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Dinotefuran-induced neurotoxicity in rainbow trout brain and the protective potential of aloe-emodin: a multi-biomarker assessment

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ABSTRACT

This study evaluated dinotefuran (DTF)-induced neurotoxicity and the potential protective effects of aloe-emodin (Al-EMD) in the brain tissue of rainbow trout (*Oncorhynchus mykiss*). The 96-h LC50 values were 154.89 mg/L for DTF and 3.16 mg/L for Al-EMD; 50% of these values (77.5 and 1.58 mg/L, respectively) were used in the subsequent experiment. Fish were assigned to control, DTF, Al-EMD, and DTF+Al-EMD groups. Exposure was conducted for 10 days under semi-static conditions, followed by an untreated recovery period from days 11 to 21. Brain tissue was evaluated using neurotoxicity (AChE and BDNF), oxidative stress and antioxidant (SOD, CAT, GPx, MDA, MPO, and ROS), inflammatory (TNF- α and IL-6), Nrf2, oxidative DNA damage (8-OHdG), apoptosis (caspase-3), and histopathological endpoints. DTF exposure was associated with decreased AChE, BDNF, SOD, CAT, GPx, and Nrf2 levels and with increased MDA, MPO, ROS, TNF- α , IL-6, 8-OHdG, and caspase-3. Histopathological examination showed neuronal degeneration, necrosis, and vascular hyperemia. Co-administration of Al-EMD attenuated several DTF-associated biochemical and histological alterations, and partial normalization was observed during the recovery period. Under the present experimental conditions, these findings indicate that DTF induces marked neurotoxic and oxidative injury in rainbow trout brain tissue and that Al-EMD has potential protective effects. Further studies with greater tank-level replication, environmentally representative

exposure concentrations, and pathway-specific analyses are required before broader mechanistic or applied conclusions can be drawn.

Keywords: Pesticide, Aloe Emodin, rainbow trout, neurotoxicity

INTRODUCTION

Fish and other aquaculture products from freshwater and marine environments are critical to achieving food security goals and potentially providing more environmentally sustainable animal foods (Gephart et al., 2021). Freshwater ecosystems are the most polluted environments worldwide (Atamanalp et al., 2022). It is known that aquatic organisms are exposed to many stressors such as acidic substances, climate changes, sewage pollution and chemicals, which are the most common sources of pollution in fresh waters. The indiscriminate use of chemicals, especially pesticides in agriculture, pollutes the ecosystem and poses a serious risk to all aquatic life, including fish.

Neonicotinoid insecticides are widely used in food products because they are less toxic to mammals and have a greater affinity for binding to insects (Jeschke et al., 2011; Yan et al., 2016; Gül et al., 2019). Dinotefuran (DTF), a new class of neonicotinoid insecticides, is a third-generation neonicotinoid with high insecticidal activity, selectivity and broad spectrum of two enantiomers (R-dinotefuran and S-dinotefuran) with different bioactivity and toxicity (Wang et al., 2018; Di et al., 2021). DTF has high solubility and mobility in water with a half-life of 50-100 days in soil. May pass into the water column via runoff, rainfall/surface runoff, causing damage to aquatic organisms (Liu et al., 2018; Di et al., 2021). While the direct potential risks of DTF on fish and amphibians are considered low, the potential for indirect risks has been reported to exist (U.S. Environmental Protection Agency, 2020).

Studies on the toxic effects of neonicotinoid insecticides on non-target organisms have reported negative effects on reproduction/growth, affecting the nervous system, disrupting metabolic balance, and damaging DNA (Qi et al., 2020). Recognizing that pollutants are a major threat to the aquatic ecosystems and organisms, it is important to take sustainable measures for decontamination. In particular, the use of effective, efficient and economical products that are assumed to be curative has brought about environmentally friendly prophylactic approaches (Rodrigues et al., 2020). The sustainable use of medicinal plants as alternative therapeutic agents in intensive aquaculture systems has gained increasing scientific interest in recent years. Among these plants, Aloe vera has attracted considerable attention

67 because of its diverse biological activities, including anti-inflammatory, antioxidant,
68 immunostimulatory, hepatoprotective, and gut health-promoting properties. Previous studies
69 have demonstrated that dietary supplementation with appropriate concentrations of *A. vera*
70 products, particularly at levels below 2%, can enhance growth performance, improve general
71 physiological condition, and increase resistance to environmental and physiological stressors
72 in fish species. Despite these promising outcomes, further toxicological evaluations and
73 species-specific investigations are still required to validate its growth-promoting and
74 immunomodulatory effects. In addition, more comprehensive experimental designs
75 incorporating different biological and environmental factors are necessary to better understand
76 the potential applications of *A. vera* in aquaculture practices. In recent years, research has
77 focused on the application of new environmentally friendly and alternative supplements to
78 improve fish growth, immune responses and disease resistance (Doan et al., 2019). However,
79 *A. vera* polysaccharides act as immunostimulants that exhibit adjuvant activity and increase
80 the release of different types of cytokines (Peng et al., 1991) and stimulate hematopoiesis
81 (Egger et al., 1996). It also triggers the release of interleukins (ILs), TNF- α , and modulates
82 immune signaling through upregulation (Devi et al., 2019). Neurotoxicity is an etiologic
83 phenomenon of alteration in the structure and function of the central nervous systems by a
84 biological, chemical or physical agent (Salvi et al., 2020). Therefore, it can be assumed that
85 water pollutants that cause oxidative stress or alter AChE activity act similarly in a wider range
86 of aquatic species. Such changes act at the level of metabolism, leading to a decrease in the
87 physiological state of living organisms. Based on this, it has been reported that some pollutants
88 can affect relationships within ecosystems through mechanisms such as oxidative stress and
89 disruption of neurotransmission (Formicki et al., 2025). The homeostasis of an organism
90 depends on bidirectional communication between the neuroendocrine system and the immune
91 system to mount adaptive responses to physiological disturbances and pathogenic challenges.
92 Although the neuroendocrine system and the immune system were initially thought to act
93 independently, recent publications recognize that a pervasive communication network controls
94 a harmonious neuroendocrine-immune interaction. Acetylcholinesterase (AChE) is an
95 essential enzyme involved in cholinergic neurotransmission by catalyzing the hydrolysis of
96 acetylcholine (ACh) and regulating its concentration within the synaptic cleft (Serafini et al.,
97 2019). Through this mechanism, AChE terminates excitatory signaling at the postsynaptic
98 membrane and ensures the proper transmission of nerve impulses (Zhang et al., 2017).
99 Acetylcholine-mediated neurotransmission represents one of the primary functional pathways
100 of the nervous system. Besides functioning as a preganglionic neurotransmitter in the

sympathetic nervous system and a postganglionic neurotransmitter in the parasympathetic nervous system, ACh also acts as an excitatory signaling molecule (Olivares-Rubio and Espinosa-Aguirre, 2021). Due to their sensitivity to toxic compounds, cholinesterases are widely accepted as valuable biomarkers for evaluating environmental pollution (Umar and Aisami, 2020).

BDNF (Brain-derived neurotrophic factor), a member of the neurotrophin family together with neurotrophins (NT) and nerve growth factor (NGF), plays significant roles in neuronal differentiation, neurogenesis, synaptic plasticity, and neuronal maturation (Topal et al., 2021). Oxidative stress develops when the production of ROS exceeds the antioxidant defense capacity of cells, leading to cellular dysfunction and tissue injury. Reactive species including H₂O₂ - hydrogen peroxide, OH⁻-hydroxyl radicals, and O₂⁻-superoxide anions are naturally produced during metabolic and immune processes. Nevertheless, excessive ROS accumulation disrupts antioxidant defense systems, while also promoting lipid peroxidation, DNA damage, apoptosis-related pathways, and cellular injury. Therefore, these parameters are considered important indicators of oxidative damage.

