

Biochemical and histological alterations induced by UV-328 in the gill tissue of Zebra fish (*Danio rerio*) and its reversal by Dimethoxy curcumin

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The presence of Benzotriazole UV Stabilizer-328 (UV-328) in different biological and natural systems is of fast, regular concern in recent days due to their exuberant use in sunscreens. To learn more about its role of oxidative damage in the gills and to uncover the protective nature of DiMC, we evaluated its sub-lethal toxicity in Zebra fish gill tissue and also evaluated the potential defensive role of Dimethoxy curcumin (DiMC). Grown-up Zebra fish were exposed to 55 µg/L of UV-328 and 50mg/kg of DiMC for four weeks. After the completion of 28 days, gill tissues were dissected out and assessed for their response to oxidative stress, antioxidant enzymes status, and histopathological changes. Our results demonstrated that antioxidant such as glutathione (GSH) levels and the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and Glutathione S-transferase (GST) were all diminished in the gill tissue along with the profound increase in lipid peroxidation (measured as TBARS) in the UV-328-treated group. Histopathological lesions, for instance, inflammatory cell infiltration with a minimal congestion in primary lamellae, diffuse epithelial hyperplasia and fusion of secondary lamellae, multifocal mucus cell hyperplasia were seen in the UV-328-treated gill tissue of Zebra fish. The UV-328-induced oxidative, biochemical, and histological alterations were almost reverted back to normal in the DiMC enhanced group, suggesting its remedial efficacy against UV-328-induced respirotoxicity. Based on our findings, exposure to UV-328, even at a low level, could be toxic, causing oxidative stress, antioxidant depletion, and pathological gill damage in Zebra fish. And these alterations were almost recuperated in DiMC supplemented group which signifies its protective influence against UV-328 toxicity.

Keywords: UV-328; Oxidative stress; Antioxidants; Histopathology; Zebra fish

INTRODUCTION

Sunscreen organic UV filters, particularly benzotriazoles, are the common ecological pollutants that are representing a mounting serious health concern because of their rising presence in all parts and parcel of the environment. These are the most regularly involved organic UV filters for their capacity to safeguard from sun related burns by engrossing an expansive range of UV radiation. Benzotriazole UV filters (BUVSs) are projecting synthetic compounds used to safeguard against UV radiation, which could retain the range of both UV-A and UV-B. It has been reported that UV filters entered the aquatic system via either direct input of recreational activities (e.g., washing and swimming) or indirect input from the wastewater treatment plants. BUVSs have likewise been broadly utilized in grouped scope of commercial and industrial items, like plastics, building materials, corrective items, paints, coatings, etc.¹ Because of its high-volume of manufacture and use, BUVSs have been viewed as universally scattered in different biotic and abiotic settings, including air, soil and water as well as in human examples like milk, fat, urine and serum.^{2,3}

On account of the boundless and high focuses in numerous biotic and abiotic interfaces, BUVs have continuing toxicological interests for its unfavorable impacts on

ecological and human wellbeing. In 2021, researchers proposed to add UV-328 in the list of Persistent Organic Pollutants (POP's) of the Stockholm Show on Tenacious Natural Poisons, because of its qualities of determination, bioaccumulation, potential for long-range ecological vehicle and unfavorable impacts to people as well as the ecosystem.⁴ Some other BUVSs like UV-327 and UV-350 were remembered for the applicant rundown of exceptionally high worry by European Synthetic substances Organization.⁵ In spite of the information about its boundless defilement, the toxicological impacts and system toxic effects of UV-328 on different animals remain underexplored. Concentrates on distinguished that UV-328, is the potential endocrine upsetting synthetic compounds,⁶ dysregulated thyroid chemical framework in Zebra fish,⁷ could interweave nuclear receptors and acted as an endocrine disruptor.⁸

Ecological impurities can set off the toxicity related to oxidative pressure. Oxidative stress is a hurtful impact brought about by cytotoxic reactive oxygen species (ROS), delivered during metabolic change in living beings. Organism's antioxidant defense systems are able to remove ROS and shield complex biological macromolecules from ROS attack under normal conditions. However, the balance will be destroyed when pollutant-induced ROS levels exceed the antioxidant defense system's scavenging capacity, thereby

reducing the activity of antioxidant enzymes. Organic UV filters will experience oxidative pressure, bringing about lipid peroxidation with upgraded degree of malondialdehyde (MDA), DNA chain breakage, protein oxidation, carbonylation, and even cell death or canceration. Cell reinforcement guard frameworks comprise of an assortment of enzymatic (e.g., catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferase (GST)) and non-enzymatic cell reinforcements, for example, reduced glutathione (GSH).⁹⁻¹¹

