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# Effects of spirulina (*Arthrospira maxima*) on teratogenicity and diclofenac-induced oxidative damage in *Xenopus laevis*

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## Abstract

Diclofenac (DCF) is a medication that is highly consumed and eliminated worldwide; it is constantly detected in the environment (primarily in water) and resists conventional degradation processes. It was included in the European Union watch list for the water framework. There are no regulations for this compound in Mexico. Therefore, this study evaluated the protective effect antioxidant activity of spirulina (*Arthrospira maxima*) against DCF-induced toxicity in *Xenopus laevis* at early life stages. *X. laevis* oocytes were exposed at the medium blastula stage for 96 h to three different mixtures: DCF+S 2 (149 μg $L^{-1}$ DCF plus 2 mg $L^{-1}$ spirulina), DCF+S 4 (149 μg $L^{-1}$ DCF plus 4 mg $L^{-1}$ spirulina), DCF+S 10 (149 μg $L^{-1}$ DCF plus 10 mg $L^{-1}$ spirulina). Other groups of oocytes were also exposed to DCF 149 μg $L^{-1}$ and a control group. The mortality and malformation rate, growth, lipid peroxidation, and antioxidant enzymatic activity (superoxide dismutase and catalase) were determined. Spirulina at 4 and 10 mg $L^{-1}$ reduced DCF-induced mortality by 80% and reduced malformations in severity and frequency. The abnormalities were malformations of the eye, tail, notochord, intestine, and rectum. All spirulina exposure groups showed an increase in total body size compared to those exposed to DCF. Regarding oxidative damage, the groups exposed to the

mixture with spirulina decreased lipid peroxidation levels and diminished antioxidant activity. Spirulina reduced DCF-induced damage in *X. laevis* at early life stages and decreased mortality, frequency, and severity of abnormalities, growth inhibition, and oxidative damage. Further research is needed to evaluate the effects of spirulina against toxicity induced by xenobiotics in the early stages of development.

**Keywords:** Pharmaceuticals, diclofenac, spirulina, teratogenesis, oxidative stress, *Xenopus laevis*

## INTRODUCTION

In recent decades, aquatic environment pollution has become a global issue; domestic and industrial discharges can cause detrimental effects, even at trace concentrations, primarily on aquatic organisms. Among the compounds that have been identified as environmental pollutants are emerging contaminants (a group of unregulated contaminants present in different water matrices for decades ago), such as personal care compounds, synthetic hormones, steroids, and endocrine-disrupting chemicals, among others, which represent only a tiny fraction of the total chemical pollution[1].

Pharmaceuticals are used extensively in human and veterinary medicine[2]. Hundreds of tons of pharmaceuticals are consumed worldwide annually[3]. Their physicochemical properties constitute a significant class of emerging environmental pollutants threatening aquatic organisms and ecological health[3-5]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used in Mexico and worldwide[4,6]. Diclofenac (DCF, a member of the phenylacetic acid class with analgesic, anti-inflammatory, and antipyretic effects), since its introduction in 1973, has been one of the most prescribed worldwide due to its effectiveness in treating a variety of acute and chronic pain and inflammatory conditions[7-11]. DCF exerts its action by inhibiting prostaglandin synthesis; it binds to cyclooxygenase (COX)-1 and COX-2 and inhibits the conversion of arachidonic acid into pro-inflammatory prostaglandins through chelation; nevertheless, unlike many NSAIDs, DCF inhibits COX-2 with greater potency than it does with COX-1, making it able to inhibit tumor angiogenesis. Several studies found that DCF activity extends beyond COX inhibition, including multimodal and novel mechanisms of action[7-9].

DCF was included on the First Watch List of the EU Water Framework Directive. This inclusion was done because of its ubiquitous nature in the aquatic environment, mainly in surface waters such as rivers, lake canals, estuaries, and seas; it also frequently occurs in groundwater and wastewater effluents. Due to its potential toxicity reported over the last 20 years, it harms aquatic biota such as fish and mussels[4,12-15].

DCF has been detected in several aquatic matrixes in concentrations ranging from ng $L^{-1}$ to µg $L^{-1}$. For example, in surface water, it has been found at < 100 ng $L^{-1}$[16,17] to 364 ng $L^{-1}$[18], 419 ng $L^{-1}$[19], 10 200 ng $L^{-1}$[20], and 7.76 µg $L^{-1}$[21]; in groundwater, the reported concentrations vary from < 10 ng $L^{-1}$[16,22] to 48.1 ng $L^{-1}$[23], 518 ng $L^{-1}$[19], and 2.77 µg $L^{-1}$[24]. In drinking water samples, DCF has been found from < 10 ng $L^{-1}$[25,26] to 16-18 ng $L^{-1}$[27,28]; while in seawater, it has been found at 0.021, 48, 11.6, 880 ng $L^{-1}$[29-32], and 10.2 and 31.9 ng $L^{-1}$[33,34]; in municipal wastewater influent/effluent, it has been detected at < 500 ng $L^{-1}$[35-37] to 812 ng $L^{-1}$[38] and 2.5 µg $L^{-1}$[39].

Other matrixes include soil, where DCF has been detected from 0.3 to 0.35 mg $kg^{-1}$[40,41], 257 mg $kg^{-1}$[42], and 0.2 ng $g^{-1}$[43]; in sediments, it was found from 3.95, 6.8, 10.6, and 13.88 ng $g^{-1}$[18,44-46]; in suspended solids, it has been detected from 119 ng $g^{-1}$[47] to 1.3 mg $g^{-1}$[18]; in sewage sludge; it has been detected from < 1 ng $g^{-1}$[48] to >10 ng $g^{-1}$[49,50], even at 35.3 mg $Kg^{-1}$[51] and 4,968 mg $Kg^{-1}$[42]. Finally, DCF has been detected in the leachate at 40 and 613.3 ng $L^{-1}$[52,53] and 108.34 mg $L^{-1}$[24].

