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RESEARCH ARTICLE

Protective efficacy of naringenin against cadmium-induced redox imbalance in *Labeo rohita*: an integrated biomarker approach

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Abstract

The protective efficacy of dietary naringenin (NG) has been investigated against the toxicity caused by cadmium chloride ($CdCl_2$) using biomarkers of oxidative stress in the liver, gills and kidney of *Labeo rohita*. The fish were exposed to environmentally relevant concentrations of $CdCl_2$ (0.37 and 0.62 mg/L) and simultaneously orally administered with NG (50 mg/kg bw/day) for 60 days. Tissue (gills, liver and kidney) samples were collected on days 15, 30 and 60 of the experiment and analysed for endogenous antioxidants and oxidative stress biomarkers. $CdCl_2$ exposure for 15 and 30 days induced the development of adaptive mechanism as demonstrated by the enhanced activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase in all three tissues. However, on the 60th day, $CdCl_2$-induced oxidative damage was stipulated by a decline in the enzyme activities and reduced glutathione (GSH) content significantly ($p < 0.05$) below control levels along with enhanced levels of lipid peroxidation. Oral administration of NG in toxicant exposed fish significantly restored the altered levels of antioxidants, oxidative enzymes and lipid peroxidation. Besides, integrated biomarker response (IBR) analysis was applied by combining all the biomarkers to indicate the overall stress response index. IBR analysis confirmed the altered levels of biomarkers, the oxidative stress induced by $CdCl_2$ exposure and the ameliorative potential of NG. The present study suggested that NG might have protective role against Cd-induced oxidative insult which might be ascribed to the ability of NG to chelate metals and scavenge free radicals.

Keywords Cadmium chloride · Oxidative stress · Antioxidants · Lipid peroxidation · Integrated biomarker response

Introduction

The protective antioxidant defence system of aquatic organisms continuously experience many challenges as a consequence of exposure to diverse natural and anthropogenic factors capable of inducing redox imbalance (Danabas et al. 2015; Wen et al. 2018). Reactive oxygen species (ROS) are produced continuously as typical by-products of cellular metabolic processes and, at low concentrations, possess dualistic role; i.e. they are not only involved in cell signalling and biosynthetic reactions but also provide defence against pathogens and have deleterious effects on macromolecules (Rajendran et al. 2014). However, overproduction of ROS alters the redox homeostasis, overpowers the antioxidant defence system of the cell and subsequently results in oxidative damage, often termed as oxidative stress (Pisoschi and Pop 2015). Oxidative stress instigates deleterious alterations in cellular macromolecules like DNA, lipid and proteins (Naik et al. 2020). Alterations in levels of endogenous free radical scavengers can be applied as potent biomarkers for studying the oxidative damage caused by pollutants (Ziech et al. 2010; Poletta et al. 2016). Literature is replete with evidences suggesting that numerous xenobiotics, and amongst them heavy metals, may enhance ROS production which is considered an important mechanism of toxicity (Waisberg et al. 2003; Cobbina et al. 2015). Amongst heavy metals, Cd is of major concern as a consequence of its considerable toxic effects to aquatic animals at relatively low concentrations and long elimination half-life (Jindal

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and Verma 2015; Prabu et al. 2011a; McRae et al. 2019). The extensive use of Cd in modern industries and agricultural fertilisers has resulted in increased concentrations of Cd in the environment which contributes to the entry of Cd in food chain and exerts adverse effects in organisms (Gad 2014; Naik et al. 2020; Bhardwaj et al. 2021). Many studies have reported higher levels of Cd in underground water, soil, sewage, sediments, vegetation and animal tissues (El-Ghasham et al. 2008; Aulakh et al. 2009; Kumar and Singh 2010; Idrees et al. 2018; Abougabal et al. 2020; Tamele and Vázquez Loureiro 2020). Elevated Cd levels such as 0.04 mg/L in fresh water fish farm at Qassim Region, K.S.A. (El-Ghasham et al. 2008), and 0.01 mg/L in water samples from Koekemoerspruit, Africa (Fernández-Luqueño et al. 2013), have been recorded in aquatic ecosystem. Bhardwaj et al. (2017) reported the variation in the level of Cd from 0.006 mg/L (post-monsoon) to 0.11 mg/L (pre-monsoon) in Yamuna River during 2013 to 2015. Cd induces deleterious effects in animals such as nephrotoxicity, hepatotoxicity, cytotoxicity, genotoxicity, immunotoxicity, cancer and disrupted reproductive processes thus causing infertility (Kumar and Singh 2010; Bhardwaj et al. 2021). Cd is considered to augment ROS production which in turn leads to lipid peroxidation (McRae et al. 2018; Verma et al. 2020), protein modifications and altered gene expression as well as DNA damage (Valko et al. 2005).

Recently, great emphasis has laid on intervening Cd-induced oxidative stress by using naturally occurring antioxidants. In this context, a variety of compounds has been evaluated for their efficacy to reduce the detrimental effects of Cd by inhibiting lipid peroxidation or chelating the metal ions thus, easing their elimination (Karaytug et al. 2014; Bhardwaj and Panchal 2021). Flavonoids (a group of polyphenols) are prevalent in numerous fruits and vegetables, and display remarkable array of pharmacological properties. Many researchers have investigated the potential of flavanoids to alleviate oxidative damage (Cheng and Breen 2000; Prabu et al. 2011a). Naringenin (4,5,7-trihydroxy flavonone), a naturally occurring flavonoid present in citrus fruits, has attracted considerable attention because of its widespread biological applications including antiatherogenic, hepatoprotective, neuroprotective, anti-inflammatory, anticancer and antimutagenic (Choi et al. 1994; Lee et al. 2001, 2004; Amaro et al. 2009; Zhang et al. 2019; Zeng et al. 2020). In the recent years, NG as an antioxidant has been the focus of research owing to its potential to chelate metal ions and scavenge free radicals (Kapoor and Kakkar 2014; Priscilla et al. 2015; Huang et al. 2019).

Fish, being poikilothermic and sensitive, are the most susceptible to metal contamination in aquatic environment and, therefore, are extensively employed in biomonitoring studies (Çavaş and Ergene-Gözükara 2005). *L. rohita* (Indian major carp), have high growth potential, consumer preference and are widely used as an ecological indicator (Prusty et al. 2011). Taking into consideration ROS production as the key investigated mechanisms of Cd toxicity and the antioxidative property of NG, the present study hypothesised that NG possess a protective role against $CdCl_2$-induced oxidative stress in *L. rohita*. To elucidate Cd-induced toxicity and protective efficacy of NG, oxidative stress biomarkers of gills, liver and kidney were chosen because they are the most vulnerable to toxicity caused by pollutants in aquatic environment and, thus, considered an efficient tool for biomonitoring of Cd-mediated oxidative damage in aquatic fauna. The major objectives of the present study were (1) to explicate the antioxidative defence response of *L. rohita* to short- (15 days) and long-term exposure (60 days) of environmentally relevant concentrations of $CdCl_2$, (2) to elucidate the extent of toxicity caused to three different organs (gills, liver and kidney) using integrated biomarker response and (3) to evaluate the ability of NG in mitigating the toxicity induced by $CdCl_2$ through oxidative stress biomarkers.

