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Original Article

Effects of gamma-irradiated ethanolic extract of Iranian propolis on growth performance, immunological and haematological parameters in juvenile common carp, *Cyprinus carpio* L.

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Abstract: This study evaluated the oral administration of gamma-irradiated ethanolic extract of Iranian propolis on juvenile common carp. Fish were assigned into five groups and fed on the basal diet (control group) and an enriched diet containing 0.5 g kg^{-1} of GI-EEP supplements for 45 days. Growth performance, haematological indices, and immunological parameters were measured. The results showed that oral administration of GI-EEP (0, 10, and 30 kGy) significantly improved the growth performance. However, GI-EEP dietary supplements had no significant effects on the survival rate, haematological indices, catalase activity and total mucosal immunoglobulins of fish. A significant increase in lysozyme activities and total immunoglobulins was observed in fish fed on GI-EEP. The results suggested that feeding fish with 50 kGy of GI-EEP decreased certain growth performance indices and immunological parameters. The highest superoxide dismutase activity was observed in the fish fed on 30 kGy of GI-EEP, while the SOD activity significantly decreased in fish fed on 50 kGy of GI-EEP. The overall results of this study showed the use of GI-EEP at 10 and 30 kGy in the diet could improve growth indices and increase the efficiency of the innate immune and cellular antioxidant capacity. In contrast, the biological impact of EEP treated at 50 KGy gamma rays may be significantly reduced due to changes in its quality.

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Introduction

Propolis is a natural product that can be used as a dietary supplement for humans, livestock, poultry, and aquatic animals (Salleh et al., 2021). The honeybees collect nectar from flowers, buds and leaves of medicinal and aromatic plants. Nectar is then mixed with pollen, resin and β-galactosidase secreted from the salivary glands of bees; later it is partially digested, and wax is added to form propolis (Salleh et al., 2021; Song et al., 2021). Over 300 different biochemical compounds can be found in propolis depending on plant diversity, the genetic characteristics of bees, and climatic conditions (Peixoto et al., 2021; Sahlan et al., 2021). Propolis contains various biochemical compounds, including phenols, flavonoids, terpenes, fats and beeswax, trace elements, vitamins, proteins, amino acids, pigments, and sugars (Irigoiti et al., 2021; Peixoto et al., 2021; de Lima et al., 2022). The antimicrobial properties of propolis have been confirmed in studies on gram-positive and gram-negative bacteria (Peixoto et al., 2021; Vică et al., 2021). The antioxidant and anti-inflammatory properties of propolis are also related to phenols, flavonoid compounds and vitamins (Irigoiti et al., 2021; Sarıkahya et al., 2021).

Although various researchers have reported propolis' therapeutic and protective effect in fish, and also studies have shown that oral administration of propolis is safe for fish (Acar, 2018; Yonar et al., 2012). Moreover, the protective effect of propolis was reported in reducing the toxic effects of xenobiotics in fish (Yonar et al., 2011; Talas et al., 2012; Yonar et al., 2014). Also, feeding fish with propolis extract could enhance the immune system function (Acar,

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2018). Therefore, providing a standard and practical preparation method for propolis in the aquaculture industry has always been a concern of aquaculture researchers. One of the ways to improve the quality of propolis is to purify and remove biological and chemical contaminants. Therefore, researchers are trying to innovate a proper technique for sterilizing, improving the shelf-life and quality of propolis, including γ-radiation (Heidarieh et al., 2021).