Natural UV filters been found to be related with the enlistment of oxidative pressure in aquatic life forms. Inferable from the intricacy of natural UV filters in aquatic system, the stimulation of oxidative stress with the essentially, impacted cell reinforcement protections can be utilized to reflect the extensive contamination of POPs in the aquatic life and to assess the ecological dangers of contaminated water. Zebra fish (*Danio rerio*) have been broadly utilized as indicator species in toxicological investigations of ecological toxins. As the first organ reacting with waterborne pollutants, the gill filaments and lamellae have a large surface area which is indirect or constant contact with xenobiotics. Histopathological alterations of gills are widely used as indicators of environmental pollutants. Studies elucidating the toxic effects of POPs on the organ system will help to address a better understanding about their potential to induce oxidative stress mediated multiple organ dysfunction.¹²

As our previous findings demonstrated that continuous sub lethal UV stabilizers exposure in the Zebra fish model prompted assorted biochemical and histomorphological deficits in several tissues.¹³⁻¹⁵ Therefore, we postulated that adult Zebra fish would develop oxidative respirotoxicity as a result of continuing constant exposure to UV-328 at a sublethal level. In order to investigate this presumption, the Zebra fish treated with 55 µg/L of UV-328 for four weeks and to assess its toxicological impacts on gills. Specifically, markers related to oxidative affront and antioxidant pathway markers were evaluated along with histomorphological alterations to reveal a possible underlying toxicity mechanism of UV-328 -impelled respiratory deficiencies in Zebra fish. Studies with antioxidant supplementation have possibly been ended up being advantageous against xenobiotics prompted oxidative pressure intervened organ brokenness in rats.¹⁶ Dimethoxycurcumin (DiMC) is a structural analogue of curcumin in which methoxy groups are replaced with phenolic-OH groups. It has a same symmetric plan with synthetically steadier than curcumin with extended cell support, apoptotic viability as well as less destructiveness in standard cells and extended metabolic immovability (bioavailability). Plasma levels for dimethoxycurcumin were for the most part reach higher (3-times) in comparison with curcumin at same imbued level of 5 mg/kg bw in mice. In comparison with curcumin, processing of DiMC is less far reaching therefore it is possible that DiMC shows extended cell reinforcement potential over curcumin.¹⁷

Our past investigations affirmed that constant exposure to organic UV filters even at sub lethal levels altogether incited a biochemical and pathological changes in the zebra fish. In this manner, we hypothesized that persisting exposure to UV-328 at sub lethal level to cause oxidative respirotoxicity in Zebra fish. and its recovery through the supplementation of DiMC. It has proactively been accounted for to display renal protection against UV-328 in Zebra fish model.¹⁸ To test this speculation, we treated Zebra fish. to 55µg/L of UV-328 and supplemented with 50mg/kg, BW of DiMC through diet for 4 weeks to examine the gill toxicity of UV-328 and its conceivable

recuperation through DiMC supplementation. In particular, markers related with oxidative affront and antioxidative pathways were assessed alongside histological factors to uncover the conceivable subtle toxic effect of UV-328 and the defensive impact of DiMC in the gill tissue of Zebra fish.

MATERIALS AND METHODS

Ethics statement

All analyses, maintenance, and treatment dealing with Zebra fish were completed according to the rules of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Mass Biotech, Tamil Nadu. India. Endorsement number: MB/IAECCC/2022/03/06.

Chemicals

Benzotriazole UV stabilizer - 328 (UV-328; 98% purity) was obtained from Sigma-Aldrich, USA. Dimethoxy curcumin (DiMC) was supplied by Biosynth Ltd, UK as a gift sample. Dimethyl sulphoxide (DMSO) supplied by Sigma-Aldrich (USA) was utilized to prepare the stock solutions of UV-328. The other chemicals employed in the present investigation were of analytical grade and used without further purification.

