



Biochemical and gene expression alterations due to individual exposure of atrazine, dichlorvos, and imidacloprid and their combination in zebrafish

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Abstract

In environmental toxicology, combined toxicity has emerged as an important concern. Atrazine (ATZ), dichlorvos (DIC), and imidacloprid (IMD) are the major pesticides, extensively used to control insect, flies, mosquitoes, and weed. Here, we investigate whether the exposure to three different types of pesticides individually and in combination for 24 h alters anti-oxidant enzyme responses in zebrafish (*Danio rerio*). Oxidative stress parameters (biochemical and mRNA expression), acetylcholinesterase (AChE) activity, and Metallothionein-II (MT-II) mRNA expression levels were measured. Present work includes toxicological assessment of individual and combined (CMD) exposure of ATZ (185.4 μM), DIC (181 μM), IMD (97.8 μM), and CMD (ATZ 92.7 μM + DIC 90.5 μM + IMD 48.9 μM), in the liver, kidney, and brain of adult zebrafish. Lipid peroxidation (LPO), glutathione (GSH) content, AChE, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activity along with mRNA expression of SOD, CAT, GPx, and MT-II were evaluated. Briefly, LPO, GSH content, the activity of AChE, and all antioxidant enzymes enhanced significantly in individual exposure, which was further altered in the CMD group. The mRNA expression of SOD, CAT, GPx, and MT-II in the liver and kidney showed significant down-regulation in all exposed groups. In the brain, significant upregulation in mRNA expression of SOD, CAT, GPx, and MT-II was observed in DIC and IMD groups, while ATZ and CMD showed significant downregulation except for GPx. Findings postulate that the CMD group exhibits synergistic toxic manifestation. The present study provides the baseline data on the combined toxic effects of pesticides and suggests regulating the use of pesticides.

Keywords Aquatic ecotoxicology · Combined toxicity · mRNA expression · Oxidative stress · Pesticide pollution · Synergistic effects

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Introduction

The indiscriminate use of pesticides in agriculture has become a major risk to ecology as they enter the environment through spraying or surface run-off after rainfall. The small quantity of pesticide(s) can badly affect the ecosystem due to their presence, multiplicity, toxicity, and persistency (Wang et al. 2017; Nele et al. 2020; Covert et al. 2020; Satyanarayana et al. 2023). Total pesticides retain after use in agricultural fields, and transported to different water sources depends on several factors like topography, weather (especially rainfall), agricultural practices, and chemical properties of pesticides (Olwenn et al. 2021; Kruć-Fijałkowska et al. 2022). In the past few decades, the diversity of agrochemicals increases, resulting in water pollution shifts from being dominated by a single active compound to complex multi-agent polluting factors. The presence of numerous chemicals and agents shows combined effects that can be greater (synergy) or lower (antagonism) than the sum of the effects predicted by single-stressor models (Shukla et al. 2017a; Kruć-Fijałkowska et al. 2022; Falfushynska et al. 2022; Li et al. 2023). Despite this most standardized water-quality criteria and risk assessment protocols focus on the toxicity of single chemical (Van Leeuwen et al. 1996; Barata et al. 2006). Pesticides greatly influence reactive oxygen species (ROS) and cause oxidative stress, which leads to the inactivation of antioxidant enzymes which in-turns, serves as a biomarker for pesticide-induced toxicity. Free radicals/ROS scavengers such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are enzymatic and protect against their deleterious effects (Blahova et al. 2013; Narasimhamurthy et al. 2022; Ahmed et al. 2022; Ahmad et al. 2022). Glutathione (GSH) is a tripeptide with antioxidative properties because of the thiol group which acts as a reducing agent and minimizes the toxicity of free radical or ROS (Santos and Martinez 2014). Acetylcholinesterase (AChE) is the primary enzyme that catalyzes the hydrolysis of acetylcholine into choline and acetic acid (Rodríguez-Fuentes et al. 2015). AChE activity has been widely utilized in assessing aquatic pollution (Assis et al. 2007; Rodríguez-Fuentes et al. 2015; Benli and Çelik 2021; Guerra et al. 2021; Viana et al. 2022; Mendonça-Soares et al. 2023). Metallothioneins (MTs) are low molecular weight protein and helpful in scavenging ROS (Valavanidis et al. 2006; Moncaleano-Niño et al. 2022). MTs have been extensively studied in heavy metal and pesticide toxicity (Agarwal et al. 2010; Erdogan et al. 2011; Ceyhun et al. 2012; Mai et al. 2020; Moncaleano-Niño et al. 2022).

Atrazine (ATZ) (6-chloro-N-ethyl-N'-(1-methyl ethyl)-1,3,5-triazine-2,4-diamine) is a chlorinated herbicide of

triazine class which is the most extensively used herbicide mainly applied in corn, maize, wheat, sorghum grass, and sugarcane field (Jin et al. 2010; Ahmed et al. 2022; Ahmad et al. 2022). The mode of action of triazine class herbicides includes inhibition of the photosynthetic pathway, specifically the photosystem II by binding the Q_B -binding site of the D1 protein complex present in the chloroplast thylakoid membrane (Shaner 2014). It is highly persistent and resistant to degradation with a half-life in surface water is about >200 days (Ghosh and Philip 2006).

Dichlorvos (DIC) (2, 2-dichloroethyl dimethyl phosphate) is an organophosphate class insecticide widely used in household, agriculture, and livestock parasite purpose in controlling flies, aphids, mites, and caterpillars (Bui-Nguyen et al. 2015; Ogunro 2023). Unrestrained application has resulted in the detection of DIC in freshwater sources which has amplified the risk of exposure thus affecting various non-target organisms including human beings (Falfushynska et al. 2022; Narasimhamurthy et al. 2022). DIC mainly inhibits the activity of the AChE enzyme and leads to an accumulation of acetylcholine and elevated cholinergic activity in the central and peripheral nervous system, which may even lead to death (Bui-Nguyen et al. 2015; Falfushynska et al. 2022; Nachimuthu et al. 2023; Mali et al. 2023). AChE is an enzyme that hydrolyzes the neurotransmitter acetylcholine at neuromuscular junctions and brain cholinergic synapses.

Imidacloprid (IMD) 1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine) is a chloronicotinyl neonicotinoid class insecticide used to control pests in cereals, vegetables, tea, and cotton worldwide (Tomizawa and Casida 2005; Mendonça-Soares et al. 2023). It is effective against insects at low application rate and is considered as a replacement for organophosphorus pesticides. It acts as a nicotinic acetylcholine receptor (nAChR) agonist and can lead to central nervous system disorders (Tomizawa and Casida 2005). IMD has been listed by US environmental protection agency, as Class E- evidence of non-carcinogenicity for humans. Imidacloprid usage became a burning issue in environmental research due to its severe toxicity to bees (Ge et al. 2015).