Cytokines are regulatory proteins involved in immunity, inflammation, and hematopoietic processes. Among the major proinflammatory cytokines, tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) have critical physiological and pathological functions. TNF- α is a multifunctional cytokine associated with cell proliferation, survival, apoptosis, necrosis, inflammatory responses, and immune regulation, and it also contributes to the progression of chronic inflammatory disorders. Although TNF- α genes have been identified in several fish species, their precise biological activities remain under investigation. Nuclear factor erythroid-2-related factor 2 (Nrf2) is another important regulator that mediates cellular defense mechanisms against oxidative stress caused by environmental contaminants. IL-6, a pleiotropic cytokine, contributes to humoral immunity by stimulating B-cell differentiation into plasma cells and promoting antibody production (Uçar et al., 2022). Pollutant-induced oxidative stress can severely impair cellular macromolecules, including lipids, proteins, and DNA, ultimately leading to lipid peroxidation, protein oxidation, DNA fragmentation, and cell death through apoptotic or necrotic pathways (Wang et al., 2016). Today, contamination of aquatic environments with insecticides is a global concern. There are limited studies on the adverse effects of Dinotefuran (DTF), one of these insecticides, on rainbow trout (*Oncorhynchus mykiss*). However, since there are gaps in these studies to elucidate the mechanism, the present study was designed to investigate the effect of DTF application on

oxidative status, cytotoxicity, DNA damage, apoptotic status indicators in brain tissue of *O. mykiss* by determining the species-specific LC₅₀ value. The study aims to fill this gap by evaluating the damage caused by DTF in rainbow trout brain tissue and the protective benefits of Aloe vera (as Aloe-emodin) against dinotefuran-induced toxicity with multidimensionally designed parameters and to provide valuable information for its potential use in sustainable aquaculture practices.

MATERIAL AND METHOD

Fish material and source

Oncorhynchus mykiss (average weight of 22±3g according to OECD report 210) obtained from Atatürk University Faculty of Fisheries, Inland Fish Application and Research Center were used. The fish is rainbow trout, the necessary permissions for this study were obtained from the Ministry of Agriculture and Forestry and the Local Animal Ethics Committee of Ataturk University Local Ethical Committee for Animal Studies (approved in 25.05.2023, Acceptance No:91). The manuscript does not contain clinical studies or patient data.

Determination of LC₅₀ Value

For the LC₅₀ determination, 100 fish were used across five concentrations for each test compound. Each concentration was tested in two replicate groups with 10 fish per replicate. The 96-h LC₅₀ values were estimated by probit analysis. The replicate structure and fish allocation are reported here to distinguish the concentration groups from the subsequent sublethal exposure experiment.

Trial design

The sublethal experiment included four groups: control, DTF, AI-EMD, and DTF+AI-EMD, with two replicate tanks per group. Treatments were applied for 10 days using a semi-static exposure design, with exposure media renewed every 24 h. Days 11–21 constituted the recovery period, during which no further DTF or AI-EMD was administered. Brain tissues were sampled at the end of the exposure period (day 11) and after the recovery period (day 21) (Haghighat et al., 2021). Because fish within the same tank shared the exposure environment, the limited number of replicate tanks should be considered when interpreting inferential statistics and generalizing treatment effects.

Chemical materials and exposure details

DTF (dinotefuran) and Al-EMD (aloe-emodin) were obtained from Sigma-Aldrich® (MO, USA). A semi-static design was used for chemical exposure. OECD 305 (OECD, 2012) was used as procedural guidance for aquatic exposure, but the present 96-h LC50 determination should not be interpreted as an OECD 305 acute-toxicity test. Based on previous zebrafish toxicity information (Ran et al., 2021) and the physicochemical persistence of DTF (Zhang et al., 2023), the DTF concentrations tested for LC50 estimation were 50, 100, 150, 200, and 250 mg/L. Al-EMD was tested at 1, 2, 3, 4, and 5 mg/L. LC50 values were estimated using the EPA Probit Analysis Program (V.1.5) (Uçar et al., 2022). The subsequent experiment used 50% of the estimated 96-h LC50 for each compound (77.5 mg/L DTF and 1.58 mg/L Al-EMD). These concentrations represent experimentally derived sublethal challenge concentrations and are not presented as measured environmental exposure levels.

Monitoring of Water Quality Parameters: Throughout the experimental period, water quality variables including temperature, pH, and dissolved oxygen concentration were measured and recorded on a daily basis.

Brain Tissue Collection and Homogenate Preparation

During both the exposure phase (days 1–10) and recovery phase (days 11–21), brain tissues were excised from the fish and rinsed with cold phosphate-buffered saline (PBS, pH 7.4) to eliminate residual blood contamination. Tissue homogenization and centrifugation procedures were carried out with slight modifications following the method of Alak et al. (2021). Homogenates were centrifuged at 4000 rpm for 15 min, and the obtained supernatants were subsequently used for biochemical analyses.

Neurotoxicity Biomarkers

Acetylcholinesterase (AChE) activity was quantified according to the method of Ellman et al. (1961). The assay is based on the enzymatic hydrolysis of acetylcholine to acetate and thiocholine, followed by the reaction of thiocholine with DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)], producing a yellow-colored compound. Absorbance changes were monitored spectrophotometrically at 412 nm. Brain-Derived Neurotrophic Factor (BDNF) concentrations in brain homogenates were determined using a commercial ELISA kit (YL Biant, YLA0091FI) in accordance with the manufacturer's instructions.

Antioxidant and Oxidative Stress Biomarkers

Catalase (CAT) activity was evaluated by initiating the reaction with hydrogen peroxide (H₂O₂). Absorbance values were monitored at 240 nm at 30 s intervals for 3 min, and enzyme activity was expressed as U/mg protein following the procedure of Aebi (1974). Glutathione Peroxidase (GPx) activity was determined by measuring the conversion of NADPH to NADP⁺ at 340 nm according to the method described by Beutler (1984). Superoxide Dismutase (SOD) activity was analyzed using a reaction mixture containing xanthine, EDTA, bovine serum albumin, and nitroblue tetrazolium (NBT). Supernatant samples or standard solutions were added to the mixture, followed by xanthine oxidase addition at 30 s intervals. After incubation at 25 °C for 20 min, the reaction was terminated using CuCl₂ solution. The reduction of NBT by superoxide radicals generated from the xanthine/xanthine oxidase system was measured spectrophotometrically at 560 nm (Sun et al., 1988). Malondialdehyde (MDA) levels, indicative of lipid peroxidation, were measured based on the formation of a pink chromogen produced by the reaction between thiobarbituric acid (TBA) and MDA (Luo et al., 2016). Tissue supernatants were mixed with phosphate buffer, BHT, and TCA, followed by incubation at –20 °C for 2 h and centrifugation. Subsequently, EDTA and TBA solutions were added to the collected supernatants, and samples were incubated in a water bath at 90 °C for 15 min. After cooling, absorbance values were recorded at 532 nm to determine MDA concentrations. Reactive Oxygen Species (ROS) production was assessed according to (Gupta et al., 2007) with minor modifications. Tissue samples were homogenized in Tris-HCl buffer, and homogenates were incubated with DCFDA at 37 °C for 30 min. Fluorescence intensity was measured using excitation and emission wavelengths of 485 nm and 525 nm, respectively.

