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Mitigation potential of zingerone and rutin on toxicity mechanisms of nickel to zebrafish based on morphological, DNA damage and apoptosis outcome analysis

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ABSTRACT

Although nickel (Ni) is an important cofactor for various enzymes in biological systems, it can cause serious problems when insufficient or excessive in an organism. Therefore, it is very important to investigate Ni in biological systems, especially in cells with its related pathogenic mechanism. This study was carried out to demonstrate the effects of zingerone (ZO) and rutin (RN) administration against nickel chloride ($NiCl_2$) toxicity on neurobehavioral performance and brain oxidative status in zebrafish (*Danio rerio*) embryos/larvae on histological perspective. The experimental design of the study, which included twenty groups of fish, each containing 10 embryos, was prepared as semi-static and the trial continued for 96 hpf. In the obtained findings, it was determined that ZO and RN had a mitigating effect in this toxicity table where Ni caused oxidative stress in zebrafish larvae, induced DNA damage and apoptosis. A similar picture is valid for malformation processes as well as survival and hatching rates. These results showed that nickel is toxic to developing embryos via acting different mechanisms. In conclusion, we observed that ZO and RN have a greater effect on physiology, DNA damage and apoptosis than gross morphology, with a significant ameliorative effect.

1. Introduction

Heavy metals are important sources of pollution in aquatic ecosystems. One of these, nickel (Ni) can cause problems in aquatic environments due to its widespread use (all anthropogenic processes such as melting, refining, mining and production of stainless steel batteries) in different fields [5,11].

Due to their high molecular weight and stability, heavy metals can remain in the environment for a long time and are magnified along the food chain. Numerous researches had reported the tissue accumulation of heavy metals and their toxic effects on the biological processes of aquatic organisms (Alak et al., 2013; Ucar et al., 2018; [4,5,11,21]). The negative effects of heavy metals on vital processes such as growth, reproduction, metabolism and immunity in fish had been demonstrated by various studies (Alak et al., 2013; Topal et al., 2013; [10,11,19]).

In recent years, there has been increased interest in the herbal agents (Phytotherapeutics) usage as alternative, environmentally friend additives in the modulation of possible toxicities of pollutants in aquatic ecosystems (Gholampour et al., 2020; Kuriana et al., 2020, Hamed et al., 2021). Among these phytotherapeutics, as a lipophilic component rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) is also called rutoside. It is soluble in organic solvents such as pyridine, methanol and ethanol as a polyphenolic natural flavonoid [9,18]. This herbal origin substance has different (antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective and cardioprotective) pharmacological activities [9].

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Table 1
Experiment design.

Treatment groups (Code)	Treatment groups details
Control	E3
1 Ni	1 mg/L $NiCl_2$
5 Ni	5 mg/L $NiCl_2$
10 Ni	10 mg/L $NiCl_2$
1 RN	1 μM Rutin
1RN+ 1Ni	1 μM rutin+ 1 mg/L $NiCl_2$
1RN+ 5Ni	1 μM rutin+ 5 mg/L $NiCl_2$
1RN+ 10Ni	1 μM rutin+ 10 mg/L $NiCl_2$
10 RN	10 μM rutin
10RN+ 1Ni	10 μM rutin+ 1 mg/L $NiCl_2$
10RN+ 5Ni	10 μM rutin+ 5 mg/L $NiCl_2$
10RN+ 10Ni	10 μM rutin+ 10 mg/L $NiCl_2$
10 ZO	10 μmol/L Zingerone
10ZO+ 1Ni	10 μmol/L zingerone+ 1 mg/L $NiCl_2$
10ZO+ 5Ni	10 μmol/L zingerone+ 5 mg/L $NiCl_2$
10ZO+ 10Ni	10 μmol/L zingerone+ 10 mg/L $NiCl_2$
50 ZO	50 μmol/L Zingerone
50ZO+ 1Ni	50 μmol/L zingerone+ 1 mg/L $NiCl_2$
50ZO+ 5Ni	50 μmol/L zingerone+ 5 mg/L $NiCl_2$
50ZO+ 10Ni	50 μmol/L zingerone+ 10 mg/L $NiCl_2$

Another widely used herbal origin agent zingerone (ZO) is one of the most common active ingredients in ginger. Although ZO is an effective and inexpensive component with many pharmacological properties, it had also been stated that it is non-toxic and scavenges superoxide radicals [17]. Zingerone is also known as vanillilacetone, which belongs to the phenolic alkanone group 36. Zingerone has a vanilloid [3-methoxy-4-hydroxybenzene] group in the structural part and belongs to antioxidant phenolic compounds due to the presence of the hydroxyl group in the zingerone structure. Zingerone had been shown to have a wide variety of pharmacological properties, including antioxidant, anti-inflammatory, anticancer and antimicrobial activity. Studies on zingerone metabolism had shown that oral or intraperitoneal administration of zingerone results in side chain oxidation at all sites present, leading to excretion of glucuronide and sulfate conjugates in the urine 24 h after consumption, without any adverse effects on vital organs [14]. Although there are a few excellent reviews on ginger, no studies had been conducted on the use and beneficial role of zingerone (one of its active ingredients) in aquaculture, especially in fish, and there is no consolidated information on its pharmacological effects with a multi-biomarker approach.

Given the increasing worldwide demand and the potential of amplified anthropogenic inputs, it is essential to develop a modeling strategy to combat the effects of Ni on fish. In addition, based on the biological activities of ZO and RN, the risk of exposure to Ni as a neurotoxic agent in aquatic organisms and the curative effect of this phytotherapeutic in zebrafish were evaluated by behavioral, biochemical and histopathological analyzes.

2. Materials and methods

2.1. Chemicals

All chemicals of the research (Rutin CAS No: 153–18–4, PhytoLab, Germany), Zingerone (vanillylacetone, CAS No: 122–48–5, ≥ 96 %, Sigma-Aldrich, Cat. No. 02870), Nickel (II) chloride ($NiCl_2$) (98 % purity, Sigma-Aldrich) were purchased from Merck.

2.2. Zebrafish care and maintenance

The live material of the research (Zebrafish) was obtained from İzmir Biomedicine and Genome Center. Fish were kept (at 28 °C water temperature with a 14/10 h light dark cycle), and bred according to standard procedures (Westerfield 1995). Fish were fed twice a day (at 9 am–3 pm) and the morning feeding was done with flake food (TetraMin Tropical Flakes 48 % protein, 8 % fat, and 2 % fiber), the second one was with *Artemia salina*. The study does not require any license since using younger than 5-day-old (Directive 86/609/EEC and EU Directive, 2010/63/EU). The selected fish for breeding were transferred to special tanks at 6 pm with 1:1 female: male ratio and kept overnight. The next morning, zebrafish were stimulated by light for laying eggs. Embryos were collected and transferred to petri dishes and kept at 28 °C until analyses (Köktürk et al., 2021b).

