ich, an up-regulation of C3 complement gene expression was observed in fish fed irradiated propolis. Exposure to Ich led to an increase in IgM mRNA levels. Furthermore, the gene expression of IgM in fish fed with GI-EEP and subsequently challenged with Ich exhibited a substantial increase compared to fish treated solely with Ich. The

significant increase in MCH-II gene expression observed in fish exposed to Ich suggested an elevated localized inflammation response. Furthermore, fish fed GI-EEP and subsequently challenged with Ich exhibited significantly higher levels of MCH-II gene expression compared to the referenced fish. This result indicated that the combination of feeding with GI-EEP and exposure to Ich increased the immune response characterized by the increased MCH-II gene expression (Fig. 1).

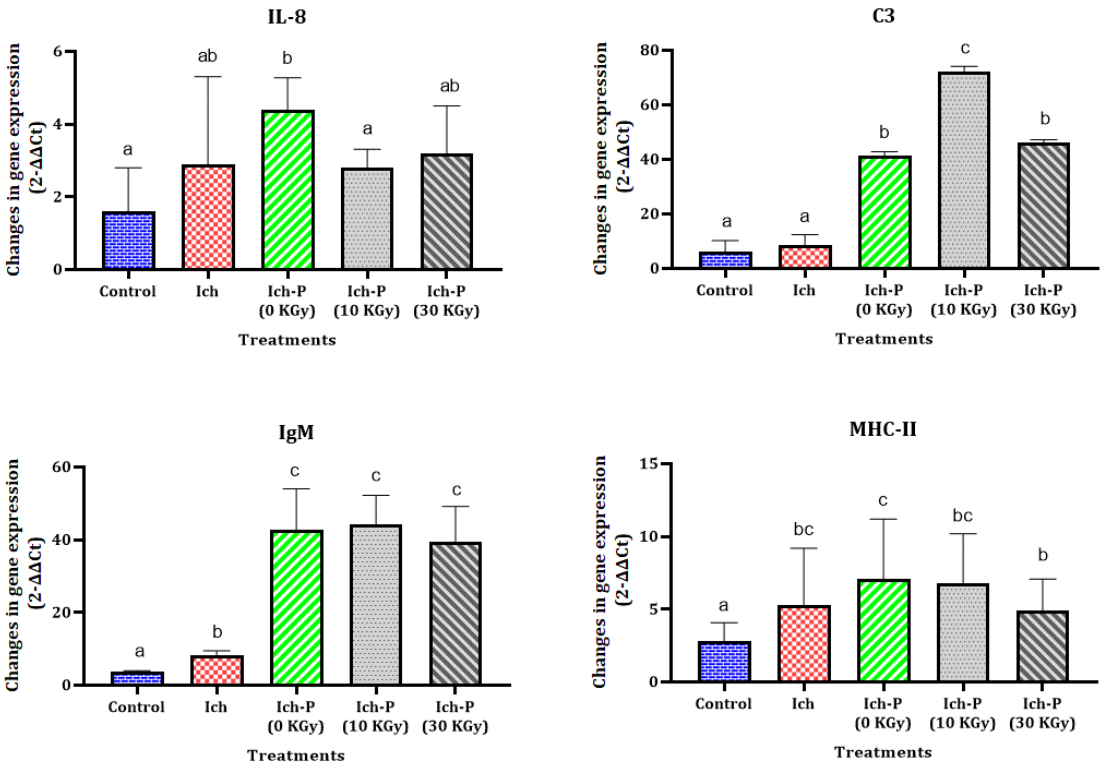

Figure 1: The effects of irradiated propolis supplements on the gene expression related to the immune system of fish exposed to *I. multifiliis*

