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The protective role of ferulic acid against imidacloprid-induced oxidative stress in liver and brain of *Cyprinus carpio*

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ABSTRACT

This study evaluated the potential protective effect of ferulic acid (FA) on stress induced by imidacloprid (IM) in *Cyprinus carpio* (mean weight 60.10±2.22 g). Therefore, *C. carpio* was exposed to sublethal IM concentration (2.80 mg/L) for 10 days. During the experimental period, fish were fed a basal (control) diet and a basal diet supplemented with 5 g/kg of ferulic acid. The fish were divided into four groups (n = 6 in each group). The control group was exposed to tap water (the absence of imidacloprid) fed with control diet. The FA group was exposed to tap water (the absence of IM) and was fed with FA supplemented diet. The liver and brain tissues of both control and treated fish were dissected. Tissues samples were obtained from an individual fish and prepared for analysis. The variations in catalase (CAT), superoxide dismutase (SOD) activities, and levels of malondialdehyde (MDA) with protein carbonyl (PCO) in liver and brain tissues of *C. carpio* were investigated in experimental groups. CAT activities were significantly increased whereas SOD activities were decreased in liver and brain tissues of treated fish by exposure to imidacloprid. Tissue MDA and PCO levels in the IM supplemented experimental groups were increased compared to the control. At the end of the experiment, it was determined that FA application had the effect of improving tissue enzyme activities and levels of MDA and PCO.

KEYWORDS: *Cyprinus carpio*, imidacloprid, ferulic acid, antioxidant system, protein carbonyl

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1. Introduction

Neonicotinoids are the newest insecticide class developed in recent years and have replaced organophosphorus, organochlorine and pyrethroid compounds in the against agricultural pests. Imidacloprid is a new insecticide belonging to the neonicotinoid class and has a rapidly increasing use worldwide (El-Gendy et al., 2010). Imidacloprid (IM) is a neurotoxic insecticide, its mode of action is similar to that of nicotine and competes with acetylcholine for the receptor site (Tyor and Harkrishan, 2016). Various studies have shown that imidacroplide has adverse effects on non-target organisms (Desai and Parikh, 2013; Topal et al., 2017).

One of the toxic effects of pesticides and environmental chemicals is the formation of free radicals. It can cause oxidative stress and stimulate reactive oxygen species (ROS) production by creating changes in antioxidant enzyme systems. The main damages of these molecules, known ROS, are the structural changes of cellular macromolecules such as membrane lipids (lipid peroxidation), DNA and protein. These structural changes can lead to loss of biological function and pathophysiological conditions may occur (Jablonska-Trypuc, 2017). Direct or indirect environmental changes can cause varying degrees of stress in aquatic organisms, disrupting the balance between ROS production and elimination (Gonzalez et al., 2015). Organisms have a system that deactivates ROS. Intracellular and extracellular, enzyme and nonenzymic defense mechanisms are called antioxidant defense system (ASS) and antioxidants are important for the conversion of ROS into non-harming compounds and for the cell to perform its normal functions (Jablonska-Trypuc, 2017). Catalase (CAT), Superoxide dismutase (SOD) are enzymatic ASS elements capable of destroying free radicals. Like other vertebrates, fish also have enzymatic and non-enzymatic ASS (Trenzado et al., 2006).

Lipids are molecules sensitive to oxidation reactions by free radicals. Unsaturated fatty acids, especially in cell membranes, react easily. Lipid peroxidation (LPO) is the function of oxidative degradation of polyunsaturated fatty acids (PUFAs) (Repetto et al., 2012). Malondialdehyde (MDA) is one of the LPO products formed as a result of oxidative damage to cell membrane phospholipids and circulating lipids, and its level is indicative of the oxidative damage caused. Measurement of these parameters can provide information about the potential of possible toxic substances to cause oxidative damage (Vlahogianni et al., 2007).

All reactions and agents that cause the production of ROS can lead to protein oxidation (Davies, 2016). Oxidative changes in proteins affect a variety of cellular functions in which proteins play a role. Cellular events including structural proteins, receptors, transduction regulation and enzymes can be affected by oxidative protein damage (Davies, 2016). As a result of the interaction of ROS with proteins, many amino acid residues such as, arginine, lysin, histidine and proline or peptide bonds are damaged, resulting in protein carbonyl oxidation (PCO) products. Protein carbonyl compounds are the most commonly measured product of protein oxidation. Oxidative protein modification resulting from the oxidant effect of free radicals and the resulting excess accumulation of oxidized proteins can cause various pathological conditions due to cell and tissue damage (Kehm et al., 2021).