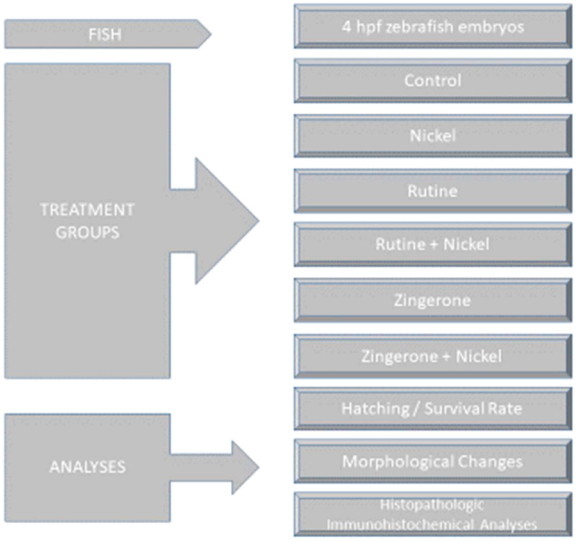

Fig. 1. Flowchart showing the procedures.

2.3. Experimental design

Since it was reported in previous studies that the LC_{50} (Lethal concentration) value of nickel chloride in zebrafish embryos and larvae was more than 10 mg/L, the concentrations in our study were determined according to these values [12,15]. Similarly, previous studies were used to determine the rutin and zingerone (vanilylacetone) doses for our study [8,16]. All application groups in our experiment were prepared with E3 medium (0.17 mM KCl, 0.33 mM $MgSO_4$, 5 mM NaCl and 0.33 mM $CaCl_2$). Trial design was constructed in Table 1 and Fig. 1.

The experiment was carried out on 6 plates, repeated 3 times and 10 embryos were used for each group. The trial was started at the 4th hour after fertilization and continued for 96 h, and all applications were carried out under the same conditions (28 °C). Survival rate, hatching rate and morphological alterations were monitored at 24–96 hpf (hours post fertilization) intervals. Changes in embryos and larvae were monitored daily with a stereomicroscope (SZX16 Olympus microscope + SC50 Olympus camera) (Kokturk et al., 2022). The experiment was terminated at 96th hour and 6 larvae from each group were fixed in 10 % neutral formalin for histopathology and immunohistochemical analysis.

2.4. Histopathological examination

Larva samples taken after the evaluation were fixed 10 % neutral formalin solution for 48 h and embedded in paraffin blocks at the end of routine tissue follow-up procedures. Sections of 4 μm thickness were taken from each block, and the preparations prepared for histopathological examination were stained with hematoxylin-eosin (HE) and examined with a light microscope (Olympus BX 51, Japan). The sections were evaluated as absent (-), very mild (+), mild (++), moderate (+++), severe (++++), and very severe (++++++) according to their histopathological features.

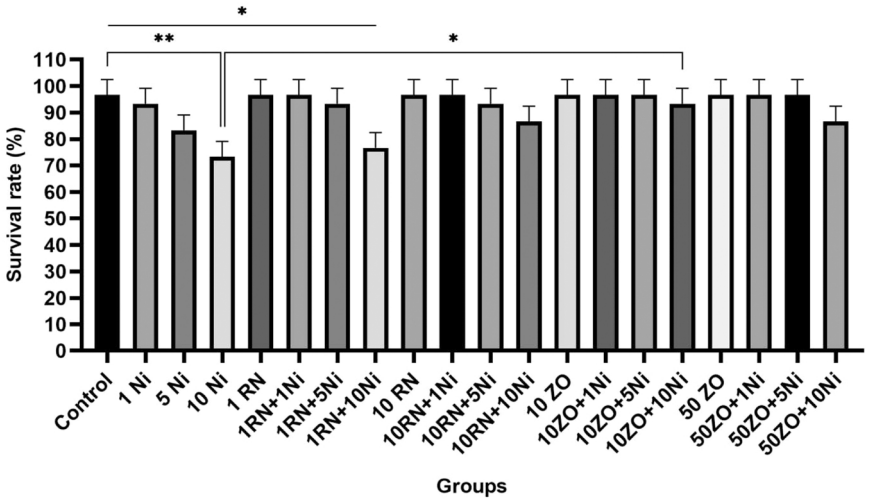

Fig. 2. Survival rates in zebrafish embryos and larvae exposed to different doses nickel and nickel+rutin, nickel+zingerone. Treatment groups were determined using one-way ANOVA with Tukey's post hoc test. $^{**}p < 0.01$, and $^{*}p < 0.05$.

2.5. Immunofluorescence examination

Tissue sections taken on adhesive (poly-L-Lysin) slides for immunofluorescence examination were deparaffinized and dehydrated. Then, endogenous peroxidase was inactivated by keeping it in 3 % H_2O_2 for 10 min. Then the tissues were boiled in 1 % antigen retrieval (citrate buffer (pH+6.1) 100X) solution and kept at room temperature. The sections were incubated with protein block for 5 min to prevent nonspecific background staining in tissues. Then, primary antibody (1. 8-OHdG Cat No: sc-66036, Dilution Ratio: 1/100, US; 2. Caspase 3 Cat No: sc-7272, Dilution Ratio: 1/100, US) was dripped onto the tissues and incubated according to the manual. Immunfluorescence secondary antibody was used as a secondary marker (FITC Cat No: ab6785 Diluent Ratio: 1/1000, UK) and kept in the dark for 45 min. Then, the other primary antibody (1. Bax Cat No: sc-7480, Dilution Ratio: 1/100, US; 2. Tnf-α Cat No: sc-52746, Dilution Ratio: 1/100, US) was added and incubated according to the instructions. Immunfluorescence secondary antibody was used as a secondary marker (Texas Red Cat No: ab6787 Diluent Ratio: 1/1000, UK) and kept in the dark for 45 min. Then, DAPI with mounting medium (Cat no: D1306, Dilution Ratio: 1/200, UK) was dripped onto the sections and kept in the dark for 5 min, and the sections were covered with a coverslip. The stained tissues were examined under a fluorescent microscope (Zeiss Axio Germany).

2.6. Data analysis

In this study, statistical analyzes (survival rate, morphological changes, and hatching rate) were performed using GraphPad Prism 8. Post-hoc analyses were conducted using Tukey's multiple comparison tests after one-way ANOVA and two-way ANOVA. All values are presented as mean ± standard deviation (SD), and differences were considered statistically significant at $^{****}p < 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$. SPSS 13.0 program was used for statistical analysis for immunofluorescent findings and the data were evaluated with $p < 0.05$ considered significant. Duncan test was used to compare groups, non-parametric Kruskal-Wallis test was used to determine group interaction, and Mann Whitney U test was used to determine differences between groups. In order to determine the intensity of positive staining from the pictures obtained because of immunofluorescent staining; 5 random areas were selected from each image and evaluated in the ZEISS Zen Imaging Software program. Data were statistically defined as mean and standard deviation (mean±SD) for % area. Mann-Whitney U test was performed to compare positive immunoreactive cells and immunopositive stained areas with healthy controls. Because of the test, P value was considered as < 0.05 significant and the data were presented as mean ± SD.