The individual toxic potential of ATZ, DIC, and IMD has been extensively studied (Celik and Suzek 2009; El-Gendy et al. 2010; Kapoor et al. 2010; Jin et al. 2010; Zhu et al. 2011; Ceyhun et al. 2012; Blahova et al. 2013; Tao et al. 2020; Khaled et al. 2023; Mendonça-Soares et al. 2023). All three pesticides are known to cause oxidative stress, liver ailment, sperm abnormalities, and neurobehavioral abnormalities, alter the antioxidant capacity of the cell, affect the normal functioning of the cell, and even lead to death (Jin et al. 2010; Bui-Nguyen et al. 2015; Ge et al. 2015; Guo-Ping et al. 2020; Azpiazu et al. 2021; Ahmed et al. 2022; Ahmad et al. 2022; Mali et al. 2023). ATZ is known to cause several health effects

like ovarian cancer, DNA methylation changes, breast cancer, DNA damage, decreased semen quality, and birth defects. (Zhu et al. 2011; Blahova et al. 2013; Abarikwu et al. 2017; Demirci et al. 2018; Kruć-Fijałkowska et al. 2022). Tao et al. (2020) assessed the individual and combined toxicity of three organophosphate pesticides on freshwater green algae *Chlorella pyrenoidosa*. They observed a reduction in the malondialdehyde (MDA) content and SOD activity. Similarly, a sub-lethal dose of IMD can alter LPO, GSH, AChE, and ribonucleic acid (RNA) in Japanese quail (Khaled et al. 2023).

The combined toxicity of these pesticides has not been explored yet. However, we have reported oxidative stress caused by the combination of ATZ, DIC, and IMD (commercial grade) in zebrafish (Shukla et al. 2017a). The reason behind this work is to estimate the initiation of toxic manifestations of commonly used pesticides to predict the risk of environmental and health hazards so that proper measures can be taken to prevent toxicity. Therefore, the present work was executed to evaluate the toxic effects caused by ATZ, DIC, and IMD individually and in combination. mRNA expression analyses were carried out for SOD, CAT, GPx, and MT-II along with LPO, GSH, AChE, SOD, CAT, and GPx enzymatic activity in adult zebrafish.

Materials and methods

Chemicals, reagents, kits, and primers

High-purity analytical grade (99% or above) chemicals and reagents were used in this study. Pesticide standards, atrazine (99.6%), dichlorvos (98.8%), and imidacloprid (99.9%) were purchased from Sigma Aldrich USA. RiboZol™, Amresco, USA (Fig. 1 showing the structure of atrazine, dichlorvos, and imidacloprid). qPCR Green Master with UNG-blue dye and Verso cDNA synthesis kit for PCR analysis were acquired from Thermo Fischer Scientific, India. Specific

primer sequences for SOD, CAT, GPx, MT-II, and β -Actin were obtained from Eurofins Genomics, India Pvt. Ltd.

Zebrafish

Certified wild species of adult zebrafish (*Danio rerio*) (4–5 months; length $2.7 \text{ cm} \pm 0.5 \text{ cm}$; weight $0.285\text{--}0.385 \text{ g}$) were procured from a pet store and handled following good animal practice as defined by animal welfare bodies. The study was approved by University Research Committee (No. PhD/FS/RA/02). Zebrafish were acclimatized for 15 days to the laboratory environment by keeping them in glass aquaria containing 50 lt of de-chlorinated tap water maintained under controlled temperature ($26 \pm 2 \text{ }^\circ\text{C}$) with constant aeration and maintaining 12 h alternate light and dark cycle. Zebrafish were fed daily on dried blood worms (Taiyomax™) and commercial pellets (Otohime™ A1, size; $75\text{--}150 \text{ }\mu\text{m}$). The tank was cleaned every day; feces and other residue were removed from the water on time and no feeding was given 24 h before experimentation.

Experimental protocol

Water physiochemical parameters

Water conditions examined by American Public Health Association (APHA 2005) are as follows: temperature — $26 \pm 1.0 \text{ }^\circ\text{C}$, pH — 7.0 ± 0.1 , dissolved oxygen — $7.2 \pm 0.2 \text{ mg/L}$, hardness — 109.0 mg/L , conductivity — $0.2 \text{ }\mu\text{hm}$, alkalinity — 0.27 g/L .

Animal exposure

The acute toxicity test was conducted in a static condition; the experiment was designed as per Organization for Economic Co-operation and Development (OECD 2004) guidelines, and zebrafish were fastened during the experimentation period.

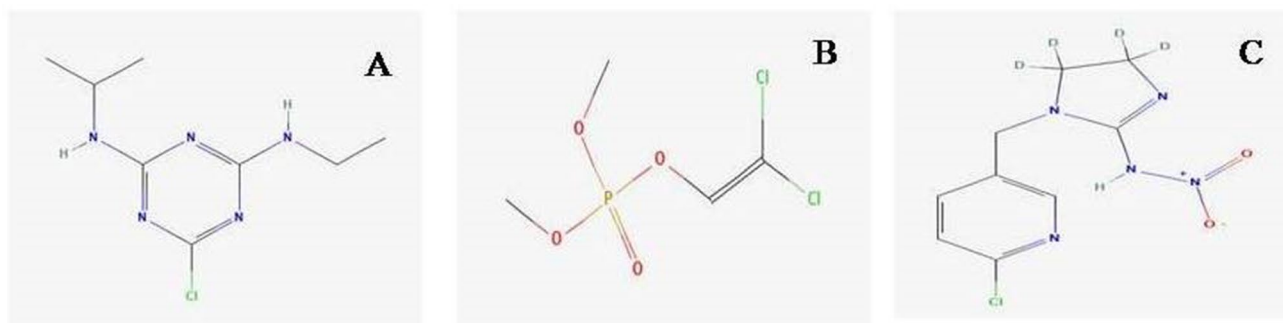


Fig. 1 Structure of atrazine (A), dichlorvos (B), and imidacloprid (C) (source Pubchem)

Total 180 adult zebrafish (mixed sex) were divided into five groups (36 fish randomly selected in each group, 18 fish used for biochemical, and 18 for mRNA expression, with random pooling of 06 fish); all experiments were conducted in duplicates. Group I: animals were held in de-chlorinated water, served as control (CTRL) Group II: ATZ, 185.4 μM , Group III: DIC, 181 μM , Group IV: IMD, 97.8 μM , and Group 5: CMD (ATZ 92.7 μM + DIC 90.5 μM + IMD 48.9 μM) (half concentration of each single compound exposure). The study was based on our earlier reported work with these three pesticides (Shukla et al. 2017a).