The liver is an important organ in the detoxification function in organisms. It therefore faces the threat of maximum exposure to harmful compounds and their metabolic byproducts. The central nervous system is very sensitive to the effects of ROS due to its easily oxidized substances such as polyunsaturated fatty acids and its highly oxidative metabolic function (Mehta et al. 2009). The brain is among the most vulnerable organs due to its high oxygen consumption and the fact that cell membrane

lipids are rich in oxidizable polyunsaturated fatty acids (Gupta 2004). For this, both tissues are very sensitive to oxidative stress.

The aquaculture sector, which is one of the fastest growing sectors in the world, modern aquaculture practices cause various alternatives in nutritional formulations. As a result of the increasing intensification of agricultural practices, polluting agents and disease outbreaks in the aquaculture sector have increased their importance (Body et al., 2020). A wide range of chemicals are used in the aquaculture industry, including antibiotics and therapeutic drugs. Due to the risks that may occur with the accumulation of chemical substances, fish health is the most important issue in the fish farming sector and is directly related to public health.

In terms of fish health, especially researching ways to prevent diseases increases the importance of natural feed sources day by day. For these reasons, the concept of functional food has become a topical issue. Functional foods can generally be obtained by adding various components with functional properties from the outside into the food (Mohan et. al., 2022). Antioxidants are one of the most used functional food ingredients today (Dawood et al., 2020). In recent years, there has been great interest in the discovery and use of new antioxidants due to their potential applications in the context of disease occurrence due to oxidative stress and imbalance between ROS production and antioxidant defense (Santos and Ramos, 2018; Dawood et al., 2020).

Phenolic compounds constitute the most important group of water-soluble antioxidants. It is found in high amounts of fruits and vegetables and has a positive effect on health. Phenolic compounds are mainly known as phenolic acids and flavonoids. Phenolic acids are the simplest phenolic compounds found in plants. It includes two subgroups; hydroxybenzoic acid and hydroxycinnamic acid. These are the precursors of flavonoids (Bourne and Rice-Evans, 1998). Ferulic acid (FA) (4-hydroxy-3-methoxycinnamic acid; $C_{10}H_{10}O_4$), a compound found in plant tissues, is a phenolic compound formed during the metabolism of phenylalanine and tyrosine (Yu et al., 2017). FA is a powerful membrane antioxidant and has been proven to end free radical chain reactions. FA is considered one of the most important types of additives that work to raise immunity and improve the physiological condition in animals (Ahmadifar et al., 2019).

Cyprinus carpio used in this study is an economically important species in aquaculture worldwide and was chosen because of its adaptive response to water pollution (Vinodhini and Narayanan, 2008). CAT, SOD activities, MDA (for LPO) and PCO levels were investigated in liver and brain tissues of *C. carpio* after 10 days of Imidacloprid application, which is the experimental period. In addition, the potential protective effect of ferulic acid as an antioxidant in imidacloprid-induced oxidative stress was investigated.

2. Material and Methods

2.1. Chemicals

A technical formulation of the organophosphate insecticide Imidacloprid was used pure of 99%. All of chemicals and reagents were purchased from Sigma-Aldrich Chemical Corporation (USA).

2.2. Test animals and treatment

C. carpio (mean weight 60.10±2.22 g) were obtained from Mersin University, Fisheries Faculty, Aquaculture Department and reassigned to laboratory where the temperature was kept at 23 ± 1°C (12:12 L:D). Throughout the experiments, dechlorinated tap water with pH value of 7.35, an alkalinity of 332 mg/L $CaCO_3$, and oxygen concentration of 6.80 mg/L was used. The fish were allowed to acclimatize to these conditions for 4 weeks. The fish were fed at a rate of 3% body weight/day with a commercial pellet during the acclimation period. Commercial fish diet, Camli

Yem/Bioaqua, Turkey (44% crude protein, 18% crude fat, 12% moisture, 12% ash and 3% fiber), was used as the basal control diet. The pellet was ground, and ferulic acid was added at 5 g per kg to a commercial fish diet. The pellets were air-dried at 40°C in an oven and stored at 4°C during the experiment. Each diet was given to carp by hand at 3% of fish body weight (at 09:00 and 16:00 h) for 10 days. During this experiment, fish were fed unsuplemented diet (Control) or suplemented diet with ferulic acid.

