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PII: S1382-6689(20)30070-3

DOI: https://doi.org/10.1016/j.etap.2020.103394

Reference: ENVTOX 103394

To appear in: *Environmental Toxicology and Pharmacology*

Received Date: 28 October 2019

Revised Date: 7 April 2020

Accepted Date: 9 April 2020

Please cite this article as: Bio S, Nunes B, Acute effects of diclofenac on zebrafish: indications of oxidative effects and damages at environmentally realistic levels of exposure, *Environmental Toxicology and Pharmacology* (2020), doi: https://doi.org/10.1016/j.etap.2020.103394

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Acute effects of diclofenac on zebrafish: indications of oxidative effects and damages at environmentally realistic levels of exposure

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Highlights

- Diclofenac induced oxidative stress in larvae of *Danio rerio*

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- The acute exposure to diclofenac resulted in peroxidative damage
- Organisms might be subjected to oxidative stress in their natural habitat
- Some of these alterations occurred for low, realistic levels of diclofenac

Abstract

With the increasing awareness about the contamination of the aquatic environment by pharmaceuticals, there is a growing need to study their adverse effects on aquatic organisms. Diclofenac is a non-steroidal anti-inflammatory drug (NSAID), whose wide use contributes for its presence in freshwater ecosystems increasing the probability of causing deleterious changes in aquatic biota. This study evaluated possible oxidative stress effects in *Danio rerio* embryos and larvae when exposed to a range of ecologically relevant concentrations of diclofenac. It was possible to conclude that diclofenac caused a scenario of oxidative stress, since all tested toxicological parameters were responsive to the drug. In general diclofenac caused not only significant anti-oxidant adaptive responses for most levels of exposure, but also peroxidative damage. This work evidenced the responsiveness of *D. rerio* towards diclofenac in environmentally relevant concentrations, which shows that these organisms might face a scenario of oxidative stress in their natural habitat.

Keywords

Danio rerio, pharmaceuticals, catalase, glutathione-S-transferases, glutathione peroxidase, lipid peroxidation

1. Introduction

Pharmaceutical contamination of the aquatic ecosystems is considered an emerging problem, due to the ever increasing human population with access to healthcare services, and intense animal feeding practices (Kim et al., 2007; Brandão et al., 2013; Correia et al., 2016). From these scenarios, pharmaceutical drugs are increasingly used, a trend which is not encompassed by investment and development/implementation of wastewater techniques and methodologies (Ferrari et al., 2003; Correia et al., 2016). Consequently, there is the need to increase the monitoring of the presence and putative adverse effects of pharmaceuticals, as they are being systematically found in waste

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water treatment plants (WWTPs) effluents and in surface waters, in concentrations which range from ng/L to µg/L (Quesada et al., 2019). Among other characteristics, pharmaceuticals are necessarily bioactive, and designed to have slow metabolism, being also moderately lipophilic, a feature that allows drugs to cross cellular membranes and favouring their absorption (Quesada et al., 2019; Oliveira et al., 2015b). Most drugs are not completely removed in WWTPs (Iglesias et al., 2014), so they become persistent in the aquatic environment after being released by WWTPs effluents (Bila and Dezotti 2003). Furthermore, there are not enough studies about the effects of the drugs in non-target organisms in the aquatic environment (Quesada et al., 2019), especially those that are used in large amounts.