Tissue collection procedure and homogenization

Zebrafish were sacrificed after 24 h of exposure under a stereomicroscope. For total RNA isolation, the liver, kidney, and brain were removed immediately and stored at $-20\text{ }^{\circ}\text{C}$ in 200 μL of ice-cold ribozol for 48 h. For biochemical studies, the liver, kidney, and brain were minced, and homogenized (2.5% w/v) with ice-cold 0.15% KCl–0.1 M phosphate buffer (pH 7.4) using Potter–Elvehjem homogenizer and stored at $-20\text{ }^{\circ}\text{C}$.

Biochemical assays

All biochemical parameters were done on Multiskan EX Plate Reader, Thermo Fisher Scientific, Inc., USA, using standard methodologies. The levels of MDA, an end product of lipid peroxidation, were measured with the absorbance coefficient of the malondialdehyde-thio-barbituric acid (MDA-TBA) complex at 532 nm using 1,1,3,3-tetraethoxypropane as the standard (Ohkawa et al. 1979). GSH levels were determined using 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) for color development at 412 nm (Ellman 1959). SOD enzyme activity was measured by the modified method of NADH-phenazine-methosulfate-nitroblue-tetrazoliumformazan inhibition reaction at 550 nm (Kakkar et al. 1984). GPx enzyme activity was measured using GSH as substrate and DTNB as standard by the method of (Flohe and Gunzler 1984) at 420 nm.

CAT activity was measured at 570 nm by the method of (Sinha 1972). AChE activity was measured by Ellman et al. (1961), Protein levels were assayed by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard at 690 nm.

Gene expression study

Total RNA extraction and reverse transcription

Isolation and extraction of total RNA were done by Shukla et al. (2017b), and the qualitative and quantitative analyses were performed on NANOGENIUS photometer MAPADA instruments, China. The optical density 260/280 ratio for entire samples ranges from 1.9 to 2.0. Total RNA was reverse transcribed using Verso cDNA synthesis kit as per manufacturer's instructions using thermal cycler Veriti™, Applied Biosystems, Thermo Fischer Scientific, USA.

Real-time polymerase chain reaction (RT-PCR)

PCR amplification and quantitation were carried out for SOD, CAT, GPx, MT-II, and β -Actin as housekeeping gene (reference gene) were executed with 100–150 basepairs (bp). β -Actin remains unchanged in exposed groups during the whole procedure. The primer sequence for SOD, CAT, GPx, MT-II, and β -Actin was taken from Jhamtani et al. (2018) (Table 1). RT-PCR was performed by qPCR GreenMaster, UNG-blue dye detection kit using Applied Biosystem ABI@ 7500 QPCR System, USA. All the reactions were carried out in a 10- μL reaction mixture in triplicates as per the maker's instructions.

Data analysis

The data obtained by real-time RT-PCR are presented as threshold cycle (Ct), which is the cycle at which the intensity of fluorescence was determined to be statistically significant Pfaffl (2001). For fold change calculation in target gene expression levels, relative quantification by the “delta-delta Ct Method” was used for comparing relative expression levels between treatments in RT-PCR. The method is based on the relative expression of a target gene vs a reference gene.

Table 1 Primer sequences of genes used in study

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Size (BP)
SOD	CGCATGTTCCAGACATCTA	GAGCGGAAGATTGAGGATTG	100
CAT	AGTGCTCCTGACGTCCAGCCA	TGAAGAACGTGCGCACCTGGG	115
GPx	GGACGATCCAAGCGTGGTGG	CAGCCGTACACGTCTGGGC	148
MT-II	GCCAAGACTGGAACCTGCAAC	CGCAGCCAGAGGCACACT	124
β -Actin	CAACAGAGAGAAGATGACACAGATCA	GTCACACCATCACCAGAGTCCATCAC	138

Statistical evaluation

The data of individual exposed groups were presented as the mean ± standard error ($n = 6$), and the statistical significance of mean value in the examined tissue for different groups, viz., control, ATZ, DIC, IMD, and CMD was examined by two-way analyses of variance (ANOVA). A probability of ≤ 0.05 was considered to be significant. The signs *, #, \$, and † indicate significant difference from the CTRL, ATZ, DIC, and IMD exposed groups, respectively, which represents as *, #, \$, † $p < 0.05$; **, ##, \$\$, †† $p < 0.001$; ***, ###, \$\$\$, ††† $p < 0.005$ levels.

Results

Lipid peroxidation levels (LPO), glutathione content (GSH), and acetylcholinesterase (AChE) activity

Considerable enhancement in LPO was observed in all examined tissues of entire exposed groups in comparison with the control group Fig. 2(i). Significant ($p < 0.005$) enhancement of LPO was observed in the CMD group in all three examined tissue, the group followed by DIC, IMD, and ATZ. Brain suffered with maximum LPO followed by liver and kidney.

In the liver, reduced ($p < 0.005$) GSH content was found in individual exposed groups, and no change in the CMD group when compared with control Fig. 2(ii). The ATZ group showed maximum reduction followed by DIC and IMD. In the kidney, the DIC exposed group shows significant ($p < 0.005$) elevated GSH content, while considerable reduction in the CMD group. However, no alteration was observed in ATZ and IMD groups. In the brain, reduced GSH content was observed in ATZ ($p < 0.005$) and CMD ($p < 0.001$), while DIC showed a significant ($p < 0.005$) increase and no change in the IMD group in comparison with the control group.

In the liver, kidney, and brain, considerable increase in AChE activity was found in the entire exposed groups when compared with control Fig. 2(iii). The CMD group showed significant ($p < 0.005$) enhancement in AChE activity in comparison with individual exposed groups. The brain shows maximum AChE activity than the liver and kidney.

Antioxidant enzymes activities and gene expression

SOD enzyme activity and mRNA expression

In the liver, a progressive increase in SOD enzyme activity was found in the entire exposed groups to ATZ < DIC < IMD < CMD in comparison with the control