Experiments were conducted in glass aquaria containing 100 L test solution. Fish were exposed to 2.80 mg/L sublethal concentrations of imidacloprid for 10 days. Sublethal concentration of imidacloprid was chosen outstanding to the earlier studies (Tyor and Harkrishan, 2016). The water was refreshed every 2 days to compensate for the insecticide lost in the exposure medium. The fish were divided into four groups (n = 6 in each group). The control group (Cont) was exposed to tap water (the absence of imidacloprid) fed with control diet. The FA group was exposed to tap water (the absence of IM) and was fed with FA supplemented diet. The IM group was exposed to imidacloprid concentration of 2.80 mg/L and was fed with control diet. The IM+FA group was exposed to 2.80 mg/L concentration of imidacloprid and was fed with FA supplemented diet.

At the end of exposure period, six fish were removed from each tank and sacrificed by transaction of the spinal cord. The liver and brain tissues of both control and treated fish were dissected. Tissues samples were obtained from an individual fish and prepared for analysis. Tissues were homogenized to 1/5 (w/v) ratio in physiological saline solution (0.8% NaCl) with homogenizer and then centrifuged at 13500 rpm for 10 min in a Sigma 2-16 K centrifuge at +4 °C, and supernatant was used for biochemical analyses.

2.3. Biochemical Assays

The CAT activities of liver tissues were determined according to the method of Aebi (1974). The enzymatic decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm. The difference in absorbance per unit time was used as a measure of CAT activity. The enzyme activities are given in U/mg protein. The SOD activity was measured by the inhibition of nitroblue tetrazolium (NBT) reduction due to oxygen generated by the xanthine/xanthine oxidase system (Sun et al., 1988). One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the NBT reduction rate. The reduction in NBT by superoxide anion to blue formazan was measured at 560 nm. The enzyme activities are given in U/mg protein.

The levels of MDA homogenized tissue, as an index of LPO, were determined by TBA reaction using the method of Yagi (1998). Determination of carbonyl groups formed on proteins as a result of oxidative stress Levine et al. (1990) method was used. According to this method, protein carbonyl groups react with 2,4-dinitrophenylhydrazine (DNPH) to form stable 2,4-dinitrophenyl (DNP) hydrazone.

The tissue protein contents were measured only to determine the specific activity of antioxidant enzymes and levels of MDA and PCO according to the method developed by Lowry et al. (1951) using bovine serum albumin as standard. Absorbance of samples were measured at 750 nm wavelength by spectrophotometer.

2.3. Statistical Analysis

Data were expressed as mean ± standard error (SE) and analyzed using with SPSS 10.0 for Windows computer program. ANOVA and Duncan's multiple range tests were used to analyze differences between groups. The differences were defined as statistically significant when $P<0.05$.

3. Results

In this experiment, no mortality was observed. The CAT activities in tissues of fish are given in Figure 1. Compared to the control values, there was no significant change in the CAT activities of the tissues treated with only FA. The CAT activity in liver and brain tissues of fish were significantly increased (32%, 50% respectively) by exposing the concentration of imidacloprid (2.80 mg/L) at the end of the experiment. In addition, FA application in the IM+FA group had an improvement effect on CAT enzyme activity in liver and brain tissues compared to the IM group.

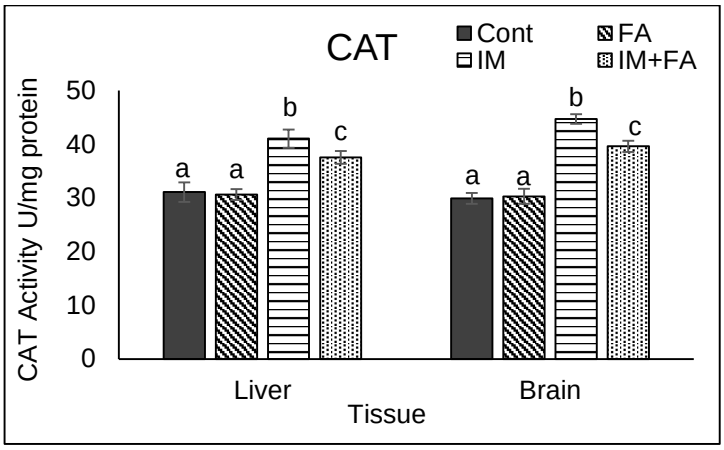

Figure 1. CAT activity in liver and brain tissues of *C. carpio* exposed to sublethal concentrations of imidacloprid (2.80 mg/L), with or without a dietary supplementation of FA. Each value is the mean ± SE (n = 6). Multiple comparisons were made separately for each tissue, and means with different superscript in tissues are significantly different ($p<0.05$)