Discussion

Ich (*I. multifiliis*) infection significantly affects fish's immune system, triggering an inflammatory response that causes tissue damage and activates immune cells. Fish initiate a cellular immune reaction involving various immune cells to eliminate the parasites. Moreover, they develop an adaptive immune response, potentially inducing resistance through antibodies and memory cells. However, the infection can lead to immune suppression, making fish susceptible to secondary infections, and the parasites may evade the immune response (Buchmann *et al*., 2022; Huang *et al.,* 2022; Gholamhosseini *et al.,* 2023), and clinical signs, including physical and behavioral indicators observed during sampling. The degree of harm and skin injuries observed in the treated fish with IEP was noticeably decreased compared to the infected group. These results demonstrated that propolis effectively treated Ich-infected fish. The study by Qu *et al.* (2024) demonstrated the effectiveness of catechol compounds—

quercetin, luteolin, caffeic acid, and chlorogenic acid—as antiparasitic agents against *I. multifiliis*, a significant pathogen affecting aquaculture. Moreover, Fu *et al.* (2021) found that extracts from *Cynanchum atratum*, *Psoralea corylifolia*, *Zingiber officinale*, *Sophora flavescens*, *Homalomena occulta*, *Curcuma longa*, and *Morus alba* show antiparasitic properties against *I. multifiliis*. Kumar *et al.* (2022) found that *Curcuma longa* (turmeric) essential oil significantly increased the survival of *Pangasianodon hypophthalmus* against co-infections with *I. multifiliis* and *Aeromonas hydrophila* by improving anti-stress and antioxidative responses. Fu *et al.* (2022) highlighted the efficacy of sophoraflavanone G (SG), an active compound extracted from *Sophora flavescens*, in combating the theronts of *I. multifiliis*.

Reduced blood sodium levels in infected fish may result from increased skin permeability and osmotic imbalances. Damage to the skin and histopathological alterations in the gills following experimental infection with *I. multifiliis* can disrupt the osmotic pressure of biological fluids, potentially leading to hyponatremia (Garcia *et al.,* 2007; Rahanandeh, 2020). The symptoms that manifest in infected fish, including loss of energy, restlessness, irritability, lethargy, and muscle spasms, may correlate with diminished blood sodium levels.

Although the provision of essential extracted proteins (EEP) did not significantly alter sodium levels in fish, the administration of gamma-irradiated EEP (GI-EEP) at doses of 10 and 30 KGy demonstrated the ability to restore blood sodium levels to baseline values. Furthermore, the gamma-ray treatment of EEP appears to enhance the bioavailability of sodium and improve its therapeutic efficacy in mitigating injuries associated with Ich. A significant decrease in chloride (Cl^-) levels was observed in the blood of fish exposed to *I. multifiliis*, indicating an osmotic imbalance. Cl^- ions play a crucial role in acid-base balance. Therefore, reduced blood Cl^- levels could affect the relationship between blood Na^+ and Cl^- levels, any Na^+ fluctuations can impact Cl^- levels. The regulation of Cl^- in the blood of fish fed GI-EEP could enhance the protective properties of EEP in reducing *I. multifiliis* virulence. Potassium (K^+) is an essential electrolyte that supports cell, muscle, and nerve functions (Garcia *et al.,* 2007; Rahanandeh, 2020; Huang *et al.,* 2022).

Results showed that the experimental infection of fish with *I. multifiliis* decreased blood K^+ levels. Declined K^+ levels, or hypokalemia may also be associated with increased infected fish's skin permeability. Furthermore, hypokalemia may also be significantly correlated with reduced blood magnesium levels. The slight decrease in K^+ levels could be due to gastrointestinal dysfunction, impaired osmoregulation, and adrenal dysfunction. A severe decline in blood K^+ levels can lead to lethargy, muscle cramps, and heart failure. Thus, inactivity and lethargy observed in infected fish may be related to a significant reduction in K^+. The results demonstrated that oral administration of gamma-irradiated-EEP (10 and 30 KGy) could adjust K^+ levels in the blood of infected fish with *I. multifiliis*. Previous studies have shown that the purity

and quality of antioxidants in EEP increased after gamma radiation treatment (Heidarieh *et al.,* 2021). This may explain the regulation of K^+ levels in fish fed GI-EEP, likely due to increased cell membrane stability.