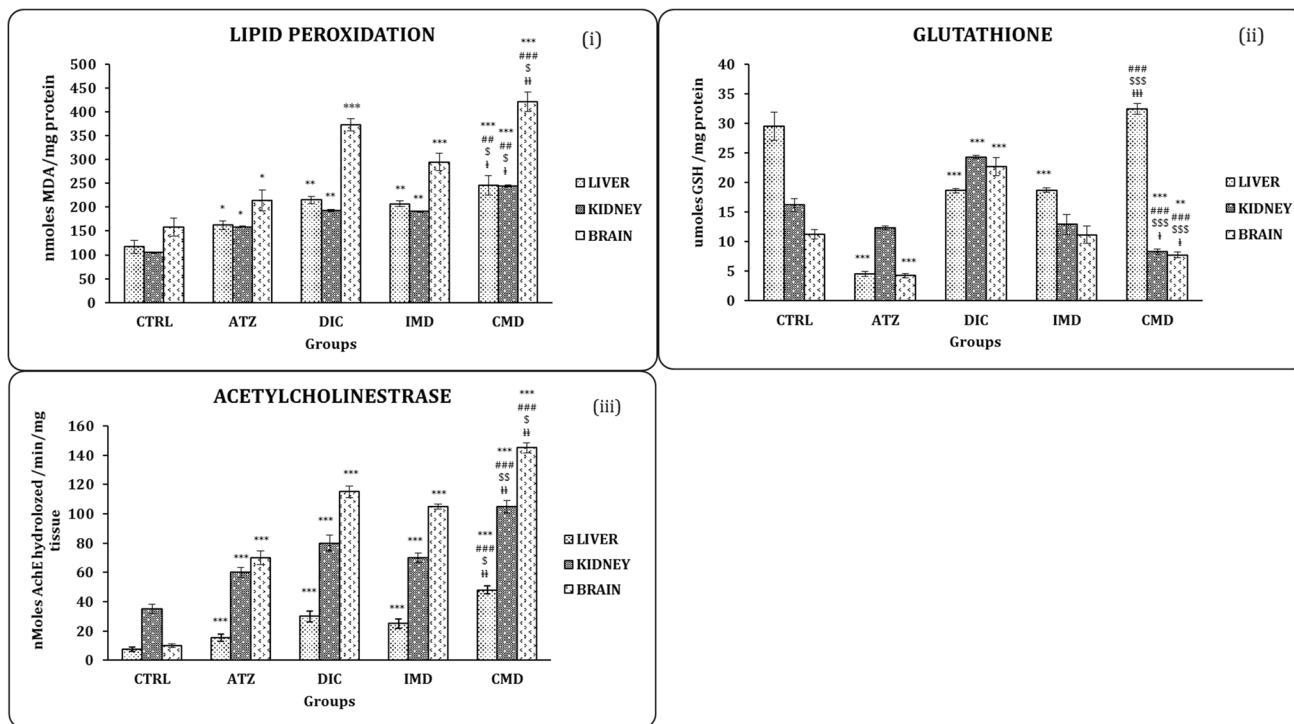


Fig. 2 (i–iii) Lipid peroxidation, glutathione content, and acetylcholinesterase activity in the liver, kidney, and brain of zebrafish exposed to ATZ, DIC, IMD, and CMD group for 24 h, presented as mean ± SE ($n = 6$). Difference between groups were considered as

significant when $p < 0.05$. Signs, viz., *, #, \$, and †, indicate significant difference from the CTRL, ATZ, DIC, and IMD exposed groups, respectively, which represents *, #, \$, † $p < 0.05$; **, ##, \$\$, †† $p < 0.001$; ***, ###, \$\$\$, ††† $p < 0.005$ levels

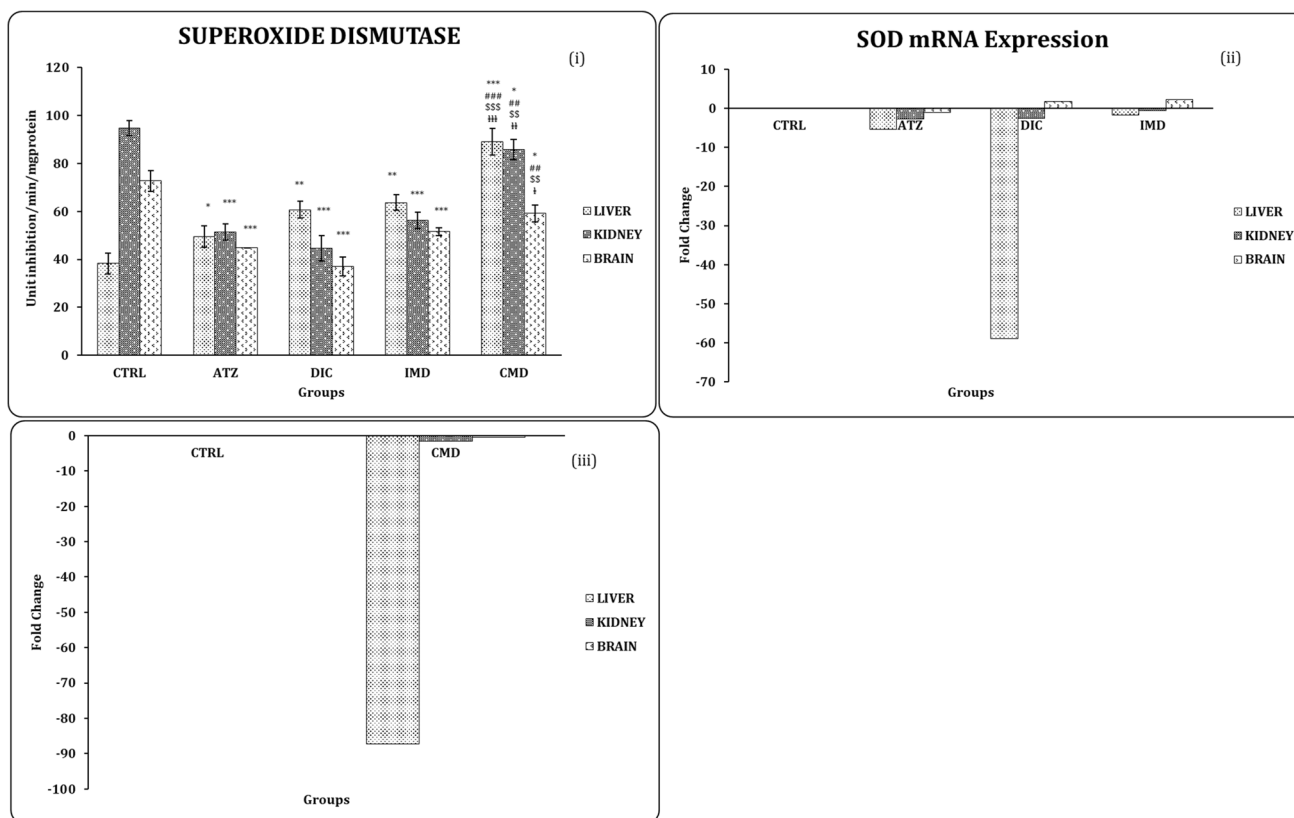


Fig. 3 (i) Superoxide dismutase activity in liver, kidney, and brain of zebrafish exposed to ATZ, DIC, IMD, and CMD group for 24 h, presented as mean \pm SE ($n=6$). Difference between groups were considered as significant when $p < 0.05$. Signs, viz., *, #, \$, and †, indicate significant difference from the CTRL, ATZ, DIC, and

IMD exposed groups, respectively, which represents $^{*},\#, \$, \dagger p < 0.05$; $^{**},\#\#, \$\$, \ddagger p < 0.001$; $^{***},\#\#\#, \$\$\$, \ddagger\ddagger p < 0.005$ levels. (ii–iii) Fold change in SOD mRNA expression in individual exposed groups vs control and in the CMD group vs control, respectively

group Fig. 3(i). In the kidney, a considerable decline in SOD activity was observed in individual exposed groups, while significant changes ($p < 0.05$) in the CMD group when compared with the control group. In the brain, significantly diminished SOD activity was noticed in all exposed groups in comparison with the control group. In the CMD group, significant ($p < 0.001$) elevated SOD activity was observed in comparison with individual exposed groups.