The SOD activities of liver and brain tissues in experimental fish are shown in Figure 2. Only the FA treated group did not significantly change the SOD activities of the tissues compared to the control values. IM administration with a sublethal concentration of 2.80 mg/L caused a significant decrease in enzyme activity of 30% in the liver and 32% in the brain ($p<0.05$). With this, However, when the IM+FA group was compared with the IM group, it was found that the addition of FA improved brain tissue SOD activity in fish.

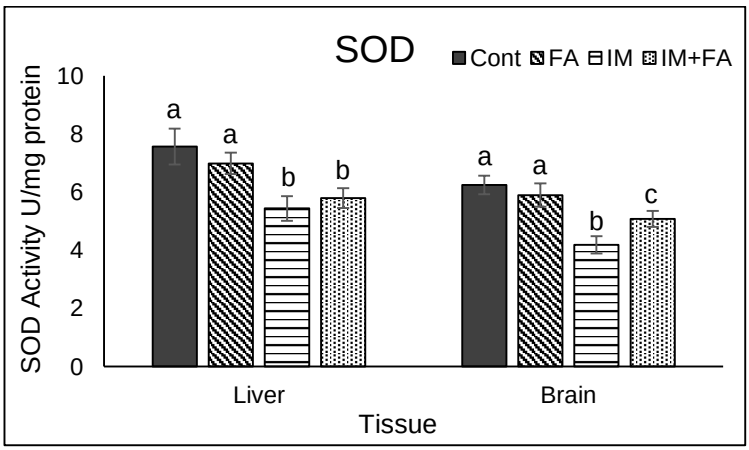

Figure 2. SOD activity in liver and brain tissues of *C. carpio* exposed to sublethal concentrations of imidacloprid (2.80 mg/L), with or without a dietary supplementation of FA. Each value is the mean ± SE (n = 6). Multiple comparisons were made separately for each tissue, and means with different superscript in tissues are significantly different ($p<0.05$)

The MDA levels in tissues are given in Figure 3. The tissue MDA level of the FA applied group decreased compared to the control group. The liver and brain tissue MDA levels of the fish in the IM-administered experimental group were significantly increased by 92% and 107%, respectively, compared to the control group ($p<0.05$) (Figure 3). In the IM+FA group, supplemented FA administration significantly decreased the MDA content in the tissues compared to the IM group, without reaching the control values.

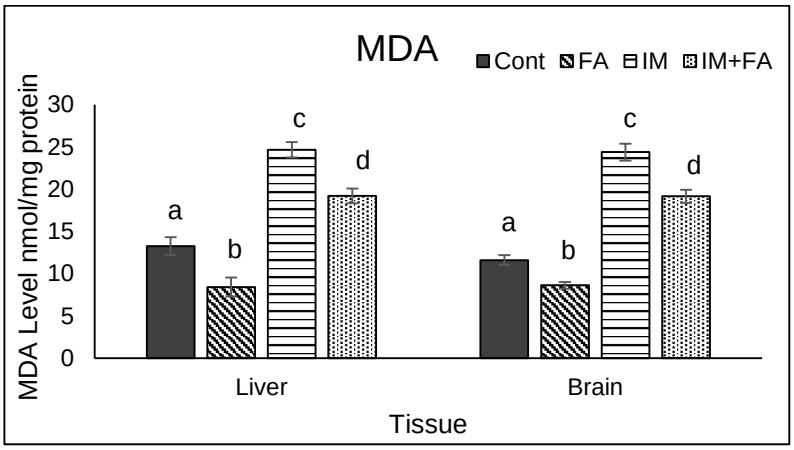

Figure 3. MDA level in liver and brain tissues of *C. carpio* exposed to sublethal concentrations of imidacloprid (2.80 mg/L), with or without a dietary supplementation of FA. Each value is the mean ± SE (n = 6). Multiple comparisons were made separately for each tissue, and means with different superscript in tissues are significantly different ($p<0.05$)

The levels of PCO in tissues are given in Figure 4. Compared to the control values, there was no significant change in the PCO levels of the tissues treated with only FA. Liver and brain tissue PCO levels were significantly increased by 77% and 90%, respectively, compared to the control group, with the effect of IM administration ($p<0.05$) (Figure 4). In the IM+FA group, supplemented FA administration significantly decreased the MDA content in the tissues compared to the IM group, without reaching the control values.