DCF causes toxic effects, even at trace concentrations for humans and aquatic organisms, including significant inhibition of acetylcholinesterase (as a biomarker of neuronal regulation) in *Daphnia magna* (0.08-18.4 $ngL^{-1}$)[54]. A significant decrease in growth was observed in *Danio rerio* (at 30 and 60 $mgL^{-1}$)[55], with alterations in hematologic parameters including higher mean corpuscular hemoglobin concentration, mean corpuscular volume, and white blood cell, with significantly lower hemoglobin, hematocrit, red blood cell and mean corpuscular hemoglobin in *Clarias gariepinus* (at 1.57, 3.14, and 6.28 $mgL^{-1}$)[2]. It modulated genes associated with kidney repair and regeneration in *Pimephales promelas* (at 25 $\mu gL^{-1}$)[10] and reduced larval developmental growth and several morphological abnormalities such as altered body axis and organ and visceral abnormalities, including cardiac hypoplasia and miscoiling guts in *Trachycephalus typhonius* and *Physalaemus albonotatus* (at 125 and 250 $\mu gL^{-1}$)[56]. It affected the hatching rate, development rate, survival, growth, and histopathological changes in *Oncorhynchus mykiss* and *Danio rerio* (3.2 to 1,000 $\mu gL^{-1}$)[57].

Due to its biotransformation leading to the formation of reactive metabolites and reactive oxygen species (ROS) can induce oxidative stress and damage in diverse biomolecules[11,58,59]. It has been highlighted that relevant environmental concentrations may result in adverse effects, mainly chronic exposure, in aquatic and marine organisms[12,14,60]. The toxicities reported by several working groups worldwide include increased oxidative stress biomarkers such as lipid peroxidation (LPX), hydroperoxide content, protein carbonyl content, and the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase, and glutathione peroxidase activities. They also include teratogenic effects, malformations in the tail and notochord, edema, and stunted growth in *Xenopus laevis* and *Lithobates castesbeianus* at 1, 4, 8, 16, 32, and 62.5 $mgL^{-1}$[61]. These effects occur in *Daphnia magna* at 0.08-18.4 $ngL^{-1}$[54] and 2.9 $mgL^{-1}$[6], in *Danio rerio* at 5, 15, 30, and 60 $mgL^{-1}$[55] and 0.5, 5, 50, and 500 $mgL^{-1}$[15], and in *Cyprinus carpio* at 7.098 $mgL^{-1}$[62] and 70.98 $mgL^{-1}$[59].

Spirulina (*Arthrospira maxima*) is a unicellular blue-green cyanobacterium microalga. It has been used as a nutrient supplement because of its nutritional value, medicinal properties, and pharmacological activity. Spirulina is an excellent source of protein, vitamins, fatty acids, and amino acids. It is also a potent immune system stimulant; it increases phagocytic and natural killer activity[63], increases IL-1 levels and activates antibodies[64]. Other properties include antiviral, anticancer, hypoglycemic, and antihyperlipidemic[65-68]. Spirulina's antioxidant and protective effects have been demonstrated against toxins such as mercury[69,70], D - galactosamine[71], acetaminophen[72], copper[73], carbon tetrachloride[74], beta - cypermethrin[75], and tetracycline[76].

Spirulina significantly improved the activity of glutathione, glutathione peroxidase, glutathione reductase, and glutathione S transferase in rabbits[77], rats[78], hamster[79], C*yprinus carpio*[80], Nile tilapia[81,82], and *Danio rerio*[83,76]. However, there is no evidence of benefits in *X. laevis*. Therefore, this study evaluated the protective effects of spirulina against mortality, malformations, growth inhibition, and oxidative damage induced by DCF in *X. laevis* at early life stages.

## MATERIALS AND METHODS

All procedures were carried out at the Faculty of Chemistry of the Autonomous University of the State of Mexico, approved on January 14, 2022 (project code 6453/2022CIB), as indicated in the official letter D.I./021/2022. The ethical protocols for the care, use, and management of laboratory species established in the American Society for Testing Materials (ASTM) Guide E-1439-12 were followed, as were the specifications in the official Mexican standard (NOM-062-ZOO-1999, Technical specifications for the production, care, and use of laboratory animals).

### Chemicals and reagents

All reagents used were analytical grade (> 99% purity). Diclofenac sodium salt (CAS # 15307-79-6, 99% purity), 3-amino-benzoic acid ethyl ester (MS-222), NaCl, $NaHCO_3$, KCl, $CaCl_2$, $CaSO_4 \cdot 2\ H_2O$, $MgSO_4$, and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. Spirulina dried powder was purchased from a local supplier (AEH Spiral Spring, Mexico).

### Organism selection and maintenance

Adult *X. laevis* males and females were obtained from an aquaculture center (Aquanimals) in Queretaro, Mexico. The selection criteria for males were 8 to 10 cm in length and two years of age (for females, 10 to 12.5 cm and three years). Differentiation criteria were the presence of visible cloacal labia and a larger size in females.

Males and females were housed separately in 60 L aquariums filled to 80% capacity with dechlorinated water. The following conditions were maintained: temperature 21 ± 2 °C, pH 6.5 to 9, and 12-h light-dark photoperiods. Total organic carbon < 10 mg $L^{-1}$, alkalinity, and hardness (by determination of $CaCO_3$ 16 to 400 mg $L^{-1}$) were determined monthly. *X. laevis* were fed three times a week ad libitum with *Chrisotoma sp.* (0.5 ± 0.3 cm in length) or commercial food NUPEC pellets (Purina).

### FETAX assay

This study followed the American Society for Testing Materials Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX)[84].

*FETAX and test solutions*

The FETAX medium formulation was 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2\ H_2O$, and 75 mg $MgSO_4$ per liter of deionized water. The final pH was 7.6-7.9. All reagents were purchased from Sigma-Aldrich.