Magnesium is one of the most abundant essential minerals in fish, playing a role in over 300 metabolic reactions, including protein synthesis, production and storage of cellular energy, cell stabilization, DNA synthesis, the transmission of nerve signals, bone metabolism, regulation of heart function, glucose metabolism, and regulates osmotic blood pressure. Therefore, a reduction in blood Mg levels could lead to various physiological disorders in infected fish with parasitic (Garcia *et al.,* 2007; Rahanandeh, 2020; Huang *et al.,* 2022). For example, the lethargy and anorexia observed in fish infected with *I. multifiliis* may be associated with low blood Mg^{+2} levels. Although administration of non-irradiated EEP (0 kGy) showed no significant effect on regulating blood Mg levels, GI-EEP (10 and 30 KGy) restored Mg^{+2} concentrations to normal levels in infected fish. Mg^{+2} is one of the essential elements in propolis. However, its concentration depends on the specific extracts and nectar of plants collected by bees. The results indicated that oral administration of GI-EEP could regulate Mg^{+2} content in the blood of fish exposed to *I. multifiliis*.

Although experimental infection of fish with *I. multifiliis* significantly reduced blood calcium (Ca^{+2}) levels, feeding fish with EEP could regulate Ca^{+2} levels, with the best result obtained in the fish fed with GI-EEP at 30 KGy. A significant increase in blood phosphorus was observed in fish challenged with *I. multifiliis*, which can negatively affect Ca^{+2} levels. Oral administration of GI-EEP (at 10 KGy) helped regulate phosphorus levels in these fish.

The experimental infection also led to a significant increase in blood glucose which can serve as a bio-indicator of stress in fish. Elevated glucose levels in fish exposed to Ich may be attributed to stress response, inflammation or infection, hormonal imbalance, and increased energy demand. Factors such as stress hormones, inflammatory signals, disrupted glucose metabolism, and the need for energy during immune responses and tissue repair can contribute to elevated glucose levels (Garcia *et al.,* 2007).

Creatinine levels in treated fish with *I. multifiliis* were significantly higher than in the control group. The results indicated that dietary supplementation with GI-EEP could help regulate blood creatinine levels. Increased blood creatinine levels in fish may be associated with kidney dysfunction, muscle damage, dehydration, or exposure to medications or toxins. Impaired kidney function, muscle breakdown, fluid imbalance, and renal toxicity can all contribute to elevated creatinine levels in the bloodstream. It is essential to consider other clinical signs and perform further diagnostic tests to accurately identify the underlying cause and determine appropriate treatment options for affected fish (Adel *et al.,* 2021).

Elevated total protein in fish indicated that *I. multifiliis* may stimulate protein synthesis or alter protein metabolism in affected fish (Gholamhosseini *et al.,* 2023). This

response could be to the infection and the subsequent need for tissue repair or immune system activation. The findings indicated that the fish given the IG-EEP supplement and exposed to Ich had a significantly lower total protein level than those exposed to Ich without any treatment. This reduction in total protein levels demonstrated that IG-EEP had a protective effect on fish exposed to Ich. However, the protective effectiveness of both EEP and 10 KGy IG-EEP in regulating total protein was considerably less than that of 30 KGy IG-EEP. Interestingly, the administration of irradiated propolis at a dosage of 30 KGy resulted in the restoration of total protein levels in fish exposed to Ich. This suggests that the propolis treatment at this specific dosage had a positive effect on protein synthesis or improved the overall health status of the fish, leading to the restoration of protein levels.

No significant differences were noted in the levels of albumin and globulins in fish exposed to *I. multifiliis*, regardless of whether they consumed irradiated propolis. This indicates that Ich infestation and propolis treatment did not significantly impact these specific protein fractions in the studied fish.