Experimental set-up

Adult wild-type (AB strain) Zebra fish (*Danio rerio*) with a body length of 2.46 ± 0.04 cm and a mean body weight of 0.28 ± 0.04 g were obtained from Mass biotech Zebra fish facility, Chennai. The fishes were adjusted to the research facility conditions for a week time in glass aquarium going before to the trials and the testings were conducted as per the guidelines of OECD,203 for the testing of chemicals (OECD,2022). The fishes were raised in re-circulating circulated air through freshwater kept up with at 26 ± 1 °C, with a photo-period time of 12:12 h (light/dark) routine. During the acclimatization time frame, they were taken care of with fish food at not obligatory and water reestablishment was done one time each day. After acclimation, fish (150 numbers) were arbitrarily isolated into three trial gatherings, for example, water control group, UV-328 treated group at centralization of 55µg/L. Each group was kept up with in three duplicates and each recreate contains 50 fish in 25 L test arrangement. Stock arrangements of UV-328 were arranged newly in DMSO. In order to maintain the aquarium's water quality and the appropriate concentrations of UV-328, the test solutions were changed every 24 hours. Furthermore, UV-328 with DiMC treated group were treated with a similar grouping of UV-328 alongside DiMC 50mg/kg BW through the feed regimen. Prior to the experiment, they were starved for 24 hours. On day 28, fish were haphazardly chosen from openness and control and treated tanks (n = 15/repeat) and gill samples were gathered and utilized promptly for biochemical examination. One more arrangement of gill tissues was fixed in 10% formalin for histological perception.

Biochemical analysis

The gill tissues were flushed, homogenized with 50 mM ice cold potassium phosphate buffer (pH 7.0), centrifuged for 10 min (10,000 rpm) and the clear supernatant was gathered to gauge the protein content, chemical exercises (SOD, CAT, GPx GR and GST) GSH and MDA level. Each examine was acted in sets of three. GSH level was examined by the method of Moron and expressed at a density of g/mg protein.¹⁹ SOD activity was assessed by estimating the hindrance of pyrogallol autooxidation at 420 nm, and the catalyst action was communicated as Units/mg protein.²⁰ CAT movement was assessed by estimating the absorbance of hydrogen peroxide at 590 nm and communicated as µmol H₂O₂ of

consumed/min/mg protein.²¹ GST was assayed after the complexation of glutathione (GSH) with 1-chloro-2, 4-dinitrobenzene CDNB at 340 nm, and the outcome was given in μmol of CDNB form shaped/min/mg protein.²² GPx movement was assessed by Rotruck after the oxidation of glutathione (GSH) within the sight of H_2O_2 at 412 nm and the information was communicated as μg of GSH consumed/min/mg protein.²³ The activity of glutathione reductase is used as indicator for oxidative stress. The activity can be monitored by the NADPH consumption, with absorbance at 340 nm, or the formed GSH can be visualized by Ellman's reagent and expressed as μmol GSH utilized/min/mg protein.²⁴ MDA content was assessed by the method of Devasagayam at 532 nm, which depends on 2-thiobarbituric corrosive (4,6-dihydropyrimidine-2-thiol; TBA) reactivity, and the outcome was communicated as nmol/mg protein.²⁵ Lowry et al. method was used to determine the protein concentration with bovine serum albumin as the standard.²⁶

Histopathological investigation

Gill tissues were at first fixed in 10% unbiased cradled formalin. The decent tissue tests were dried out in a progression of reviewed ethanol, implanted in paraffin wax, segmented at $5\mu\text{m}$ thickness, and stained with hematoxylin and eosin (H&E) for histopathological examination.²⁷⁻²⁹ The sections were examined and photographed using a light microscope (Leica, DME light microscope).

Statistical analysis

Statistical investigation was completed by utilizing GraphPad Crystal 5.0 programming bundle (GraphPad Programming Inc., San Diego, CA). The outcomes acquired from each exploratory groups were exposed to one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc correlation. Means with different superscripts of $P < 0.05$ were viewed as genuinely significant between the compared groups.

RESULTS

No mortality was seen during the acclimatization and exposure period, and there was no tremendous distinction between the blank (water) and dissolvable control (DMSO) for any of the biomarkers during the exposure. Thus, the water control group was kept up with as the reference group.

Impact of UV-328 on cellular antioxidant and lipid peroxidation

The activity of SOD, CAT, GPx, GR and GST and the degree of GSH in the gill of Zebra fish. presented to UV-328 are portrayed in the Table.1 and in the Fig. 1 separately. It is clear that when contrasted with the control, the activities of antioxidant enzymes and the GSH level were altogether diminished when exposed to UV-328 for 28 days. These adjusted variables were essentially recovered in DiMC treated group means the expected defensive antioxidant role of DiMC against UV-328 prompted gill oxidative stress and keeps up with attuned antioxidant status. The fact that the lipid peroxidation level was significantly higher in UV-328-treated gill tissue and significantly lower in DiMC-supplemented gill tissue (Fig.1) demonstrated the antilipoperoxidative property of DiMC.