A stock solution was prepared daily by dissolving 1 g in 1 L of FETAX medium for DCF exposure. Later, DCF solutions were ready to have a final concentration of 1, 4, 8, 16, 32, and 62.5 mg $L^{-1}$[61,85] for $LC_{50}$ and lowest observed adverse effect level (LOAEL) determination. Spirulina and DCF mixtures were prepared by dissolving 2, 4, and 10 mg of spirulina in a 149 µg$L^{-1}$ DCF solution. The entire procedure was performed under a laminar flow hood.

The concentration of DCF 149 µg$L^{-1}$ in mixtures was previously determined to be the lowest adverse effect level (LOAEL) in *X. laevis*, and spirulina doses were 2, 4, and 10 mg$L^{-1}$ based on previous experiments[86]. No spirulina concentration had been tested previously in embryonic stages in amphibians.

The final test solutions for evaluating protective effects are in Table 1. New solutions were prepared daily to avoid degradation and protected from light at 4 °C.

*Ovulation induction and fertilization*

The night before the assay, one male and one female were placed in a 40-L aquarium with a plastic mesh suspended 3 cm over the bottom to separate the embryos from the adult organisms with opaque sides at 21 ± 2 °C, pH 6.5-9.

In the dorsal lymph sac, ovulation and spermatogenesis were induced by human chorionic gonadotropin hormone (previously dissolved with a NaCl 0.9% sterile solution; CHORAGON®, Ferring) using 1-mL

**Table 1. Exposure groups**

| Test solution | Composition |
|---|---|
| Control | FETAX medium |
| Spirulina | 10 $mgL^{-1}$ |
| Diclofenac | 149 $\mu gL^{-1}$ of diclofenac |
| DCF+S 2 | 149 $\mu gL^{-1}$ of diclofenac plus 2 $mgL^{-1}$ of spirulina |
| DCF+S 4 | 149 $\mu gL^{-1}$ of diclofenac plus 4 $mgL^{-1}$ of spirulina |
| DCF+S 10 | 149 $\mu gL^{-1}$ of diclofenac plus 10 $mgL^{-1}$ of spirulina |

DCF: Diclofenac; FETAX: Frog Embryo Teratogenesis Assay-*Xenopus*.

hypodermic syringes fitted with long 26-gauge needles; males were administered 300 IU and females were administered 700 IU.

*Oocyte selection*

On the morning of the following day, the aquarium was inspected for oviposition. Oocytes were extracted from the aquarium with sterile Pasteur pipettes and placed in separate containers for examination under a Zeiss Stemi 305 stereoscopic microscope to select oocytes with a spherical shape, homogeneous cell division, and blastula stage (8-10).

*Exposure*

(1) Diclofenac

For DCF exposure, 10 mL of each solution (1, 4, 8, 16, 32, 62.5 mg $L^{-1}$) were placed in 50-mm Petri dishes under a laminar flow hood; twenty oocytes were placed in each Petri dish using Pasteur pipettes and a stereoscopic microscope. A control group exposed to FETAX medium was prepared at the same conditions, 21 ± 2 °C for 96 h. All experiments were performed in triplicate.

(2) Diclofenac and spirulina mixtures

As mentioned previously (2.3.4.1), 10 mL of each solution (Diclofenac, D+S 2, D+S 4, D+S 10) were placed in 50-mm Petri dishes under a laminar flow hood. Twenty oocytes were collected and placed in each Petri dish using Pasteur pipettes and a stereoscopic microscope. A control group exposed to FETAX solution was prepared at the same conditions, 21 ± 2 °C for 96 h. All experiments were performed in triplicate.

*Culture monitoring*

Diclofenac, mixtures, and control group solutions were replaced daily under a laminar flow hood. Sterile 50-mm Petri dishes were filled with 10 mL of each test concentration, mixture, and control solution. They were added and maintained for 1 h 30 min at room temperature to ensure that solutions were 20 ± 2 °C before transferring oocytes. Every 24 h, cultures were inspected, and live embryos were transferred to a new Petri dish. A daily record was taken, and the number of dead larvae and residues (if any) in each culture were documented.

*Examination of larvae*

At 96 h of exposure, we checked larvae for swimming; if there were none, this was noted in a developmental parameter sheet used to record malformations. Precipitates (if any) and dead larvae were also recorded.

After 96 h, larvae were euthanized by placing them in a 50-mm Petri dish containing 0.06% (w/v) ethyl 3-aminobenzoate methanesulfonate (lethal dose).

Each larva was measured head-to-tail using Zen Blue Zeiss software, and we registered values to determine the minimum concentration to inhibit growth. Each larva was observed and evaluated in the microscope fitted with a Zeiss Axiocam 5s camera to identify developmental abnormalities following the Atlas of Abnormalities[87] and other resources. After examination, the disposal of larvae followed the institutional standards for eliminating biological samples.

### Oxidative damage assessment

The FETAX assay was performed as in 2.3 to 2.3.5, extending the exposure period to 192 h to ensure the larvae were feeding[88,89]; afterward, larvae were weighed and homogenized with a phosphate buffer solution (pH 7.2) 4 °C in a 1:4 (w/v) proportion, then centrifuged at 2,500 rpm for 15 min.

*Determination of total protein*

Total protein was determined using Bradford's 1976 method[90]. 25 µL of supernatant were added in a microtube plus 75 µL deionized water and 2.5 mL Bradford's reagent [0.05 g Coomassie blue dye (Sigma-Aldrich), 25 mL of 96 % ethanol (Sigma-Aldrich), and 50 mL $H_3PO_4$ (Sigma-Aldrich) in 500 mL deionized water]. The microtubes sat for 5 min in darkness, and absorbance was read at 595 nm. Total protein concentration was determined by interposing the results on a bovine albumin standard curve (Sigma-Aldrich). The total protein concentration was used to express the results of lipid peroxidation, SOD, and CAT.