Cytokine Analyses

Tumor Necrosis Factor-Alpha (TNF- α) levels were measured using a commercial ELISA kit (YLBiant, YLA0053FI). Absorbance was recorded at 450 nm following incubation procedures recommended by the manufacturer. **Interleukin-6 (IL-6)** concentrations were quantified using an ELISA-based assay, and absorbance values were measured at 450 nm. **Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2)** levels were determined using a commercial ELISA kit, and absorbance readings were obtained at 450 nm after incubation.

Assessment of DNA Damage and Apoptosis

8-hydroxy-2'-deoxyguanosine (8-OHdG) and Caspase-3 were evaluated using commercial assay kits (Alak et al., 2018). A fish-specific ELISA kit (BT LAB Bioassay Technology Laboratory, Cat. No: EA0004FI) was utilized. Prior to analysis, all reagents and standards were

equilibrated to room temperature. Blank wells received substrate solutions A and B together with stop solution. For experimental wells, 50 μ L of sample and 50 μ L of biotin antigen were added and incubated at 37 °C for 60 min on a plate shaker. Following incubation, wells were washed five times with washing buffer. Streptavidin-HRP solution was then added, and plates were incubated again at 37 °C for 60 min. After another washing step, substrate solutions A and B were introduced, and the plates were kept in the dark at 37 °C for 10 min. The reaction was terminated by adding stop solution, and absorbance was measured at 450 nm using an ELISA plate reader within 10 min. Sample concentrations were calculated from the standard calibration curve generated from standard optical density values.

Histopathological Examination

At the end of the experimental period, brain tissues were fixed in 10% formaldehyde for 48 h. Following routine histological processing, tissues were embedded in paraffin blocks, and 4 μ m sections were prepared. Slides were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus BX51, Japan). Histopathological alterations were graded semi-quantitatively as absent (–), minimal (+), mild (++), moderate (+++), or severe (++++), based on tissue damage severity.

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics. Data distributions were assessed for normality before parametric testing. Parametric outcomes were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test when an overall group effect was detected (Alak et al., 2017). Results are presented as mean $\pm$ standard error (SE), with $p < 0.05$ considered statistically significant. The experimental design included two replicate tanks per treatment; therefore, statistical significance should be interpreted cautiously because fish sharing a tank are not fully independent exposure units. This limitation is explicitly considered in the Discussion and should be addressed with greater tank-level replication in confirmatory studies.

Histopathological data were analyzed using GraphPad Prism. Statistical significance was accepted at $p < 0.05$. Since histopathological scores were nonparametric, group comparisons were performed using the Kruskal–Wallis test, while intergroup differences were evaluated with the Mann–Whitney U test.

Findings and Results

Determining the LC50 value of DTF and AI-EMD

For DTF, five concentrations (50, 100, 150, 200, and 250 mg/L) were included in the 96-h toxicity assessment. Probit analysis yielded a 96-h LC50 of 154.89 mg/L. A concentration corresponding to 50% of this LC50 (77.5 mg/L) was selected for the subsequent sublethal exposure experiment (Figure 1).

For AI-EMD, mortality at 1, 2, 3, 4, and 5 mg/L was used for probit analysis. The estimated 96-h LC50 was 3.16 mg/L, and 50% of this value (1.58 mg/L) was used in the subsequent experiment (Figure 2).

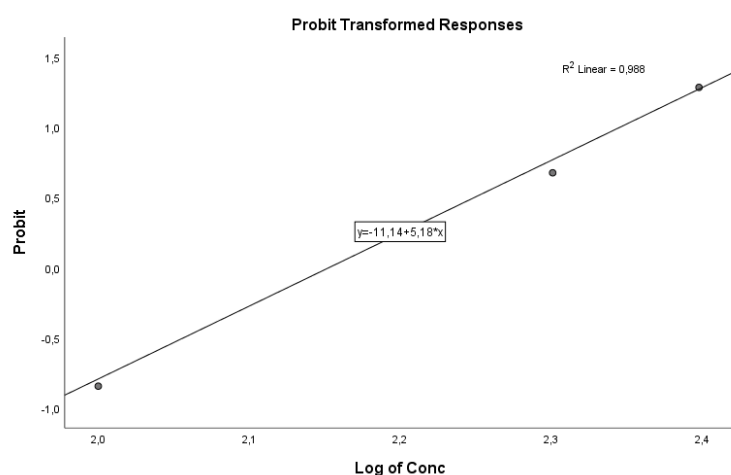


Figure 1. The probit analyses of Dinotefuran (DTF) pesticide

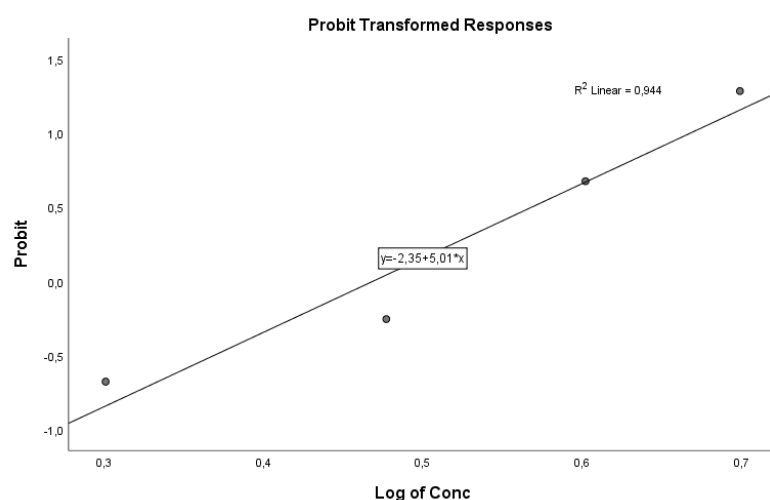


Figure 2. Probit analysis of Aloe-emodin (Al-EMD)

Neurotoxic biomarkers (AChE and BDNF)

DTF exposure was associated with lower AChE activity and BDNF levels than the control. Co-administration of Al-EMD attenuated these reductions, whereas Al-EMD alone did not reproduce the DTF-associated inhibition (Figure 3). After the recovery period, AChE and BDNF values were generally closer to those of the control group, indicating partial normalization after exposure ceased.

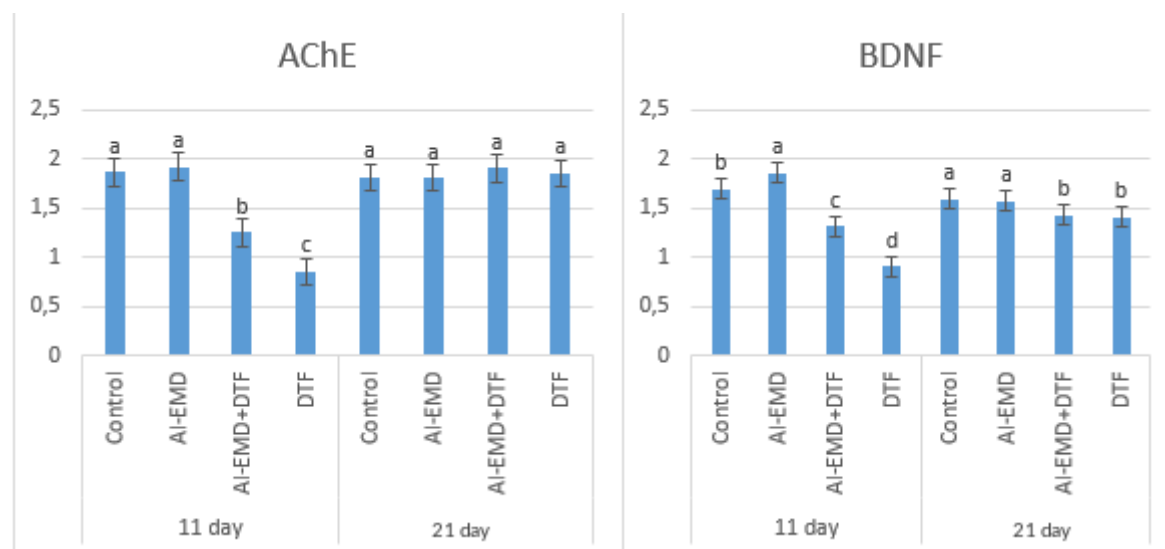


Figure 3. Effects of DTF and/or Al-EMD on AChE activity and BDNF levels in *O. mykiss* brain tissue. Data are presented as mean $\pm$ SE. Different superscript letters (a–d) indicate statistically significant group differences ($p < 0.05$).

