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Protective effects of green tea (*Camellia sinensis*) extract against cypermethrin-induced neurotoxicity in rainbow trout (*Oncorhynchus mykiss*) brain tissues

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Abstract: This study was conducted to evaluate the protective impacts of green tea extract (GT) against cypermethrin-induced (CYP) neurotoxicity parameters such as acetylcholinesterase activity, oxidative stress, immune response, histopathology, apoptosis, and DNA damage in the brain tissues of rainbow trout (*Oncorhynchus mykiss*). The trial was divided into four groups (group 1, 2, 3 and 4). Group 1 was the control, while group 2 was treated with 0.1 µg/l of CYP, group 3 was treated with 0.1 µg/l of CYP + 100 mg/l of GT and group 4 was treated with 0.1 µg/l of CYP + 200 mg/l of GT for 14 days. While the level of malondialdehyde increased, the exposure to CYP in group 2 resulted in a reduction in acetylcholinesterase, lysozyme, total immunoglobulin, white blood cell, superoxide dismutase, and catalase levels in the brain tissues, as compared to group 1. Furthermore, CYP exposure in group 2 resulted in severe hyperaemia in meningeal and parenchymal vessels, accompanied by significant degeneration and necrosis. In addition, neurons in group 2 exhibited pronounced cytoplasmic expressions of 8-OHdG and caspase-3. On the contrary, both doses of 100 mg/l and 200 mg/l of GT demonstrated remarkable neuroprotective impacts against CYP toxicity across all parameters as mentioned above. In summary, this study conclusively showed that the administration of 200 mg dose of GT yielded more pronounced neuroprotective impacts, surpassing the impacts observed with the 100 mg dose of GT. The higher dosage effectively reduced CYP-induced oxidative stress, apoptosis and DNA damage, while exhibiting an enhanced immune response.

Keywords: fish; extract; pesticide; oxidative stress; immunity; histopathology

Cypermethrin (CYP) is a synthetic pyrethroid insecticide widely used in the management of ectoparasites in public health and livestock (Abdel-Tawwab and Hamed 2020). CYP, known for its high toxicity to fish, has a 96-hour LC_{50} value of 0.5 µg/l for rainbow trout, 0.2 µg/l for crayfish (Lidova et al. 2016) and 13.8 µg/l for common carp (Stara et al. 2013) and it exhibits a water solubility of 0.01 mg/l and a log Kow value of 6.06, with a half-life of over fifty days in water (Shelley et al. 2009).

Increased reactive oxygen species (ROS), including radicals (superoxide anion, singlet oxygen, hydroxyl radical, and their derivatives) can irreversibly modify ketone groups, thereby causing protein aggregation, deactivation, or degradation, which includes aldehydes, amino acid side chains, and protein function (Koubova et al. 2022). These biochemical changes occur as a consequence of oxidative stress (Amin and Hashem 2012). Pesticides play an important role in detecting the impacts

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of stress, which encompasses membrane lipids, proteins, and DNA damage (Parvez and Raisuddin 2005). Oxidative DNA damage can induce modifications in organisms like hydroxyl radical target bases and deoxyribose residues within the DNA structure (Arslan et al. 2017). Thus, 8-OHdG emerges as a vital marker for assessing the impacts of pesticide-induced oxidative DNA damage and stress (Topal et al. 2017).

Apoptosis, regulated by genes, proteins (caspases), and organelles, is the normal process of eliminating unnecessary or abnormal cells in the body in response to stress (Kumar et al. 2014).

Caspases, enzymes belonging to the cysteine-protease group, play a vital role in numerous cellular and morphological changes that occur in the complex process of apoptosis (Gurtu et al. 1997; Gao et al. 2013). Eukaryotic cells have initiator and effector caspases that affect the apoptotic pathway (Arslan et al. 2017). Initiator caspases, including caspases 2, 8, 9, and 10, activate effector caspases such as caspases 3, 6, 7 (Arslan et al. 2017). Caspase-3 is very important in the mitochondrial and death receptor pathways of apoptosis (Karats et al. 2023).