γ-Irradiation of foods, especially herbs, natural drugs, and food additives can improve their quality and durability (Fatemi et al., 2012; Jan et al., 2020; Ic and Cetinkaya, 2021). Many environmental and biological pollutants such as pesticides, aflatoxins, etc. can be removed in radiation. Reducing bacterial load, sterilizing food, and the natural drug is another benefit of γ-irradiation, while the chemical quality of food is well preserved (Alsager et al., 2018; Park et al., 2020; Robichaud et al., 2021). Therefore, the use of radiation in increasing the shelf life and quality of food is an approved routine method in many countries. Despite the usefulness of γ-irradiation in maintaining the quality of food and medicine, our knowledge about the effect of γ-irradiation on the biological properties of dietary supplements in aquatic animals' feed is limited. Therefore, this study aimed to investigate the biological effects of a treated-ethanolic extract of Iranian propolis at different doses of γ-irradiation on common carp, *Cyprinus carpio*.

Materials and Methods

Fish: The healthy common carp were obtained from a local fish farm, Karaj, Iran, and transferred to the Nuclear Agricultural Research School, Nuclear Science and Technology Research Institute, Karaj, Iran. Then, the specimens were temporarily kept in1000L fiberglass tanks equipped with aerators (temperature: 24±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 (Banaee et al., 2019).

Ethanol extract of propolis (EEP): Propolis (a sticky dark-coloured hive product) was collected manually using the scraping method in the bee-hive of the Marvdasht-farm, Fars, Iran. All of the samples were clean and cooled with liquid nitrogen. Then samples were ground before extraction. The EEP was prepared based on de Lima et al. (2022). About 10 g of crude propolis was extracted in 95% (v/v) ethyl alcohol in a hermetically closed glass vessel for seven days at 37°C, under occasional shaking. The ethanol extract was filtered through a Whatman filter paper no. 4, and the remained ethanol was evaporated under vacuum. The dried residual powder was stored at -20°C in a closed container, protected from light, and exposed to gamma irradiation.

Irradiation of EEP: The EEP was irradiated using the gamma cell model PX-30 (Russia) at a dose rate of 0.02 Gys–1 at the Nuclear Agricultural Research School, Nuclear Science and Technology Research Institute, Karaj, Iran. The doses of 10, 30, and 50 kGy are considered for EEP irradiation. After the irradiation, the samples were stored at 4°C for further experiments.

Experimental design and sampling: The fish (19.45±0.35 g) were randomly allocated into five experimental groups in 15 fiberglass tanks (300 L): a control group and three irradiated propolis groups were fed the basal diet supplement with 0.5 g kg^{-1} of irradiated ethanolic extract of Iranian propolis at different doses of 0, 10, 30, and 50 KGy of gamma-ray for 45 days, respectively. Each treatment contained 45 fish and was performed in triplicate (15 fish per tank). Fish feeding was stopped one day before sampling. The length and weight of all fish were measured to calculate the growth performance. Then, nine fish were sampled from each treatment and anesthetized using 150 mg L^{-1} clove powder. Afterward, the blood and mucous samples were obtained.

Growth performance: The growth performance was estimated according to the following equations (Mohiseni et al., 2017):

Weight gain (g) = final body weight - initial body weight

Specific growth rate (SGRs) = [(final mean body weight-initial mean body weight (g))/time interval (days)]

Feed conversion ratio (FCR) = feed intake (g)/weight gain (g)

Blood sampling: The blood samples were collected by puncturing the tail vein with a 2.5 cc syringe. The blood samples were aliquoted into two parts. The first part was used for haematological analysis, and the second part was utilized for immunological and oxidative biomarker analysis. The 2nd samples were immediately centrifuged at 6000 rpm at 4°C for 15 min. Finally, the serum was collected and transferred into 2 ml microtubes and stored in a freezer at -80°C until analysis.

Mucous sampling: After anesthesia, the fish were placed individually in sterile plastic bags containing 10 ml of 50 mM sodium chloride. After 2 min, the fish were taken out of the bags and placed in oxygenated water. Next, the mucous was collected into 15 ml sterile tubes and centrifuged at 15000 rpm at 4°C for 15 min. Finally, the supernatant was collected and transferred into 2 ml microtubes and placed in a freezer at -80°C until analysis.