Among drugs that are frequently found in the aquatic ecosystems, one may find antibiotics, beta-adrenergic antagonists and non-steroidal anti-inflammatory drugs (NSAIDs) (Gómez-Oliván et al., 2014) as the most prominent classes. Diclofenac belongs to the pharmacotherapeutical group of the non-steroidal anti-inflammatory drugs (NSAIDs), which also includes acetylsalicylic acid, paracetamol, ibuprofen and naproxen, and act as an inhibitor of the cyclooxigenase responsible for the prostanoids biosynthesis (Islas-Flores et al., 2013; Chen et al., 2014; Gómez-Oliván et al., 2014). Diclofenac acts by inhibiting reversibly or irreversibly, one or two forms of the cyclooxygenase enzymes, COX-1 and COX-2, which are involved in the synthesis of different prostaglandins from arachidonic acid (Oliveira et al., 2015a; Vane and Botting, 1998). It is one of the NSAIDs most commonly used, being prescribed in cases of inflammation, arthritis, gout and rheumatic pain (Chen et al., 2014; Oliveira et al., 2015). Considering this wide use, its environmental concentrations range from 2.2-5.1 µg/L in WWTPs effluents in Germany (Stülten et al., 2008). In Mexico City-Mezquital Valley concentrations up to 0.55 µg/L were reported in untreated wastewater in the canals of the Valle del Mezquital Dendhó channel (Siemens et al., 2008). Iglesias et al. (2014) detected diclofenac in Spanish surface waters in concentrations up to 46 ng/L. In Karachi, Pakistan, diclofenac was found in rivers in concentrations of 4.4 µg/L (Scheurell et al., 2009). In Brazil, Américo-Pinheiro et al. (2017) studied the diclofenac occurrence in an urban stream in Três Lagoas and obtained concentrations up to 8.25 µg/L.

Most ecotoxicological effects of realistic levels of diclofenac are unknown at present. However, the metabolism of diclofenac seems to result in oxidative stress

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modifications, including a significant increase in levels of lipid peroxidation and glutathione peroxidase as shown by Gómez-Oliván et al. (2014) when exposing individuals of *Daphnia magna* to a different range of diclofenac concentrations. Guiloski et al. (2017) exposed the fish *Rhamdia quelen* to different concentrations of diclofenac and also obtained differences in the levels of catalase, superoxide dismutase, glutathione S-transferase and lipid peroxidation.

To address this issue, it is important to select appropriate test organisms that represent species from putatively impacted freshwater systems. The organism test used in this assay was *Danio rerio*, also known as zebrafish. This is a freshwater tropical fish species, which is native from the Indian and South Asian rivers (Chen et al., 2014; Hanisch et al., 2010). It is commonly used in (eco)toxicology because it is easy to maintain in the laboratory, has a rapid development and growth, is small sized and has high fecundity (Chen et al., 2014; Hanisch et al., 2010; Hill et al., 2005).

The study of chemical responses in organisms, namely, biomarkers of oxidative stress (which evaluate the imbalance between pro-oxidant effects and antioxidant intracellular defence mechanisms), provide early-warning signals about the toxic effects caused by xenobiotics (Nunes et al., 2017). Therefore, the aim of this study was to evaluate whether diclofenac induces oxidative stress and lipid peroxidation to embryos and larvae of *D. rerio* when acutely exposed to a range of relevant environmental concentrations of this drug.

2. Methods and Materials

2.1. Chemicals

Diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl) acetic acid) was purchased from Sigma Aldrich.

2.2. Test organisms

Danio rerio embryos used in this study were obtained from the natural mating of adult individuals of *D. rerio* which were maintained in a specialized facility in the Biology Department of the University of Aveiro. Animals were kept in aquaria with carbon-filtered dechlorinated water, at 26 ± 1ºC and with a photoperiod of 16:8h light/dark.

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Conductivity was maintained at 750 ± 50 µs, pH at 7.5 ± 0.5 and dissolved oxygen at 95% saturation. This test was performed in compliance with the Organisation for Economic Co-operation and Development (OECD) guideline on Fish Embryo Toxicity Test (OECD, 2013). Male and female individuals of *D. rerio* were kept in aquaria with marbles in the bottom in order to prevent them from eating their eggs, 24 hours before the beginning of the assay. The zebrafish eggs were then collected 30 minutes after natural mating, rinsed in water and checked under a stereomicroscope. Unfertilized eggs or with any malformations were discarded. 500 eggs with no more than 5 hours postfertilization were used in this test.