Antioxidant system and immune system biomarkers (SOD, CAT, GPx, MDA, MPO, ROS, and TNF- α , IL-6, Nrf-2)

DTF exposure altered the oxidative balance in brain tissue, with lower SOD, CAT, and GPx activities and higher MDA, MPO, and ROS values relative to the control. Co-administration of Al-EMD attenuated several of these changes, including reductions in lipid peroxidation and partial preservation of antioxidant enzyme activities (Figure 4). These findings are consistent with a protective antioxidant effect of Al-EMD under the conditions tested, although they do not by themselves establish a specific molecular pathway.

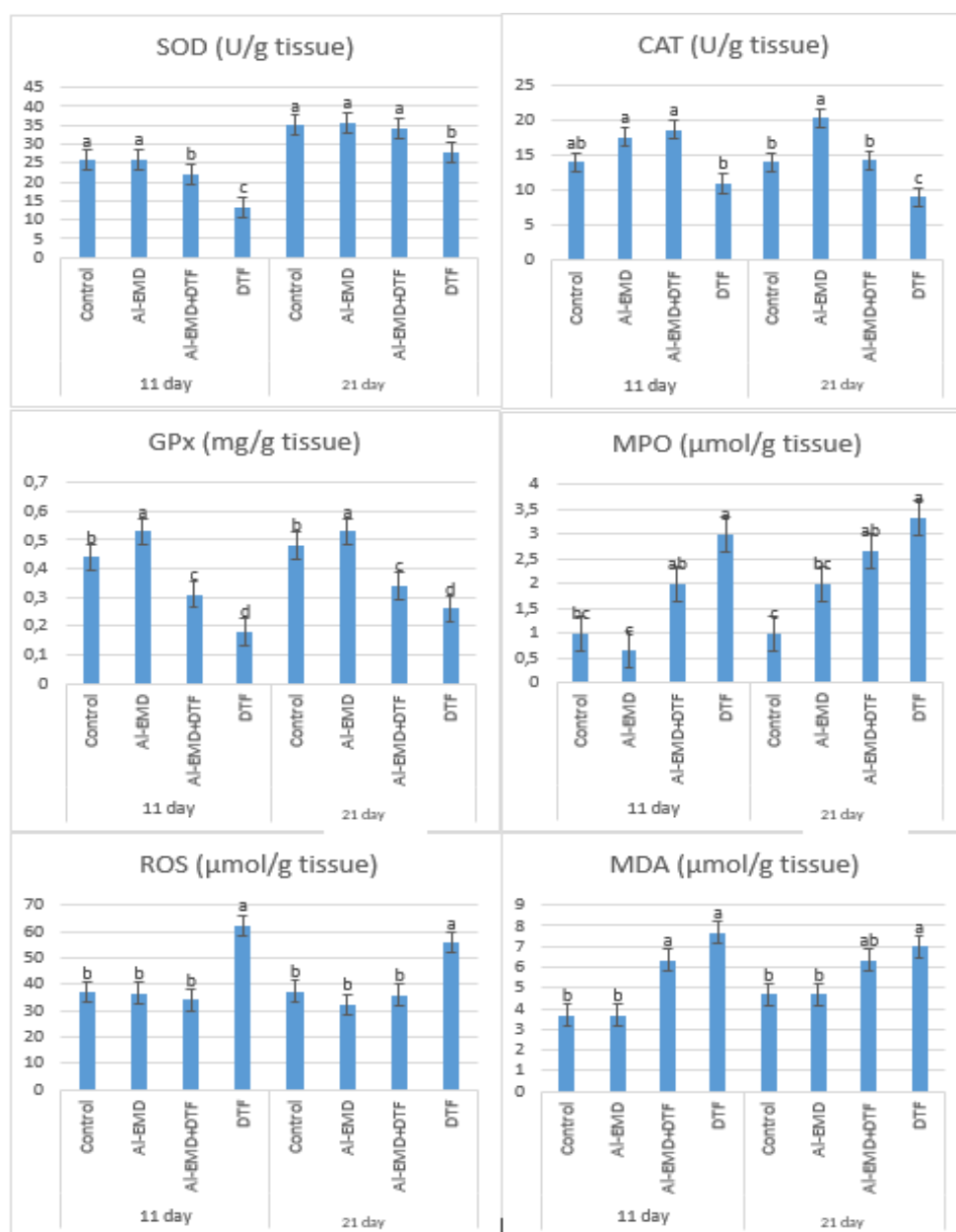


Figure 4. Effects of DTF and/or Al-EMD on SOD, CAT, GPx, MDA, MPO, and ROS in *O. mykiss* brain tissue. Data are presented as mean $\pm$ SE. Different superscript letters indicate statistically significant group differences ($p < 0.05$).

TNF- α levels were higher in the DTF group than in the control group. Co-administration of Al-EMD attenuated the DTF-associated increase, indicating a partial reduction in the inflammatory response ($p < 0.05$; Figure 5).

IL-6 levels were also elevated following DTF exposure. In the DTF+Al-EMD group, IL-6 values were lower than in the DTF-only group and were closer to control values, suggesting that Al-EMD partially attenuated the DTF-associated inflammatory response (Figure 5).

Nrf2 levels decreased in the DTF group relative to the control. The pattern across the AI-EMD-containing groups indicates treatment-related modulation of Nrf2; however, because downstream ARE activation and Nrf2 target-gene expression were not measured, the present data should be interpreted as changes in Nrf2 level rather than direct evidence of Nrf2-pathway activation (Figure 5).