In the liver, mRNA expression of SOD is significantly downregulated in all exposed groups (Fig. 3ii (fold change, ATZ, DIC, and IMD), iii (fold change, CMD)). Maximum downregulation was observed in the CMD group (87.29 fold) followed by DIC (58.97 fold), ATZ (5.32 fold), and IMD (1.7 fold). In the kidney, significant downregulation was observed in all exposed groups. The highest downregulation was observed in the ATZ group (2.7 fold) followed by DIC (2.60 fold), CMD (1.56 fold), and IMD (0.61 fold). In the brain, upregulated in DIC (1.83 fold) and IMD (2.33 fold) while downregulated in ATZ (1.04 fold) and CMD group (0.43 fold) were observed.

CAT enzyme activity and mRNA expression

In the liver and kidney, diminished CAT activity was observed in all exposed groups when compared with the control Fig. 4(i). In the CMD group, noteworthy enhanced activity was observed in the liver when compared with individual exposed groups. In the same way, the CMD group shows elevated activity in the kidney when compared with ATZ and DIC. However, no alteration was observed in comparison to IMD. In the brain, a noteworthy increase in CAT activity was noticed in the DIC, IMD, and CMD groups and a decrease in the ATZ exposed group when compared with the control. In the CMD group, a significant increase in CAT activity was observed when compared with ATZ, while decrease with DIC and no significant change when compared to the IMD group.

In the liver, mRNA expression of CAT is significantly downregulated in all exposed groups (Fig. 4ii (fold change, ATZ, DIC, and IMD), iii (fold change, CMD)). Maximum downregulation was observed in CMD (29.50 fold) followed

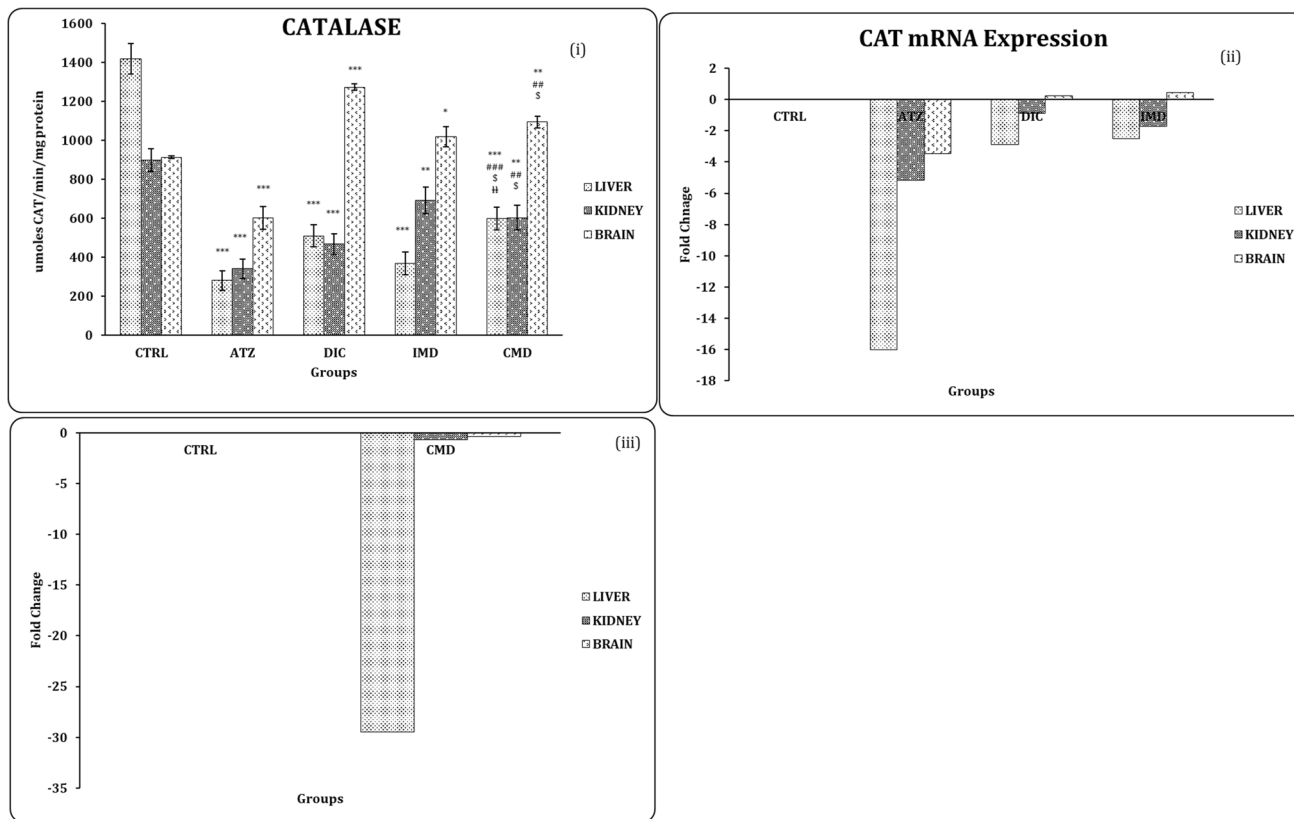


Fig. 4 (i) Catalase activity in liver, kidney, and brain of zebrafish exposed to the ATZ, DIC, IMD, and CMD groups for 24 h, presented as mean ± SE (n=6). Difference between groups were considered as significant when p < 0.05. Signs, viz., *, #, \$, and ‡, represent significant difference from the CTRL, ATZ, DIC, and IMD exposed groups,

respectively, which represents *, #, \$, ‡ p < 0.05; **, ##, \$\$, †† p < 0.001; ***, ###, \$\$\$, ††† p < 0.005 levels. (ii–iii) Fold change in CAT mRNA expression in individual exposed groups vs control and in CMD group vs control, respectively

by ATZ (16.01 fold), DIC (2.87 fold), and IMD (2.5 fold). In the kidney, significant downregulation was observed in the ATZ group (5.18 fold) followed by IMD (1.69 fold), DIC (0.87 fold), and CMD (0.67 fold). In the brain, upregulation in DIC (0.24 fold) and IMD (0.43 fold) while downregulation in ATZ (3.44 fold) and CMD (0.37 fold) were observed.