2.3. Test solutions and exposure

D. rerio eggs were put in petri dishes with 14 mL of the testing solution. Each petri dish contained 20 eggs. Four different concentrations of diclofenac were tested, each with five replicas. The chosen concentrations were 0.5 µg/L, 5 µg/L, 50 µg/L, 500 µg/L and the control treatment (without diclofenac). These concentrations were all sub-lethal (below the established LC_{50} for the *D. rerio* embryonic stage of 6.11 ±2.48 mg/L; Praskova et al., 2011)) and below 0.54 µg/L which, according to Hallare et al. (2004), is the predicted environmental concentration (PEC) in Central European surface waters for this drug. All the test solutions were prepared from a stock solution of diclofenac (4 g/L) which was diluted in water from the zebrafish facility system. An acute exposure during 96 hours was selected to test the effects of diclofenac along two developmental stages of *D. rerio* (from the embryo to the larvae; Parichy et al., 2009). Embryos/larvae were daily monitored and dead individuals were immediately discarded. The exposure conditions were similar to those mentioned above.

2.4. Tissue processing

After 96 hours of exposure, the larvae were isolated in Eppendorf microtubes, in groups of 20 larvae, with as less medium as possible, and were sacrificed by being subjected to low temperatures (-20ºC) during 24 hours. After the sacrifice, 1 mL of phosphate buffer 50 mM, pH 7.0, with Triton X-100 0.1% was added to each replica of 20 larvae and then stored at -80ºC until the determination of oxidative stress biomarkers (catalase (CAT), glutathione-S-transferases (GST) and glutathione peroxidase (GPx), and lipid peroxidation (thiobarbituric acid reactive substances, (TBARS)). Before the determination of biomarkers, all samples were homogenized with a Branson sonifier,

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model 250 and centrifuged for 10 minutes at 15 000 g, at 4ºC, with a refrigerated Thermo Scientific Heraeus Megafuge 8R centrifuge.

2.5. Enzymatic biomarkers

Catalase activity determination was based on the degradation rate of the substrate H_2O_2, monitored at 240 nm (Aebi, 1984). Results were expressed knowing that one enzymatic activity unit is equal to the mole number of H_2O_2 degraded per minute, per milligram of protein.

Both forms of GPx were determined (selenium-dependent and total GPx). The GPx activity quantification was based on the principle that glutathione peroxidase participates in the oxidation of reduced glutathione (GSH) and hydrogen peroxide (H_2O_2) to oxidized glutathione (GSSG) and H_2O. Simultaneously, GPx mediates the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH), which can be monitored at 340 nm (Flohé and Gunzler, 1984). For the determination of the activity of both forms, two substrates were used: hydrogen peroxide (determination of selenium-dependent GPx) and cumene hydroperoxide (determination of total GPx). Results were expressed in nanomoles of oxidized NADPH per minute, per milligram of protein.

The enzyme GSTs catalyses the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, forming a thioether. This can be observed by an increase in absorbance at 340 nm (Habig et al., 1974). Activity was expressed in nanomoles of thioether produced per minute, per milligram of protein.

The TBARS quantification measures the extent of lipid peroxidation (LPO), based on the reaction between the LPO subproducts and the 2-thiobarbituric acid (Buege and Aust, 1978). Activity was expressed in nanomoles per milligram of protein.

Protein concentration of each sample was determined based on the Bradford's method (1976), using ɣ-globulin as a standard to express the enzymatic as a function of the protein. It was determined spectrophotometrically at 595 nm.

2.6. Statistical analyses

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The results obtained from the biochemical determinations were statistically analysed using a unifactorial ANOVA, followed by a Dunnett's test. Before performing the ANOVA test, the basic assumptions of the analysis of variance were demonstrated. The equality of variances was proved by the Brown-Forsythe test, and the normal distribution of values was proved by the Shapiro-Wilk test. The significance value chosen to all the statistical analysis was 0.05.