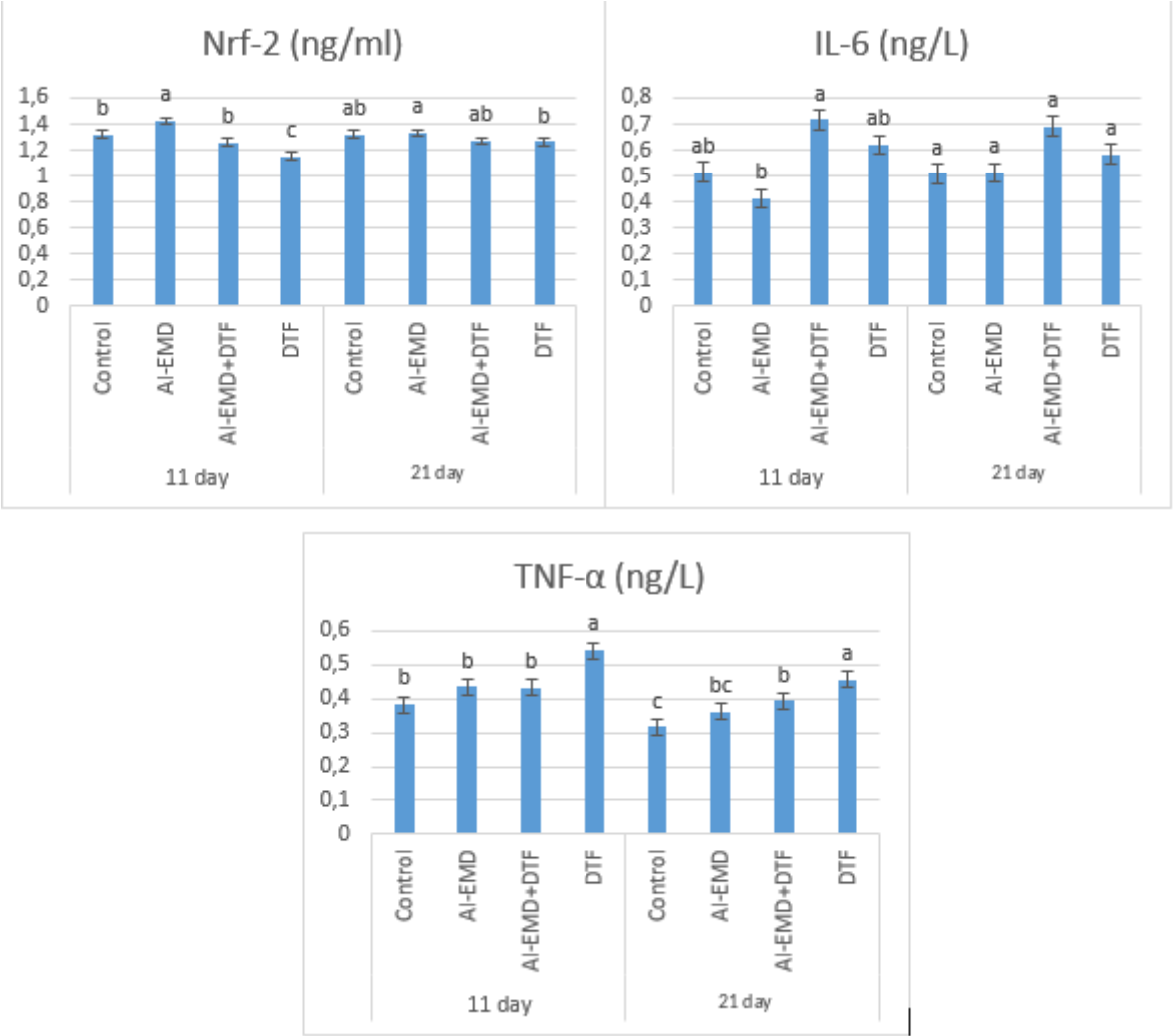


Figure 5. Effects of DTF and/or AI-EMD on TNF- α , IL-6, and Nrf2 levels in *O. mykiss* brain tissue. Data are presented as mean $\pm$ SE. Different superscript letters indicate statistically significant group differences ($p < 0.05$).

DNA and apoptosis biomarkers

DTF exposure increased 8-OHdG levels relative to the control, consistent with greater oxidative DNA damage (Figure 6). Co-administration of AI-EMD attenuated this response, and values

after the recovery period were generally closer to the control than those observed following DTF exposure.

Caspase-3 levels were highest in the DTF-exposed group, whereas co-administration of AI-EMD attenuated the DTF-associated increase. During the recovery period, caspase-3 values showed partial normalization relative to the exposure phase (Figure 6). Group differences were statistically significant where indicated by the superscript lettering ($p < 0.05$).

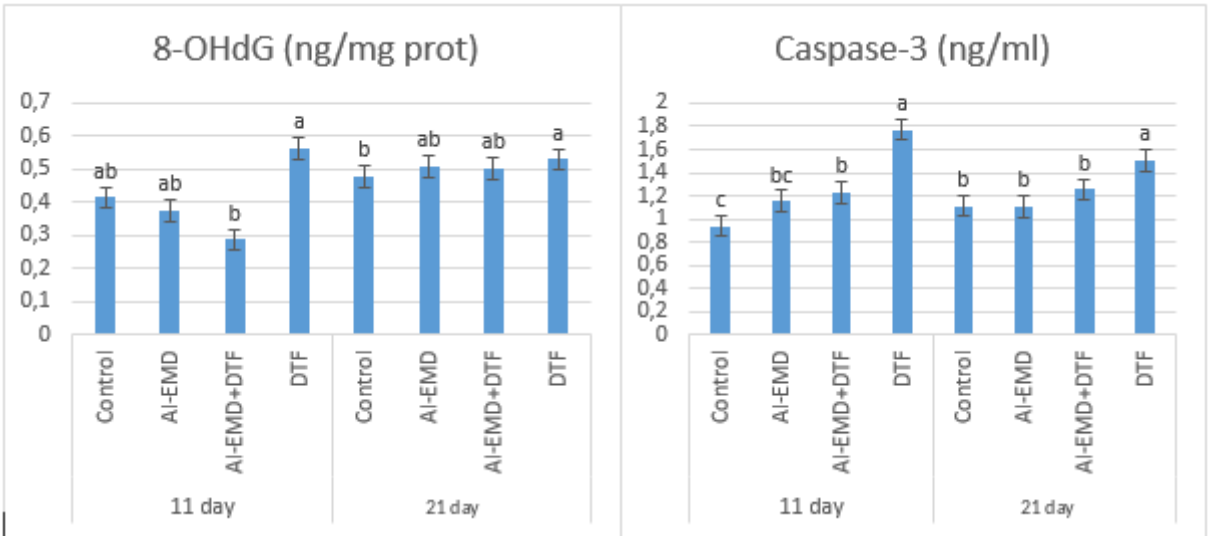


Figure 6. Effects of DTF and/or AI-EMD on 8-OHdG and caspase-3 levels in *O. mykiss* brain tissue. Data are presented as mean $\pm$ SE. Different superscript letters indicate statistically significant group differences ($p < 0.05$).

Histopathologic Findings

Exposure period (Days 1–10; sampling on Day 11)

Control: Histopathologic analysing of rainbow trout brain tissues revealed normal histologic structure (Figure 7-8).

AI-EMD: Histopathologic analysing of rainbow trout brain tissues revealed normal histologic structure. (Figure 7-8).

DTF: Histopathological analysing of rainbow trout brain tissues revealed severe degeneration and necrosis in neurons and hyperemia in vessels (Figure 7-8).

AI-EMD+DTF: Histopathological analysing of rainbow trout brain tissues revealed mild degeneration and necrosis in neurons and hyperemia in vessels (Figure 7-8).