The results showed that experimental infection of fish with *I. multifiliis* led to increased alkaline phosphatase activity. Presbitero *et al.* (2018) found that when fish challenge with pathogens, ALP could prevent inflammation by dephosphorylating inflammatory triggers (ITMs) such as bacterial lipopolysaccharides and extracellular nucleotides (Presbitero *et al.,* 2018; Gholamhosseini *et al.,* 2023). Therefore, increased ALP activity in infected fish may relate to its role in controlling inflammation. Furthermore, ALP is a transmembrane enzyme, and any damage to the cell membrane can lead to changes in ALP activity. The increase in ALP in the blood may be due to its release following damage to the cell membrane (Banaee, 2020).

Buchmann (2020) and Shiota *et al.* (2021) found that freshwater fish activate both inflammatory and adaptive immune defenses in response to infection by *I. multifiliis*. Exposure of fish to *I. multifiliis* decreased lysozyme activity, whereas oral administration of GI-EEP could increase lysozyme activity in the serum of rainbow trout challenged with *I. multifiliis*. Increased lysozyme activity may enhance the fish's immune system's ability to combat the pathogenicity of *I. multifiliis*. A significant decrease in lysozyme activity could indicate a reduced immune response or impaired bacterial clearance. Moreover, the physical damage caused by Ich on fish tissues, such as skin and gills, can directly affect lysozyme production by disrupting normal cellular processes (Jorgensen *et al.,* 2018).

The results showed that fish exposure to *I. multifiliis* could significantly increase white blood cells (WBC) counts. The increased leukocyte counts in fish challenged with *I. multifiliis* indicated that the fish's immune system was stimulated. Changes in blood cells, including neutrophils, were observed in zebrafish following infection with *I. multifiliis* (Von Gersdorff Jørgensen, 2016).

A significant decrease in red blood cell (RBC) counts was observed due to the

experimental infection of fish with parasites. However, the administration of EEP increased the number of erythrocytes. However, the increase in the erythrocytes in fish fed EEP to fish exposed to the parasite could indicate the effect of EEP on increasing the biosynthesis of RBC in hematopoietic tissues. Furthermore, phenolic and antioxidant compounds in EEP may increase the half-life of RBC. Also, the ionic compounds of the EEP may play a key role in inhibiting cutaneous bleeding in fish infected with the *I. multifiliis*. There is no significant difference in the Hct% between different groups of fish.

Studies showed that pathogens could indeed suppress the immune system of fish by altering the gene expression of proteins and enzymes involved in the immune response (Elumalai *et al.,* 2021; Huang *et al.,* 2022). In response to theronts and trophonts of *I. multifiliis*, there may be an increase in the expression of immune-related genes, such as IgM, IgD, cytokines, immune cell receptors, and complement factors in fish (Xu *et al.,* 2016; Buchmann, 2020; Heidarieh *et al.,* 2021b; Shiota *et al.,* 2021).

The results indicated that the exposure of rainbow trout to *I. multifiliis* did not result in significant changes in IL-8 mRNA levels. However, when fish were fed EEP and subsequently challenged with *I. multifiliis*, it had a notable effect on the gene expression of IL-8 compared to the control group. Interleukin 8 (IL-8) is a chemoattractant cytokine synthesized by various tissues and leukocytes in response to inflammation. Increased levels of IL-8 secretion can activate neutrophils and stimulate them to migrate to the site of inflammation. In response to IL-8, neutrophils undergo intracellular changes and release granular enzymes to oppose the inflammatory agent (Garcia-Castillo *et al.,* 2002; Wang *et al.,* 2017). The absence of IL-8 upregulation did not necessarily mean the fish is defenseless against *I. multifiliis*. Other immune pathways or components might have been activated to counteract the parasites. The fish might rely on different chemokines or cytokines to recruit immune cells and combat ectoparasites such as *I. multifiliis*.