3. Results and Discussion

In general, the here-obtained data support the occurrence of oxidative alterations that were closely followed by an adaptive response to counteract this effect. The metabolism of diclofenac has been studied in a wide variety of biological systems (Grillo et al., 2009), including in fish species (Kallio et al., 2010). These studies demonstrated that diclofenac is extensively metabolized in all studied species, from humans to rodents, and also fish (Sarda et al., 2011). Diclofenac is metabolized in mammals by being converted in two metabolites, 4'-hidroxidiclofenac and 5'-hidroxidiclofenac, two highly reactive intermediaries, whose formation might result in an increased formation of reactive oxygen species (ROS) (Oviedo-Goméz et al., 2010). This pathway corresponds to a bioactivation route, mediated by the cytochrome P450, which decisively contributes to the frequently observed oxidative stress that results from diclofenac exposure, by favouring the formation of p-quinoneimine. This chemical corresponds to a derivate of the above mentioned hydroxylated metabolites, being the main responsible for the alteration of normal redox cycles and oxidative toxicity (Oliveira et al., 2015b).The here-obtained results point to the occurrence of a significant disturbance of the physiology. Regarding the CAT activity, it was possible to observe a statistically significant increase of its activity only in organisms exposed to the highest concentration tested (One Way ANOVA: $F_{(4,17)}=20.093$; $p<0.001$; Fig. 1). This is a clear indication about the putative occurrence of pro-oxidative alterations, which are likely to occur if one considers the known metabolism of diclofenac. Our results are comparable to data already published in the literature. Isla-Flores et al. (2013) also had an increase in CAT activity after exposing *Cyprinus carpio* to diclofenac for different exposure periods. The same results were obtained from Oviedo-Goméz et al. (2009) when exposing *Hyalella azteca* to diclofenac-enriched artificial sediment for different

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exposure times. An increase in CAT levels might indicate that the cell is trying to defend itself from the scenario of oxidative stress induced by the increase in H_2O_2 (Saucedo-Vence et al., 2014), as a result of diclofenac exposure.

An important contribution for the assumption about the establishment of pro-oxidative conditions results from the analysis of biomarkers related to the glutathione metabolism. Our study evidenced an increase in the activities of selenium-dependent GPx (One Way ANOVA: $F_{(4,15)}$=4.586; p=0.013; Fig. 2) and of total GPx (One Way ANOVA: $F_{(4,11)}$=29.572; p<0.001; Fig. 2), however, there were only statistically significant differences between the control and the two highest tested concentrations. These results are in agreement with the study of Goméz-Oliván et al. (2014) who exposed *Daphnia magna* to different diclofenac concentrations and obtained an increase in GPx levels. Pandey et al. (2017) exposed *Oreochromis niloticus* to a range of diclofenac concentrations during up to 60 days and also obtained an increase in GPx levels. GPx is involved in the reduction of hydrogen peroxide and lipid peroxide and also prevents oxygen to form radical intermediates by mechanisms of oxygen reduction (Pandey et al., 2017).When an increase in GPx activity is observed, it might mean that the enzyme is trying to reduce the impact of increased ROS formation (John et al., 2010; Pandey et al., 2017). Saucedo-Vence et al. (2014) also obtained an increase of GPx activity in the work mentioned above, which is in agreement with the results here obtained.

Detection and identification of reactive intermediates generally involves the analysis of conjugates with glutathione (Braver et al., 2017). An increase in ROS levels may cause oxidative damage to aquatic organisms, including fish and invertebrates (Oliveira et al., 2015b). Reduced glutathione (GSH) intervenes directly in ROS elimination through the action of GPx and GSTs. Our data showed a significant decrease in the activity levels of the enzyme GSTs (One Way ANOVA: $F_{(4,14)}$=53.904; p<0.001; Fig. 3). Statistically significant differences were found between animals exposed to all the tested concentrations and those from the control. This result is in agreement with the results obtained from Stancova et al. (2017) when exposing early life stages of *Tinca tinca* to different concentrations of diclofenac, which showed that fish exposed to the concentration of 500 μg/L of diclofenac had significantly lower levels of GSTs compared to control organisms. These results are also in line with those obtained by Diniz et al. (2015) after exposing for seven days adult organisms of *D. rerio* to