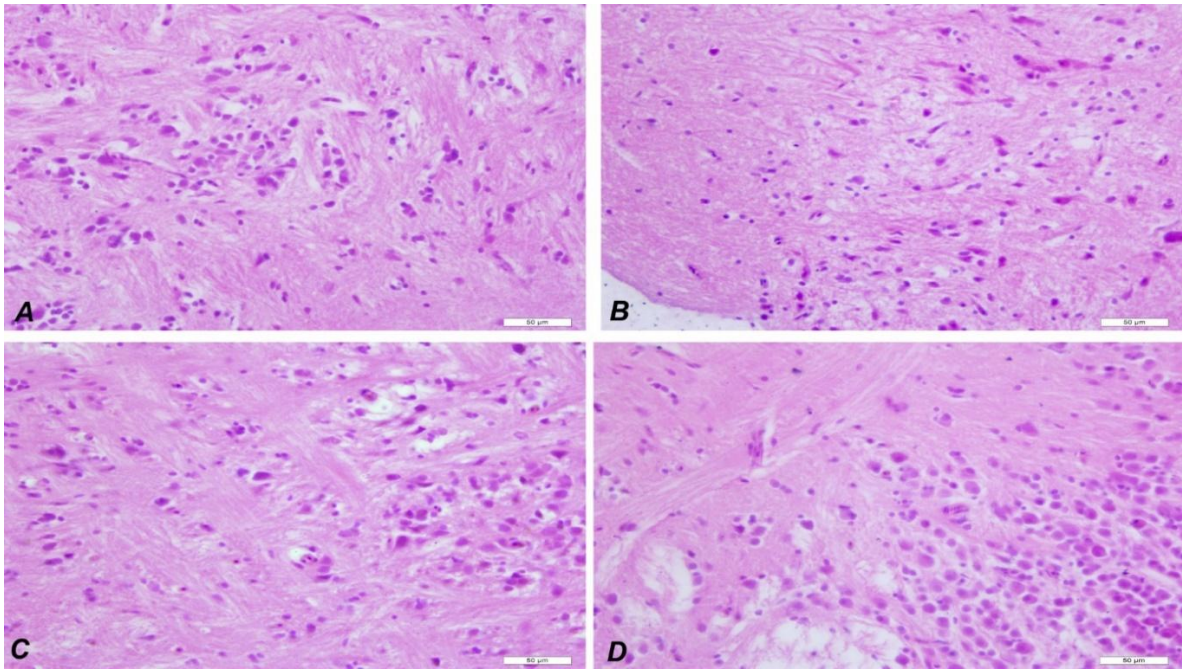
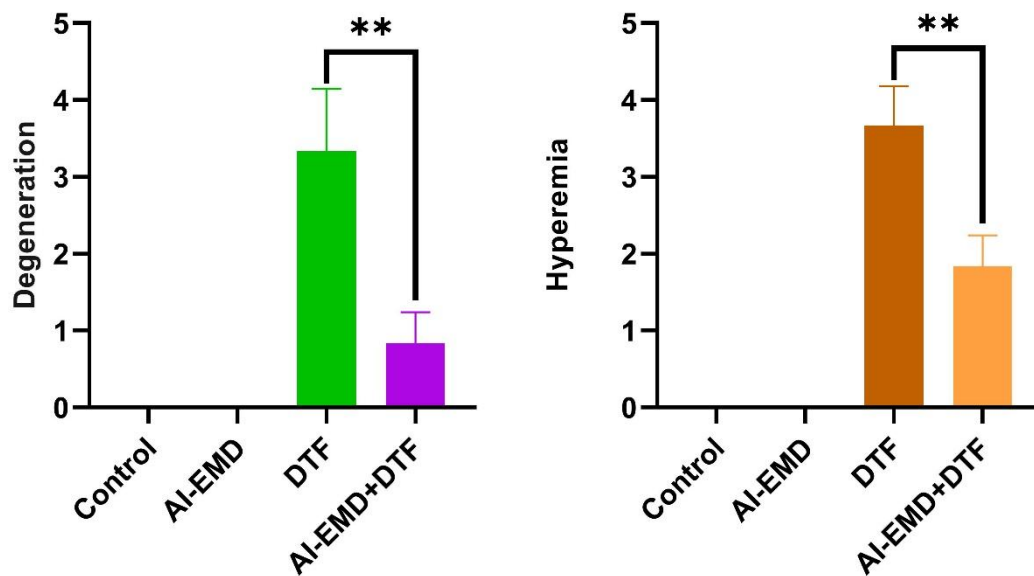


Figure 7. Brain tissue, Control (A), AI-EMD (B), DTF (C), DTF+ AI-EMD (D), degeneration and necrosis of neurons, hyperemia of vessels, H&E, Bar:50μm.



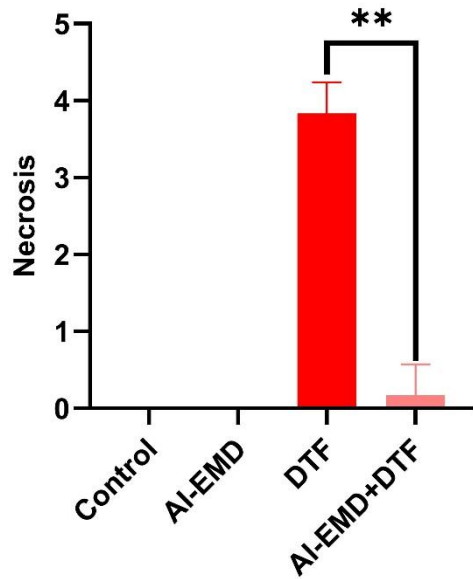


Figure 8. Statistical analysis data of histopathological staining results seen in brain tissue. Degeneration (** $P < 0.0022$) Necrosis (** $P < 0.0022$) Hyperemia (** $P < 0.0022$), (Day 11).

Recovery endpoint (Day 21)

Control: Histopathologic analysing of rainbow trout brain tissues revealed normal histologic structure (Figure 9-10).

AI-EMD: Histopathologic analysing of rainbow trout brain tissues revealed normal histologic structure (Figure 9-10).

DTF: Histopathological analysing of rainbow trout brain tissues revealed severe degeneration and necrosis in neurons and Purkinje cells in the cerebellum, and hyperemia in the vessels (Figure 9-10).

AI-EMD+DTF: Histopathological analysing of rainbow trout brain tissues revealed mild degeneration of neurons and Purkinje cells and hyperemia in vessels (Figure 9-10).