GPx enzyme activity and mRNA expression

In the liver, considerable elevated GPx activity was seen in ATZ and CMD group and no alterations in the DIC and IMD when compared with the control Fig. 5(i). Correspondingly, in the kidney, significant elevated activity was observed in individual exposed to the ATZ (p < 0.05) and CMD groups in comparison to the control. However, the suppressed activity was observed in individual exposed to DIC and IMD in comparison with the control. In the brain, significant elevated GPx activity was observed in the DIC, IMD, and CMD groups in comparison to the control. Decreased activity was observed in the ATZ exposed group in comparison with the control. In the CMD group, considerably increased

GPx activity was noticed in all three tissues in comparison with the individual exposed group.

In the liver, mRNA expression of GPx is considerably downregulated in all exposed groups (Fig. 5ii (fold change, ATZ, DIC, and IMD), iii (fold change, CMD)). The highest downregulation was observed in CMD (31.84 fold) followed by ATZ (22.34 fold), DIC (9.07 fold), and IMD (0.67 fold). In the kidney, significant downregulation in DIC (7.99 fold) followed by ATZ (7.36 fold), CMD (4.2 fold), and IMD (1.4 fold). In the brain, GPx mRNA expression significantly upregulated in DIC (14.45 fold), IMD (36.62 fold), and CMD (0.53 fold) while downregulated in ATZ (0.30 fold) were observed.

MT-II mRNA expression

In the liver, mRNA expression of MT-II is significantly downregulated in all exposed groups (Fig. 6i (fold change, ATZ, DIC, and IMD), ii (fold change, CMD)). The highest downregulation was observed in CMD (55.91 fold) followed by ATZ (6.32 fold), DIC (4.5 fold), and IMD (1.01 fold). In

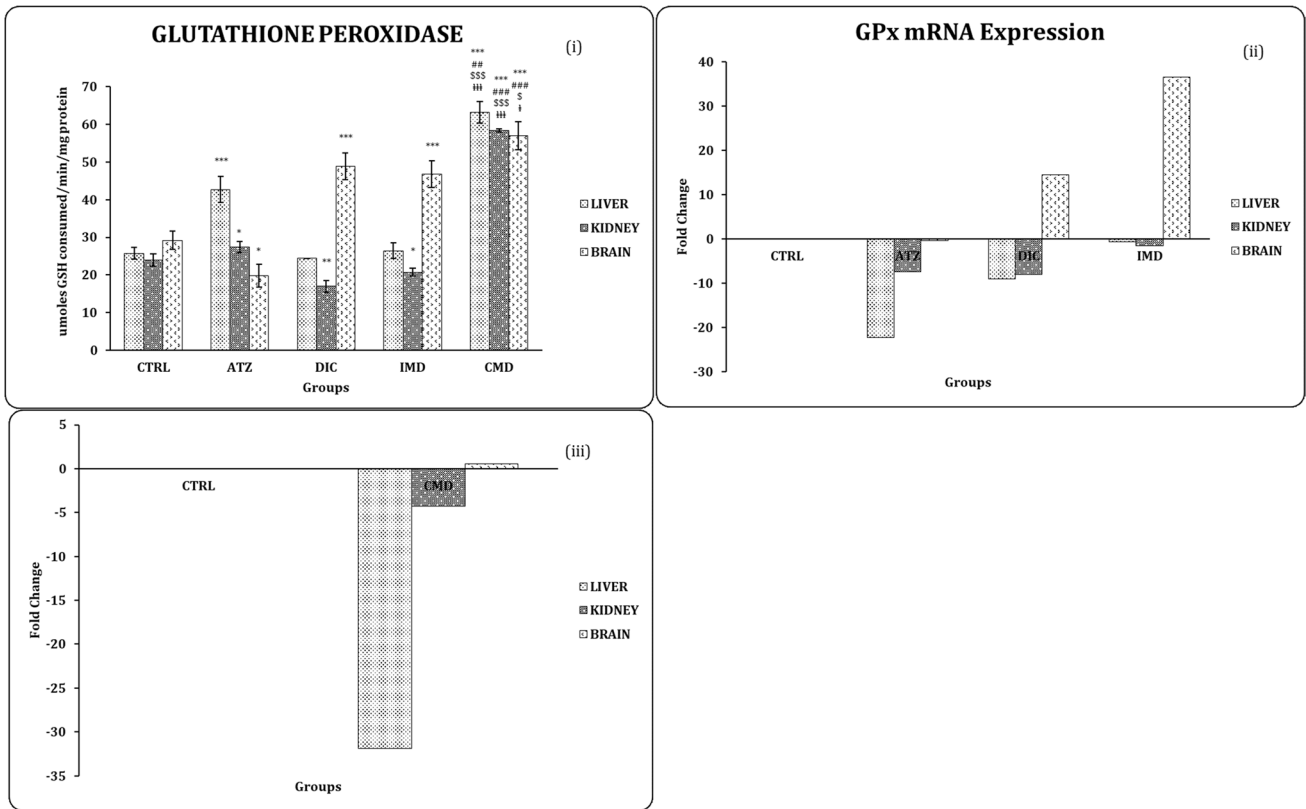


Fig. 5 (i) Glutathione peroxidase activity in liver, kidney, and brain of zebrafish exposed to the ATZ, DIC, IMD, and CMD groups for 24 h, presented as mean ± SE (n=6). Difference between groups were considered as significant when $p < 0.05$. Signs, viz., *, #, \$, and ‡, represent significant difference from the CTRL, ATZ, DIC, and

IMD exposed groups, respectively, which represents $*.#.S.‡ p < 0.05$; $**.#.#.SS.‡‡ p < 0.001$; $***.###.SSS.‡‡‡ p < 0.005$ levels. (ii–iii) Fold change in SOD mRNA expression in individual exposed groups vs control and in the CMD group vs control, respectively

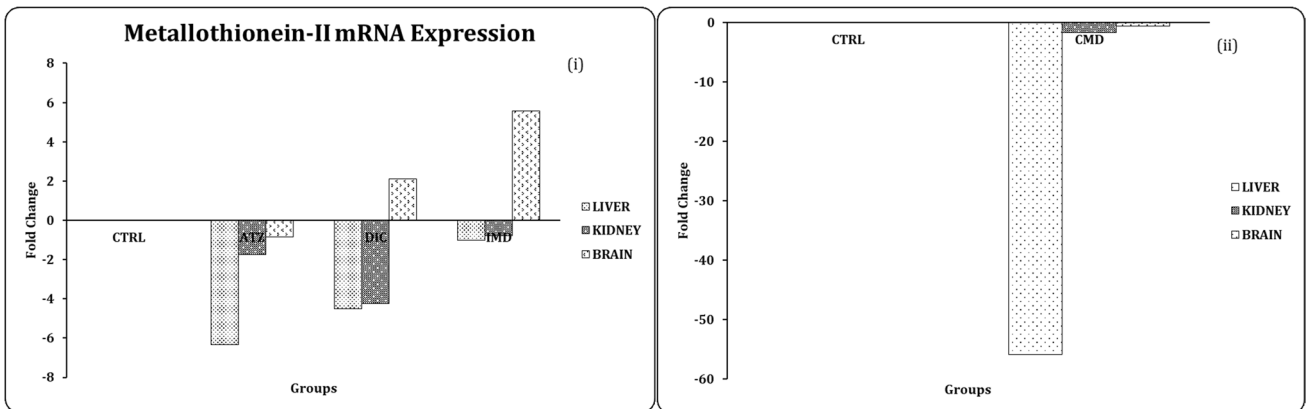


Fig. 6 (i–ii) Fold change in MT-II mRNA expression in individual exposed groups vs control and in the CMD group vs control, respectively