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diclofenac and to its photolysis byproducts. Guiloski et al. (2015) related a decrease of the GSTs levels with the inhibition of diclofenac biotransformation, which impairs the excretion process. The inhibition of GSTs observed in this study could also be related to their denaturation by direct reaction with ROS (Lushchak et al., 2009; Diniz et al., 2013). This direct effect by ROS is suggested based on the assumption that ROS were indeed overproduced following exposure to DCF, as suggested by the increments of both CAT and GPx activities. In these cases of ROS overexpression, it is possible to hypothesize that ROS may act directly on GSTs, thereby denaturing these isoenzymes, rendering them inactive. In addition, this augment of the activities of both forms of GPx suggests an increase of GSH oxidation, which may be a critical factor in the analysis of the obtained data. Furthermore, diclofenac is conjugated not only with glucuronic acid, but also with glutathione in its reduced form, GSH (Tang et al., 1999; Boelsterli, 2003; Alkimin et al., 2019). Both uses of GSH (as antioxidant, and as conjugation factor) might lead to its depletion; this effect may result in an impairment of GSTs activity, which is likely to be restricted by low amounts of intracellular GSH (Monteiro et al., 2006). Consequently, the activity of GSTs can be further impaired, due not only to its denaturation by ROS, but also to limitations in the amount of available GSH to serve as cofactor for the conjugation activity of these isoenzymes (Monteiro et al., 2006).

Modification of cellular macromolecules by reactive drug metabolites plays an important role in tissue damage caused by various drugs (Braver et al., 2017). Lipid peroxidation is a biomarker of damage that occurs when the activated antioxidant defences are not effective enough to protect the tissue that is being attacked (Quinn et al., 2011). Peroxidative damage is a frequent outcome of oxidative stress, and may contribute for irreversible tissue damage. This was a pattern that was reported in our study, since TBARS levels were increased, from the lowest to the highest concentration (One Way ANOVA: $F_{(4,13)}=1.823$; $p=0.185$; Fig. 4), however, no statistically significant differences where found between any tested concentration and the control. This result is partially in agreement with the findings of McRae et al. (2017) who obtained an increase in lipid peroxidation levels in the fish *Galaxias maculatus* after being acutely exposed to different concentrations of diclofenac. Oviedo-Gómez et al. (2010), in the already mentioned study, also obtained an increase over time in LPO levels quantified in *Hyalella azteca*. In their study, they related the increase in LPO levels with the formation of 4'-hydroxydiclofenac and 5'-hydroxydiclofenac followed

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by their biotransformation to benzoquines which increases ROS formation. Another hypothesis is that the increases in CAT activity was not enough to protect against cellular damage, so this scenario culminated in the occurrence of oxidative damage in membrane lipids, reflected by an increase in LPO levels (McRae et al., 2017). Our results suggest the occurrence of a mild augment of lipid peroxidation, which follows an already described pattern. Despite the triggering of defensive antioxidant mechanism, these were only hardly effective enough to cope with the oxidising effect of diclofenac. Considering the onset of oxidative defense response, it is possible to suggest that CAT and GPx were activated to degrade H_2O_2 to water and oxygen (Djordjević, 2004; Crichton, 2019). However, GPx activity depends upon the existence of a supply of GSH, which was depleted by oxidation, forming the biologically inactive corresponding disulphide (GSSG). Along the time course of the intoxication process, levels of GSH were systematically depleted, strongly conditioning the activity of GSTs, which were progressively reduced (Monteiro et al., 2006). This reduction of GSH levels (and consequently of GSTs activity) was indeed progressive, and inhibitory effects of GSTs were observed for all tested concentrations; on the contrary, antioxidant enzymes were only significantly enhanced for the higher tested levels of DCF. This scenario of increased levels of GPx and CAT, and concomitant reduction of GSTs, one of the most enduring conjugation pathways of DCF, resulted in a compromised efficacy of the phase II metabolism and antioxidant system, resulting in enhanced lipid peroxidation. Despite being clearly activated, the increases of CAT and GPx activities that were observed in animals exposed to the higher level of DCF were not enough to totally protect cells against oxidative damage.