*Determination of LPX*

LPX activity was determined according to Buege and Aust[91]; 50 µL of supernatant, 450 µL Tris-HCl buffer solution (150 mM) pH 7.4, and 1 mL of 0.38% thiobarbituric acid (Fluka, Sigma-Aldrich, Toluca) in 15% tricarboxylic acid were added to a glass tube (10 × 75 mm) and incubated at 37 °C for 45 min. Absorbance was determined at 535 nm. Results were expressed as mM malondialdehyde/mg protein using the $1.56 \times 10^5$ /M/cm molar extinction coefficient.

*Determination of SOD activity*

SOD activity was determined according to Misra and Fridovich[92]; 40 µL of supernatant, 260 µL carbonate buffer solution [50 mM sodium carbonate,0.1 mM EDTA (Sigma-Aldrich)], pH 10.2, and 200 µL of adrenaline (30 mM,) were added to a quartz cuvette. Absorbance was measured at 480 nm at 30 seconds and 5 minutes. SOD activity was determined with the molar extinction coefficient 21 M $cm^{-1}$, and results were expressed as IU SOD/mg of protein.

*Determination of CAT activity*

CAT activity was determined according to Radi *et al.*[93]; 30 µL of supernatant were placed into a quartz cuvette plus 420 µL of isolation buffer solution[0.3M sucrose (Vetec, Sigma-Aldrich), 1 mM EDTA, 5 mM HEPES, 5 mM $KH_2PO_4$ (Sigma-Aldrich)] and 300 µL of 20 mM $H_2O_2$ solution (Sigma-Aldrich). Absorbance was read at 240 nm, at 0 and 60 s. CAT activity was estimated using the molar extinction coefficient of $H_2O_2$ 0.093 mM/cm.

### Statistical analysis

The data were analyzed using the software Stat graphics Centurion XIX. All results were expressed as the mean of three experiments performed under the same conditions. To determine normality, the Shapiro-

Wilk and Kolmogorov-Smirnov tests were performed. To calculate the values of median lethal concentration ($LC_{50}$), we performed a probitanalysis ($P < 0.05$); for the LOAEL, we performed a Dunnett's test ($P < 0.05$). To determine the differences in growth, each larva was measured from head-to-tail, and the mean values were compared using one-way analysis of variance (ANOVA) and Fisher's multiple comparisons ($P < 0.05$). LPX, SOD, and CAT were analyzed using one-way ANOVA and Fisher's multiple comparisons ($P < 0.05$).

## RESULTS

### FETAX assay

The median lethal concentration ($LC_{50}$) of 14.905 mg $L^{-1}$ was determined (PROBIT analysis $P < 0.05$), and LOAEL was calculated at 149 µg $L^{-1}$.

*Mortality and malformations*

Mortality and malformation data are shown in Figure 1. Survival in spirulina-treated embryos from 60% to 80%; the highest increased in surivival was on D+S 4 and D+S 10, and neither group showed significant differences from the control. Nevertheless, there was a significant increased in survival to DCF exposure; malformations were reduced by 6% to 16%, and D+S 4 demonstrated a higher reduction. The most frequent malformations observed were bent tail, bent notochord, gut and rectum malformation, eye abnormalities, microcephaly, and cardiac edema [Figure 2]. All spirulina mixtures significantly reduced malformation severity [Figure 3].

*Growth inhibition*

Diclofenac exposure reduced the total larval body size, spirulina mixtures increased full body size, and enhanced growth, as shown in head-to-tail measurements in Figure 4. Larvae exposed to D+S 4 and D+S 10 had similar total body size as the control; D+S 10 was the most effective in reducing growth inhibition.

### Oxidative damage

*Lipid peroxidation*

Lipid peroxidation data are shown in Figure 5. Diclofenac induced a significant increase in malondialdehyde levels compared to the control; on the other hand, all spirulina mixtures demonstrated a significant reduction in lipid peroxidation compared to DCF, and D+S 10 achieved the highest reduction.

*SOD activity*

Figure 6 shows SOD activity; DCF exposure induced an increase compared to control; spirulina mixtures significantly reduced SOD activity. The most effective decrease is observed in D+S 10; this mixture has similar SOD activity levels to the control group.

*CAT activity*

DCF also increased CAT activity [Figure 6]; however, all spirulina mixtures achieved CAT levels lower than DCF. The mixture with the most effective reduction was D+S 10; this mixture reached similar values to the control group.

## DISCUSSION

Pharmaceuticals have been detected in aquatic environments. Because these products are biologically active and are constantly released into water, they represent a risk to organisms and human health. DFC is an NSAID that detects aquatic environments in concentrations ranging from ng $L^{-1}$ to µg $L^{-1}$[13,14,94]. Although DFC has a relatively short half-life in water (8 days)[95], its constant elimination can be a problem because