The presence of natural antioxidants in medicinal plants is vital in mitigating the adverse impacts of free oxygen radicals and serving as inhibitors of oxidative stress. Green tea, renowned for its rich antioxidant content, holds a prominent position among the most important herbal remedies. Green tea is rich in polyphenolic compounds and flavonoids, with catechins being its main component (Ogaly et al. 2015). It has been determined that catechins in green tea have antioxidant, anti-carcinogenic, anti-inflammatory, antimicrobial, anti-mutagenic and neuroprotective impacts (Wu et al. 2021; El Tabaa et al. 2022). In the past decade, research has revealed that green tea possesses significant antioxidant properties and demonstrates a high potential in eliminating free oxygen radicals, as well as capturing free hydroxyl and superoxide anion radicals (Bagherpour et al. 2018). Previous studies have demonstrated the protective impacts of green tea extract against 4-nonylphenol in *Clarias gariepinus* (Sayed and Soliman 2018), silver nanoparticles in *Ceriodaphnia cornuta,* and *Poecilia reticulata* (Banumathi et al. 2017), and ammonia stress in juvenile *Megalobrama amblycephala* (Guo et al. 2020). Considering the limited number of studies on the efficiency of green tea extract against pesticides in fish, there are no studies showing its impacts on brain tissue. Therefore, this study was conducted to determine the protective efficiency of green tea (*Camellia sinensis*) extract on oxidative stress, immunity, histopathology, apoptosis, and DNA damage in brain tissues of rainbow trout (*Oncorhynchus mykiss*) against neurotoxicity induced by CYP.

MATERIAL AND METHODS

Experimental organisms

Rainbow trout of 70 ± 2.58 g in weight were placed in a storage tank for a 15-day adaptation period. During this period, the fish were fed a daily amount equivalent to 3% of their body weight (Abalıoğlu, Turkey, size 4 mm, crude protein 480 g/kg, moisture 120 g/kg, crude fat 180 g/kg, ash 120 g/kg, crude cellulose 30 g/kg and digestible energy 4 400 kcal/kg) and their metabolic waste was cleaned daily (Karatas et al. 2020). Fish were kept in 380 l tanks with a flow rate of 0.5 l/minute. Throughout the trial, the water temperature was maintained at 11.4 °C, dissolved oxygen levels remained at 7.90 mg/l, and pH was consistently measured at 6.7 (Karatas et al. 2019a). The study was performed within the ethical rules determined by Agri Ibrahim Cecen University (Writing and decision number: 42163/133).

Green tea extract

The green tea (GT) extract was acquired from a local commercial enterprise, and each portion contained 500 mg. The nutrient composition comprised 90% polyphenols and 45% epigallocatechin gallate (EGCG). The fresh stock solution was prepared with a concentration of 1 000 mg/l of green tea extract and used in the experiment.

Experimental design and toxicity

Cypermethrin, $C_{22}H_{19}C_{l2}NO_3$ (CAS Number 52315-07-8, ≥ 98%, molecular weight 416.30 g/mol) was purchased from Sigma-Aldrich (Burlington, MA, USA). After a 15-day adaptation period, rainbow trout were divided into four groups with eight fish in each group. In the literature, the LC_{50} value of CYP for rainbow trout was determined

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as 0.5 µg/l (Shelley et al. 2009). Group 1 was the control, while group 2 was treated with 0.1 µg/l of CYP, group 3 was treated with 0.1 µg/l of CYP + 100 mg/l of GT and group 4 was treated with 0.1 µg/l of CYP + 200 mg/l of GT. The trial was conducted with three repetitions, and each group underwent a two-week exposure.