Table 1: Effect of DiMC on UV-328 induced changes in the gill enzymatic antioxidant enzymes of Zebra fish

Experimental Groups	Gill
SOD (Units/mg protein)	
Control	30.07 ± 1.32^a
UV-328	23.52 ± 1.07^b
UV-328+ DiMC	27.76 ± 1.54^c
CAT (μmol of H_2O_2 consumed /min/mg protein)	
Control	21.42 ± 0.51^a
UV-328	17.25 ± 1.37^b
UV-328+ DiMC	19.36 ± 1.24^c
GPx (μmol of GSH oxidized/min/mg protein)	
Control	10.67 ± 0.46^a
UV-328	6.82 ± 0.34^b
UV-328+ DiMC	8.34 ± 0.57^c
GST (μmol of CDNB conjugate formed/min/mg protein)	
Control	0.051 ± 0.008^a
UV-328	0.027 ± 0.002^b
UV-328+ DiMC	0.042 ± 0.005^c
GR (μmol GSH utilized/min/mg protein)	
Control	10.78 ± 0.42^a
UV-328	6.23 ± 0.27^b
UV-328+ DiMC	8.41 ± 0.63^c

Values are expressed as mean $\pm$ SE. The superscript letters (a and b) indicate significant difference from the control and experimental groups determined by one way analysis of variance followed by Dunnett's post-hoc comparison, $p < 0.05$ (DMRT).

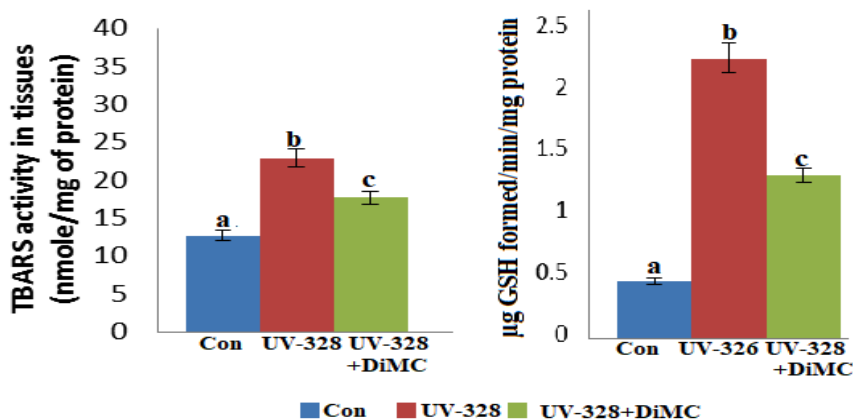


Fig. 1

Figure 1: Effect of UV-328 and DiMC on the LPO and GSH level in the gill tissue of zebra fish.

Values are expressed as mean $\pm$ SE. The superscript letters (a,b and c) indicate significant differences from the control and experimental groups determined by one way analysis of variance followed by Dunnett's post-hoc comparison, $p < 0.05$ (DMRT).

Histopathological changes

The gills structure in the control and experimental groups were shown in Fig. 2. In the control fish, gill structure was normal with the primary lamellae, secondary lamellae, blood vessels, and cartilage. The primary gill lamellae arranged alternatively on either side of the intrabronchial septum. Secondary gill lamellae arranged on either side of the primary gill lamellae. Primary gill lamellae comprised of blood vessels, lining epithelial cells and a central cartilaginous rod. Mucous cells were observed at the base of the gill filament. The squamous epithelium, pillar cell, and erythrocyte were orderly arranged in the secondary lamellae, which was the site for

gaseous exchange (Fig.2B). However, in the UV-328 treated group, gills appeared more fragile and disorganized. The most common changes included epithelial lifting and sloughing, intraepithelial edema, necrosis, vacuolization, fusion of adjacent secondary gill lamellae, curving, shortening and rupture of gill filaments were also observed (Fig.2B). Furthermore, the sections from the DiMC supplemented UV-328 group displayed a better recuperation of gill histoarchitecture with normal lamellar structure with regular cellular arrangements without any pathological alterations (Fig. 2C), which clearly indicates the potential salutary impact of DiMC against UV-328 induced oxidative gill injury.