the kidney, significant downregulation was observed in DIC (4.25 fold), ATZ (1.73 fold), CMD (1.72 fold), and IMD (0.79 fold). In the brain, significant downregulation in ATZ (0.84 fold) and CMD (0.55 fold) while upregulation in DIC (2.11 fold) and IMD (5.58 fold) were observed.

Discussion

MDA has been widely used for many years as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids because of its facile reaction with TBA (Jin

et al. 2010; Blahova et al. 2013; Shukla et al. 2017a; Yanhua et al. 2021). The results showed significant MDA generation showing lipid peroxidation in all three examined tissue caused by pesticide exposure. Fascinatingly, in the combined exposure group, maximum lipid peroxidation was observed when compared with the individual exposed groups. An increase in lipid peroxidation due to pesticide exposure may ascribe to triggering ROS. ROS are known to enhance the oxidation of polyunsaturated fatty acids (PUFA) leading to lipid peroxidation and altering endogenous enzyme activity (Jin et al. 2010; Blahova et al. 2013; Shukla et al. 2018; Falfushynska et al. 2022). The liver is a vital organ to metabolize xenobiotics; it encompasses supplementary unsaturated lipids, which experience swift oxidative degradation thus resulting in high MDA generation (El-Gendy et al. 2010; Jhamtani et al. 2018; Olwenn et al. 2021). The kidney is responsible for the filtration and elimination of xenobiotics from the body, therefore may show elevated MDA levels (Abarikwu et al. 2017). We have observed significant LPO in the combined exposed groups as compared with the individual exposed groups. Increased LPO represents the oxidative injury induced by the combination of pesticides. Similar results were reported by other researchers Santos and Martinez (2014) and Ojha et al. (2011). In our earlier reported work with commercial-grade pesticides, we observed similar results of significant LPO in the combined exposed groups (Shukla et al. 2017a). Our results also exhibit significant LPO in the individual exposed groups in comparison with the control groups. Similar results were observed by Celik and Suzek (2009), Jin et al. (2010), Kapoor et al. (2010), and Guerra et al. (2021). The brain is susceptible to oxidative stress due to elevated oxygen utilization with high levels of lipids, which enhances PUFA (Jhamtani et al. 2018). In the brain tissue of combined exposed groups, we have observed a significant increase in LPO, which might be due to the synergistic effects shown by the combination of three pesticides on the occurrence of LPO in the brain. Our findings are in line with the results documented by Bacchetta et al. (2014). We have observed significant LPO in the individual exposed groups when compared with the control group, which demonstrates the neurotoxic nature of the undertaken pesticides. Similar results of increased LPO were reported by other authors caused by individual and combined exposure to pesticides (Celik and Suzek 2009; Kapoor et al. 2010; Blahova et al. 2013; Falfushynska et al. 2022; Ahmed et al. 2022; Ahmad et al. 2022).

GSH is a vital tripeptide with antioxidative properties having a thiol group; it acts as a reductant and can be reversed back in the cell. The depleted GSH content in the liver shows it is utilized by glutathione peroxidase to scavenge ROS on individual exposure to pesticides. Similar results were also reported by Jin et al. (2010) and Kapoor

et al. (2010). In combined exposure, the GSH content is near to control which may be due to the protection provided by antioxidant enzymes. An increased GSH content under combined exposure shows its presence in the cell in adequate amounts as required by antioxidant enzymes. Similar results were documented by other authors (Abarikwu et al. 2017; Jhamtani et al. 2018). In the kidney and brain, significantly depleted GSH levels were noticed in the CMD group which is an early effect of ROS generation due to pesticide exposure. In the present work, GSH might be utilized by antioxidant enzymes to eliminate ROS due to combined exposure to pesticides. Analogous to our findings, Demirci et al. (2018), Abarikwu et al. (2017), and Ahmed et al. (2022) have reported a decrease in GSH content.

The assessment of AChE enzyme activity is used as a biomarker for aquatic pollution. It is an endogenous enzyme found in all tissue and abundantly in the brain, which makes it a target enzyme for different xenobiotics (Rodríguez-Fuentes et al. 2015; Benli and Çelik 2021; Viana et al. 2022). Pesticide causes oxidative stress which in turn alters AChE activity. Effects of dichlorvos and other organophosphates pesticides on AChE activity have been studied (Gultekin et al. 2001; Lionetto et al. 2003; Assis et al. 2007; Rodríguez-Fuentes et al. 2015; Benli and Çelik 2021; Viana et al. 2022), while the combined toxic effects of pesticides on AChE activity have not been explored. We have observed the induction of AChE enzyme activity in the entire examined tissue due to pesticide exposure. The changes were more prominent in the brain than liver and kidney in all exposed groups, which shows the neurotoxic nature of DIC, IMD, and ATZ individually as well as in the combined group. The increased AChE activity due to pesticide exposure indicates the initiation of oxidative injury in the examined tissue. However, the results are not parallel with the findings of other authors wherein they reported a decrease in AChE activity after pesticide exposure in zebrafish (Assis et al. 2007; Rodríguez-Fuentes et al. 2015; Guerra et al. 2021). However, the combination of fipronil (insecticide) and 2,4-D (herbicide) increases the activity of the AChE enzyme in zebrafish (Viana et al. 2022). Similarly, sulfoxaflor (insecticide) exposure causes a significant increase in AChE activity in the brain of zebrafish (Benli and Çelik 2021). In the same way, a significant increase in AChE activity was observed due to IMD exposure in the zebrafish brain (Mendonça-Soares et al. 2023). In our work, individual and combined exposure shows the initiation of toxic manifestations due to ROS generation along with the commencement of endogenous detoxification process as shown by AChE activity in all examined tissue in zebrafish. This is further correlated with elevated lipid peroxidation levels and altered endogenous enzyme activity in individual and combined pesticide exposure. Therefore, AChE can be regularly used as an important biomarker in the determination of pesticide-induced toxicity.