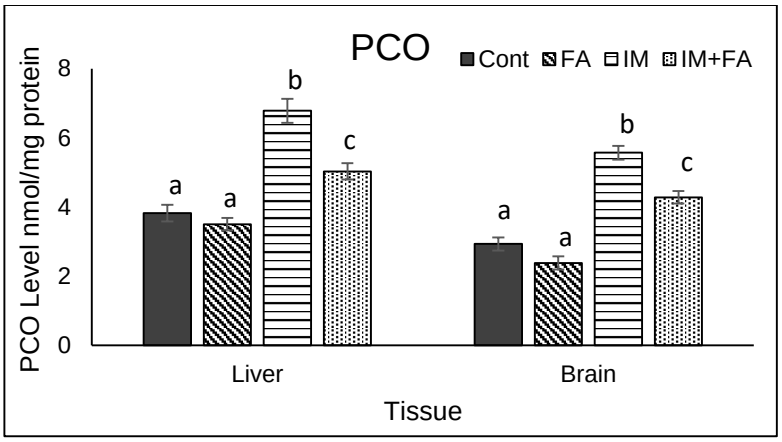

Figure 4. PCO level in liver and brain tissues of *C. carpio* exposed to sublethal concentrations of imidacloprid (2.80 mg/L), with or without a dietary supplementation of FA. Each value is the mean ± SE (n = 6). Multiple comparisons were made separately for each tissue, and means with different superscript in tissues are significantly different ($p<0.05$)

4. Discussion

In this study, CAT enzyme activities in liver and brain tissues of *C. carpio* increased with the effect of 2.8 mg/L sublethal concentration of imidacloprid. SOD activities were decreased in both tissues. Various studies have reported different effects on antioxidant enzymes in fish treated with imidacloprid. Ge et al. (2015), determined that liver tissue CAT and SOD activities of *Danio rerio* increased significantly in the first days with the effect of different concentrations of imidacloprid (0.3, 1.25, and 5 mg/mL), but decreased towards the end of the exposure time. It has been reported by researchers that there is an increase in radical production with the effect of a stress factor and that enzyme synthesis may be increased to protect against oxidative stress (Ge et al., 2015). Shukla et al. (2017), reported findings of an increase in CAT enzyme activities and a decrease in SOD activity in *D. rerio* liver and brain tissues with the effect of IM medium concentration. The SOD-CAT system is important and is the first defense against oxidative stress. It is known that the SOD enzyme protects against free radical-induced damage by converting the produced superoxide radicals to hydrogen peroxides. Generally, a simultaneous induction response in SOD and CAT activities is observed when exposed to contaminants (Pandey et al., 2003). Increased SOD activity in organisms indicates that ROS levels are in the range that they can withstand oxidative stress. As the exposure time increases, SOD activity is inhibited due to the increased amount of ROS (Shao et al., 2012). The activity of antioxidant enzymes can be induced or inhibited under the pressure of contaminants. Under oxidative stress, differences in enzyme activities can be observed. At the beginning of stress, activities increase, but if the stress lasts for a long time, enzyme inhibition occurs (Berrahal et al., 2007). This situation may vary depending on the stress intensity and the sensitivity of the species (Awoyemi et al., 2014). In this study, the increase in CAT and SOD enzyme activities indicates the possibility that superoxide radicals and hydrogen peroxide formation may be increased in the liver and brain tissue of *C. carpio* with the effect of IM concentration. At the end of the exposure period (10^{th} day), CAT activity increased, SOD activity decreased compared to the control group and even inhibited, indicating that the CAT enzyme is required to eliminate excess H_2O_2 in the cell (Ge et al., 2015; Shukla et al., 2017).