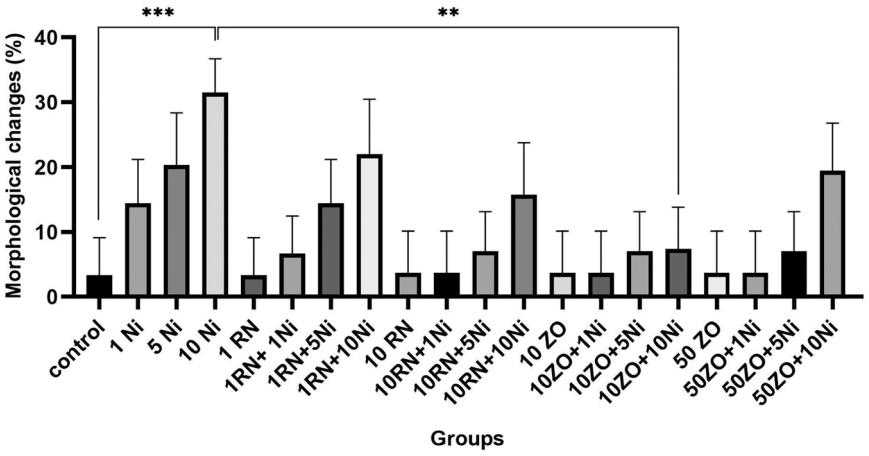

Fig. 3. Morphological changes in zebrafish embryos/larvae exposed to different doses nickel and nickel+rutin, nickel+zingerone. Data are expressed as means ± S. D. from three independent experiments (n = 10) one-way ANOVA with Tukey's post hoc test. $^{***}p < 0.001$, and $^{**}p < 0.01$.

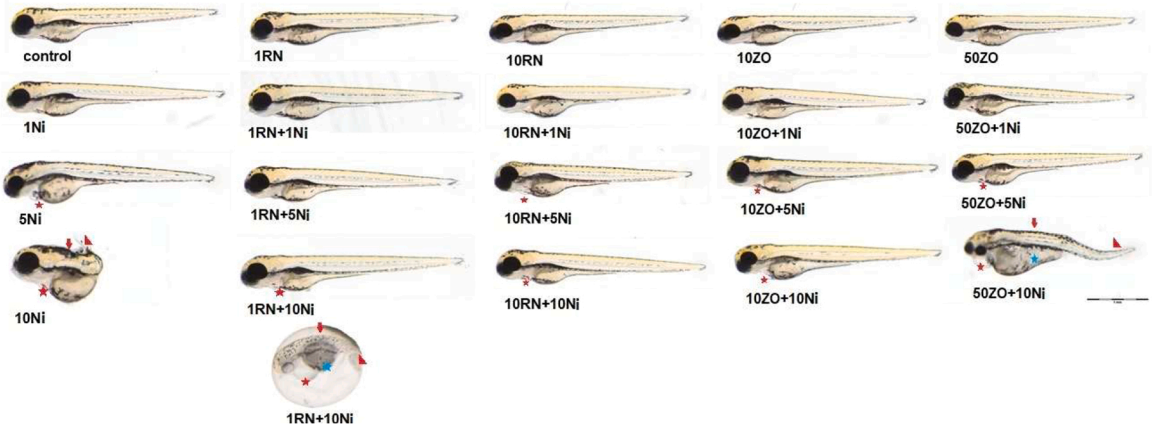

Fig. 4. Microscopic images of the abnormalities observed in zebrafish embryos/larvae at 96 hpf. Blue star: Yolk sac edema, red arrow: Lordosis/kyphosis, red star: Pericardial edema, red arrowhead: Tail malformation. Scale bar: 1 mm (2.5X).

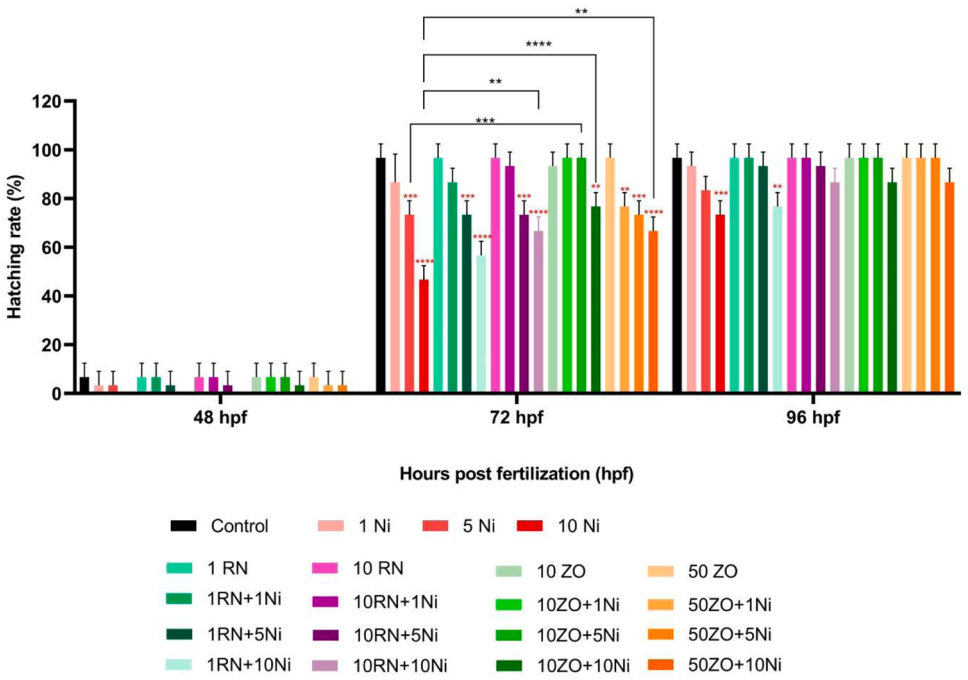

Fig. 5. Hatching rate of zebrafish embryos exposed to different doses of nickel and nickel+rutin, nickel+zingerone. Data are expressed as means ± S.D. from three independent experiments (n = 10) two-way ANOVA with Tukey's post hoc test. ****p < 0.0001, ***p < 0.001, and **p < 0.01, red star: comparison of control with other groups and black star: comparison between groups).

3. Results

3.1. Zingerone reduced the nickel-induced mortality rate in zebrafish embryos and larvae

Zebrafish embryos were exposed to different concentrations of nickel and two important active substances (rutin and zingerone) at different concentrations. A significant difference was found between the control and the highest concentration nickel application group (**p < 0.01) (Fig. 1). A significant difference was determined between the control group and the 1RN+ 10Ni treatment group (*p < 0.05) (Fig. 2). Considering the positive effect on the toxicity of the applied active substance, a significant difference was observed in the survival rate between the 10 Ni group and the 10ZO+ 10Ni group (*p < 0.05) (Fig. 2). In our study, it was revealed that 10 μmol/L ZO reduced the mortality rate due to 10 mg/L Ni toxicity. In zebrafish embryos, the survival rate was 73 % in the groups treated with only 10 mg/L Ni, while it was 97 % in the 10 mg/L nickel treatment groups with 10 μmol/L zingerone (10ZO" +10Ni)(Fig. 2).