Immunity parameters

Blood from the fish was collected in serum tubes and allowed to clot for 7 minutes. Subsequently, the clotted blood was separated into serum after centrifugation at 3 000 rpm for approximately 10 minutes. The enzyme lysozyme and total immunoglobulin (T. Ig) levels in the serum of fish were measured using the methods described by Ellis (1990) and Siwicki (1993), respectively. The measurement of white blood cell (WBC) was performed using the Sysmex XN9500 modular system (Karatas et al. 2023).

Malondialdehyde, antioxidant enzyme and protein analysis

For malondialdehyde (MDA) and protein analyses, brain tissues powdered using TissueLyser II were reconstituted with 1.15% KCl and homogenized in TissueLyser II (Qiagen, Hilden, Germany). Then they were centrifuged at 3 500 rpm for approximately 15 min (Karatas et al. 2021). The protein concentration in brain tissues was determined at 650 nm using the Lowry method with standard bovine serum albumin (Beijing, China) as a reference (Lowry et al. 1951). MDA levels were assessed at 532 nm following the method reported by Placer et al. (1966). Superoxide dismutase (SOD) levels were determined at 560 nm based on the procedure outlined by Sun et al. (1988). Catalase (CAT) levels were described at 405 nm using the method established by Goth (1991).

Determination of acetylcholinesterase activity

The brain tissues were homogenized by centrifugation at 3 500 rpm for 10 min in a 0.05 M phosphate buffer solution. Acetylcholinesterase levels in brain tissues were determined according to the method described by Ellman et al. (1961). In this method, a mixture consisting of phosphate buffer, homogenate, 0.01 M DTNB [5,5'-dithiobis-(2-nitro-benzoic acid)], and 1.25 M acetylthiocholine iodide was prepared, and the resulting mixture was measured spectrophotometrically at 412 nm for 4–6 minutes. Protein concentrations in brain tissues were determined following the procedure described by Lowry et al. (1951).

Histopathological examination

The brain sections fixed in 10% formalin (pH 2.8–4.2) for 48 h were routinely observed by an automated tissue monitoring device (Shormo Citadel 2000; Thermo Fisher Scientific, Waltham, MA, USA). Then, 4-µm sections were taken from paraffin-embedded brain tissues. Haematoxylin-eosin (H&E) staining was used to stain the sections, and the levels of tissue damage were then assessed under a microscope (Olympus BX 51; Olympus, Tokyo, Japan) at a magnification of 50 µm (Karatas et al. 2021).

Immunohistochemical examination

The deparaffinized and dehydrated tissues were mounted on adhesive (poly-L-lysine) slides and exposed to 3% H_2O_2 for 10 minutes. The sections were subjected to boiling in an antigen retrieval solution for two rounds of 5 min at 500 W, followed by cooling to retrieve the antigens. 8-OHdG antibody (Cat. No. SC-66036) was then incubated for 60 min at 37 °C according to the manufacturer's instructions supplied with the Abcam HRP/DAB Detection IHC kit. The colour stain development was assessed using the chromogen 3,3'-diaminobenzidine (DAB). Haematoxylin was used for counterstaining the sections. The tissue examination was conducted based on the degree of immune positivity, categorized as no, mild, moderate, and severe (Karatas et al. 2021).

Immunofluorescence examination

The deparaffinized and dehydrated tissues were mounted on adhesive (poly-L-lysine) slides and

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subjected to 3% H_2O_2 for 10 minutes. The sections were subjected to boiling in an antigen retrieval solution. After incubating the sections for 5 min, the primary antibody (Caspase-3, Cat. No.: sc-7272, dilution ratio: 1/100) was applied by dropping onto the sections. After dropping the immunofluorescent secondary antibody (FITC, Cat. No.: ab6785, dilution ratio: 1/1 000) onto the sections, they were held in a dark room for 45 minutes. Subsequently, after application of DAPI (Cat. No.: D1306, dilution ratio: 1/200) the sections were kept in the dark for 5 minutes. Finally, the tissues were observed and analyzed using a Zeiss AXIO fluorescence microscope (Zeiss, Oberkochen, Germany).