Hematology: The blood was immediately used to estimate the number of erythrocytes (RBC) and leukocytes (WBC) using a haemocytometer slide (Improved Neubauer type) at 400x magnification. Thus, blood was diluted to 10^{-2} and 10^{-3} in PBS at pH 7.2 (Gholamhosseini et al., 2021). Haemoglobin (Hb) concentration was assayed using the cyanohaemoglobin method (Banaee et al., 2008). To calculate mean corpuscular volume (MCV), the hematocrit (Hct) is divided by the concentration of RBCs (Banaee et al., 2008; Gholamhosseini et al., 2020).

Lysozyme activity: The lysozyme activity was assayed according to Gholamhosseini et al. (2020). Consequently, serum (40 µl) was mixed to 2 ml of a suspension of Micrococcus lysodeikticus (0.2 mg ml^{-1}) in a 0.05 M sodium phosphate buffer (pH = 6.2). The reaction was performed at 25°C, and absorbance was recorded at 530 nm after 0.5 and 4.5 min on a spectrophotometer. A unit of lysozyme activity was expressed as the sample amount causing a decrease in absorbance of 0.001 per min.

Total immunogluboline levels: First, total protein content was estimated using the biochemical reagents (ParsAzmun Co. Iran). Then, total immunoglobulin was determined (Banaee et al., 2021). In this method, 100 µg of the plasma was dissolved in a ratio of 1 to 100 in PBS buffer and mixed with an equivalent volume of 12% polyethene glycol solution. The resulting mixture was incubated for 2 hours at room temperature. After centrifugation of the sample (at 5000 rpm at 4°C), the immunoglobulin molecules were removed from the sample. Finally, the protein concentration in the sample treated with polyethene glycol was subtracted from the initial protein concentration. Total immunoglobulin level was expressed as mg ml^{-1}.

Superoxide dismutase: Superoxide dismutase (SOD) activity was determined according to the instructions provided in the SOD activity kit (KSOD96-Kiazist Co. Iran). The test kit was comprised of a mixed substrate (xanthine and 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) (R1a reagent), buffer (R1b reagent), xanthine oxidase (R2 reagent) and a reference standard (R3 reagent). These reagents were prepared according to the instructions found in KSOD96-Kiazist manual provided with the test kit. Then, the change of absorbance per minute at 570 nm in each sample or standard was calculated using the formula:

$$(A2 - A1)/ 3 = \Delta A/\text{min of standard or sample}$$

The following equation was then used to calculate the inhibition percentage of the reaction in each of the samples and standards:

$$\% \text{ Inhibition} = 100 - (\Delta\text{std or sample/min} \times 100) / (\Delta\text{blank/min})$$

Next, the percentage of inhibition for each standard dilution was plotted to create a standard curve. The percentage of inhibition for each sample was then plotted on the standard curve to obtain the measurement of SOD activity. The activity of SOD could then be expressed as U mL^{-1}.

Catalase: Catalase (CAT) activity was measured according to the instructions provided in the CAT activity kit (KCAT96-Kiazist Co. Iran). The test kit comprised of a mixed substrate (H_2O_2, methanol, periodate, chromogen, catalase assay buffer, sample

Table 1. growth performance of juvenile common carp fed with 0.5 g kg^{-1} of GI-EEP at different doses.

Growth performance indices	Basal diet	0 kGy GI-EEP	10 kGy GI-EEP	30 KGy GI-EEP	50 KGy GI-EEP
Initial weight (g)	18.12±0.43^{a}	18.57±0.35^{a}	19.48±0.29^{a}	19.76±0.45^{a}	18.98±0.41^{a}
Final weight (g)	41.21±1.09^{a}	51.25±0.23^{b}	53.42±0.11^{b}	49.31±0.15^{b}	40.65±0.18^{a}
Weight gain (g)	23.09±1.66^{a}	32.68±1.59^{bc}	33.94±1.47^{c}	29.55±1.39^{b}	21.67±1.45^{a}
Specific growth rate (%)	1.82±0.07^{a}	2.24±0.03^{c}	2.23±0.03^{c}	2.02±0.02^{b}	1.61±0.05^{a}
Food conversion rate	2.89±0.07^{d}	2.09±0.07^{b}	1.88±0.04^{a}	2.21±0.05^{bc}	2.44±0.08^{c}

Different alphabet letters indicated a significant difference between experimental groups. Data are shown as mean±S.D.