3.2. Zingerone alleviate the nickel-induced morphological changes in zebrafish embryos and larvae

Morphological abnormalities such as yolk sac edema, lordosis/kyphosis, pericardial edema, and tail malformation were observed in groups treated with nickel and active agents to zebrafish embryos (Figs. 3–4). When the morphological changes were evaluated, a significant difference was found between the control group and the 10 Ni group (***p < 0.001) (Fig. 3). Again, a significant difference was determined between the 10 Ni (32 %) group and the 10ZO+ 10Ni (7 %) application groups (**p < 0.01) (Fig. 3).

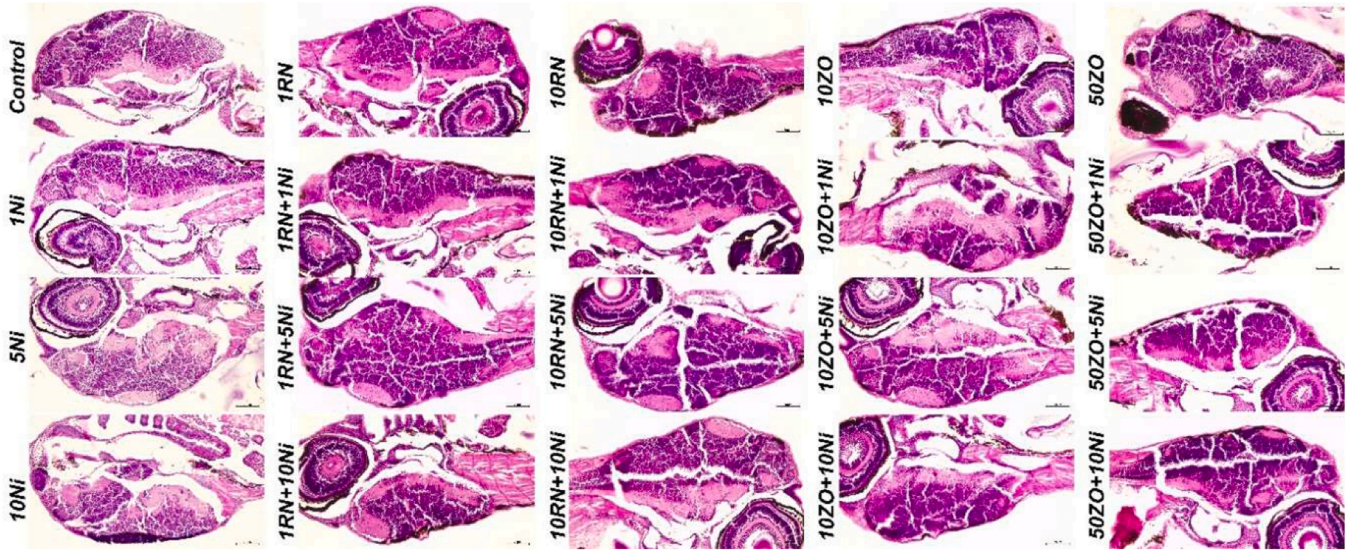

Fig. 6. Degeneration and necrosis of larval brain tissues, neurophiles, H&E, Bar:70 μm.

Table 2
Scoring histopathological findings of larval samples' brain tissues.

	Degeneration in neurophiles	Necrosis in neurophiles
Control	-	-
1Ni	+ ++	+ ++
5Ni	+ ++ +	+ ++ +
10Ni	+ ++ ++	+ ++ ++
1RN	-	-
10RN	-	-
1RN+ 1Ni	+ ++	+ +
1RN+ 5Ni	+ ++	+ ++
1RN+ 10Ni	+ ++ +	+ ++ +
10RN+ 1Ni	+	-
10RN+ 5Ni	+ +	+
10RN+ 10Ni	+ ++	+ +
10ZO	-	-
50ZO	-	-
10ZO+ 1Ni	+ ++	+ +
10ZO+ 5Ni	+ ++ +	+ ++
10ZO+ 10Ni	+ ++ ++	+ ++ +
50ZO+ 1Ni	+ +	-
50ZO+ 5Ni	+ ++	+
50ZO+ 10Ni	+ ++	+ +

3.3. Zingerone and Rutin attenuated the nickel-induced delay of hatching rate in zebrafish embryos

It had been determined that some concentrations of zingerone (10ZO+10Ni and 50ZO+10Ni) and rutin (10RN+10Ni) alleviate the delay of larvae emergence caused by the toxic effect of nickel (Fig. 5). A significant difference was determined between the 5Ni (73 %) group and the 10ZO+ 5Ni (97 %) group at 72 h from the nickel application groups (***$p < 0.001$) (Fig. 5). When the highest nickel application group (10Ni) (47 %) and 10RN+ 10Ni (67 %), 10ZO+ 10Ni (77 %) and 50ZO+ 10Ni (67 %) groups were compared, a significant difference was found between larval hatchability rates (****$p < 0.0001$ and **$p < 0.01$) (Fig. 5).

4. Histopathological and immunohistochemically examination

4.1. Histopathological findings

When the brain tissues of the control group larvae samples were examined histopathologically, it was seen that the tissues had a normal histological structure. Only in the Ni administration groups (1Ni, 5Ni, and 10 Ni) neurophiles degeneration and necrosis intensified with increasing dose. Moderate degeneration and necrosis were detected in the 1Ni group, severe in the 5Ni group, and very severe in the 10Ni group. In only rutin (1RN and 10RN) and zingerone (10 ZO and 50 ZO) treatments, it was seen that the tissues had a normal histological structure. (Fig. 6).

The following findings were determined when rutin and nickel were used together.

1RN+ 1 Ni group: When the brain tissues of the larvae samples were examined histopathologically, moderate degeneration and mild necrosis were detected in the neurophiles. 1RN+ 5 Ni group: Moderate degeneration and necrosis were observed in neurophiles. 1RN+ 10 Ni group: Severe degeneration and necrosis were detected in neurophiles. 10RN+ 1 Ni group: Very mild degeneration was observed in neurophiles. 10 RN+ 5 Ni group: Mild degeneration and very mild necrosis were observed in neurophiles. 10RN+ 10 Ni group**:** When the brain tissues of the larval samples were examined histopathologically, moderate degeneration and mild necrosis were detected in the neurophiles (Fig. 6). A statistically ($p < 0.05$) significant difference was detected when compared with the 1RN+ 10Ni, 5Ni and 10Ni groups. Scoring of histopathological findings is summarized in Table 2.