Statistical analysis

Biochemical, immunohistochemistry and immunofluorescence data were carried out with SPSS v13.0 statistical program (SPSS Inc., Chicago, IL, USA). Biochemical and enzymatic data were analysed by one-way analysis of variance (ANOVA) and Duncan multiple comparison tests were used for group differences. Differences between semi-quantitative data in histopathological examination were determined by non-parametric Kruskal-Wallis test, while Mann-Whitney *U*-test was used for differences in paired groups. For the evaluation of immunohistochemical and double immunofluorescent staining, five random regions were selected to assess the intensity of positive staining, and the analysis was conducted using the Zeiss Zen Imaging software (Zen core 2, Dusseldorf, Germany). Data are presented as the mean ± SD and *P*-value < 0.05 was accepted as being statistically significant.

RESULTS

Effects on oxidative stress of GT extract against CYP toxicity in rainbow trout brain tissues

CYP exposure caused a reduction in SOD, CAT and acetylcholinesterase (AChE) levels in brain tissues of group 2 compared to group 1, while causing an enhancement in levels of MDA ($P < 0.05$) (Table 1). Conversely, in group 3 and 4 treated with GT extract, MDA levels decreased while SOD, CAT, and AChE activities increased compared to group 2 ($P < 0.05$). Both doses of GT extract demonstrated a reduction in the impacts of oxidative stress on brain tissues against CYP toxicity (Table 1).

Effects on the immunity of GT extract against CYP toxicity in rainbow trout brain tissues

CYP exposure led to important decreases in lysozyme, WBC and T. Ig levels in group 2 compared to the immune parameters of group 1 ($P < 0.05$). Conversely, substantial increases were detected in the immune parameters of groups 3 and 4 treated with GT extract compared to group 2 ($P < 0.05$). Group 4, treated with GT extract, exhibited greater effectiveness in boosting the immunity compared to group 3 (Table 2).

Table 1. Effects on oxidative stress biomarkers of green tea (GT) extract against cypermethrin (CYP) toxicity in rainbow trout brain tissues

Parameters	Group 1	Group 2	Group 3	Group 4
Protein (mg/g tissue)	2.38 ± 0.02^a	1.87 ± 0.01^b	2.13 ± 0.03^c	2.34 ± 0.02^a
AChE (EU/mg protein)	1.01 ± 0.02^a	0.81 ± 0.03^b	0.93 ± 0.02^c	1.04 ± 0.02^a
MDA (mmol/g tissue)	62.9 ± 0.34^a	80.5 ± 0.70^b	70.2 ± 0.16^c	63.6 ± 1.34^a
SOD (EU/g protein)	58.4 ± 1.16^a	46.9 ± 0.24^b	51.3 ± 0.50^c	57.6 ± 0.40^a
CAT (kU/g doku)	29.7 ± 1.52^a	19.7 ± 0.51^b	25.4 ± 0.85^c	28.6 ± 0.94^a

AChE = acetylcholinesterase; CAT = catalase; MDA = malondialdehyde; SOD = superoxide dismutase

Group 1 = the control; Group 2 = treated with 0.1 µg/l of CYP; Group 3 = treated with 0.1 µg/l of CYP + 100 mg/l of GT; Group 4 = treated with 0.1 µg/l of CYP + 200 mg/l of GT

$^{a–c}$Different symbols show the group differences ($P < 0.05$)

The results are given as mean ± standard deviation

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Table 2. Effects of green tea (GT) extract on immunity against cypermethrin (CYP) toxicity in rainbow trout

Immunity	Group 1	Group 2	Group 3	Group 4
Lysozyme (IU/ml)	93.4 ± 1.08[a]	71.1 ± 2.39[b]	80.3 ± 0.71[c]	91.8 ± 0.81[a]
T. Ig (mg/ml)	2.92 ± 0.05[a]	2.27 ± 0.02[b]	2.57 ± 0.02[c]	2.88 ± 0.01[a]
WBC (10^4 mm^{-3})	58.3 ± 0.97[a]	42.7 ± 1.31[b]	49.1 ± 0.92[c]	57.3 ± 0.71[a]