Table 2. Heamatological indies of juvenile common carp fed with 0.5 g kg-1 of GI-EEP at different doses.

Heamatological indies	Basal diet	0.0 kGy GI-EEP	10 kGy GI-EEP	30 KGy GI-EEP	50 KGy GI-EEP
Erythrocytes (10^{6} μL^{-1})	1.51±0.45^{a}	1.44±0.42^{a}	1.48±0.39^{a}	1.36±0.23^{a}	1.57±0.31^{a}
Haemoglubin (g dL^{-1})	3.77±0.66^{a}	4.04±0.7^{a}	4.33±0.85^{a}	4.18±0.54^{a}	3.95±0.74^{a}
Mean corpuscular volume (fL)	212.3±35.1^{a}	195.1±37.2^{a}	189.3±30.5^{a}	201.5±41.7^{a}	192.8±50^{a}
Leukocytes (10^{4} μL^{-1})	7.1±1.3^{a}	6.7±0.99^{a}	6.7±1.8^{a}	7.1±1.06^{a}	7.1±1.63^{a}

Different alphabet letters indicated a significant difference between experimental groups. Data are shown as mean±S.D.

buffer, formaldehyde standard, stop solution,). These reagents were prepared according to the instructions found in the KCAT96-Kiazist manual provided with the test kit. Then, the change of absorbance per minute at 540 nm in each sample or standard was calculated using the formula of CAT activity = (μM of sample / 20) × (0.24 / 0.02) × sample dilution coefficient. The activity of SOD could then be expressed as U mL^{-1}.

Statistical analysis: All data were analysed by one-way analysis of variance (ANOVA) using SPSS software (24) after data normality was checked through the Shapiro-Wilks test. Next, Tukey's test was used to compare means. Differences were considered significant at $P<0.05$.

Results

During this trial, no mortality was observed. The results of growth performance are shown in Table 1. A significant difference was observed in the FW, WG, SGR, and FCR ($P<0.05$) between treatments. All treatment groups except for the 50 kGy GI-EEP group showed a significantly higher FW, WG, and SGR than that of the control group ($P<0.05$). Similarly, a dietary supplement of GI-EEP (0, 10, and 30 kGy) significantly decreased the FCR compared with the control group ($P<0.05$).

Table 2 shows the effect of dietary supplement of GI-EEP on haematological indices in the *C. carpio*. The results revealed no significant changes in the haematological parameters ($P>0.05$) of fish feed on dietary supplement of GI-EEP compared with the control group.

The effect of GI-EEP supplement on lysozyme activities is illustrated in Figure 1. A significant increase was observed in serum lysozyme activities in fish fed on dietary supplements of 10 and 30 KGy GI-EEP ($P<0.05$). Lysozyme activities in mucous in all treatment groups, except for the 50 KGy GI-EEP group, significantly increased compared to the control ($P<0.05$).

The GI-EEP groups revealed no difference in total Ig in mucous on day 45th of the experiment, while total Ig levels in serum of fish fed on 10 and 30 KGy Kg^{-1} of GI-EEP were significantly higher than the control group ($P<0.05$) (Fig. 2).

A significant increase was observed in SOD activity in serum of fish fed on 30 KGy Kg^{-1} of GI-EEP, whereas feeding fish with 0.0 and 50 KGy Kg^{-1} of GI-EEP significantly reduced SOD activity ($P<0.05$) (Fig. 3). Although SOD activity increased in mucous of fish fed on 10 and 30 KGy Kg^{-1} of I-EPE, feeding fish with 50 KGy^{-1} of GI-EEP significantly decreased SOD activity ($P<0.05$) (Fig. 3). Fish treated with GI-EEP showed no significant changes in CAT activities in serum and mucous after 45 days ($P>0.05$) (Fig. 4).