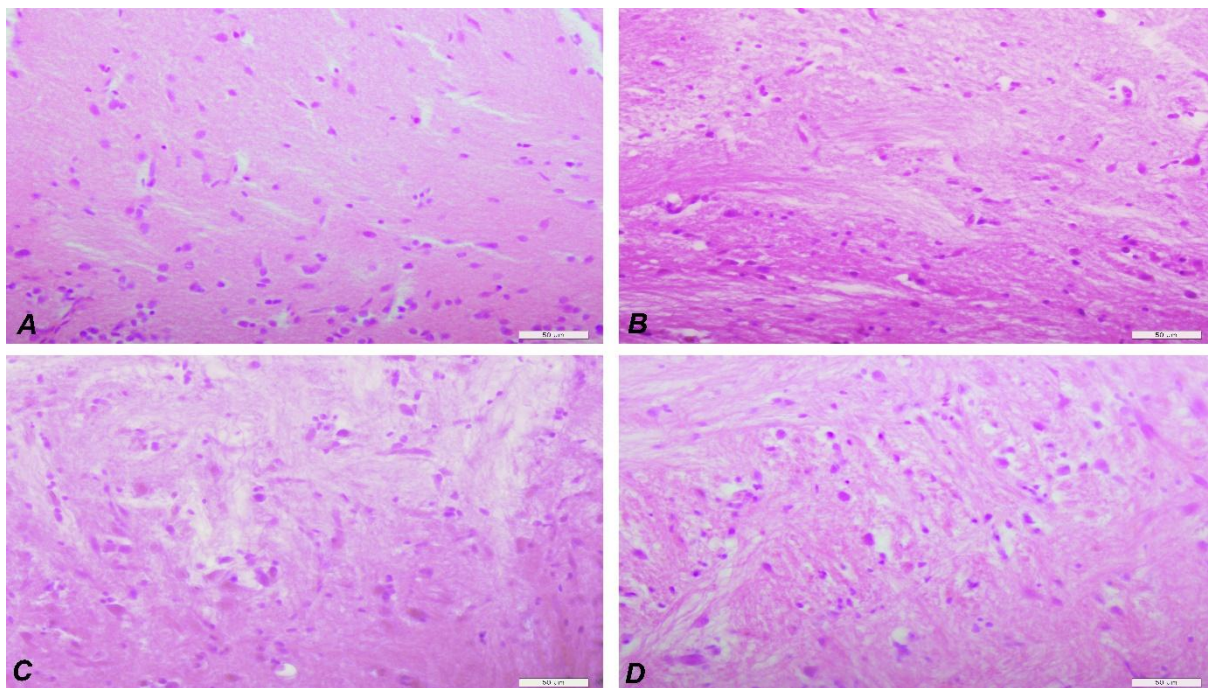


Figure 9. Brain tissue, Control (A), Al-EMD (B), DTF (C), DTF+ Al-EMD (D), degeneration and necrosis of neurons, hyperemia of vessels, H&E, Bar:50μm.

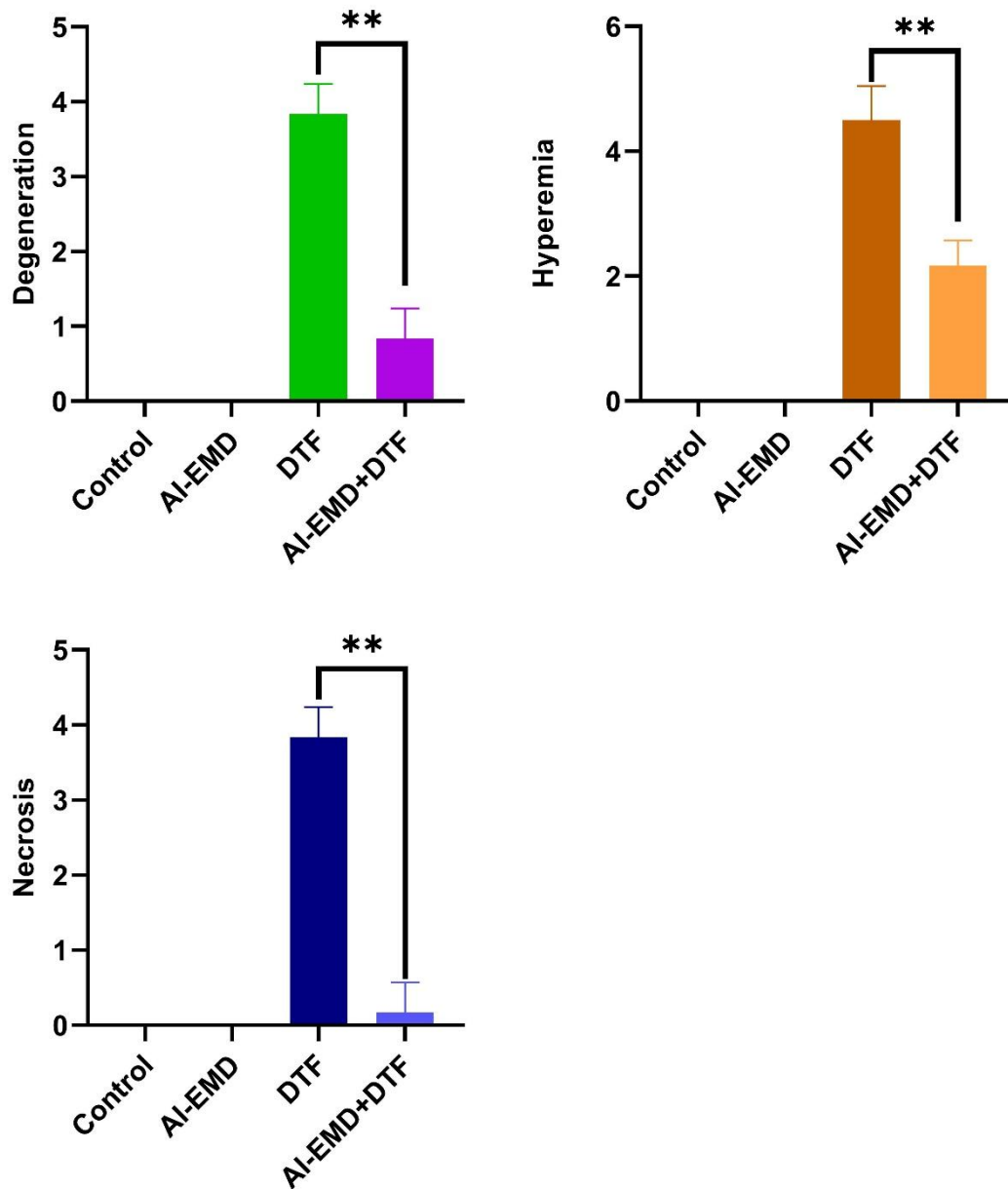


Figure 10. Statistical analysis data of histopathological staining results seen in brain tissue. Degeneration (** P<0.0022) Necrosis (** P<0.0022) Hyperemia (** P<0.0022). (Day 21 recovery)

Discussion

The brain, which is vital in the regulation of physiological functions in fish, is highly vulnerable to ROS produced by oxidative stress due to its metabolic activity and lipid-rich composition. ROS-induced oxidation of proteins and lipids in neuronal membranes can impair the fluidity, structural integrity, and permeability of brain cells (Alak et al., 2024). ROS can also reduce ATP generation in mitochondria and trigger apoptosis in neurons, thereby impairing brain

function. The blood-brain barrier (BBB) is a selective barrier that protects the brain from toxins and provides a stable internal environment for neuronal function (Uçar et al., 2022). Pesticide damage to the BBB has been reported to cause leakage of ROS into brain tissue and exacerbate oxidative stress and neuronal damage (Abou Diwan et al., 2023).

By triggering protein oxidation and inducing enzymes such as the vital acetylcholinesterase (AChE), pesticides cause i) acetylcholine accumulation (Faria et al., 2021), ii) neuromuscular impairments for neurotransmission, and iii) Causes decreased detoxification capacity of antioxidants such as GST/SOD (Moezzi et al., 2025). In this process, AChE inhibition causes acetylcholine accumulation; nerve transmission at cholinergic synapses is disrupted, which can result in hyperactivity, neuromuscular impairments and ultimately death of the organism (Mukherjee et al., 2025).

In this study, DTF exposure was associated with significant reductions in AChE activity and BDNF levels in *O. mykiss* brain tissue. These findings support a neurotoxic effect of DTF but do not identify a single molecular binding mechanism. The partial normalization of AChE and BDNF during the recovery period suggests that at least some DTF-associated neurochemical alterations may be reversible after exposure ceases. Co-administration of Al-EMD attenuated the reductions observed during DTF exposure, supporting a potential protective effect that requires confirmation in experiments with greater independent replication.

Histopathological reactions of the fish showed the extent of damage to brain tissue caused by pesticides. The amount of damage and the gradual changes that DTF poisoning induces in the brain of the fish over the exposure period indicate that the histopathological responses depend not only on the pesticide concentration but also on how long the fish is exposed. As a result, histopathological changes such as vacuolization, necrosis and cerebral edema caused by ROS in the brain impair brain function, leading to neurological disorders and reduced survival of fish (Hawkey et al., 2023).