Materials and methods

Experimental fish

The live specimens of *L. rohita* (12.05 ± 0.19 cm long and 18.05 ± 0.98 g, mean ± SE) were obtained from Sultan Fish Seed Farm, Haryana, India. After disinfection (0.01% $KMnO_4$ solution for 1 min), the specimens were shifted to glass aquaria (300-L capacity). The fish were then acclimatised for 15 days in dechlorinated tap water at 24–25 °C temperature with dissolved oxygen content of 8.07 ± 0.50 mg/L, pH 6.9–7.1 and hardness 92.675 as $CaCO_3$ mg/L.

The experiments carried out on fish and the protocols included in the study followed the IAEC guidelines (Panjab University, Chandigarh, IAEC/527).

Chemicals

The anhydrous $CdCl_2$ and NG were of technical-grade (Sigma-Aldrich, USA). Other reagents used in the study were of analytical grade (Merck, Mumbai, India, and Hi Media India Ltd.).

Feed preparation

The basal diet was prepared by thoroughly mixing locally available ingredients (see Table S1 in supplementary material). Water was added to prepare thick dough and pellets were formed. The pellets were sun-dried and stored in air tight container at − 20 °C. The fish were fed (two times/day) with the basal diet at 2% of body weight (bw).

Determination of LD_{50} of NG

To determine LD_{50} of NG in *L. rohita*, 'Acute toxic class method' (Fig. 1) was used according to OECD guidelines (Organization for Economic Cooperation and Development 2001). It is a sequential method involving use of three animals in each group at each step with a particular dose level. All the experiments were performed in triplicates to ensure reproducibility of results. As there is no information available on the toxicity of NG to fish, 5 mg/kg bw was selected as the initial dose to carry out the study. Three fish in a plastic tank (40-L capacity) were fed with a basal diet of 5 mg/kg bw of NG at 2% of body weight. The remnants of feed were removed after 2 h by siphoning. The fish were observed every half an hour for 4 h and then after 24 h for any signs of mortality. According to Acute Toxic Class method, if the death of 2 or 3 fish occurs then, the experiment has to be stopped immediately and LD_{50} falls in level 1 (GHS 2013). On the other hand, if there is no mortality or only 1 fish dies, the above step is repeated with the identical dose. Since no fish died at this step, then the next step was conducted with 50 mg/kg bw dose. Further, the above procedure was repeated with a 300 and 2000 mg/kg bw dose and the range of 24 h LD_{50} was obtained (Fig. 1).

Exposure protocol

Fish were randomly distributed into six groups ($n = 6$) as depicted in Table 1 and maintained in the plastic tanks (40-L capacity) filled with dechlorinated water at experimental conditions as mentioned in the "Experimental fish" section. All the experiments were conducted in triplicates with the same no. of fish in each ($n = 6$, total 108), and experimental

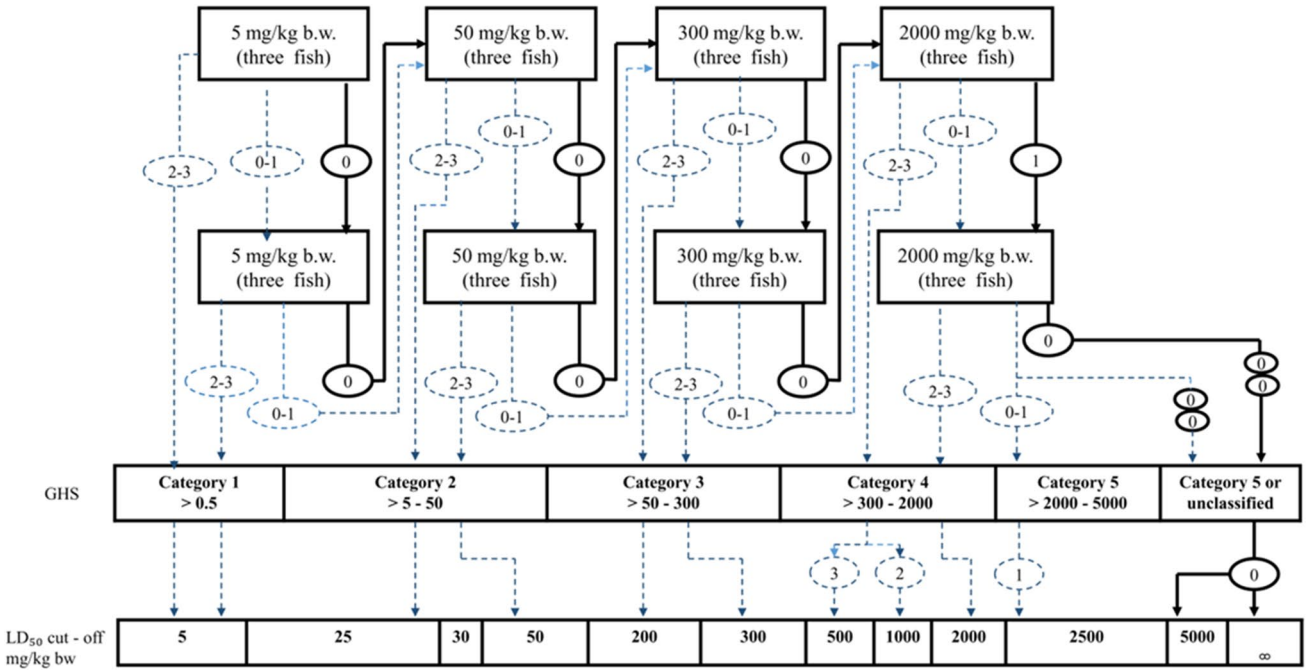

Fig. 1 Estimation of LD_{50} of NG to *L. rohita* by oral administration (in diet) through acute toxic class method based on OECD guidelines 423 (Organization for Economic Cooperation and Development 2001). Dotted lines represent the procedure given in the guidelines, while bold lines show the results obtained during the study. Oval boxes represent the no. of fish died during the experiment

Table 1 Experimental groups

Groups	Treatment
I	Control (dechlorinated tap water), fed with basal diet without NG
II	NG (orally administered in diet) at dose of 50 mg/kg bw/day
III	0.37 mg/L $CdCl_2$ (in medium)
IV	0.37 mg/L $CdCl_2$ (in medium) + 50 mg/kg bw/day NG (orally administered in diet)
V	0.62 mg/L $CdCl_2$ (in medium)
VI	0.62 mg/L $CdCl_2$ (in medium) + 50 mg/kg bw/day NG (orally administered in diet)

tanks (capacity 50 L) were provided with aerators and heaters (to maintain temperature).

The $CdCl_2$ exposure concentrations were selected based on our previous study (Jindal and Verma 2015), while the dose of NG (50 mg/kg bw/day) was chosen according to the effective dose used by earlier workers for amelioration of metal-induced toxicity in experimental models (Wang et al. 2012; Mershiba et al. 2013). Test media was replaced every 2 days and the concentration of $CdCl_2$ was examined using ICP-AES (Thermo Electron Corporation, iCAP 6000 series) on random basis during the experimental period.

Sample collection and preparation

On days 15, 30 and 60 of the experiment, 2 fish from each replicate (6 fish/group) were selected at random and sacrificed by cervical dislocation. Liver, gills and kidney were immediately removed using sterile forceps on ice-cold plates, rinsed (with chilled 0.9% NaCl), dried in filter paper and weighed separately. The 10% (W/V) homogenate of tissues was prepared in 100 mM potassium phosphate buffer (pH 7.4) using Potter-Elvejhem homogeniser. Then centrifugation of homogenate was done at 4 °C (10,000 × *g* for 30 min.). Afterwards, the supernatant was carefully separated and stored at − 30 °C. All the antioxidant levels and enzyme activities were analysed within 3 days of sampling.