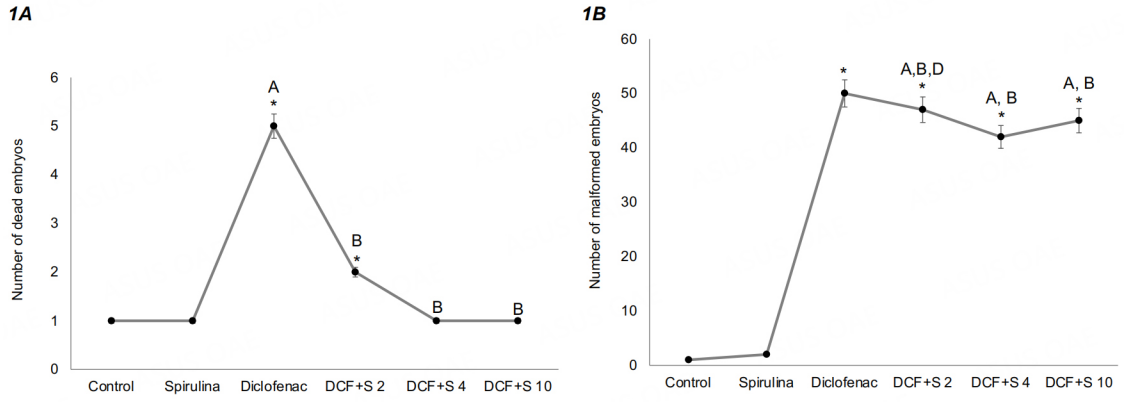


**Figure 1.** (1A) Total number of dead embryos of *X. laevis* after exposure 96 h to control, spirulina 10 $mgL^{-1}$, DCF 149 $\mu gL^{-1}$, DCF+S 2 (diclofenac 149 $\mu gL^{-1}$ + spirulina 2 $mgL^{-1}$), DCF+S 4 (diclofenac 149 $\mu gL^{-1}$ + spirulina 4 $mgL^{-1}$), DCF+S 10 (diclofenac 149 $\mu gL^{-1}$ + spirulina 10 $mgL^{-1}$); (1B) total number of *Xenopus leavis* embryos with malformation, after exposure 96 h to control, spirulina 10 $mgL^{-1}$, diclofenac 149 $\mu gL^{-1}$, DCF+S 2 (diclofenac 149 $\mu gL^{-1}$ + spirulina 2 $mgL^{-1}$), DCF+S 4 (diclofenac 149 $\mu gL^{-1}$ + spirulina 4 $mgL^{-1}$), DCF+S 10 (diclofenac 149 $\mu gL^{-1}$ + spirulina 10 $mgL^{-1}$). Significant differences relative to: *: control; A: spirulina; B: DCF; C: DCF+S 2; D: DCF+S 4; E: DCF+S 10 (One-way ANOVA and Fisher's test, $P < 0.05$).

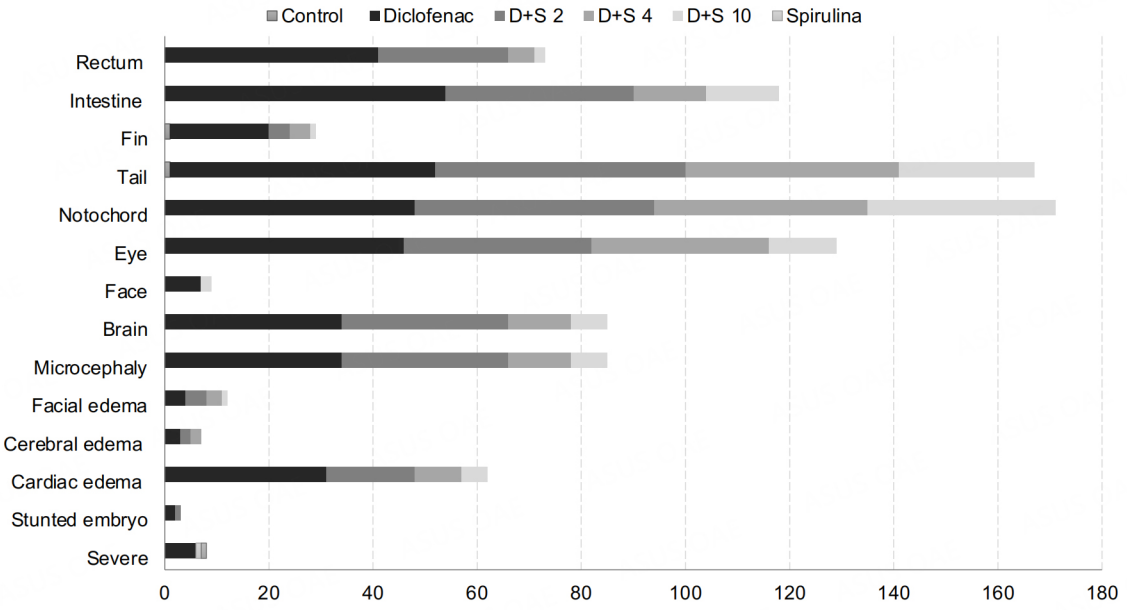


**Figure 2.** Frequency histogram for malformations induced in *X. laevis* embryos after 96 h exposure to control, spirulina 10 $mgL^{-1}$, diclofenac 149 $\mu gL^{-1}$, DCF+S 2 (diclofenac 149 $\mu gL^{-1}$ + spirulina 2 $mgL^{-1}$), DCF+S 4 (diclofenac 149 $\mu gL^{-1}$ + spirulina 4 $mgL^{-1}$), DCF+S 10 (diclofenac 149 $\mu gL^{-1}$ + spirulina 10 $mgL^{-1}$).

most of the standard remotion processes in wastewater treatment plants are ineffective in eliminating it[96]. Their continuous introduction to the environment can compensate for their transformation-remotion rates.

In Mexico, studies about DCF concentrations in water are scarce. However, it was detected in the influent of a wastewater treatment plant in Ciudad Juarez at 160 parts per billion[97]. Unfortunately, Mexico has no regulations stipulating the maximum permissible limit for the emission of pharmaceutical products into the aquatic environment. This absence can lead to the generation of adverse in aquatic organisms induced by pharmaceutical products; therefore, we must explore alternatives to reduce the toxic effects of