The findings of treatment groups were as; 10ZO+ 1Ni group: When the brain tissues of the larvae samples were examined histopathologically, moderate degeneration and mild necrosis were detected in the neurophiles. 10ZO+ 5Ni group: Severe degeneration and moderate necrosis were detected in neurophiles. 10ZO+ 10Ni group: Too severe degeneration and severe necrosis were observed in neurophiles. 50ZO+ 1Ni group: Mild degeneration was seen in neurophiles. 50ZO+ 5Ni group**:** Moderate degeneration and very mild necrosis were detected in neurophiles. 50ZO+ 10Ni group**:** Moderate degeneration and mild necrosis were observed in neurophiles. A statistically ($p < 0.05$) significant difference was detected (Fig. 6) when compared with the 10ZO+ 10Ni, 1Ni, 5Ni and 10Ni groups. Scoring of histopathological findings is summarized in Table 2.

5. Immunofluorescent findings

When the brain tissues of the larval samples were examined by immunofluorescence staining method, 8-OHdG, Bax, Caspase-3 and Tnf-α expression levels were negative in the control group (Figs. 7–8).

Intracytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions intensified with increasing dose in alone Ni administration groups (1Ni, 5Ni and 10 Ni) (Figs. 7–8). Moderate degeneration and necrosis were

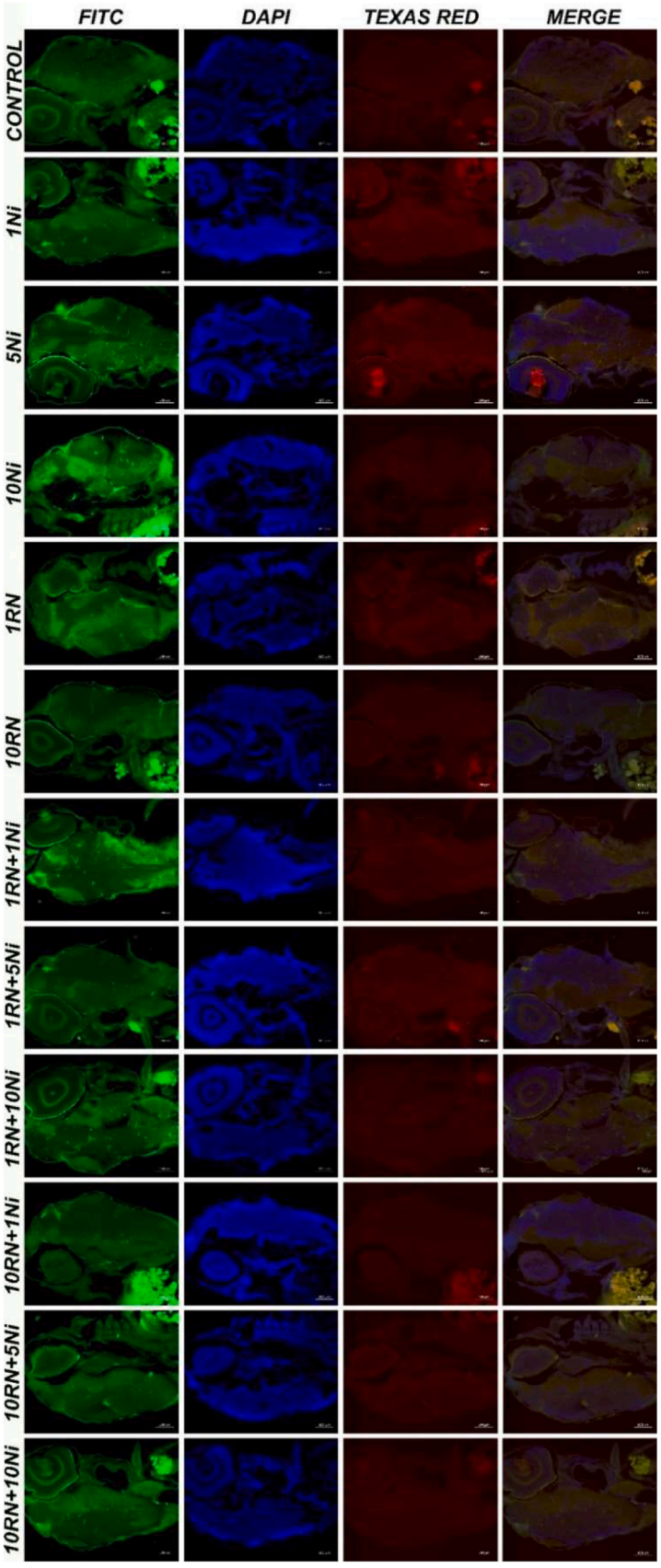

Fig. 7. Larval tissue, 8-OHdG expressions (FITC), Bax expressions (Texas Red), IF, Bar:100 μm.