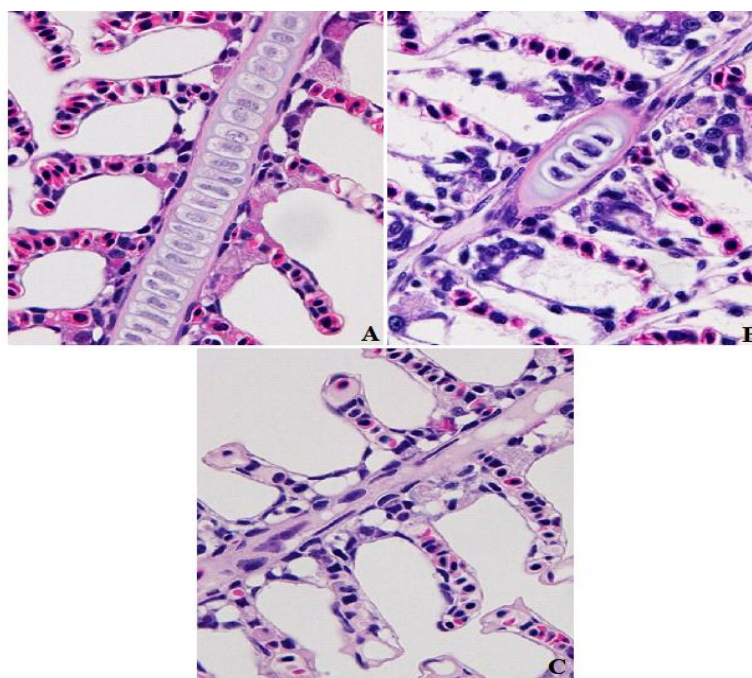


Figure 2: Light micrographs of sections through gill of Zebra fish showing histological structure of the control and experimental groups. Samples were stained with hematoxylin and eosin and photomicrographs were taken using 200x magnification. **(A).** Control group showing the rigid morphological features of gill with primary and secondary lamellae. **(B).** UV-328 exposed gill tissue showing the altered pathological features like necrosis, desquamation, inflammation of gill structure. **(C).** UV-328 + DiMC treated gill tissue of Zebra fish showing almost normal features of the gill lamellae and cellular structure.

DISCUSSION

UV-328 is a component of many personal care products and one of the most commonly used organic UV filters in the industry. It is lipophilic, light-stable, and bio accumulative. As life forms that are delicate to ecological changes, fish are to be the best bioindicators and have been utilized to evaluate the nature of aquatic ecosystems and to determine whether ecological contamination is harmful. The gills and integument are significant organs for a few physiological capabilities, as they are delicate to changes in water quality caused by xenobiotics due to their immediate contact with the water. The gills are multifunctional organs that are critical for breath, acid-base equilibrium, osmoregulation, and immune protection and address an incredible model for ecotoxicological studies.³⁰⁻³² The state of disequilibrium between the antioxidant defense mechanism and reactive oxygen species (ROS), or free radicals, is what defined as oxidative stress. Reduced GSH and the major antioxidant enzymes viz, SOD, CAT, GPx, GR and GST help the organism to reduce the harmful effects of ROS when it is exposed to pollutants or adverse environmental conditions. The gill antioxidant system is the primary defense mechanism against ecotoxicological stress, since it can suppress the ROS that can impair many of the intracellular macromolecules.³³

Findings of the present investigation, indicate that a significant elevation in the level of gill TBARS and a marked decline in the level of reduced GSH and antioxidant enzymes clearly signifies the excess ROS generation and its mediated lipid peroxidation and oxidative stress in the gill tissue of Zebra fish exposed to UV-328. The activities of antioxidant enzymes were decreased than the levels of the control fish upon UV-328 exposure, which can be interpreted as the inability of the antioxidant enzymes to overcome extremely high oxidative stress. Similar findings were reported in Zebra fish exposed to organic UV filters exhibited oxidative stress and antioxidant erosion.¹¹ The primary capability of GPx is to take out the extreme species like H₂O₂ and keep up with the membrane protein thiols status. Lined up with the CAT and SOD enzymes exercises, the GPx activity was likewise expanded at first yet diminished in the later stages. The decreased GPx activity after 4 weeks of UV-328 exposure demonstrates expanded creation of H₂O₂, which likewise corresponds with the high lipid hydroperoxide level responsible for UV-328-induced oxidative harm. At a lower concentration of UV-328, the opposing effects of the stimulative and suppressive effects may account for the unaltered GPx activity. The depressed GPx and GR action may be because of the excess measure of H₂O₂ and diminished level of reduced glutathione (GSH). Moreover, diminished CAT