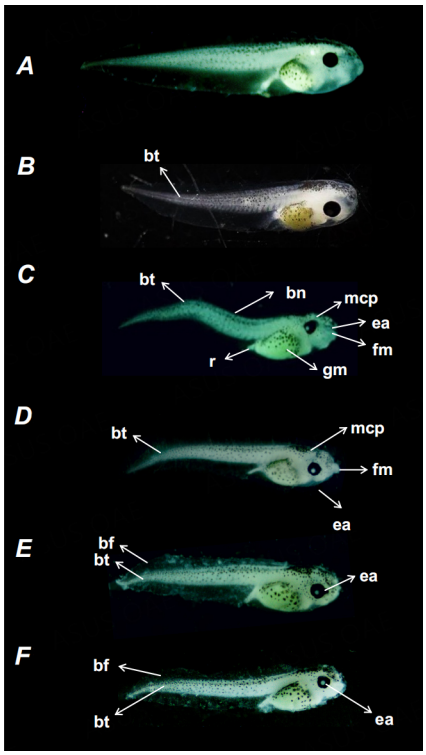


**Figure 3.** Representative, and most frequent malformations observed in *X. laevis* exposed for 96 h to (A) control; (B) spirulina 10 $mgL^{-1}$; (C) diclofenac 149 $\mu gL^{-1}$; (D) DCF+S 2 (diclofenac 149 $\mu gL^{-1}$ + spirulina 2 $mgL^{-1}$); (E) DCF+S 4 (diclofenac 149 $\mu gL^{-1}$ + spirulina 4 $mgL^{-1}$); (F) DCF+S 10 (diclofenac 149 $\mu gL^{-1}$ + spirulina 10 $mgL^{-1}$). bn: bent notochord; bt: bent tail; ea: eye abnormality; fm: face malformation; gm: gut malformation; mcp: microcephaly; r: rectum.

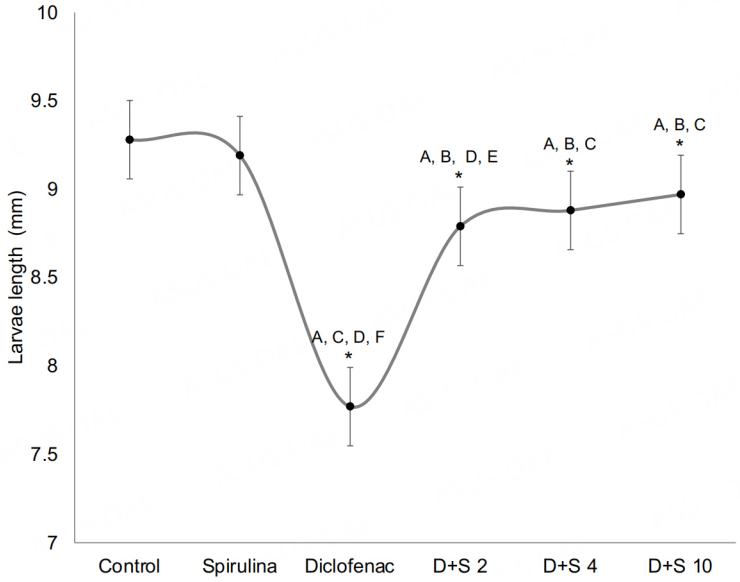


**Figure 4.** *X. laevis* larvae total body length head-to-tail after 96 h exposure to control, spirulina 10 $mgL^{-1}$, 149 $\mu gL^{-1}$, DCF+S 2 (diclofenac 149 $\mu gL^{-1}$ + spirulina 2 $mgL^{-1}$), DCF+S 4 (diclofenac 149 $\mu gL^{-1}$ + spirulina 4 $mgL^{-1}$), DCF+S 10 (diclofenac 149 $\mu gL^{-1}$ + spirulina 10 $mgL^{-1}$). Significant differences relative to: *: control; A: spirulina 10 $mgL^{-1}$; B: diclofenac 149 $\mu gL^{-1}$; C: DCF+S 2; D: DCF+S 4; E: DCF+S 10 (One-way ANOVA and Fisher's test, $P < 0.05$).

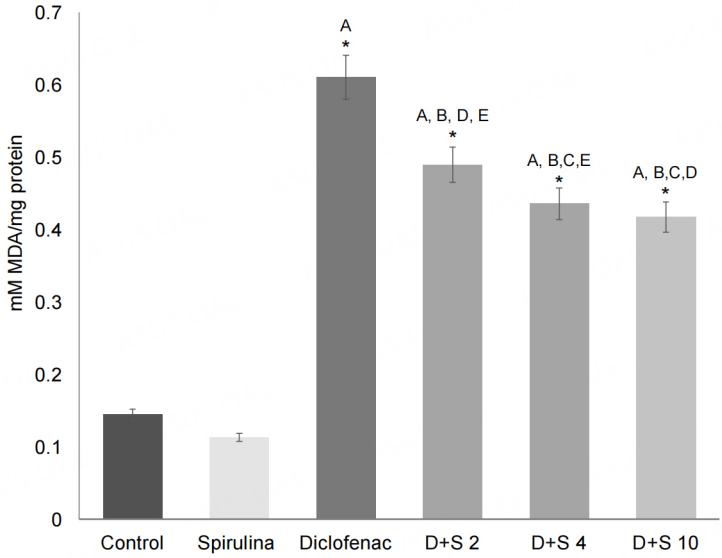


**Figure 5.** Lipid peroxidation level assessed in *X. laevis* after 192 h exposure to control, spirulina 10 $mgL^{-1}$, diclofenac 149 $\mu gL^{-1}$, DCF+S 2 (diclofenac 149 $\mu gL^{-1}$ + spirulina 2 $mgL^{-1}$), DCF+S 4 (diclofenac 149 $\mu gL^{-1}$ + spirulina 4 $mgL^{-1}$), DCF+S 10 (diclofenac 149 $\mu gL^{-1}$ + spirulina 10 $mgL^{-1}$). Significant differences relative to: *: control; A: spirulina 10 $mgL^{-1}$; B: diclofenac 149 $\mu gL^{-1}$; C: DCF+S 2; D: DCF+S 4; E: DCF+S 10 (one-way ANOVA and Fisher's test, $P < 0.05$).