Biochemical analysis

The superoxide dismutase (SOD) activity was determined following the method of Kono (1978). Briefly, the reaction mixture containing 1.2 ml of 50 mM sodium carbonate in 0.1 mM EDTA (pH 10.8), 0.5 ml of 96 µM nitro blue tetrazolium (NBT) and 0.1 ml of 0.6% Triton X-100 were incubated at 37 °C for 10 min. Reaction was initiated by adding 0.1 ml of 20 mM hydroxylamine hydrochloride. The rate of NBT dye reduction by superoxide radical generated due to photoactivation of hydroxylamine hydrochloride was recorded at 560 nm for 3 min for blank. Then immediately 0.1 ml supernatant was added to it. After mixing thoroughly, 50% inhibition in the rate of NBT reduction by SOD present in the enzyme source was recorded at 560 nm for 3 min. The enzyme activity was expressed as U/mg protein.

The activity of catalase (CAT) was assessed as change in the absorbance due to H_2O_2 decomposition as described by Luck (1965). The reaction mixture consisted of 2.9 ml of H_2O_2-phosphate buffer and 0.1 ml of supernatant. The decrease in absorbance/30 s at 240 nm was read for 3 min using double-distilled water as blank. The enzyme activity was expressed as µmole H_2O_2 decomposed/min/mg protein.

The activity of glutathione peroxidase (GPx) was assayed at 340 nm following the method of Mohandas et al. (1984), which involves the oxidation of NADPH to NADP. A total of 2 ml reaction volume consisted of 0.1 ml EDTA (1 mM), 0.1 ml sodium azide (1 mM), 1.49 ml phosphate buffer (0.1 M, pH 7.4), 0.05 ml glutathione reductase (1 IU/ml), 0.05 ml reduced glutathione (1 mM), 0.1 ml NADPH (0.2 mM) and 0.01 ml H_2O_2 (0.25 mM) and 0.1 ml supernatant. The depletion of NADPH at 340 nm was recorded. The enzyme activity was represented as µmol NADPH oxidised/min/mg protein.

The activity of glutathione-S-transferase (GST) was estimated according to Habig et al. (1974) by measuring the change in absorbance due to formation of GST catalysed CDNB-GSH conjugates at 340 nm. The reaction mixture contained 0.1 ml of 30 mM GSH, 0.1 ml of 30 mM CDNB, 2.7 ml of potassium phosphate buffer (pH 6.5) and 0.1 ml of supernatant. The enzyme activity was expressed as µmol conjugate formed/min/mg protein.

The reduced glutathione (GSH) level was estimated spectrophotometrically at 412 nm according to Moron et al. (1979) by monitoring the reaction of GSH and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) resulting in yellow-coloured complex formation. The GSH content was expressed as µmol conjugate formed/g protein.

Malondialdehyde (MDA) level as marker of lipid peroxidation was estimated following the method of Buege and Aust (1978) and the results were expressed as µmol/mg protein. 0.25 ml supernatant was incubated in 0.25 ml each of 150 mM Tris–HCl (pH 7.4), 1.5 mM ascorbic acid and 1.0 mM ferrous sulphate in a final volume of 1.0 ml at 37 °C for 15 min. Then 1.0 ml of 10% TCA and 2.0 ml of 0.375% TBA was added to it, and kept in boiling water bath for 15 min. The contents were then centrifuged at 3,000 rpm for 10 min. The absorbance of the clear supernatant was measured against reference blank at 532 nm.

The protein content quantification was carried out according to Lowry et al. (1951) using BSA as a standard.

Statistical analysis

SPSS 18.0 statistical software was used for the analysis. The data was analysed by Kolmogorov–Smirnov test and was found to be for normally distributed. The data was then subjected to one-way ANOVA followed by Tukey's post hoc test to analyse the statistical differences amongst the groups. P value < 0.05 was considered statistically significant.

Integrated biomarker response analysis

Integrated biomarker response (IBR) analysis was applied to evaluate an integrated response of *L. rohita* towards treatment with $CdCl_2$ and NG by combining the results obtained from the biomarkers' analysis (described in the "Oxidative stress biomarkers" section) into general stress index. IBR was calculated for each experimental group at various

exposure periods according to Beliaeff and Burgeot (2002) and Guerlet et al. (2010).

For IBR calculation, the hierarchy of biological organisation of biomarkers from the subcellular to individual level, i.e. SOD, CAT, GPx, GSH and MDA, was used for their clockwise arrangement (Serafim et al. 2012).

Results

Determination of LD_{50} of NG

Twenty-four-hour LD_{50} of NG on *L. rohita* was found to be more than 5000 mg/kg based on the 'Acute toxic class method' (Fig. 1) performed according to OECD toxicological protocols. Accordingly, NG was considered to be safe up to 5000-mg/kg dose for the fish and ranked at level five (Organization for Economic Cooperation and Development 2001).

Oxidative stress biomarkers

No mortality was observed during the course of chronic toxicity tests. Furthermore, in general, non-significant differences in the activities of antioxidant enzymes and levels of GSH and MDA were observed in the three tissues of fish administered with NG alone in comparison to control. The numerical data of all the biomarkers in the form of mean ± SE is provided in the supplementary material (Table S2-S7).

SOD activity

The SOD activity in the liver, gills and kidney of *L. rohita* upon treatment with $CdCl_2$ and NG is depicted in Fig. 2(A). A concentration-dependent enhancement in SOD activity in the gills, liver and kidney was noticed in $CdCl_2$-exposed groups on the 15th day in comparison to corresponding control. Afterwards, the decreasing trend in SOD activity in all tissues exposed to both the concentrations was observed and on 60th day, SOD activity reached below control. However on the 30th day, fish exposed to lower concentration of $CdCl_2$ revealed increase in activity of hepatic SOD. The results also showed the maximum inhibition of the SOD activity (45.79%) in the gills of fish exposed to $CdCl_2$ (at higher concentration) on the 60th day of exposure (Table S2). Co-administration of NG to fish exposed to $CdCl_2$ (at both concentrations) revealed significant ($p < 0.05$) reduction in the activity of SOD in the three tissues at the 15th day as compared with $CdCl_2$ alone treated groups. Further on the 30th and 60th day, treatment with NG and $CdCl_2$ (at both concentrations) provided a marked normalisation ($p < 0.05$) of SOD activity in the three tissues. However, gills of fish co-treated with NG and higher concentration of the $CdCl_2$ on the 60th day showed significant ($p < 0.05$) lesser enzyme activity in comparison to the control. Thus, indicating that NG supplementation in fish intoxicated with higher concentration of $CdCl_2$ could not completely normalise the SOD activity in the gills on the 60th day.

CAT activity

The effects of $CdCl_2$ and NG treatment alone and in combination, on CAT activity in the gills, liver and kidney of *L. rohita*, are displayed in Fig. 2(B). Exposure to low concentration of $CdCl_2$ resulted in increased CAT activity in the gills and kidney on the 15th and 30th day followed by a sharp decline on the 60th day. However, the liver showed increase in CAT activity throughout the experiment. Administration of NG along with $CdCl_2$ exposure restored the CAT activity in the three tissues.