4. Conclusions

In conclusion, diclofenac induced an oxidative stress scenario to *Danio rerio* embryos and larvae when acutely exposed to a different range of concentrations. As the used concentrations were chosen according to the ones found in the environment, it means that these organisms suffer oxidative stress in their natural habitats. However, to take more accurate conclusions about what happens at a biochemical level in these animals, a bigger array of tests should be done and more concentrations should be tested.

Credit author statement

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Sofia Bio - Formal analysis; Investigation; Methodology; Validation; Writing - original draft.

Bruno Nunes - Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing - review & editing.

Declaration of interests

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

Acknowledgments

Bruno Nunes is hired by “ECO-R-pharmplast - Ecotoxicity of realistic combinations of pharmaceutical drugs and microplastics in marine ecosystems”, Fundação para a Ciência e a Tecnologia, FCT (reference POCI-01-0145-FEDER-029203). Thanks are also due for the financial support to CESAM (UID/AMB/50017/2019), to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020.

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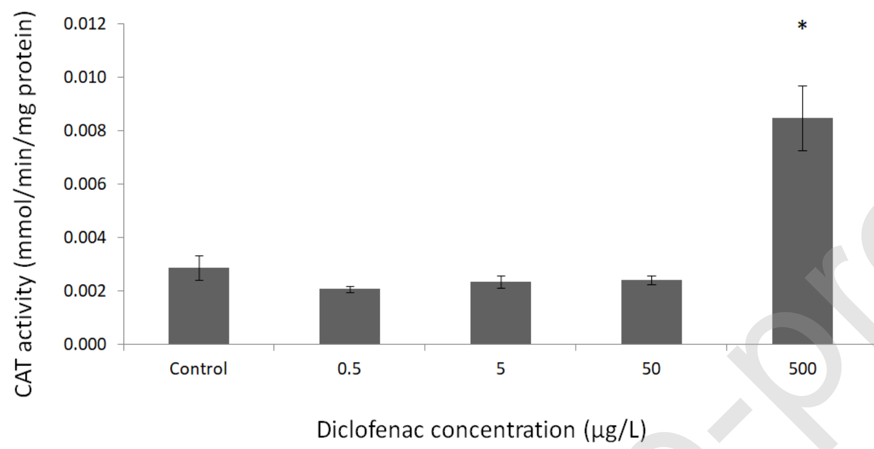

Fig. 1 CAT activity; results correspond to the average of 5 replicates per concentration, with standard error bars; *- significant statistical differences when compared to the control, $p<0.05$

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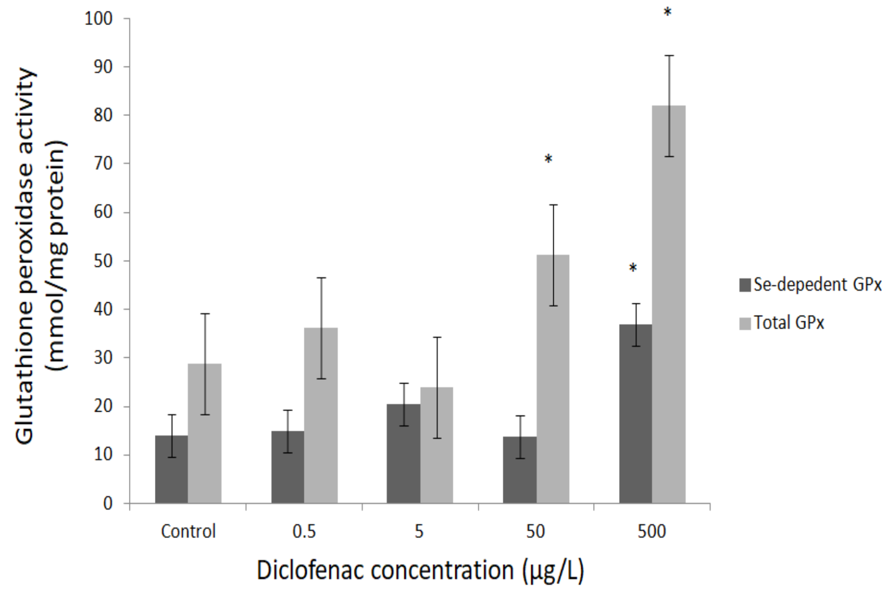