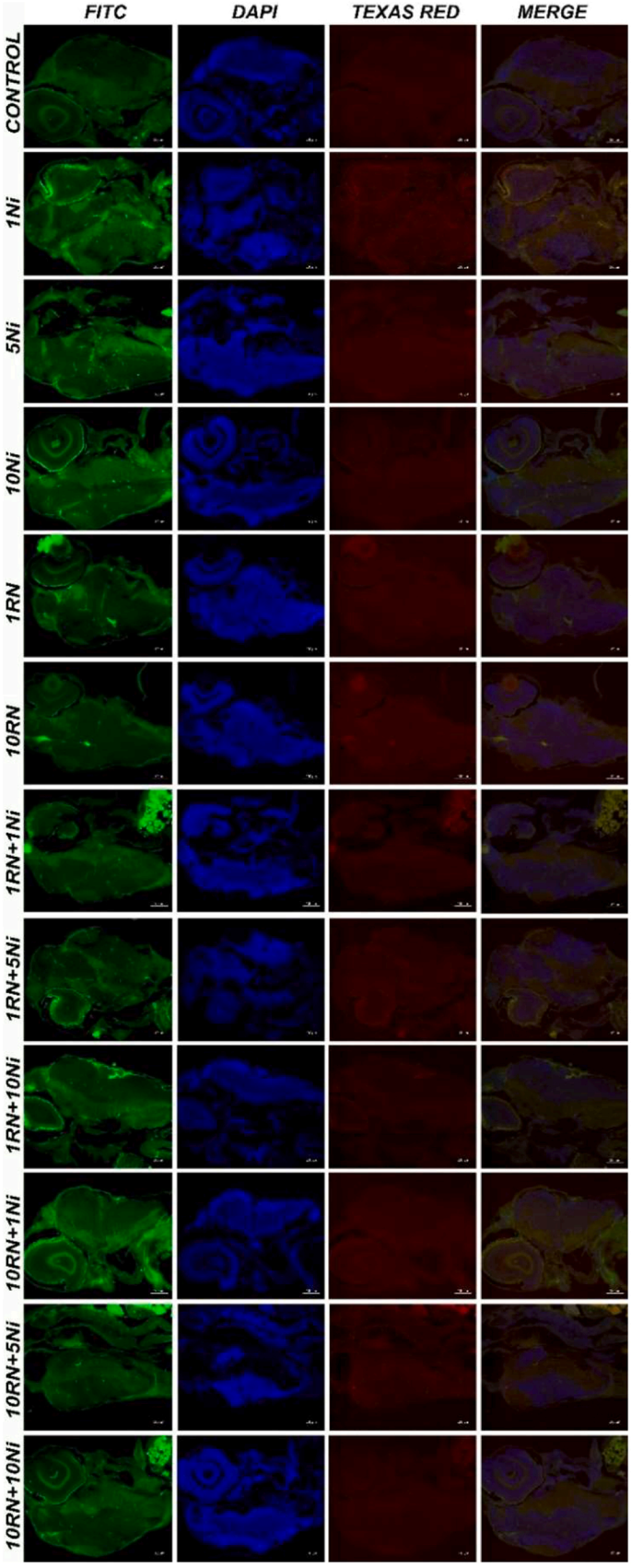

Fig. 8. Larval tissue Caspase-3 expressions (FITC), Tnfα expressions (Texas Red), IF, Bar:100 μm.

Table 3
Data and statistical analysis of immunofluorescent findings.

	8-OHdG	Caspase 3	Bax	Tnf-α
Control	22.16 ± 1.06[a]	20.09 ± 1.38[a]	18.74 ± 1.37[a]	17.48 ± 1.09[a]
1Ni	62.48 ± 2.91[b]	57.63 ± 2.43[b]	51.28 ± 1.19[b]	50.28 ± 0.91[b]
5Ni	82.16 ± 2.36[c]	76.39 ± 1.77[c]	67.16 ± 2.48[c]	69.35 ± 1.74[c]
10Ni	102.29 ± 2.9[d]	94.56 ± 0.33[d]	82.13 ± 1.59[d]	79.59 ± 2.96[d]
1RN	24.58 ± 1.39[a]	19.76 ± 1.66[a]	18.18 ± 0.74[a]	18.2 ± 0.85[a]
10RN	24.63 ± 1.66[a]	21.18 ± 0.97[a]	19.33 ± 1.08[a]	18.99 ± 1.48[a]
1RN+ 1Ni	43.39 ± 2.26[e]	43.6 ± 1.85[e]	36.81 ± 1.44[e]	33.33 ± 2.07[e]
1RN+ 5Ni	59.63 ± 2.34[b]	57.44 ± 1.03[b]	52.1 ± 2.26[b]	48.74 ± 2.66[b]
1RN+ 10Ni	79.39 ± 2[c]	74.58 ± 2.4[c]	64.39 ± 1.85[c]	66.34 ± 2.18[c]
10RN+ 1Ni	31.44 ± 1.74 [f]	28.43 ± 1.18 [f]	24.12 ± 0.26 [f]	23.57 ± 1 [f]
10RN+ 5Ni	40.29 ± 3.08[e]	41.14 ± 2[e]	35.33 ± 2.18[e]	32.19 ± 1.59[e]
10RN+ 10Ni	61.48 ± 1.85[b]	56.38 ± 1.2[b]	50 ± 1.34[b]	51.45 ± 2.22[b]
10ZO	25.74 ± 1.26[a]	18.49 ± 2.05[a]	18.35 ± 1.57[a]	19.39 ± 2.09[a]
50ZO	25.48 ± 1.43[a]	20.2 ± 1.54[a]	20.69 ± 1.96[a]	20.85 ± 1.74[a]
10ZO+ 1Ni	45.48 ± 2.5[e]	44.51 ± 2.38[e]	39.55 ± 2.59[e]	35.98 ± 2.53[e]
10ZO+ 5Ni	63.39 ± 2.66[b]	60.5 ± 2.26[b]	55.39 ± 2.64[b]	50.45 ± 2.28[b]
10ZO+ 10Ni	82.74 ± 2.38[c]	75.37 ± 2.18[c]	65.74 ± 2.67[c]	68 ± 2.35[c]
50ZO+ 1Ni	33.51 ± 2.31 [f]	30.09 ± 2.59 [f]	24.67 ± 1.97 [f]	25.43 ± 1.97 [f]
50ZO+ 5Ni	44.25 ± 2.74[e]	43.29 ± 2.87[e]	38.66 ± 2.4[e]	34.3 ± 2.09[e]
50ZO+ 10Ni	65.89 ± 2.37[b]	58.69 ± 2.09[b]	50.03 ± 2.06[b]	52.33 ± 2[b]

a,b,c,d,e,f: Different letters in the same column represent statistical difference ($p < 0.05$).

detected in the 1Ni group, severe in the 5Ni group, and very severe in the 10Ni group.

8-OHdG, Bax, Caspase-3 and Tnf-α expression levels were evaluated as negative in alone rutin (1RN and 10RN) and zingerone (10 ZO and 50 ZO) applications. The following findings were determined when nickel and zingerone were used together. 1RN+ 1Ni group: When the larvae samples' brain tissues were examined by immunofluorescence staining method, mild cytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were detected in the neurophiles. 1RN+ 5Ni group: Moderate intracytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were detected in neurophiles. 1RN+ 10Ni group: Severe cytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were observed in neurophiles. 10RN+ 1Ni group: Very mild intracytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were observed in neurophiles. 10RN+ 5Ni group: Mild cytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were detected in neurophiles. 10RN+ 10Ni group: Moderate intracytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were detected in neurophiles. A statistically ($p < 0.05$) significant difference was detected when compared with the 1RN+ 10Ni, 5Ni and 10Ni groups. Scoring and statistical data of immunofluorescent staining findings are presented in Table 3 and Figs. 7–8. 10ZO+ 1Ni group: When the brain tissues of the larvae samples were examined by immunofluorescence staining method, mild intracytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were detected in the neurophiles (Figs. 9–10). 10ZO+ 5Ni group: Moderate intracytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were observed in neurophiles (Figs. 9–10). 10ZO+ 10Ni group: Severe intracytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were detected in neurophiles (Figs. 9–10). 50ZO+ 1Ni group: Very mild intracytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were observed in neurophiles (Figs. 9–10). 50ZO+ 5Ni group: Mild cytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were detected in neurophiles (Figs. 9–10). 50ZO+ 10Ni group: Moderate cytoplasmic 8-OHdG, Bax, Caspase 3 and Tnf-α expressions were detected in neurophiles (Figs. 9–10). Scoring and statistical data of immunofluorescent staining findings are presented in Table 3.