Group 1 = the control; Group 2 = treated with 0.1 µg/l of CYP; Group 3 = treated with 0.1 µg/l of CYP + 100 mg/l of GT; Group 4 = treated with 0.1 µg/l of CYP + 200 mg/l of GT; T. Ig = total immunoglobulin; WBC = white blood cell

[a–c]Different symbols show the group differences ($P < 0.05$)

The results are given as mean ± standard deviation

Histopathological, immunohistochemical and immunofluorescence results of GT extract against CYP toxicity in rainbow trout brain tissues

The histopathology of brain tissues in group 1 appeared normal. In contrast, CYP exposure caused severe degeneration and necrosis in neurons, along with severe hyperaemia in meningeal and parenchymal vessels. Group 3, treated with a dose of 100 mg/l GT, exhibited moderate degeneration and mild necrosis in neurons, as well as moderate hyperaemia in vessels. Group 4, treated with a dose of 200 mg/l GT, showed mild hyperaemia in cerebral vessels (Table 3 and Figure 1).

The intensity of 8-OHdG and caspase-3 expressions in the brain tissue of group 1 was negative in immunohistochemistry and immunofluorescence, respectively. CYP exposure led to severe cytoplasmic expressions of 8-OHdG and caspase-3 in neurons of group 2, showing significant differences compared to group 1 ($P < 0.05$) (Table 4 and Figure 1). In contrast, the expressions of 8-OHdG and caspase-3 in brain tissues were at a moderate level in group 3 and at a mild level in group 4, compared to group 2 ($P < 0.05$) (Table 4 and Figure 1).