Upon exposure to higher concentration of $CdCl_2$, CAT activity revealed significant ($p < 0.05$) elevation in the gills (103.61%), liver (88.64%) and kidney (71.91%) on the 15th day; afterwards, a decreasing trend in its activity was observed with maximum decrease of 40.06% in the gills on the 60th day (Table S3). On the contrary, NG treatment along with $CdCl_2$ restored the altered levels of CAT activity in the three tissues but these significantly differ from the control levels.

GST activity

The GST activity in the gills, liver and kidney of the fish treated with $CdCl_2$ and NG is shown in Fig. 3(A). Non-significant alteration in GST activity was noted in both the gills and kidney throughout the experiment. On the other hand, GST activity in the liver was significantly altered by both the concentrations of $CdCl_2$. Exposure to lower concentration of $CdCl_2$ showed significant ($p < 0.05$) time-dependent increase in GST activity by 29.93% (15th day) to 44.37% (60th day) in comparison to control. Co-treatment of NG and $CdCl_2$ (low concentration) significantly ($p < 0.05$) reduced the GST activity at each sampling day (Table S4). Exposure to $CdCl_2$ at higher concentration significantly ($p < 0.05$) elevated hepatic GST activity by 48.24% and 72.27% on 15th and 30th day, respectively. Subsequently, marked inhibition of 30.98% in hepatic GST activity over control was seen on 60th day of exposure. However, the combined treatment with NG and $CdCl_2$ (higher concentration) resulted in significant reduction in GST activity on the 15th and 30th day, as compared to the $CdCl_2$-treated group thereby normalising the enzyme activity near the control, while on the 60th day NG supplementation increased the $CdCl_2$ (0.62 mg/L)-induced fall in hepatic GST activity.

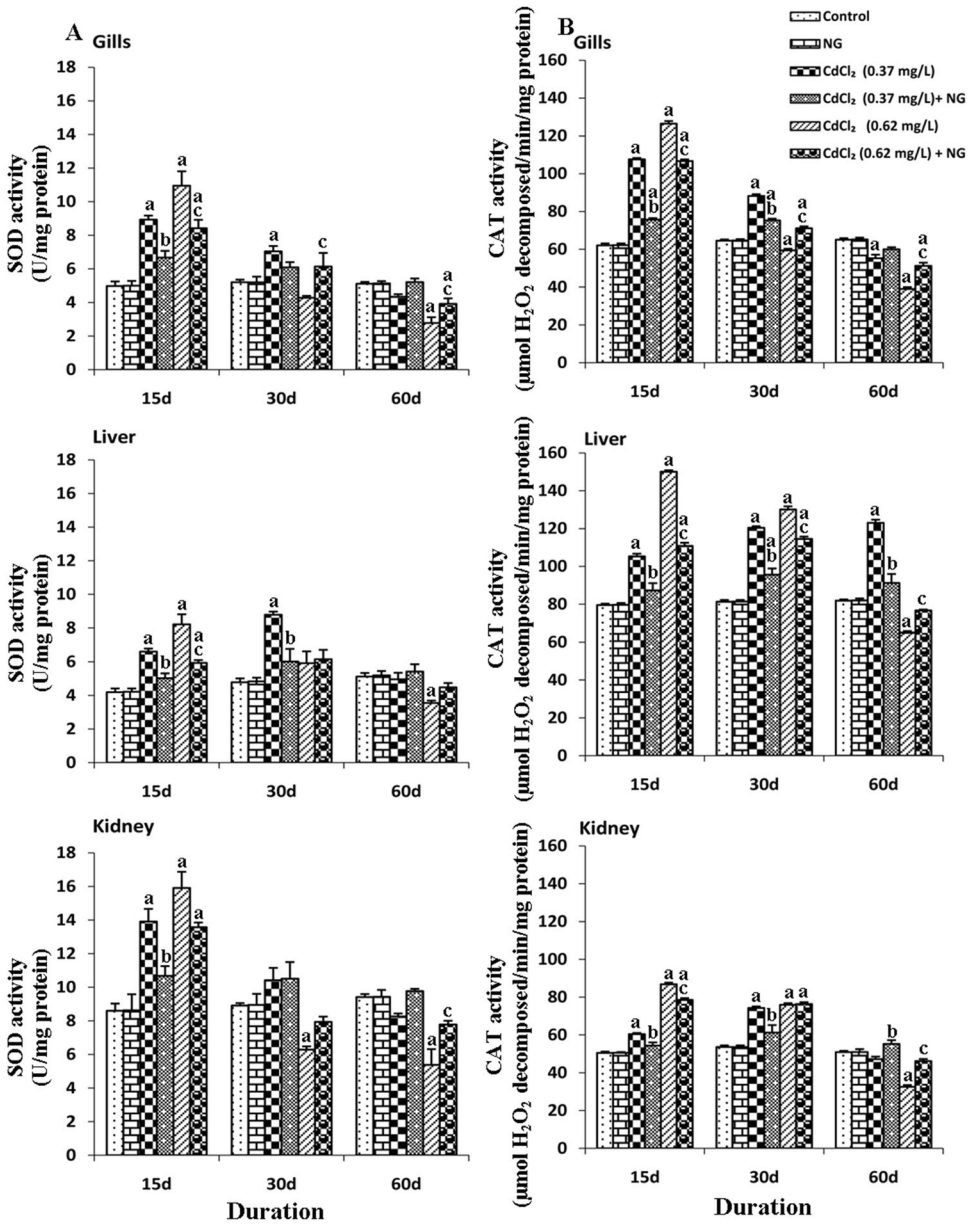

Fig. 2 Activity of SOD (A) and CAT (B) in the gills, liver and kidney of *L. rohita* exposed to $CdCl_2$ and orally administered with NG at different sampling days. Data are presented as mean ± SE on six individual estimations. Statistical significance is considered at $p < 0.05$ determined by one-way ANOVA followed by Tukey's post hoc test. 'a': significant difference with respect to control; 'b': significant difference $CdCl_2$ (0.37 mg/L) + NG with respect to $CdCl_2$ (0.37 mg/L); 'c': significant difference $CdCl_2$ (0.62 mg/L) + NG with respect to $CdCl_2$ (0.62 mg/L)

GPx activity

Figure 3(B) depicts the effects of $CdCl_2$ and NG treatment alone and in combination on GPx activity in the gills, liver and kidney of *L. rohita*. At lower concentration of $CdCl_2$, elevation in GPx activity over control in all three tissues was observed with maximum increase of 35% in the gills followed by 31.97% in the kidney and 20.93% in the liver on the 60th day (Fig. 3(B)). However, the combined treatment of NG with $CdCl_2$ (lower concentration) significantly ($p < 0.05$) decreased the GPx activity to near control levels. Conversely, groups exposed to higher concentration of $CdCl_2$ revealed enhancement in GPx activity by 43.71%, 43.98% and 39.98% in the gills, liver and kidney, on the 15th day, respectively (Table S5). Afterwards, rapid depletion in GPx activity was observed in the gills and kidney whereas the liver showed increased GPx activity on the 30th day over control. On the other hand, combined treatment of NG and $CdCl_2$ (higher concentration) provided significant ($p < 0.05$) restoration of altered GPx activity in the three tissues.