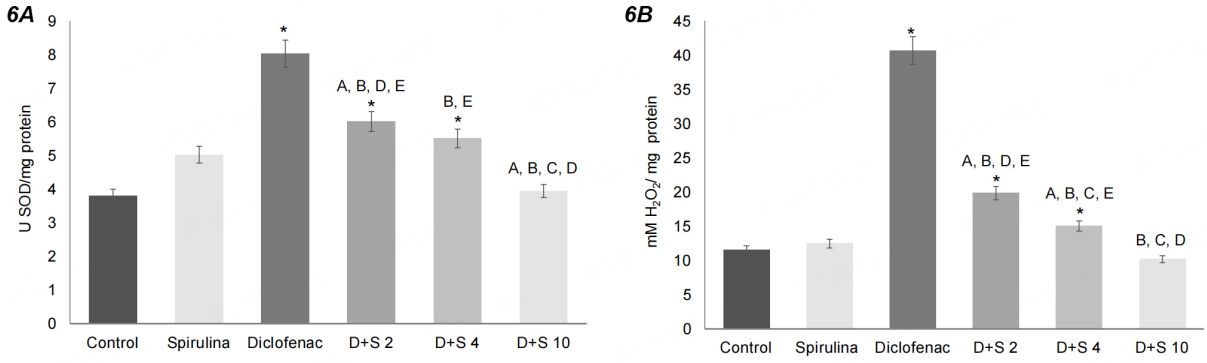


**Figure 6.** Antioxidant enzymes (6A) SOD; (6B) CAT evaluated in *X. laevis* larvae exposed 192 h to control, spirulina 10 $mgL^{-1}$, diclofenac 149 $\mu gL^{-1}$, DCF+S 2 (diclofenac 149 $\mu gL^{-1}$ + spirulina 2 $mgL^{-1}$), DCF+S 4 (diclofenac 149 $\mu gL^{-1}$ + spirulina 4 $mgL^{-1}$), DCF+S 10 (diclofenac 149 $\mu gL^{-1}$ + spirulina 10 $mgL^{-1}$). Significant differences relative to: *: control; A: spirulina 10 $mgL^{-1}$; B: diclofenac 149 $\mu gL^{-1}$; C: DCF+S 2; D: DCF+S 4; E: DCF+S 10 (one-way ANOVA and Fisher's test, $P < 0.05$).

pharmaceuticals in aquatic organisms. This is why we tested the effects of spirulina against DCF toxicity.

In the present study, as in others[85,61], DCF induced mortality [Figure 1] and malformations [Figures 2 and 3], growth inhibition [Figure 4], and caused oxidative damage in *X. laevis* in early life stages [Figures 5 and 6]. These effects may be due to oxidative stress, increased ROS, and pro-apoptotic factors, all of which cause cellular damage and death. Studies demonstrated that DCF is toxic at relatively low concentrations in aquatic organisms; it is teratogenic and embryotoxic and induces malformations (axial, edema, intestine, heart, head, eye) and growth inhibition in *X. laevis*[85]. It also causes growth restriction and malformations in viscera and skeleton, variations in acetylcholinesterase and glutathione S transferase levels, and neurotoxic and cardiotoxic damage in *T. typhonius* and *P. albonotatus*[56]. It increases mortality in embryos, variations in weight and size, increases glutathione S transferase, and reduces glutathione

reductase in *Cyprinus carpio*[98]. DCF increases the antioxidant activity of glutathione S transferase and adenosine triphosphate (ATP) binding cassette transporters and lipid peroxidation in *Danio rerio* larvae and adults[99]. It also induces oxidative stress and increases ROS levels associated with cytotoxicity in *Daphnia magna*[6,100]. Studies reported that DCF is toxic because it alters the oxidative phosphorylation in mitochondria; it induces direct depolarization of mitochondria which activates cytochrome CYP2C9 and increases cytosolic calcium levels, which leads to an increase in mitochondria membrane potential. This process generates pores and the collapse of the mitochondrial transmembrane potential, ATP depletion[99,101-103], the release of cytochrome C, which activates caspase 9 and caspase 3, and cellular death[100,103-105]. CYP2C9 activation induces the production of metabolites and ROS (mainly superoxide anion $O_2^{-\bullet}$) as superoxide dismutase is the first enzymatic mechanism of defense produced as a response when ROS is increased due to its catalysis of $O_2^-$ into $H_2O_2$. Catalase decomposes $H_2O_2$ into water and oxygen; both enzymes increase as a response to excess ROS. Because DCF induces oxidative stress, it has been reported that exposure, even at low or environmentally relevant concentrations, increases the activity of these enzymes[101].

Spirulina is a unicellular microalga with a high content of nutrients, proteins, minerals, antioxidants, and other biologically active compounds[106,107]. Its activities include antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective, and neuroprotective[108-115]; it also can reduce levels of pro-inflammatory interleukins and inflammation[108]. The protective effects of spirulina in various organisms have been reported[109,116-123].

Our results showed that exposure to spirulina reduced mortality [Figure 1], the severity and frequency of malformations [Figures 2 and 3], improved growth [Figure 4], and decreased oxidative damage [Figures 5 and 6]. Most of these effects were statistically significant at 4 and 10 mg $L^{-1}$; nevertheless, all concentrations showed positive effects against DCF-induced toxicity. These beneficial effects have been reported previously[74]. reported that spirulina restored enzyme levels and reduced histomorphological damage in the liver and kidney of Wistar albino rats exposed to DCF. The reduction of toxic effects induced by DCF may be due to the activity of spirulina components [Figure 7]. Phycocyanins possess antioxidant capacity, particularly phycocyanin C (PC), a water-soluble protein with a chromophore group (phycocyanobilin); PC scavenges ROS, including hydroxyl, alkoxy, and peroxyl[124]. It also inhibits lipid peroxidation at early stages. PC can scavenge alkoxy radicals (which propagate lipid peroxidation), inhibiting structural membrane damage[125,126].