Detoxification of xenobiotics in fish occurs through a series of enzymatic processes that are mainly divided into three phases: Phase I (functionalization), Phase II (conjugation), and Phase III (excretion) (Ensibi et al., 2010). Phytochemicals have been reported to influence these processes (Moezzi et al., 2025). In the present study, however, phase-specific detoxification enzymes were not comprehensively measured; therefore, the observed effects of Al-EMD

should be described as protective associations rather than direct evidence that AI-EMD enhanced all phases of xenobiotic detoxification. The biochemical and histological findings nevertheless indicate that AI-EMD co-administration attenuated several DTF-associated alterations and may have contributed to recovery after exposure.

Exposure to exogenous toxicants stimulates the production of both reactive oxygen species (ROS) and malondialdehyde (MDA), resulting in excessive oxidative stress (Ucar et al., 2024a). MDA, which is a major end-product of lipid peroxidation, is widely used as an indirect indicator of ROS generation, lipid membrane damage, and membrane system impairment. In the present study, DTF exposure significantly elevated ROS and MDA levels in the brain tissue of *Oncorhynchus mykiss*, indicating that DTF disrupted lipid metabolism and promoted oxidative injury in neural tissues. This increase may be associated with the progressive inability of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) to neutralize excessive ROS accumulation over prolonged exposure periods (Ucar et al., 2024b; Ucar et al., 2024a).

According to (Ullah et al., 2024), pesticide-induced ROS generation in fish may arise through several interconnected mechanisms, including mitochondrial dysfunction, inhibition of antioxidant enzymes, and activation of inflammatory pathways. Mitochondrial dysfunction can result in incomplete oxygen reduction during ATP synthesis, leading to electron leakage and ROS formation. In addition, suppression of antioxidant enzymes such as SOD, CAT, and glutathione peroxidase (GPx) further enhances oxidative stress by weakening ROS scavenging capacity. ROS production may also increase during inflammatory responses because activated immune cells, including macrophages and neutrophils, release ROS as part of host defense mechanisms. However, excessive ROS generation ultimately contributes to oxidative damage. Previous studies have demonstrated that mitochondrial oxidative phosphorylation is closely associated with ATP generation as well as inflammation-related ROS production (Song et al., 2024).

The elevated ROS and MDA levels observed in the brain tissue of DTF-exposed fish suggest enhanced lipid peroxidation and disruption of membrane integrity. These findings further indicate that endogenous antioxidant defense systems, particularly SOD and CAT activities, were insufficient to counteract oxidative injury, resulting in impaired cellular defense mechanisms. Oxidative stress appears to be intensified through multiple pathways, including

mitochondrial dysfunction, enzymatic inhibition, and depletion of non-enzymatic antioxidants (Ullah et al., 2024; Grădinariu et al., 2025).

Oxidative stress is an important mechanism by which pesticides can impair cellular and immune function in aquatic organisms (Ucar et al., 2024a). In the present study, DTF exposure was associated with increased ROS and lipid peroxidation together with reduced SOD, CAT, and GPx activities, indicating that antioxidant defenses were insufficient to counteract the oxidative challenge. After exposure ceased, several antioxidant endpoints shifted toward control values. Al-EMD co-administration also attenuated multiple oxidative changes. These patterns support an antioxidant protective effect under the tested conditions, but the study did not directly assess mitochondrial respiration, glutathione turnover, or pathway-specific gene expression; consequently, those mechanisms should be regarded as literature-supported explanations rather than mechanisms demonstrated by the present experiment.

Excessive ROS accumulation can promote inflammatory signaling and increased proinflammatory cytokine production, including TNF- α (Zeid et al., 2021). In the present study, DTF exposure increased TNF- α and IL-6 levels in brain tissue, whereas co-administration of Al-EMD attenuated these increases. Because cytokine protein levels rather than transcriptional activity were measured, these results demonstrate an inflammatory response but should not be interpreted as direct evidence of cytokine gene upregulation.

Nrf2 is a major regulator of cellular antioxidant defense and, when activated, can interact with antioxidant response elements (AREs) to regulate detoxification and antioxidant genes. This pathway provides a plausible framework for interpreting phytochemical effects reported in the literature. In the present study, however, only Nrf2 levels were measured; ARE binding, nuclear translocation, NF- κ B activity, and downstream Nrf2 target-gene expression were not assessed. Accordingly, the observed Nrf2 changes cannot establish activation of the Nrf2/ARE or NF- κ B pathways, and pathway-level interpretations remain hypotheses for future mechanistic studies.

8-Hydroxy-2'-deoxyguanosine (8-OHdG) is widely used as a marker of oxidative DNA modification. In the current study, DTF exposure increased 8-OHdG levels, consistent with oxidative DNA injury in brain tissue. This change occurred alongside increased ROS and reductions in several antioxidant enzyme activities, supporting an association between oxidative imbalance and DNA damage. Because the sublethal experiment used a single DTF

challenge concentration, the present data do not establish a concentration-response relationship for these biomarkers.

Histopathological findings were consistent with the biochemical evidence of tissue injury. H&E-stained brain sections showed persistent structural lesions after the exposure phase, while the DTF+Al-EMD group generally displayed less severe alterations than the DTF-only group. Recovery was incomplete, indicating that some structural damage persisted after exposure ceased. These observations support a potential protective effect of Al-EMD but do not establish a specific molecular repair mechanism (Abdel-Khalek et al., 2022).

Taken together, the multi-biomarker results show that DTF exposure affects neurochemical, oxidative, inflammatory, DNA-damage, apoptotic, and histopathological endpoints in rainbow trout brain tissue. The novelty of the present work lies in evaluating these endpoints together with a post-exposure recovery phase and Al-EMD co-administration. The data support a protective potential for Al-EMD under the experimental conditions, but they should not be interpreted as definitive proof of a neural signaling pathway or as evidence for field-scale remediation efficacy.

Study limitations should be considered when interpreting these findings. The treatment design used two replicate tanks per group, which limits tank-level statistical power and increases the risk of pseudoreplication if individual fish are treated as fully independent observations. The sublethal DTF concentration was derived from the experimentally estimated LC50 and should not be considered representative of typical environmental concentrations without field-exposure data. In addition, the study measured selected protein/biochemical endpoints but did not directly assess receptor binding, Nrf2 nuclear translocation, ARE or NF- κ B activity, or pathway-specific gene expression. Future studies should increase independent tank replication, report the number of fish contributing to each endpoint explicitly, use environmentally measured exposure ranges, and incorporate pathway-specific molecular assays.

Conclusion

In conclusion, DTF exposure produced marked neurochemical, oxidative, inflammatory, DNA-damage, apoptotic, and histopathological alterations in the brain tissue of *O. mykiss*. Al-EMD co-administration attenuated several of these changes, and partial normalization of multiple endpoints was observed after exposure ceased. These findings support Al-EMD as a candidate

protective phytochemical under the present experimental conditions. However, the limited tank-level replication, the experimentally derived sublethal DTF concentration, and the absence of pathway-specific molecular assays preclude definitive mechanistic, environmental, or economic conclusions. Additional independently replicated studies using environmentally relevant exposure scenarios are required to confirm the protective effect and clarify the underlying mechanisms.

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Competing interests

The authors declare no competing interests.

Ethics declarations

Ethics approval

The fish is rainbow trout, the necessary permissions for this study were obtained from the Ministry of Agriculture and Forestry and the Local Animal Ethics Committee of Atatürk University Local Ethical Committee for Animal Studies (approved in 25.05.2023, Acceptance No:91). The manuscript does not contain clinical studies or patient data.

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