GSH level

The effects of $CdCl_2$ and NG treatment on GSH levels of the gills, liver and kidney of *L. rohita* are displayed in Fig. 4(A). Exposure to $CdCl_2$ revealed significant ($p < 0.05$) concentration-dependent depletion in GSH levels in the three tissues with respect to control. Gills showed some stability in GSH

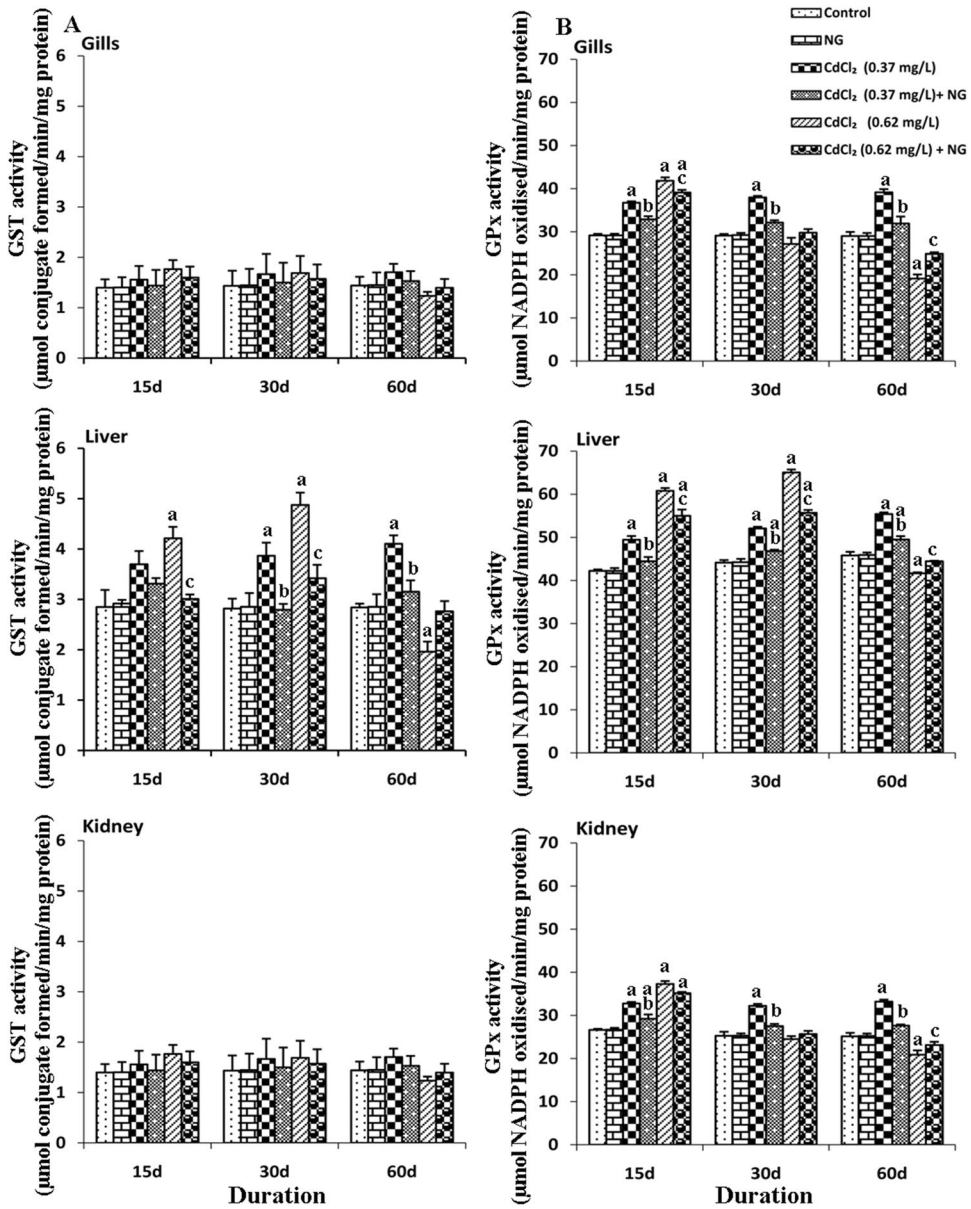

Fig. 3 Activity of GST (A) and GPx (B) in the gills, liver and kidney of *L. rohita* exposed to $CdCl_2$ and orally administered with NG at different sampling days. Data are presented as mean ± SE on six individual estimations. Statistical significance is considered at $p < 0.05$ determined by one-way ANOVA followed by Tukey's post hoc test. 'a': significant difference with respect to control. 'b': significant difference $CdCl_2$ (0.37 mg/L) + NG with respect to $CdCl_2$ (0.37 mg/L). 'c': significant difference $CdCl_2$ (0.62 mg/L) + NG with respect to $CdCl_2$ (0.62 mg/L)

content at lower concentration of $CdCl_2$ which was significantly restored to control levels by NG administration. At higher $CdCl_2$ concentration, a sharp decline in GSH content was observed overtime. The maximum decrease of 48.50% was recorded on the 60th day in the gills of fish exposed to higher concentration of $CdCl_2$ over corresponding control (Fig. 4(A)). On the contrary, co-treatment of $CdCl_2$ and NG showed increase in GSH content but the levels were not completely restored to near control groups. A much slower non-significant decrease in GSH content was observed in the liver at lower Cd concentration, whereas at higher concentration it decreased initially followed by an increase on the 30th day and then again got depleted (by 10.37%) on the 60th day (Table S6). NG administration completely rescued GSH content in the liver. The kidney showed significant depletion of GSH content at both concentrations in comparison to the respective control groups which was softened by NG.

MDA levels

Figure 4(B) shows the exposure to $CdCl_2$ resulted in concentration and duration-dependent augmentation of MDA levels in three tissues of fish. The maximum MDA level (82.47%) was recorded in the gills followed by kidney (71.87%) and liver (68%) of fish exposed to higher concentration of $CdCl_2$ on the 60th day. Co-treatment of NG and $CdCl_2$ significantly ($p < 0.05$) depleted MDA levels in the three tissues (Table S7). However, in the kidney, although co-treatment of NG with higher $CdCl_2$ concentration showed reduction