action can be considered an element in the diminished GPx activity.¹⁸

In the current study, the exposure of UV-328 notably expanded the LPO level and diminished the cellular antioxidant defense of the gill tissue of Zebra fish. It has proactively been accounted for UV-328 exposure caused abundance release of reactive oxygen free radicals. This high oxidative pressure in the gill tissue caused the peroxidation of membrane lipids, protein carbonylation and nuclear DNA damage through antioxidant depletion.¹⁵ The supplementation of DiMC had the efficacy to prevent the expansion in LPO and decline in antioxidant defense through its antioxidative capability in the gill tissue in zebra fish exposed to UV-328. It appears that DiMC through its cell reinforcement properties limits the toxic manifestations of UV-328 mediated gill oxidative stress. DiMC is chemically similar to curcumin, while the methylene group among the β -diketone structure provides them with remarkable antioxidant properties. Meanwhile, the methylation of both hydroxyl groups makes DiMC more stable and lipophilic than curcumin, further providing it with a significantly reduced degradation rate and a much-improved drug delivery system. It was also found that DiMC could respond to normal healthy cells in a way that is similar to curcumin but exerted more potent antioxidant properties.³⁴

Respiration processes across the gills and food intake are the main probable xenobiotics uptake mechanisms in aquatic biota. The perceptible structural changes in the gills of Zebra fish were considered as a nonspecific stress response to UV-328 exposure. Similar gill lesions were reported in Zebra fish exposed to organic UV filters like BP-3 and environmental pollutants.^{13,35} Likewise, UV-328-exposed Zebra fish gills showed hypertrophy, fusion, rupture, and necrosis in the lamellae coupled with increased oxidative stress and decreased antioxidant status. These unfavorable architectural changes in gills are defense mechanisms against the contaminants to reduce the entry of toxic compounds through the gills, and the toxicity of chemicals on gills is independent of the type of stress agents and fish species.³⁶ The most pronounced alterations were epithelial lifting and intraepithelial edema, which has been described as an adaptive response against contamination to increase the diffusion distance between the water and blood in the secondary lamellae.³⁷ Congruously, at biochemical levels, we found enriched pathways associated with oxidative stress and antioxidants system, which indicated that gills were susceptible to UV-328 exposure. Therefore, the potential ecological risks of UV-328 on aquatic organisms should be taken into consideration, and studies elucidating the tissues accumulation of UV-328 are also necessary. Intervention with DiMC significantly reversed all these pathological alterations in the gill tissue through its potential antioxidant action and aids in the recuperation of normal cellular architecture in the gill tissue of Zebra fish.

CONCLUSION

In conclusion, the present study demonstrates that UV-328 could cause substantial augmentation in oxidative stress (TBARS) and exhaustion of antioxidant (GSH) and antioxidant enzymes (SOD, CAT, GPx, GR and GST, in Zebra fish gills as well as the pathological modifications after exposure to 4 weeks. These phenomena demonstrate that exposure to these organic UV filters could upsurge ROS production and cause oxidative injury to Zebra fish gills. Even to the sublethal dose of this UV filter upon chronic exposure significantly heightened the oxidative harm in the gills, suggesting the toxic nature of UV-328. The observed altered variables clearly suggested that exposure to UV-328 at 55 μ g L⁻¹ for 28 days had the most severe toxicity on the morphology and antioxidative defenses

in Zebra fish gills. Numerous biochemical and histological changes were additionally seen in Zebra fish when exposed to even sub-lethal concentration of UV-328 and these huge numbers of alterations were obviously improved with the supplementation of DiMC. With the help of these findings, we can better understand the way by which the UV-328 filter could harm the Zebra fish. and the efficacy of the phytoantioxidant i.e., DiMC mitigated the respirototoxic potential through its enhanced antioxidative prospective. In a nutshell, the data obtained in this study provide a scientific foundation for the ecotoxicological risk assessment of organic filters like UV-328 and its toxicological mechanisms will be the focus of additional research in the future.

Ethical approval

All analyses, maintenance, and treatment dealing with Zebra fish were completed according to the rules of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Mass Biotech, Tamil Nadu. India. Endorsement number: MB/IAECCC/2022/ 03/06.

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Competing interests

Authors have declared that no competing interests exist.

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