Results displayed no significant difference between C3 complement gene expression rate in fish challenged with *I. multifiliis* and the control group. However, feeding fish with GI-EEP increased the gene expression of C3 complement in fish exposed to *I. multifiliis*. The C3 complement is a crucial component of the immune system in fish and plays a role in the recognition and elimination of pathogens. The findings indicated that when fish were given propolis, their immune system was stimulated in response to *I. multifiliis* exposure. This stimulation resulted in alterations in gene expression, including the C3 complement gene. The increased expression of the C3 complement may contribute to an improved immune response in fish. This protein plays a critical role in opsonization, phagocytosis, and the formation of the membrane attack complex, all of which help to eliminate pathogens (Li *et al.,* 2013; Heidarieh *et al.,* 2015; Wang *et al.,* 2017). Therefore, the upregulation of the C3 complement gene expression signifies an active immune response and suggests that the fish are mounting a

defense mechanism against potential threats.

Exposure fish to *I. multifiliis* increased IgM mRNA levels. The gene expression of IgM in fish fed with GI-EEP and challenged with *I. multifiliis* was significantly higher than those in treated fish with *I. multifiliis* only. The findings indicated that when fish were exposed to Ich, there was an increase in the expression of IgM genes, suggesting activation of the fish's immune response to the pathogen. This result implied that the fish's immune system detected the presence of Ich and initiated a defense mechanism by boosting IgM gene expression. The upregulation of IgM gene expression following exposure to Ich indicated that the fish's immune system was responding appropriately against the parasite (Garcia-Castillo *et al.,* 2002; Gonzalez *et al.,* 2007; Li *et al.,* 2013; Heidarieh *et al.,* 2015; Moreira *et al.,* 2017; Wang *et al.,* 2017; Chen *et al.,* 2018; Kumari *et al.,* 2019; Elumalai *et al.,* 2021). IgM antibodies are significant in the initial stages of immune responses and play a crucial role in identifying and neutralizing pathogens like *I. multifiliis*. When fish encounter Ich, their immune cells recognize specific antigens associated with the pathogen, leading to a series of events that result in heightened expression of IgM genes.

A significant increase in MCH-II gene expression in the fish exposed to *I. multifiliis* may indicate increased localized inflammation. The MCH-II gene expression in the fish fed with GI-EEP and challenged with *I. multifiliis* was significantly higher than MCH-II in control fish. The MHC-II mRNA levels can be upregulated in response to various stimuli, including infections, inflammatory signals, or immune stimulants. A significant increase in MCH-II mRNA levels indicates an upregulation of major histocompatibility complex class II (MHC-II) gene expression. MHC-II molecules play a crucial role in the immune system by presenting antigens to T cells, initiating immune responses, and regulating adaptive immunity (Garcia-Castillo *et al.,* 2002; Wang *et al.,* 2017). In the context of *I. multifiliis* infection, the increased MCH-II gene expression indicated an enhanced ability to present antigens associated with the parasite to T cells. Therefore, an increase in MCH-II in fish may play a role in the effective response to *I. multifiliis* infection. MHC-II molecules provide the appropriate peptides that are essential for the overall functioning of the immune system. MHC-II regulates the activity of T helper cells ($CD4^+$) and triggers B cells in response to localized inflammation (Gonzalez *et al.,* 2007; Moreira *et al.,* 2017; Chen *et al.,* 2018; Kumari *et al.,* 2019). Therefore, in the presence of localized inflammation caused by *I. multifiliis*, MCH-II expression might help trigger B cells, leading to the production of specific antibodies against the parasite.

Conclusion

According to the results of this study, fish infected with *I. multifiliis* experienced various physiological disturbances, including electrolyte imbalances, metabolic changes, altered blood cell counts, and inflammatory responses. However, supplementation with GI-EEP showed

potential in regulating electrolyte levels, improving blood parameters, and enhancing the immune system's ability to combat the infection. Based on the findings, it concluded that the optimal dose of propolis for regulating various physiological parameters and immune responses in fish exposed to *I. multifiliis* was 30 KGy.

Conflicts of Interest

The authors declare no conflict of interest.

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