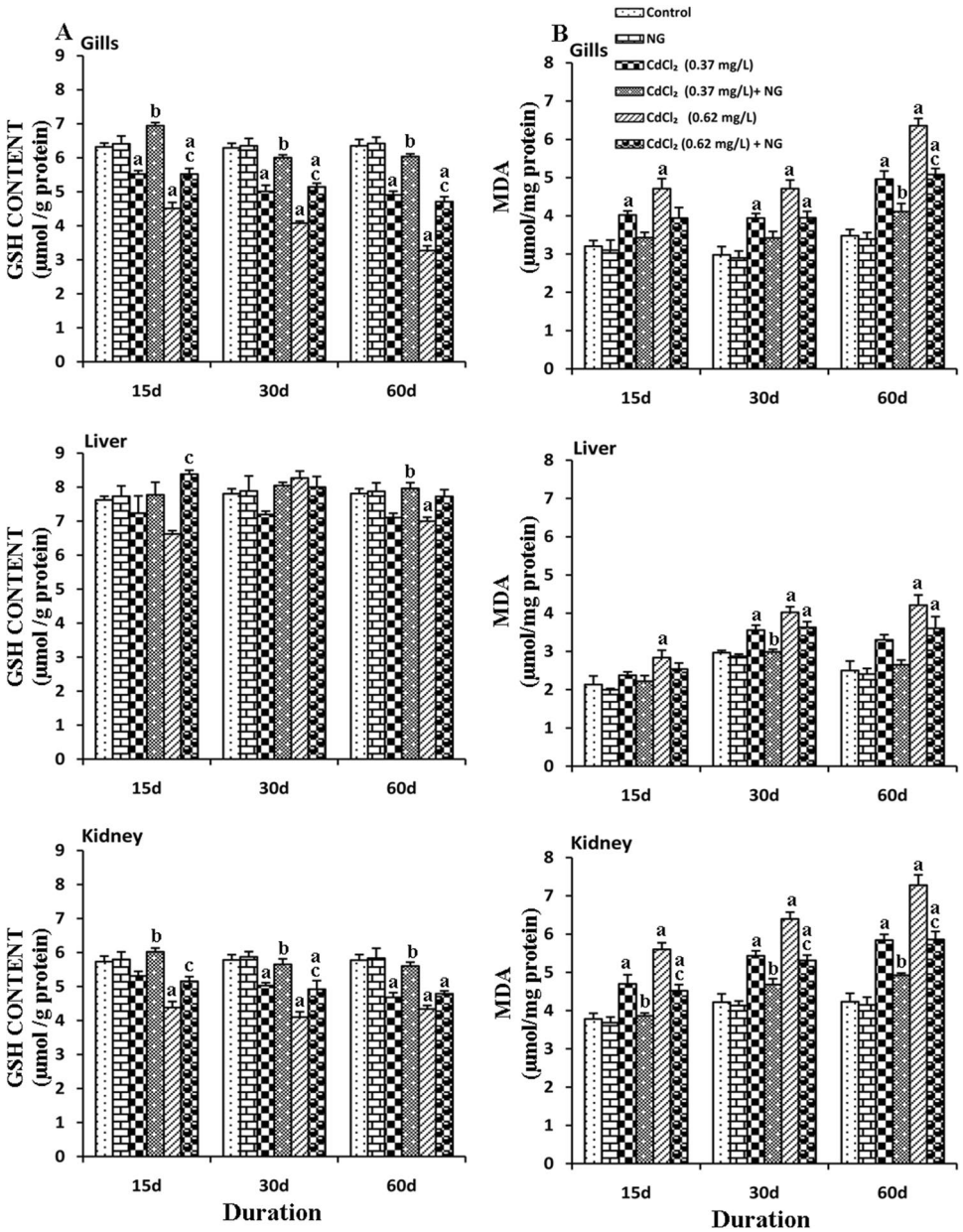

Fig. 4 GSH (A) and MDA (B) content in the gills, liver and kidney of *L. rohita* exposed to $CdCl_2$ and orally administered with NG at different sampling days. Data are presented as mean ± SE on six individual estimations. Statistical significance is considered at $p < 0.05$ determined by one-way ANOVA followed by Tukey's post hoc test. 'a': significant difference with respect to control. 'b': significant difference $CdCl_2$ (0.37 mg/L) + NG with respect to $CdCl_2$ (0.37 mg/L). 'c': significant difference $CdCl_2$ (0.62 mg/L) + NG with respect to $CdCl_2$ (0.62 mg/L)

in MDA levels, these could not be restored near the control groups.

Integrated biomarker response

The IBR index was applied to the six investigated biomarkers in the gills, liver and kidney of *L. rohita* and the findings are displayed as star plots in Fig. 5. The IBR value shows that higher concentration of $CdCl_2$ caused severe toxicity in *L. rohita* accompanied with higher values of IBR on the 60th day in all tissues. A concentration-dependent elevation in the IBR values was registered in the three tissues of $CdCl_2$-exposed fish on the 15th day, which further decreased on the 30th day of the exposure except in the liver. However, on the 60th day of exposure to $CdCl_2$ (both concentrations), a marked increase in IBR values in the gills (Fig. 5A) and kidney (Fig. 5C) was noted while the liver showed slight decrease in IBR values. Meanwhile, a remarkable decrease in IBR values was detected in the three tissues of fish treated with NG along with $CdCl_2$ exposure. Based on the IBR values (Fig. 5D), it is apparent that the most impacted organ due to Cd toxicity is the gills with a maximum IBR value of 37.62, on the 60th day of exposure to $CdCl_2$ (at higher concentration). On this basis, the organs can be ranked in terms of impact by Cd toxicity as gills > kidney > liver.