6. Discussion

Zingerone, which is a natural product of herbal origin, has antioxidant and anti-inflammatory properties. Nevertheless, especially in aquatic ecosystems, the possible protective effect of zingerone and rutin on metal toxicities has not yet been studied. This study was designed to investigate the effects of zingerone/routine on metal-induced toxicity in aquatic organisms and is the first study in its field.Insufficient or excess Ni^{2+} can lead to physiological abnormalities or serious health problems. Ni^{2+} deficiency can affect biological functions such as enzyme metabolism, respiration and protein biosynthesis [19]. Negative effects, such as Ni gas exchange disorder, inhibition of ion regulation and increased oxidative stress had been reported in freshwater biota. Understanding the embryonic toxicity of heavy metals has become imperative as environmental contamination increases. Because most of the metals bind to the chorion, they can break the chorion and accumulate in fish embryos. Thus, even a small amount of metal can pass through the chorion and adversely affect early embryonic development [2]. Our survival, hatching and morphological findings obtained as a result of Ni exposure are compatible with the previous studies. Ni exposure had been reported to cause decreased movement, muscle and gill malformations, delay in hatching and increased mortality in fish [11]. In addition, metals may reduce the hatching rate by affecting the enzyme and muscle movements associated with hatching [20]. Since there is no literature data on this subject with ZO in zebrafish in the literature reviews, our current findings are compatible with the obtained results from different herbal extracts in different aquatic organisms. Previous researches had shown that herbal extracts can reduce the mortality rate against pathogens in fish and shrimps, increase nonspecific immunity by stimulating immune stimulants, phagocytosis, bactericidal activity, and respiratory bursts and increase resistance against pathogens [7]. On the other hand, mortality rates increased and some malformations were detected in Ni application groups. It had been reported that in fish embryos, metal exposure may have a significant effect immediately after fertilization during swelling of the zygote due to water absorption in the perivitelline space containing proteins secreted by the vitelline membrane, and metal ions may change the chorion structure and permeability [2].

In the present study, high concentrations of single zingerone and rutin compounds use were accompanied by changes in immunohistochemistry parameters and histological findings close to low-dose nickel concentrations. However, the mechanism of action in these changes in both compounds has not yet been clarified in terms of aquatic toxicity. A previous study reported that zingerone reduces caspase-3 activation, so it may be antiapoptotic [1]. Single uses of both substances are thought to exert antioxidant and antiapoptic effects at low concentrations. Immunohistochemistry results, supported by histopathological findings, revealed that low concentrations of both substances prevented DNA damage, inflammation and apoptosis. In co-administration with nickel, high doses of both substances gave positive results in the prevention of toxicity, which can be explained by their antioxidative effect. Although the exact mechanism of Ni toxicity is unknown, there is indication that it may cause the generation of reactive oxygen species (ROS) and inhibit the activity of antioxidant enzymes in fish tissue [11]. Heavy metals such as Ni, Cd and Pb can generate ROS such as hydroxyl radicals (•OH),

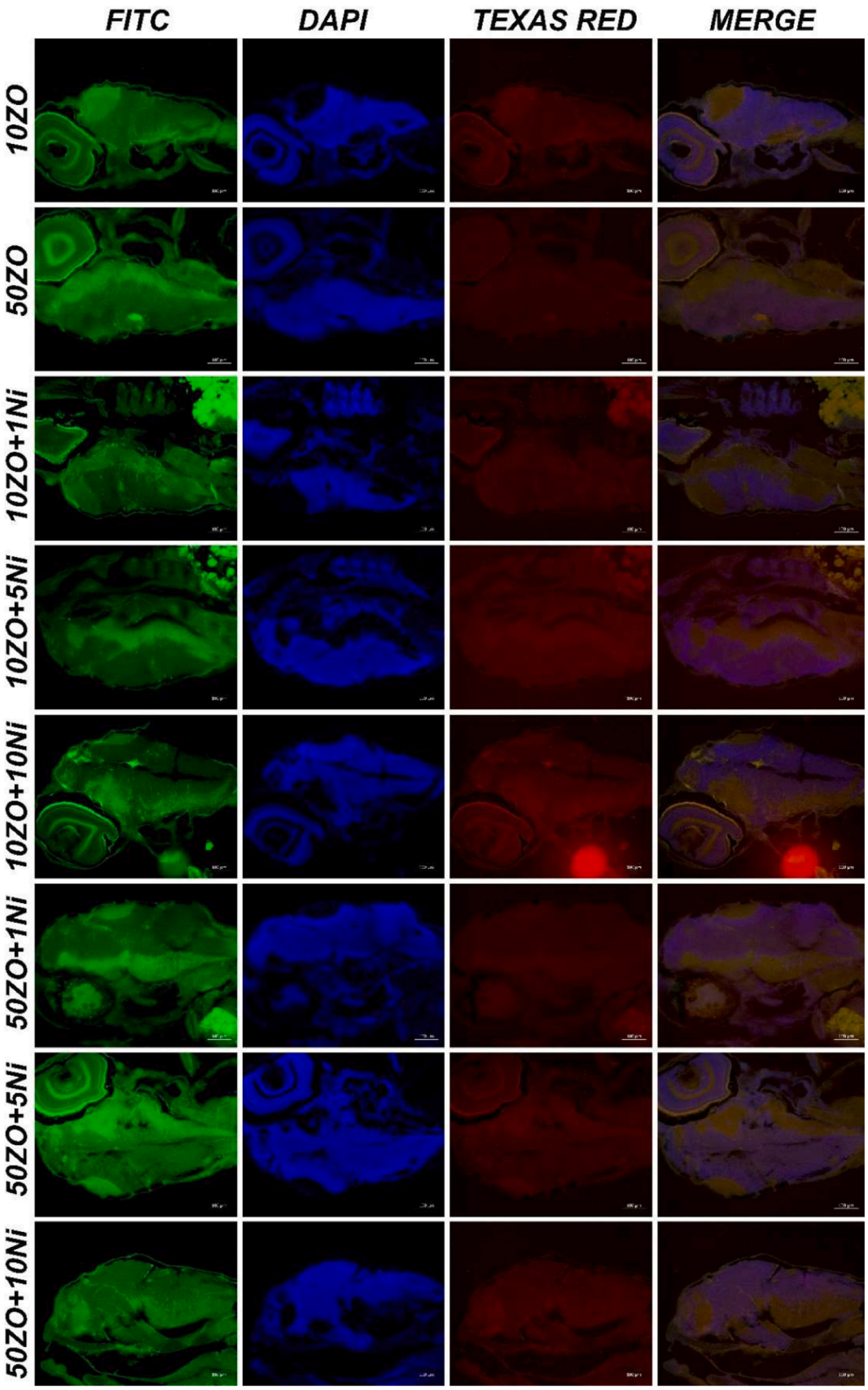

Fig. 9. Larval tissue, 8-OHdG expressions (FITC), Bax expressions (Texas Red), IF, Bar:100 μm.