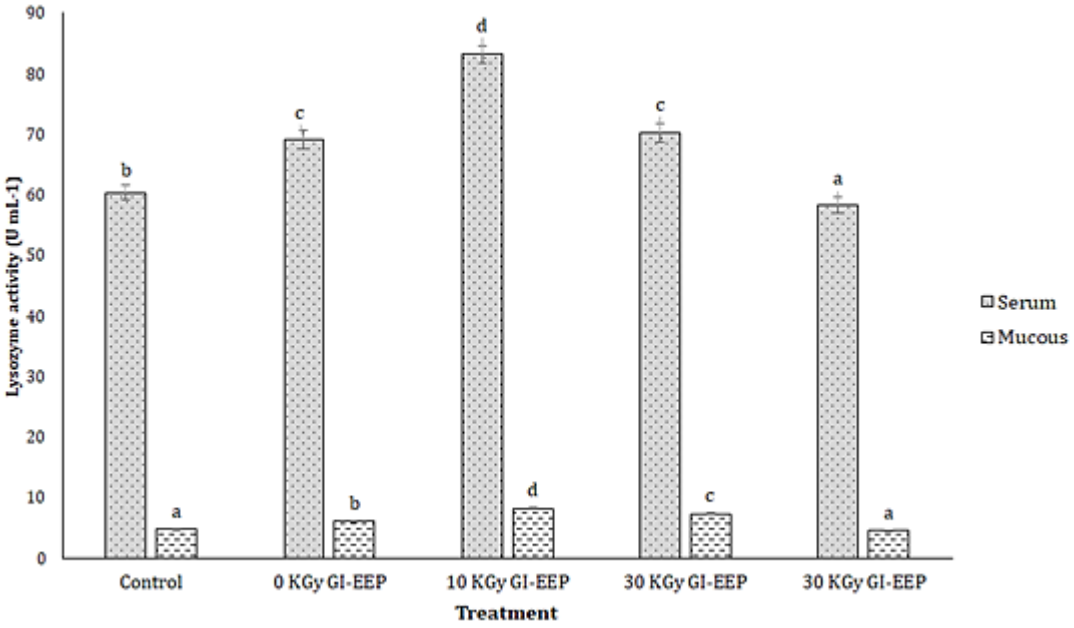

Figure 1. Lysozyme activities in the serum and mucous of juvenile common carp fed with 0.5 g kg-1 of GI-EEP at different doses. Different alphabet letters indicated a significant difference between experimental groups. Data are shown as mean±S.D.