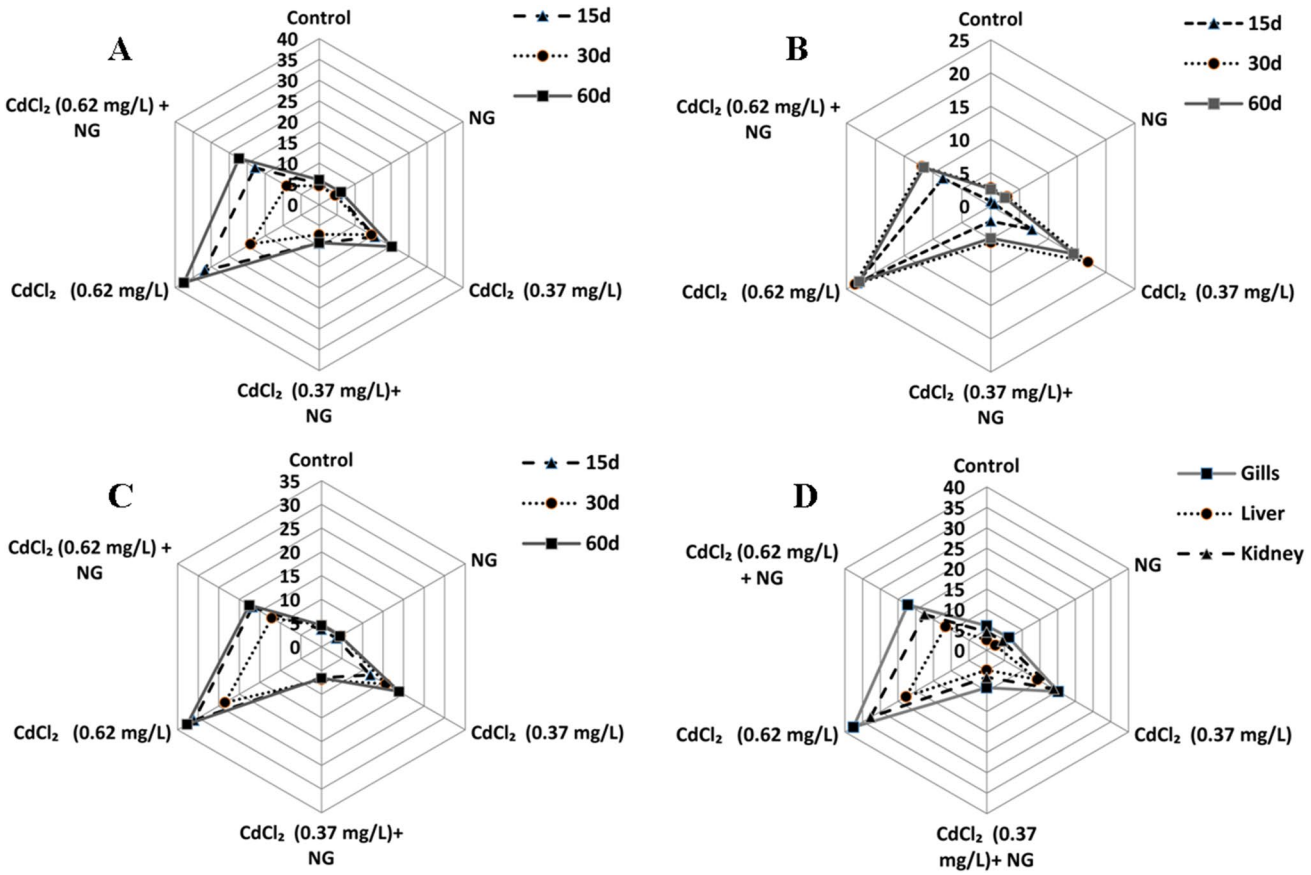

Fig. 5 Star plots for integrated biomarkers response (IBR) in **A** gills, **B** liver and **C** kidney of *L. rohita* exposed to $CdCl_2$ and orally administered with NG; **D** IBR index showing comparison of stress response in the gills, liver and kidney of treatment groups on the 60th day

Discussion

In this study, protective efficacy of NG on $CdCl_2$-mediated oxidative stress has been assessed in different tissues of *L. rohita*. Several researchers have documented the beneficial effects of flavonoids against the toxicity induced by various heavy metals in experimental animals. However, there is paucity of available scientific literature related to the effects of NG against the deleterious effects instigated due to Cd intoxication in fish. The present study is the first attempt to analyse the potential of NG to alleviate the toxicity caused by Cd in *L. rohita* through integrated response of oxidative stress biomarkers. These biomarkers such as GST, GPx, SOD and CAT form an integral components of antioxidant defence system of an organism to provide protection against oxidative stress induced by xenobiotics and are often employed as indicators of Cd toxicity (Asagba et al. 2008; Ural 2013; McRae et al. 2018). SOD, a group of metalloenzymes, catalyses scavenging of superoxide anion radicals and converts them into molecular oxygen and H_2O_2 (Liu et al. 2020) while CAT metabolises H_2O_2 into oxygen and water (Pandey et al. 2008). GPx is a selenoenzyme that scavenges the H_2O_2 by catalysing the oxidation of GSH to GSSG (oxidised glutathione), while GST belongs to a family of detoxifying enzymes involved in cellular detoxification. GST catalyses conjugation of GSH with electrophilic compounds (both endogenous and exogenous), thereby facilitating their removal from the organism (Dringen 2000).

In our study, all the three tissues showed significant concentration-dependent increase in SOD and CAT activity up to 15th day of $CdCl_2$ exposure. These findings agree with the previous observations of Asagba et al. (2008), Abdel-Rahim et al. (2014), McRae et al. (2019) and Naik et al. (2020). The enhanced SOD and CAT activity might be an adaptive mechanism of Cd-exposed animals in order to reduce oxygen radicals. Furthermore, increased exposure to $CdCl_2$, i.e. up to 60 days, caused inhibition in the both SOD and CAT activities of three tissues. Our results are supported by Basha and Rani (2003) who reported initial increase and then later inhibition of SOD and CAT activity in *Oreochromis mossambicus* treated with sublethal concentration of $CdCl_2$. This reduction in SOD and CAT activity might be attributed to overproduction of ROS by long-term Cd exposure (El-Boshy et al. 2015). SOD exists in the form of CuZnSOD (mainly

present in cytosol, nucleus and peroxisomes) and MnSOD (present in mitochondria) (Arroyo et al. 2012). Alternately, Cd causes structural instability in the enzyme by substituting manganese in MnSOD (Casalino et al. 2002) and zinc in CuZnSOD either by direct interaction between Cd and Zn via competitive inhibition or by alternative biological pathway (Huang et al. 2006). Cd-induced inhibition in CAT activity might also be attributed to peroxidative damage in the tissues, as indicated by increased lipid peroxidation observed in our study. Alternately, the metal ions may directly bind to thiol groups of CAT and thus inhibit its activity (Atli and Canli 2010).

Significant decline in SOD and CAT activities of the investigated tissues of fish co-treated with $CdCl_2$ and NG on the 15th day may be ascribed to the potential of NG to scavenge free radical (van Acker et al. 2000). However on the 60th day, fish exposed to $CdCl_2$ along with NG administration showed appreciable enhancement in the activities of SOD and CAT in relation to the metal-exposed groups. This restoration of enzyme activities might be attributed to the potential of NG to limit the accretion of free radicals generated by Cd exposure (Gnanasoundari and Pari 2006; Wang et al. 2012). Our results are supported by the findings of other authors who also observed alleviation of hepatic SOD and CAT activities by NG in Cd (Renugadevi and Prabu 2010)- and arsenic (Mershiba et al. 2013)-intoxicated animals.

GPx also increased in the three tissues of Cd-intoxicated fish (15th day) which might be to compensate the higher level of H_2O_2. Similar increase in GPx activity has also been reported in *Cyprinus carpio* treated with heavy metals, thereby suggesting the enhanced GPx activity as an indicative of its role in preventing the metal-induced lipid peroxidation and oxidative damage (Vinodhini and Narayanan 2009). Enhanced activity of hepatic GST was observed in $CdCl_2$-exposed fish due to the pivotal involvement of liver in detoxification of xenobiotics. Our results substantiate the suitability of liver as indicator in determining the response of GST to metal intoxication in studies related to environmental monitoring. Raised levels of GST may reveal that the fish attempts to reduce metal-induced stress by conjugating GSH to metals, hence decreasing their concentration (Vieira et al. 2009). Conversely, decline in GPx and GST activity in the tissues upon prolonged exposure (60th day) to $CdCl_2$ may be due to the inefficiency of tissues in neutralising the impact of peroxides, and also due to the glutathione depletion upon prolonged exposure as noticed in our study. Similar reduction in GPx activity has also been observed by Ognjanovic et al. (2008), Messaoudi et al. (2009) and Jamakala and Rani (2015) in the kidneys and liver of Cd-intoxicated animals. Treatment of NG in $CdCl_2$-exposed fish significantly restored altered levels of GPx and GST, suggesting the antioxidative potential of NG. This may be explained by the potential of NG to scavenge free radicals and hence alleviates the excess consumption of endogenous non-enzymatic antioxidants by the metal. These findings are also corroborated by Gnanasoundari and Pari (2006) and Ozkaya et al. (2016).

In the present study, significant depletion in GSH levels was recorded throughout the experimental period except elevated hepatic GSH on the 30th day. A possible explanation for the reduction is high consumption of reduced GSH by GST in detoxifying mechanisms or by GPx in reducing lipid hydroperoxides (Banerjee et al. 1999). The binding of Cd tightly to thiol groups may also result in GSH depletion (Pari and Murugavel 2005; Liu et al. 2009). These results agree with Newairy et al. (2007) and Goodarzi et al. (2020) who also found reduction in glutathione level in experimental animals intoxicated with Cd (Newairy et al. 2007; Goodarzi et al. 2020). Oral administration of NG in Cd-exposed fish restored the levels of GSH in the three tissues, which might be due to the role of NG in scavenging the free radicals and thus preventing thiol group oxidation. Moreover, the metal-chelating potential of NG augments Cd removal, inhibits iron-dependent Fenton reaction and thus reduces the consumption of non-enzymatic antioxidants (Cheng and Breen 2000).