Spirulina has other components inhibiting lipid peroxidation; carotenoids can neutralize oxygen singlets and peroxyl radicals and inhibit the production of prostaglandin E2 and nitric oxide production by suppressing inflammatory mediators[127,128]. Another component with critical activity is tocopherol because it interacts with superoxide anion, hydroxyl, and hydroperoxyl radicals; when tocopherol interacts with peroxyl radicals, it forms non-radical species that are less reactive. Thus, tocopherol reduces the lipidic peroxidation process and reduces membrane damage[129,130]. Some interactions exist between non-enzymatic antioxidants; for example, carotenoids regenerate tocopherol from its radical form (tocopheroxyl). The resulting carotenoid radical is restored by vitamin c. This interaction can also neutralize reactive nitrogen species to reduce oxidative damage[131,132].

The beneficial properties may be involved in reducing DCF-induced oxidative stress. Because oxidative stress is also to teratogenesis, the decrease in reactive radical species can offset the generation of malformations and their frequency [Figures 2 and 3], where spirulina achieved a significant restorative effect at 4 and 10 mg $L^{-1}$.

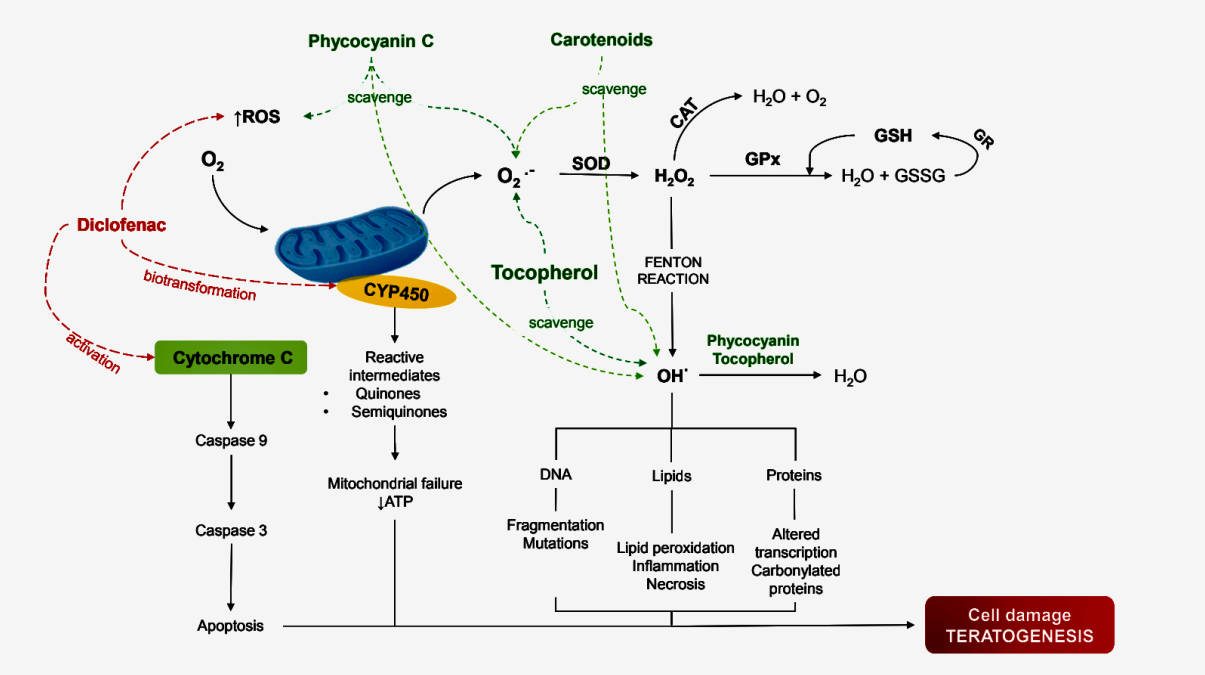


**Figure 7.** The proposed mechanism of damage reduction due to spirulina in *X. laevis* exposed to DCF. The main scavenge routes of spirulina are by some of its components. In red color the effects generated by diclofenac are shown: the production of reactive oxygen species, the activation of cytochrome CYP450 through its biotransoformation, and the activation of cytochrome C. In green color the components of spirulina and their action are shown as neutralizers of reactive oxygen species.

Spirulina also has substantial nutritional value and contains carbohydrates, proteins, minerals, and vitamins; these attributes may contribute to enhancing the growth and development of *X. laevis* exposed to DCF [Figure 4]; the total body size of embryos exposed to spirulina mixtures was similar to the control group, comparable results were reported in other organisms[81,112].

## CONCLUSIONS

DCF is a pharmaceutical found in water bodies and drinking water worldwide. Due to its physicochemical properties and mechanism of action, DCF is toxic to aquatic organisms, mainly through oxidative stress. Because DCF is a ubiquitous pollutant, it is necessary to identify compounds to reduce its adverse effects. In this work, DCF-induced oxidative stress increased SOD and CAT levels, caused malformations to the head and face, and caused growth inhibition. Spirulina, mediated by its antioxidant components that scavenge free radicals, reduces oxidative stress and the severity of malformations at all tested concentrations, with a higher effect at 4 and 10 mg $L^{-1}$. Spirulina should be considered for aquatic organism diets to protect them from toxicity induced by pharmaceutical products such as DCF. Studies on spirulina's potential protective effects against other pharmaceutical products, metals, and emerging pollutants are highly recommended.

## DECLARATIONS

### Author's contributions

Investigation, formal analysis, writing - original draft: Pérez-Alvarez I
Conceptualization, methodology, resources, writing - review & editing, supervision: Islas-Flores H
The investigation, formal analysis, writing - original draft: Sánchez-Aceves LM
Resources, writing - review & editing: Gómez-Oliván LM

Methodology, writing - review & editing: Chamorro-Cevallos G

### Availability of data and materials
Not applicable.

### Financial support and sponsorship
Not applicable.

### Conflicts of interest
All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate
All procedures followed the ethical protocols of care, use, and management of the species used in the Universidad Autónoma del Estado de Mexico. The specifications mentioned in the corresponding Official Mexican Standards were also considered (NOM-062-ZOO- 1999, Technical specifications for producing, caring, and using laboratory animals).

### Consent for publication
Not applicable.



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