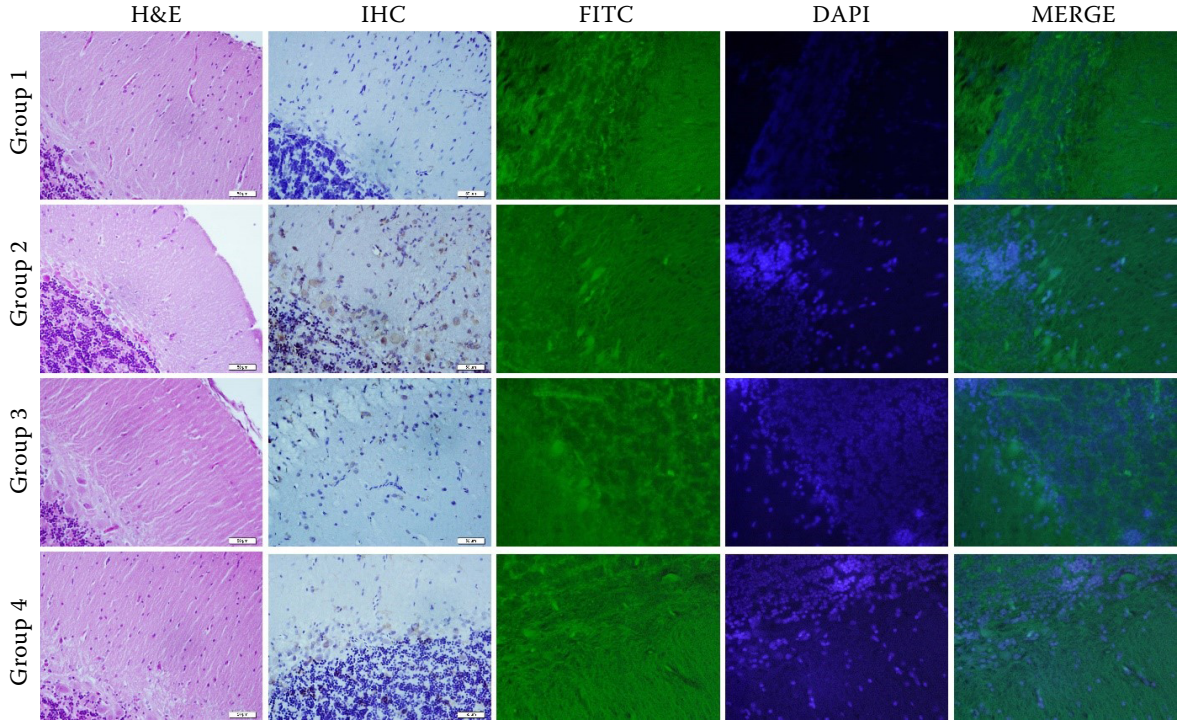

Figure 1. Brain tissues

Haematoxylin-eosin (H&E), 8-OHdG expression (IHC), caspase-3 expression (FITC), D-IF, 4',6-diamidino-2-phenylindol (DAPI), fluorescein isothiocyanate (FITC), cypermethrin (CYP), green tea extract (GT); bar = 50 µm

Group 1 = the control; Group 2 = treated with 0.1 µg/l of cypermethrin (CYP); Group 3 = treated with 0.1 µg/l of CYP + 100 mg/l of green tea (GT); Group 4 = treated with 0.1 µg/l of CYP + 200 mg/l of GT

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Table 3. Scoring histopathological results in brain tissues

	Degeneration in neurons	Necrosis in neurons	Hyperemia in the veins
Group 1	–	–	–
Group 2	+++	+++	+++
Group 3	++	+	++
Group 4	–	–	+

Group 1 = the control; Group 2 = treated with 0.1 µg/l of cypermethrin (CYP); Group 3 = treated with 0.1 µg/l of CYP + 100 mg/l of green tea (GT); Group 4 = treated with 0.1 µg/l of CYP + 200 mg/l of GT

Table 4. Statistical data on immunohistochemical and immunofluorescence results in brain tissues

	IHC-8OHdg	IF-Caspase-3
Group 1	20.12 ± 0.57^{a}	21.17 ± 0.91^{a}
Group 2	79.12 ± 4.13^{b}	82.21 ± 3.54^{b}
Group 3	60.12 ± 3.07^{c}	62.24 ± 2.76^{c}
Group 4	40.12 ± 2.18^{d}	41.31 ± 2.37^{d}

Group 1 = the control; Group 2 = treated with 0.1 µg/l of cypermethrin (CYP); Group 3 = treated with 0.1 µg/l of CYP + 100 mg/l of green tea (GT); Group 4 = treated with 0.1 µg/l of CYP + 200 mg/l of GT

[a–d]Different symbols show the group differences ($P < 0.05$)

The results are given as mean ± standard deviation

DISCUSSION

Recent studies have increased the interest in plants due to their protective properties against pesticides and diseases (Karatas et al. 2023). Green tea is one of these plants. Green tea is known to have active polyphenols and high antioxidant content. Studies have revealed that green tea has therapeutic and/or protective impacts in the regulation of all body processes as well as in the protection of living beings against pesticides and diseases (Karatas et al. 2023).

The administration of CYP resulted in a decrease in AChE activity in the brain tissues of group 2, potentially indicating the deterioration of nerve functions due to the excessive accumulation of AChE. Additionally, according to Adedara et al. (2018), the decrease in AChE activity may be associated with elevated stress levels and inadequate defence systems. In contrast, the fish treated with both 100 mg/l and 200 mg/l doses of GT extract demonstrated significant increases in AChE activities. The presence of EGCG in green tea may have contributed to a reduction in nerve damage by protecting mitochondria, suppressing oxidative stress, and inhibiting intracellular calcium increases (He et al. 2017).