It is interesting to note that hepatic GSH level significantly elevated on the 30th day of exposure to higher concentration of $CdCl_2$. Additionally, increased activity of GST and GPx was also found on the same day. These variations showed development of an adaptive mechanism in response to the metal intoxication, by strengthening antioxidative ability of liver with increased rate of GSH synthesis. Consequently, on the 60th day of exposure to $CdCl_2$, depletion in both GSH and GST was observed that indicate progressive weakening and loss of ability of tissue to respond with continuing Cd exposure (Zirong and Shijun 2007).

Lipid peroxidation is an important mechanism of cell injury which involve broad spectrum of alterations in cell triggered by free radicals and intermediate derivatives of peroxidation (Prabu et al. 2011b). MDA, a major product of peroxidation, can actively react with a variety of cellular components, and hence, impair the structural and physiological integrity of membranes (Valavanidis et al. 2006). Enhanced levels of MDA in the liver, gills and kidney of $CdCl_2$-treated fish are in line with the previous findings (Shimada et al. 2004; Dabas et al. 2012; El-Boshy et al. 2015; Verma et al. 2020). Cd imparts inhibitory effects on mitochondrial electron transfer by biding to thiols and thus generates ROS which causes peroxidative damage in the tissue (Dorta et al. 2003; Heyno et al. 2008). Reduction in antioxidant enzymes might be the major cause of enhanced lipid peroxidation as observed in the current study (El-Boshy et al. 2015). Cd intoxication has also been suggested to induce phagocytes for ROS production (Stohs

and Bagachi 1995). Alternately, Cd has been believed to displace iron from its binding sites resulting in increased generation of ROS via Fenton reaction, ultimately leading to extensive lipid peroxidation (Casalino et al. 1997). As a result, free radicals and lipid peroxidation cause extensive cellular changes by imparting oxidative damage to intracellular molecules (DNA, proteins and lipids) and cross-linking and polymerisation of membrane components. This leads to destabilisation and disintegration of cell membrane structure and function thereby inducing many pathological and physiological changes in the tissues (Gibson, 2005; El-Boshy et al. 2015). Amongst the investigated tissues, gills being in immediate contact with Cd in ambient environment and possess poor ability to scavenge ROS, and therefore showed maximum MDA level upon Cd exposure (Dabas et al. 2012). Our study showed simultaneous administration of NG in fish exposed to $CdCl_2$ significantly blunted the increased MDA levels in the tissues, which might be attributed to antilipoperoxidative ability of NG (Lee et al. 2004). Similar restoration of MDA level upon administration of NG was also documented in arsenic (Jain et al. 2011) and lead acetate (Ozkaya et al. 2016)–intoxicated animals. Another possible reason for reduction in MDA level by NG may be its lipophilic nature which enables the attachment of NG to phospholipid bilayer, thereby reducing generation of free radicals and stabilising plasma membrane (Honohan et al. 1976).

The IBR analysis which eases in visualising the biological effects of various biomarkers substantiates the above results indicating the induction of an early detoxification mechanism in *L. rohita* in response to oxidative stress generated by $CdCl_2$ exposure. IBR analysis clearly differentiated the damage with respect to oxidative stress provoked due to long-term Cd exposure to the three tissues viz. gills, liver and kidney. Amongst them, the liver was observed to be the least affected organ by Cd-mediated toxicity which might be due to the tendency of the liver to synthesise metallothioneins (protein that binds to non-essential metals for sequestration) rapidly thereby eliminating Cd efficiently (Capaldo et al. 2016). Contrarily, gills and kidney were more influenced by Cd exposure, which may be due to the fact that gills are the sensitive respiratory organ and the first point of contact with waterborne Cd, while kidneys are inefficiently equipped to metabolise Cd and therefore being the final destination for Cd accumulation (Asagba et al. 2008). The study showed reduction of enhanced IBR values in groups co-treated with NG and $CdCl_2$ as compared to $CdCl_2$ alone. In the light of these findings, IBR analysis further substantiated the protective role of NG. It has been apparent from star plot of IBR that NG is effective against lower concentration of $CdCl_2$ than higher concentration of $CdCl_2$, indicating that NG can be useful to maintain the redox homeostasis under low to moderate oxidative stress but it fails to do so if tissues are under tremendous oxidative stress as in case of higher concentration of $CdCl_2$. These findings reinforce that integrated biomarker response is a valuable tool for visualising general stress response of fish exposed to Cd than individual stress biomarkers (Qu et al. 2014) and surveying the toxicological effects of $CdCl_2$ on fish.

The current study revealed that the short-term exposure (15 days) to $CdCl_2$ at relatively low concentrations resulted in escalation of SOD, CAT and GPx activities, thus reflecting the development of compensatory response against Cd-mediated oxidative stress. Further increase in exposure to $CdCl_2$ (up to 30th day) showed decrease in their activity which may reflect the acclimatisation of the fish to the pollutant in its environment. Conversely, with further increase in exposure period (60 days), the results showed a decline in antioxidant enzyme activities below the control level, revealing the inhibition of these enzymes by $CdCl_2$ or inability of the organism to produce these enzymes which may be due to tissue damage. This is validated with increased lipid peroxidation in all three tissues and also supported by findings of IBR analysis.

Conclusions

Exposure to $CdCl_2$ in the present study showed biphasic profile of antioxidant defence system in *L. rohita*, i.e. induction in SOD, CAT, GPx and GST activities in the first phase (short-term exposure) and then progressive loss due to the oxidative stress in the second phase (long-term exposure), which was further supported by integrated biomarker response analysis. Consequently, oral administration of NG along with $CdCl_2$ exposure appreciably alleviated Cd-induced alterations in enzymatic and non-enzymatic antioxidants levels, thereby restoring the oxidative stress biomarker levels to near normal. Gills were found to be the most affected organ by Cd-induced toxicity, as indicated by IBR analysis. All these findings together confirmed the hypothetical framework, i.e. NG possesses the ability to alleviate Cd-mediated oxidative stress and facilitates restoration of oxidant-antioxidant status in *L. rohita*. The data concerning the long-term toxic effects of $CdCl_2$ to *L. rohita*, thus generated, could be useful for the environmental risk assessment of this metal. Further, the key findings related to the protective efficacy of NG could add to the development of NG as therapeutic option against Cd-induced toxicity. However, the study shows that at higher concentration of $CdCl_2$, NG could not completely restore the parameters to the levels of the control group overtime, thus indicating its inefficiency to maintain redox homeostasis under tremendous oxidative stress. Therefore, further studies are required with different concentrations of the metal and NG

doses before using NG as dietary therapeutic molecule for fish and human consumption.

Credit author contribution statement

Sakshi Verma: conceptualization; investigation; methodology; formal analysis; writing—original draft preparation; visualisation. Rajinder Jindal: supervision, resources, methodology, conceptualization, project administration. Smriti Batoye: methodology, investigation.

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Data availability All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval The experiments were performed according to the guidelines of Institutional Animal Ethics Committee, Panjab University, Chandigarh (PU/ IAEC/527).

Consent to participate Not applicable.

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Competing interests The authors declare no competing interests.

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