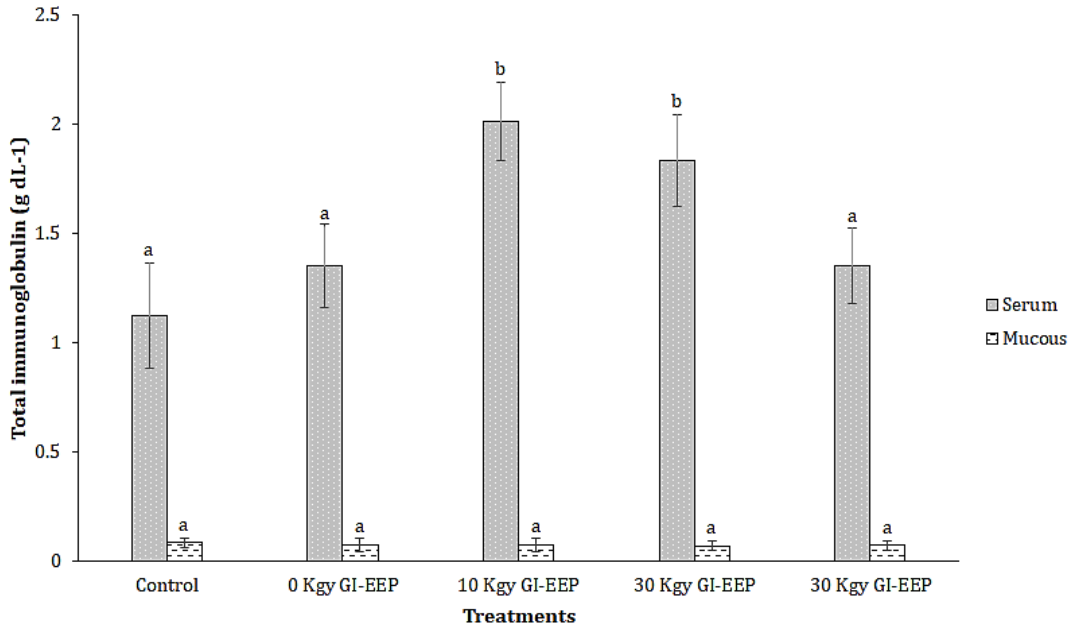

Figure 2. Total immunoglobulin levels in the serum and mucous of juvenile common carp fed with 0.5 g kg-1 of GI-EEP at different doses. Different alphabet letters indicated a significant difference between experimental groups. Data are shown as mean±S.D.

Discussions

The increase in the FW and WG, SGR, and decrease in FCR in fish fed EEP supplementation may be related to the effect of propolis compounds in increasing fish appetite. Similarly, Hassaan et al. (2019), Hamed and Abdel-Tawwab (2017), Abd-El-Rhman (2009, and Acar (2018) found that administration of EEP increased growth performance, feed intake and appetite in Nile tilapia, *Oreochromis niloticus*, and Mozambique tilapia, *O. mossambicus*. Irradiation of EEP with intensities of 10 and 30 KGy had a significant effect on improving the growth performance of fish, while irradiation with the intensity of 50 KGy led to a decrease in growth indices. Decreased growth performance of fish fed on propolis supplementation may be due to the

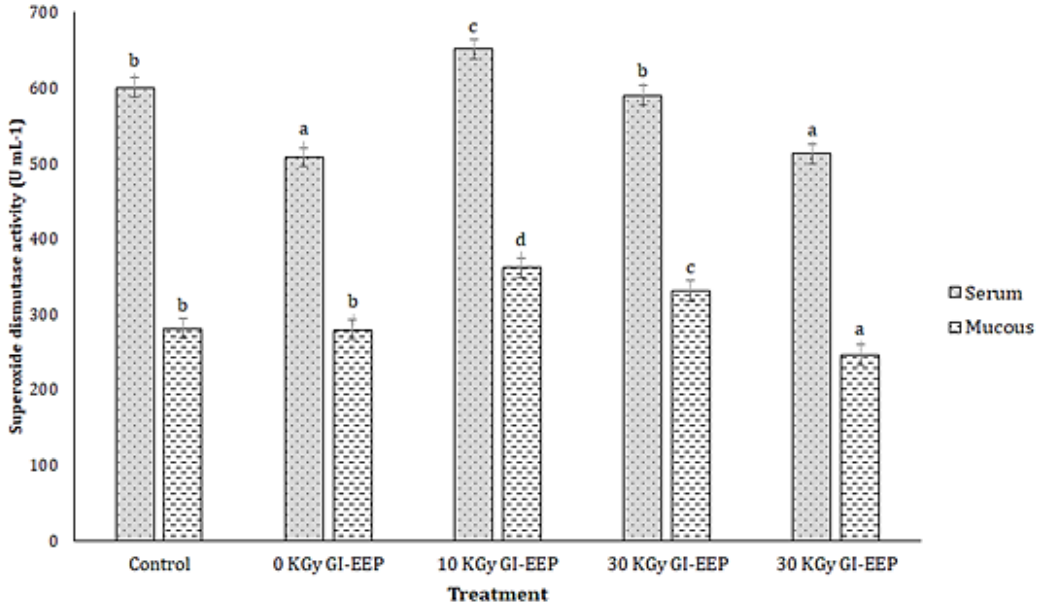

Figure 3. Superoxide dismutase activity in the serum and mucous of juvenile common carp fed with 0.5 g kg^{-1} of GI-EEP at different doses. Different alphabet letters indicated a significant difference between experimental groups. Data are shown as mean±S.D.