Reactive oxygen species (ROS), which damage cell membranes by increasing the permeability of membranes, cause physiological imbalances, necrosis and finally apoptosis in living organisms (Ullah et al. 2019). The brain, with its high oxygen consumption and PUFA content, as well as low antioxidant enzyme levels, is particularly susceptible to oxidative stress (Ogaly et al. 2015). The reduction in levels of CAT and SOD and the enhancement in MDA levels after exposure to CYP in group 2 may indicate excessive free radical production. However, reduced MDA levels and the enhancement in CAT activity in the GT-treated group 3 and 4 suggest that green tea reduces oxidative stress by inhibiting the production of reactive oxygen molecules and free radicals (Karatas et al. 2023). Furthermore, the enhancement in SOD activity in the GT-treated group 3 and 4 may be related to the detoxification of superoxide radicals (O_2^-) (Ullah et al. 2019). The enhancement in SOD and CAT activities under stress confirms the stimulating impacts of green tea extract on the defence system.

The exposure to CYP in group 2 resulted in significant reductions in lysozyme, WBC, and T. Ig levels. Previous studies have shown that long-term exposure to pyrethroids weakens the immune system of fish (Ullah et al. 2018; Yang et al. 2021; Kirici 2022). However, significant enhancements were observed in levels of lysozyme, T. Ig, and WBC levels of group 3 and 4 treated with GT extract. Our findings also confirm that GT extract reduces ROS formation in brain tissues by boosting the immune system against CYP toxicity.

8-Hydroxy-2-deoxyguanosine (8-OHdG) is a marker used to assess DNA damage levels caused by ROS (Gelen et al. 2021; Karatas et al. 2023). 8-OHdG levels in brain tissues of group 2 exposed to CYP were higher compared to group 1. Hydroxyl radicals generated by oxidative stress can react with nucleic acids, leading to the formation of 8-OHdG (Gelen et al. 2021; Karatas et al. 2023). The increase in 8-OHdG expression levels may be associated with stress and with the production of superoxide anions (O_2^-) (Karatas et al. 2019a, b) whereas the 8-OHdG levels of the GT extract-treated group 3 and 4 were significantly decreased. This decrease can be attributed to the

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inhibitory effect of polyphenol compounds present in GT extract on oxidative DNA damage. Previous studies have demonstrated that phenol compounds in green tea suppress the formation of oxidative DNA damage, including 8-OHdG (Khalaf et al. 2012; Ogaly et al. 2015).

Apoptosis can occur through various mechanisms, including signalling through cell membrane receptors, activation of caspase proteins, DNA damage, increased production of free radicals, and release of apoptogenic factors such as cytochrome C and apoptosis induction factor (AIF) (Rahmasarigumay et al. 2019). CYP exposure resulted in significant increases in caspase-3 levels in the brain tissues of group 2. The increase in caspase-3 activity in the brain tissues of group 2 may be associated with changes in mitochondrial function, including mitochondrial damage, disruption of mitochondrial permeability caused by ROS, and degradation of essential cellular proteins (El Tabaa et al. 2022). Ahmed et al. (2013) demonstrated that increased caspase-3 activity leads to DNA damage and apoptosis whereas the caspase-3 levels of group 3 and 4 treated with GT extract were significantly lower than those in group 2 exposed to CYP. It has been determined that EGCG, one of the catechins found in green tea, exhibits neuroprotective impacts, including cell death inhibition, inflammation reduction, and apoptosis inhibition (He et al. 2017). Furthermore, studies have reported that EGCG suppresses oxidative stress, lowers intracellular calcium levels, and protects mitochondria, thereby reducing nerve damage (He et al. 2017).

CONCLUSION

In conclusion, the toxic effects of CYP causing environmental pollution can be significantly reduced by pouring specified doses of green tea extract either into the water supply or into ponds. Particularly, administration of 200 mg GT extract showed greater neuroprotective impacts against CYP-induced oxidative damage, including inhibition of oxidative stress, enhancement of immune and antioxidant defence systems, regulation of inflammatory signalling pathways and anti-apoptotic impacts in brain tissues of rainbow trout compared to 100 mg GT extract. Further research is needed to determine the specific impacts of green tea catechins on fish brain tissues.

Conflict of interest

The authors declare no conflict of interest.

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