Fig. 2 GPx activity; results correspond to the average of 5 replicates per concentration, with standard error bars; *- significant statistical differences when compared to the control, $p<0.05$

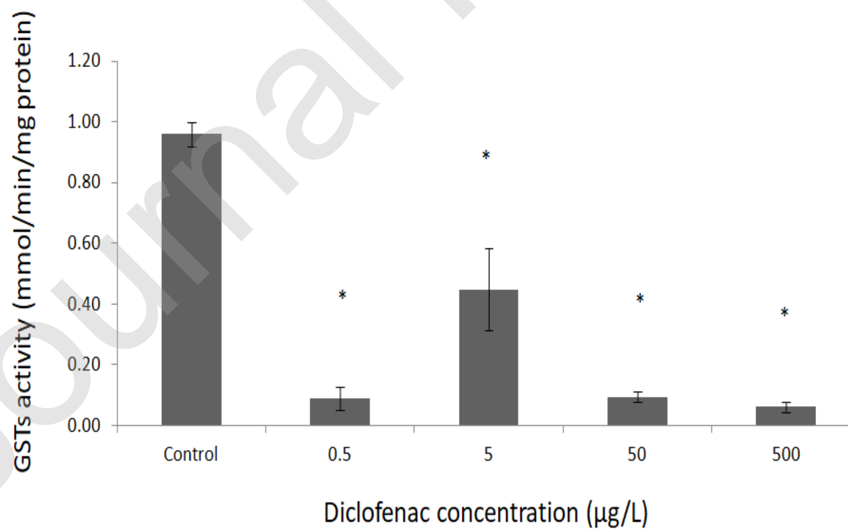

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Fig. 3 GSTs activity; results correspond to the average of 5 replicates per concentration, with standard error bars; *- significant statistical differences when compared to the control, p<0.05

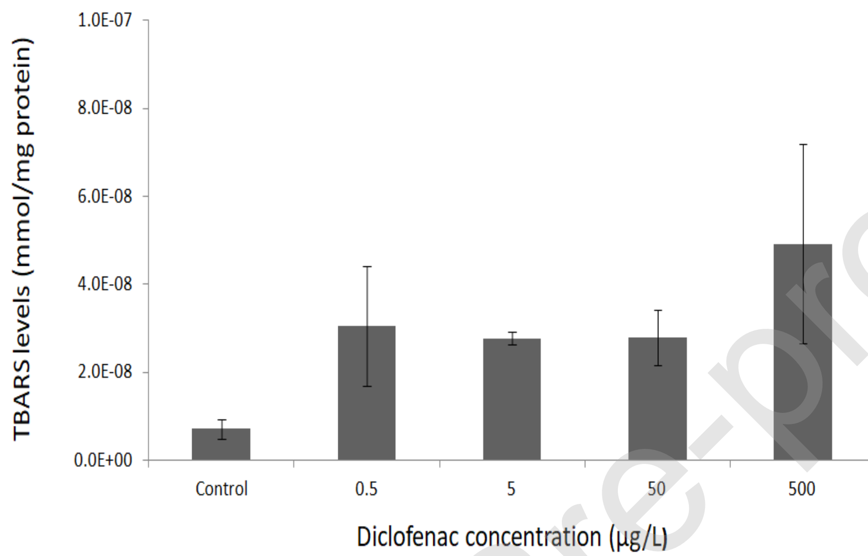

Fig. 4 TBARS levels; average of 5 replicates per concentration, with standard error bars

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