The vital antioxidant enzymes, viz., SOD, CAT, and GPx, provide the first-line of defence against free radicals induced by toxicants such as pesticides (Zhu et al. 2011; Al-Sawafi and Yan 2013; Ge et al. 2015). SOD leads to the conversion of superoxide anion ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Furthermore, CAT and GPx convert H_2O_2 into H_2O and bring down lipid hydroperoxides to their consequent alcohols.

In the liver, notable elevation in SOD enzyme activity with down-regulated mRNA levels was observed in all exposed groups. The liver is responsible for the breakdown of xenobiotics in the body. Elevated SOD activity in the liver shows compensatory effects in response to the lipid peroxidation induced by individual and combined pesticide exposure. Analogous results were postulated by Azpiazu et al. (2021); Yanhua et al. (2021), Guo-Ping et al. (2020), and observed significant alteration caused by the combination of pesticides. SOD mRNA expression in the liver on exposure shows downregulation indicating the inability to neutralize superoxide anions. Increased SOD activity and suppressed mRNA expression show inconsistent response between enzyme activity and gene expression as reported by Jhamtani et al. (2018) and Karaca et al. (2014). The decrease in SOD activity and suppressed mRNA expression in all exposed groups in the kidney may be due to high levels of ROS, which is further supported by elevated LPO. The depletion of SOD activity was maximum in the brain might be due to enhanced LPO due to pesticide exposure. Upregulated mRNA expression in the DIC and IMD exposed groups showed attenuated effects due to maximum lipid peroxidation, while the ATZ and CMD groups exhibit SOD combat-induced lipid peroxidation.

Depleted CAT activity and mRNA expression in liver and kidney tissues as well as in brain tissue on individual ATZ exposure and gene mRNA expression of the CMD group show the underlying mechanism of pesticide (oxidant) susceptibility in exposed zebrafish. Enhanced sensitivity arises as a direct consequence due to accumulation of hydrogen peroxide in tissues, i.e., an inability to maintain cellular antioxidant levels which leads to impaired activity as observed by high lipid peroxidation. Similar to our results, suppressed CAT activity was also observed by Ojha et al. (2011) and Wu et al. (2018). The elevated enzyme activity in the brain of DIC, IMD, and CMD shows the repair mechanism and elimination of H_2O_2 by CAT (Shukla et al. 2017a).

Kim et al. (2018), and Mai et al. (2020) have observed altered CAT mRNA expression and CAT enzyme activity caused by the combination of pesticides. Elevated CAT activity shows protection against the adverse effects of toxicants as it functions to reduce ROS and prevent tissue damage.

In the combined group, we observed a significant increase in the GPx enzyme activity in all the examined tissue. The overproduction of ROS has been shown to result in an attenuation of GPx in the cells. In our earlier reported work with commercial-grade pesticides, we observed similar results of enhanced GPx enzyme activity in the combined exposed groups (Shukla et al. 2017a). The mRNA levels downregulated in the liver and kidney in all exposed groups contribute to increased free radical species which lead to direct tissue damage caused by individual and combined exposure to pesticides. Upregulated mRNA expression observed in the brain of the DIC, IMD, and CMD groups represents the high ROS scavenging ability of cells to eliminate lipid hydroperoxides as a consequence of enhanced LPO. Similar results were also reported by Kim et al. (2018) on combined exposure.

MTs are known to reduce the dangerous effects of free radicals by releasing zinc into the neuronal membrane. MTs are endogenous enzymes that are shown to scavenge hydroxyl radicals because of their cysteinyl thiolate group. MT contains zinc/cadmium ions, which help in scavenging superoxide and hydroxyl produced by the xanthine oxidase reaction. MT-II mRNA expression was performed to determine its relevance in detoxifying pesticide-induced toxicity. We have observed upregulation in the MT-II mRNA levels caused by individual DIC and IMD pesticides. Upregulation in MT-II mRNA levels in response to the individual exposed brain of DIC and IMD may be considered as a defence mechanism similar to that of other examined antioxidant enzymes' mRNA levels. The results are correlated with Chen and Maret (2001) findings; they explained MT can act as an oxidative stress indicator, in which oxidation of MT release zinc to further decrease toxicity via thionein and GSH. Therefore, increased MT levels could enhance the antioxidative effectiveness of cells under stress responses. However, significant downregulation was observed in all the examined tissue of the CMD group, with a maximum in the liver. Similar downregulated results were demonstrated by the combination of different pesticides with a metal (Moncaleano-Niño et al. 2022). Fascinatingly, within our study, the levels of MT-II mRNA expression are parallel with the levels of SOD, CAT, and GPx. Our MT-II results further back the incorporation of assessing MT gene in pesticide-induced toxicity.

Similarly, other endogenous antioxidant enzymes, viz., SOD, CAT, and GPx, also helps in scavenging free radicals induced by individual and combination of pesticides. The suppression of MT-II in the present work represents impairment in the defence mechanism against the toxic effects of pesticides and the inability of the cell in MT-II protein synthesis. The results indicate a direct correlation with examined SOD, CAT, and GPx mRNA expression. In the brain, DIC and IMD show upregulation indicating the role of metallothionein in xenobiotic detoxification.

The alteration in MT mRNA expression levels in different exposed groups is similar to the antioxidant enzymes studied. Similar to our results, Ceyhun et al. (2012) and Selcuk et al. (2018) have reported upregulated MT mRNA expression due to dichlorvos and imidacloprid exposure. The result shows that MT-II plays not only a significant role in detoxifying metal toxicity but also toxicity induced by pesticide(s).

Conclusion

Our results revealed that combined exposure exhibits maximum alterations in all examined tissues. All three organs were drastically affected in individual exposure groups; however, CMD exposure further elevates the toxic manifestations; thus, it is offering support to our previously reported work in which combined pesticide toxicity causes hepatotoxicity, nephrotoxicity, and neurotoxicity rather than individual target organ toxicity. In conclusion, the combination of pesticide exposure is furthermore destructive than single pesticide exposure, even at half concentration. Further from the findings, it can be understood that the assessment of single pesticide toxicity underrates the impact of combined pesticides toxicity on aquatic system and living organisms; therefore, it is required to anticipate the risk of combined toxicity so that the utility of various pesticides can be regulated to minimize their environmental and health hazard.

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Author contribution The authors contributed to the study conception and design, material preparation, data collection, and analysis; the draft was performed by Saurabh Shukla; data collection and analysis were performed by Reena C Jhamtani; study design, editing, and manuscript were revised by Rakhi Agarwal. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability If required, the data and materials will be provided.

Declarations The experiment was designed, and animals were kept as per Organization for Economic Co-operation and Development guidelines.

Ethics approval The study was approved by the university research committee no. PhD/FS/RA/02.

Consent to participate The authors give consent to participate.

Consent for publication The authors give consent to publish the data provided in the manuscript.

Competing interests The authors declare no competing interests.

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