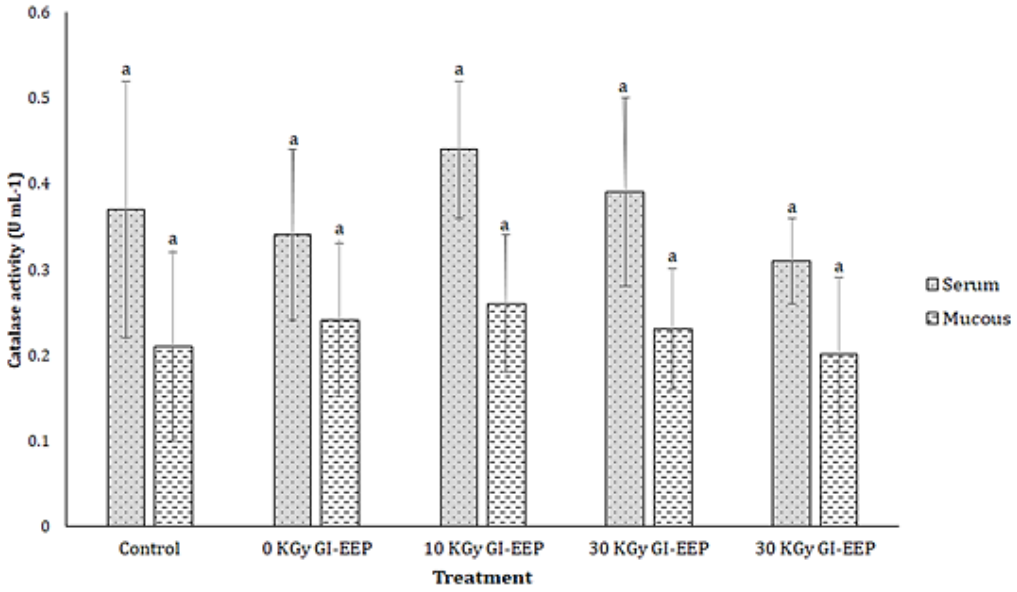

Figure 4. Catalase activity in the serum and mucous of common carp fed with 0.5 g kg^{-1} of GI-EEP at different doses. Different alphabet letters indicated a significant difference between experimental groups. Data are shown as mean±S.D.

degradation of some biochemical compounds of 30 KGy of GI-EEP affecting fish appetite.

No significant changes were observed in the haematological parameters of fish fed dietary supplement of GI-EEP, showing that the administration of EEP and irradiated EEP did not significantly affect the haematological parameters of fish. Propolis's protective and therapeutic effect on haematological parameters in fish exposed to xenobiotics has been confirmed (Talas et al., 2012). However, administration of the propolis alone may not have a significant effect on haematological parameters. Similar to the present study, propolis did not affect the haematological parameters in rats (Albokhadaim, 2015), and Nile tilapia (Dotta et al., 2014). Silva et al. (2014) showed that dietary propolis

did not affect haematological parameters (except haemoglobin) in the Blue-fronted Amazon parrot (*Amazona aestiva*, Linnaeus, 1758). Talas et al. (2011) also observed that feeding common carp with propolis did not significantly change the haematological parameters.