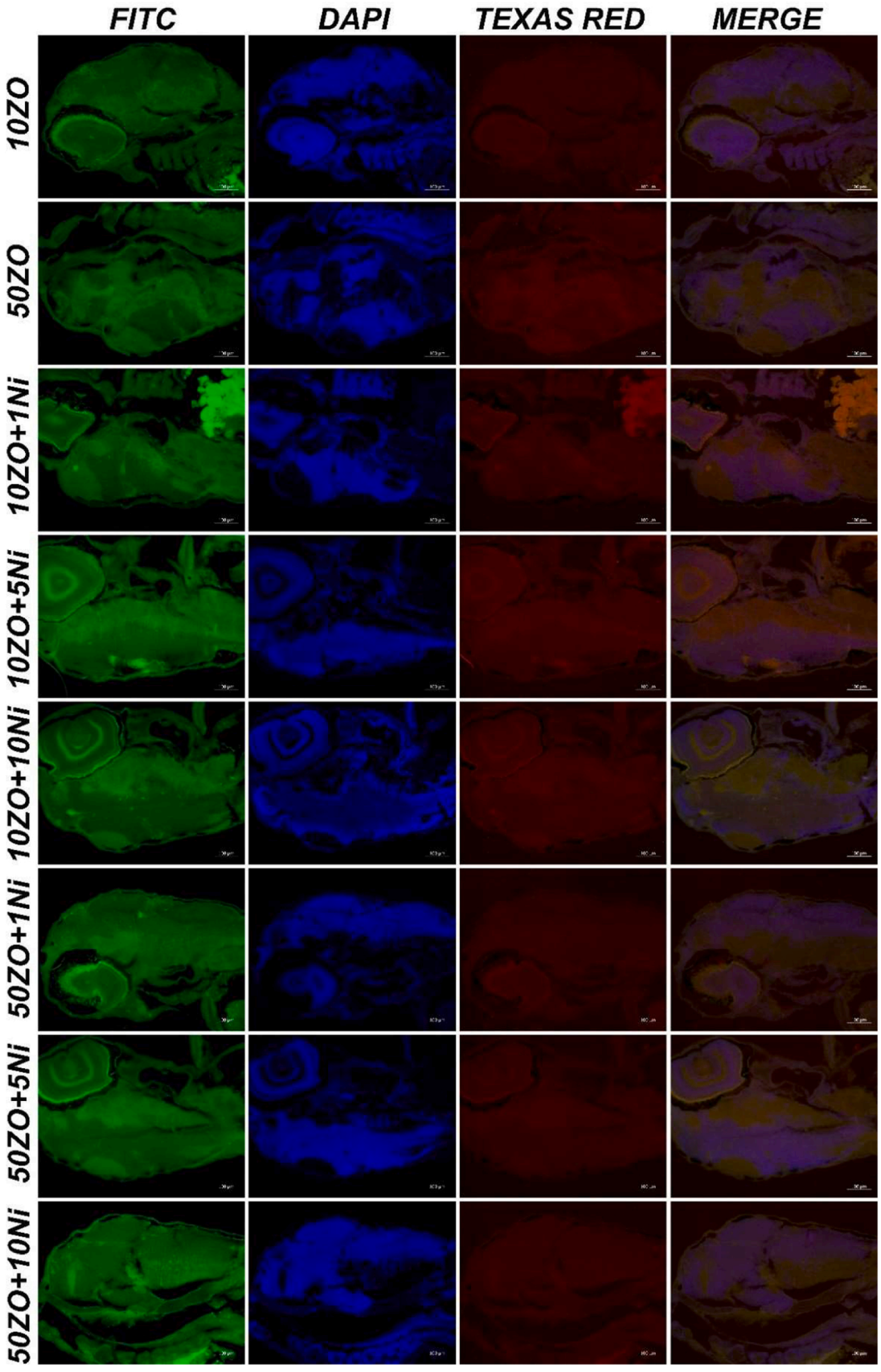

Fig. 10. Larval tissue Caspase-3 expressions (FITC), Tnfα expressions (Texas Red), IF, Bar:100 μm.

superoxide anion (O_2^-), singlet oxygen ($^1O_2$) and H_2O_2. Consequently, increased ROS production can damage DNA with the formation of 8-OHdG, the most common product of DNA lesion produced during in vivo repair of oxidatively damaged DNA [13]. In this study, it is thought that Ni activates TNF-α expression due to ROS production. However, ZO was found to reduce Ni-induced oxidative stress through its antioxidant properties, thereby lowering the levels of Bax, TNF-α, caspase 3 and 8 OHdG. Caspase-3 and Bax are pro-apoptotic proteins [22]. Various studies had demonstrated the ability of zingerone to suppress tissue damage by inhibiting caspase-3 activation, upregulating Bcl-2, and downregulating Bax proteins [14]. This study showed that Ni^{2+} exposure increased Bax expression levels; This suggested that Ni^{2+} exposure could induce damage in fish tissues by affecting mitochondrial apoptotic pathways (Zheng et al., 2014). The caspases are a family of proteins that are among the main drivers of the apoptotic process. Caspase-3 is one of the key drivers of apoptosis, capable of degrading many important proteins such as nuclear lamins, fodrin, and the nuclear enzyme poly (ADPribose) polymerase (PARP). Many studies had shown that pollutants in water induce apoptosis in fish cells through caspase-3 activation (Alak et al., 2021 [3]; Parlak et al., 2022; Ucar et al., 2022; Kökturk et al., 2022). This study showed that Ni^{2+} increased caspase-3 activity in zebrafish brain tissue. Our findings also show that caspase-3 is activated upon Ni^{2+} exposure, suggesting that the mitochondrial pathway plays an important role in Ni^{2+}-induced apoptosis (Zheng et al., 2014).

DNA is a macromolecule highly sensitive to oxidative damage and is usually followed by the 8-OHdG biomarker. It is thought that ROS plays an active role in the formation of 8-OHdG (Ucar et al., 2022). According to the obtained data from this study, Ni increased the formation of 8-OHdG by causing oxidative damage in DNA. In addition, with its antioxidant properties, ZO ameliorated Ni-induced oxidative DNA damage and brought 8-OHdG formation closer to that of the control group. Similar to our findings, Caglayan et al. [6] reported that ZO significantly reduced the level of 8-OHdG in a dose-dependent manner.

7. Conclusion

This research documents that zingerone is an immunostimulatory enhancer with biological effects against nickel toxicity through reduced expressions of 8-OHdG, caspase-3, Tnf-α and Bax. In addition, zingerone also had a positive effect on survival and hatching rates in zebrafish and suppressed morphological anomalies. No study had been found on the supplementation of zingerone extract in aquatic toxicity of heavy metal origin. The present study is the first modeling study in this area. Therefore, further research is needed to establish an optimum supplementation range and to prove the ameliorative effects of zingerone extract on survival, hatching rate, morphological abnormalities as well as DNA damage and apoptosis pathways in zebrafish at the doses administered here.

CRediT authorship contribution statement

Mine Köktürk: Conceptualization, Validation, Supervision, Resources and formal analysis. **Gonca Alak**: Formal analysis, Visualization, Writing - Original Draft, Writing - Review & Editing. **Mine Köktürk, Serkan Yıldırım, Muhammed Atamanalp, Metin Kılıçlıoğlu, Arzu Uçar, Gunes Ozhan**: Resources and formal analysis. **Muhammed Atamanalp**: Writing - Review & Editing, All the authors read and approved the final version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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