In this study, the levels of MDA in the brain and liver tissues of *C. carpio* exposed to imidacloprid was found to be higher than the control group. Some authors have published similar results. Ge et al. (2015) described elevated MDA levels in the liver of *D. rerio* depending on the time exposure to imidacloprid. It has been reported that lipid peroxidation in liver and brain tissues increases with the effect of imidacloprid concentrations applied in *Oncorhynchus mykiss* (Topal et al., 2017). Lipid peroxidation is the oxidative state of polyunsaturated fatty acids (PUFA) found in membranes. Fish have a high PUFA content in their tissue content and may be sensitive to lipid peroxidation (Stephan et al., 1995). Imidacloprid toxicity caused an increase in the level of free radicals, and as a result, the level of lipid peroxidation may have increased. In fact, this increase may have occurred as a result of changes in the antioxidant defense system (Ademuyiwa et al., 2009). Tissues are susceptible to peroxidation in terms of the substrates they contain. The increase in MDA levels of tissues may be the result of the toxicity of ROS induced by imidacloprid (Ge et al., 2015).

In this study, PCO level, which is an indicator of protein oxidation in liver and brain tissue of *C. carpio*, increased under the influence of 2.8 mg/L imidacloprid medium concentration. Similar results were found in previous studies. Vieira et al. (2018) reported that PCO levels increased with the effect of imidacloprid in different tissues of *Prochilodus lineatus*. Similarly, it has been

reported that protein carbonyl level increased in liver, kidney and gill tissues of *Channa punctata* exposed to deltamethrin, endosulfan and paraquat insecticides (Parvez and Raisuddin, 2005). Covalent modification of proteins that react directly with ROS or its byproducts is known as protein oxidation (Kehm et al., 2021). The physical and chemical properties, including conformation, structure, solubility, and enzyme activities, can undergo changes by oxidative modifications of proteins. Oxidative modification of proteins occurs as a result of oxidative stress (Berlett and Stadtman, 1997). The increase in PCO level in tissues of *C. carpio* following exposure to imidacloprid may be the result of excessive ROS production (El-Shenawy et al., 2010).

In this study, it was determined that FA administration reduced IM toxicity. Toxicity-reducing effects were detected on CAT and SOD enzyme activities and MDA and PCO levels in liver and brain tissues. In previous studies, Ferulic acid has been shown to protect from lipid peroxidation with its phenolic hydroxyl group (Maurya and Devasagayam, 2010) and to have antioxidant properties through free radical scavenging (Ghosh et al., 2017). The results displayed decreased SOD, CAT activities and increased MDA concentration during IM application while dietary FA regulated the antioxidative responses. Similar results observed *C. carpio* fingerlings (Ahmadifar et al., 2019) and *Oreochromis niloticus* (Dawood et al., 2020), when FA used in diet for functional foods. The functionality of FA is due to its composition of hydroxyl group that helps in forming the phenoxy radicals which freely rid cells of the ROS that cause LPO and oxidative stress.

The antioxidant effect of phenolic compounds is due to their properties such as scavenging free radicals (Rice-Evans et al., 1995), compounding with metal ions (metal chelation), inhibiting or reducing singlet oxygen formation, and generally the resistant stability of the phenol radical (Rice-Evans et al., 1995). These compounds can donate the hydrogen in the hydroxyl groups of their aromatic rings to prevent lipids and other biomolecules (protein, carbohydrate, nucleic acids) from being oxidized by free radicals. Phenolic compounds are considered among important antioxidants because they are reducing agents, hydrogen donors, singlet oxygen scavengers and metal chelators (Bourne and Rice-Evans et al., 1998). In this study, the amelioration of imidacloprid-induced state of supplemented FA, oxidative stress by reducing lipid peroxidation and protein oxidation, and regulating the antioxidant defense system in tissues can be attributed to the above-mentioned antioxidant properties.

In conclusion, it was determined that subletal concentrations of imidacloprid caused oxidative damage in *C. carpio* liver and brain tissues and ferulic acid had a protective feature against this damage. Brain tissue was found to be more sensitive than liver tissue. Increases or decreases in the antioxidant enzyme system and the increase in MDA and PCO levels can be evaluated as an indicator that it can be used as an indicator in the biomonitoring of the aquatic ecosystem. FA could be able to improve IM-induced oxidative stress by decreasing lipid peroxidation and protein oxidation and altering antioxidant defense system in tissues. Thus, dietary supplementation of FA may be useful in aquaculture and might be good candidate for immunological response that is occupationally exposed to insecticides. Suggesting that FA may be beneficial in preventing IM-induced oxidative stress.

Conflict of interest

The authors declare that they have no actual, potential or perceived conflict of interest for this research article.

Ethical approval

The ethics committee approval was obtained from Mersin University Animal Experiments Local Ethical Committee by decision number 2017/07 dated 27/03/2017.

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