The results also showed that EEP administration might not change lysozyme activity. Dotta et al. (2014) found that feeding fish with propolis did not significantly alter serum lysozyme activity. In contrast, Acar (2018) and Dotta et al. (2014) showed that administration of EEP increased lysozyme activity in serum of Mozambique tilapia and Nile tilapia. Radiation may increase the immune-stimulatory and bactericidal properties of EEP. Increased lysozyme activity in serum and mucous can be an indicator of enhanced serum bactericidal capacity in EEP-treated fish. Gamma-irradiated EEP (10 and 30 KGy) treatment was able to increase lysozyme activity significantly. The decrease in lysozyme activity in fish fed on 50 KGy GI-EEP supplement compared to EEP-treated fish may be due to the degradation of propolis active ingredients.

The results showed that improving the quality of EEP treated with gamma rays (10 and 30 KGy Kg^{-1}) increases the efficiency of the fish immune system in total immunoglobulin biosynthesis. Elevated total Ig in GI-EEP-treated fish may be related to its effects on humoral immunity (Yuan et al., 2012). Yonar et al. (2011) observed that administration of EEP could have a protective effect on the total Ig level in rainbow trout (*Oncorhynchus mykiss*) exposed to oxytetracycline. However, administration of EEP and gamma-treated EEP did not significantly affect the total immunoglobulin level of fish mucosa. A significant increase was reported in antibody titer and immunoglobulin levels in the serum of chicken after injection of propolis flavonoid liposome (Yuan et al., 2012). The increase in total immunoglobulin is related to the stimulation of the immune system of fish fed with irradiated EEP.

Increased SOD activity in 10 KGy^{-1} gamma-treated EEP group indicates an improvement in the efficiency of the antioxidant defense system. In contrast, the decrease in SOD activity in fish fed on 50 KGy^{-1} EEP supplements may be due to reduced flavonoids and antioxidants in EEP. In addition, oral administration of EEP or irritated-EEP did not affect CAT activities in serum and mucosa. Hamed and Abdel-Tawwab (2017) and Yonar et al. (2012) showed that EEP administration could regulate the activity of CAT and SOD in fish serum exposed to environmental pollutants. The protective effect of propolis on CAT and SOD activity was observed in rainbow trout exposed to oxytetracycline (Yonar et al., 2011). Mali et al. (2011) and Pérez et al. (2007) showed that γ-irradiation (10-30 kGy) could increase phenolic acid levels in some herbs and dietary supplements (Mali et al., 2011; Pérez et al., 2007). Increased total antioxidant and phenolic capacity in γ-irradiation-treated food products has also been confirmed by Breitfellner et al. (2002), Hagen et al. (2007), and Harrison and Were (2007). In contrast, Alothman et al. (2009) and Schindler et al. (2005) found that increasing gamma radiation dose could reduce the antioxidant properties of plant products and fruits. A significant decrease in total phenolic compounds, total ascorbate and carotenoids content in nine aromatic herbs and spices (Calucci et al., 2003), tocopherols and antioxidant capacity in cashew nuts (Sajilata and Singhal, 2006), as well as antioxidant properties in rosemary (El-Beltagi et al., 2011), was observed with increased irradiation does. Decreased total antioxidant capacity may be attributed to the degradation of phenolic compounds, vitamins, flavonoids, etc. contained in the herbal extract after the γ-irradiation (Alothman et al., 2009). Schindler et al. (2005) found that high doses of γ-irradiation could decrease the concentration of phenolic compounds in some dietary supplements (Schindler et al., 2005).

Conclusion

This study's findings indicated that Iranian EEP (0.5 g kg^{-1}) treatment with gamma rays (10 and 30 KGy) may increase the purity of EEP because the administration of 10 and 30 KGy EEP could significantly improve the efficiency of the immune system, antioxidant system, and fish growth

performance. However, treatment of EEP (0.5 g kg $^{-1}$) at 50 KGy γ-irradiation may have changed its quality. Therefore, 50 KGy EEP did not hava e significant effect on growth performance, hematological indices, and hemoural antioxidant status.

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