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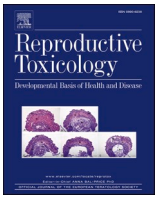

Spirulina (*Arthrospira maxima*) mitigates the toxicity induced by a mixture of metal and NSAID in *Xenopus laevis*

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ABSTRACT

Cadmium (Cd) is often detected in the environment due to its wide use in industry; also, NSAIDs are one of the most consumed pharmaceuticals, particularly diclofenac (DCF). Several studies have reported the presence of both contaminants in water bodies at concentrations ranging from ng L^{-1} to μg L^{-1}; in addition, they have shown that they can induce oxidative stress in aquatic species and disturb signal transduction, cell proliferation, and intercellular communication, which could lead to teratogenesis. Spirulina has been consumed as a dietary supplement; its antioxidant, anti-inflammatory, neuroprotective, and nutritional properties are well documented. This work aimed to evaluate if Spirulina reduces the damage induced by Cd and DCF mixture in *Xenopus laevis* at early life stages. FETAX assay was carried out: 20 fertilized oocytes were exposed to seven different treatments on triplicate, control, Cd (24.5 μg L^{-1}), DCF (149 μg L^{-1}), Cd + DCF, Cd+DCF+Spirulina (2 mg L^{-1}), Cd+DCF+Spirulina (4 mg L^{-1}), Cd+DCF+Spirulina (10 mg L^{-1}), malformations, mortality, and growth were evaluated after 96 h, also lipid peroxidation, superoxide dismutase and catalase activity were determined after 192 h. Cd increased DCF mortality, Cd and DCF mixture increased the incidence of malformations as well as oxidative damage; on the other hand, the results obtained show that Spirulina can be used to reduce the damage caused by the mixture of Cd and DCF since it promotes growth, reduce mortality, malformations, and oxidative stress in *X. laevis*

1. Introduction

Even though a wealth of evidence demonstrates the hazardous effects and toxicological interactions of pollutants, environmental risk assessments are generally based on isolated chemicals, assuming that mixtures always behave additively and therefore under-estimating the synergistic or antagonistic interactions that may occur [86]. Available data have shown that compounds such as pharmaceutical products and heavy metals are widely found in polluted water bodies due to their continuous discharges into the environment, relative persistence, and low elimination rates at WWTPs [113,47,8,9]. Among the pollutants that occur as mixtures, diclofenac (DCF) and cadmium (Cd) have received particular attention [75].

Diclofenac is one of the most prescribed non-steroidal anti-inflammatory pharmaceuticals (NSAID) worldwide due to its analgesic, anti-inflammatory, and antipyretic activity [64,85]. It is released into the aquatic system at concentrations ranging from ng L-1 to low μg L-1 through household, hospital, industrial, livestock, and wastewater discharges (A. M. [8,61,67,70,4,46,129]). Different studies on the toxicity of diclofenac in aquatic species, including fish, algae, Daphnia, and amphibians, have demonstrated that this pharmaceutical is capable of inducing oxidative damage [5], morphological deformities [69], development and growth arrest [103,104] and act as cardio- and neurotoxin [27].

While some metals are considered essential to different species due to their biological functions, including catalysis and cell signalization [130], several studies have pointed out the hazardous effects that these chemical pollutants have on wildlife and humans [7]. As a non-essential and highly toxic heavy metal, Cd is found in sediments, soil, air, food, drinking, and surface waters [83]. For decades, this metal can

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accumulate in human tissues, mainly the liver, kidney, and lungs, and induce toxic effects on aquatic species [119,126,62]. For instance, Lu et al. [68] reported that co-exposure to Cd and microplastics increased the accumulation of the metal in different tissues and led to changes in biochemical activities and histopathological endpoints on zebrafish (*Danio rerio*). Singh et al. [109] documented DNA damage, erythrocyte nuclear impairment, and lipid peroxidation following 30-day exposure to 0.05 ppm of Cd. Also, Wu et al. (2017) reported that exposure to 5 up to 500 μg L^{-1} of Cd is capable of promoting development and growth arrest, morphological malformations, histological changes, and lipid metabolism impairments on *Bufo gargarizans* as it acts as an endocrine disruptor in genes related to developmental features. On the other hand, Cd-induced mechanisms of mitochondrial dysfunction, autophagy, and oxidative damage after 30-day exposure to 0.25 up to 0.5 mg L^{-1} [37].

Available studies on the role of extrinsic supplementation with antioxidant molecules have gained particular attention in depleting oxidative damage produced by exogenous chemical substances. An antioxidant is a substance that, at low concentrations, can reduce or even inhibit the oxidation of different substrates found in cells [48,51]. Various micronutrients like carotenoids, vitamins, and natural flavonoids are considered valuable tools to counteract the action of free radicals in cells [82]. Spirulina (*Arthrospira maxima*) is an edible photosynthetic cyanobacterium of the genus *Arthrospira* commonly used as a nutraceutical product with a large number of benign nutrients including a rich protein content (almost 50–70% dry weight), vitamins (β-carotene, thiamine, riboflavin, and niacin) [55,65] and significant mineral composition, as well as bioactive compounds namely essential fatty acids and antioxidants like flavonoids, phenolic compounds and pigments (e.g., carotenoids, chlorophylls, and phycocyanin) [112]. Experimental evidence supports that Spirulina has various beneficial effects, including antioxidant [22], immunostimulatory [94], antineoplastic [36,116], antiviral [40] and an essential detoxifying activity against chemicals and heavy metals pollution [107,127]. Mahmoud et al. [71] showed that supplementation with 1% of Spirulina significantly improved the immunity of *Oreochromis niloticus* against *Pseudomonas fluorescence*. In addition, Mohanty and Samanta [78] also studied Spirulina's ameliorative effect on iron-induced oxidative stress after a 28-day diet in Indian knife fish (*Notopterus notopterus*).

Oxidative stress occurs upon uncontrolled free radical production resulting from a deficient counteracting antioxidant system. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced as by-products of oxidative phosphorylation occurring in the mitochondria are the main oxidative molecules found in cells [35,95]. In typical conditions, these products allow normal cellular functions; nevertheless, when redox generation capacity is exceeded due to the presence of extrinsic factors, antioxidant response system impairment, protein carbonylation, DNA damage, and lipid peroxidation take place, causing cellular atrophy, oxidative damage, and more so functional depletion [102]. Intrinsic antioxidant defenses include enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione (GSH) [19]. ROS, which comprises molecules such as the hydroxyl radical ($OH^{\cdot}$), superoxide anion ($O_2^{\cdot-}$), and hydroxyl peroxide (H_2O_2), play an essential role in embryonic development as they actively participate as second messengers in signaling transduction pathways and homeostatic processes during vertebrate development [21,50]. However, developmental alterations, teratogenic effects, and embryotoxic action caused by oxidative damage have been previously reported in aquatic organisms [49].

Xenopus laevis is widely used to assess developmental toxicity induced by chemical substances and multi-component mixtures in aquatic environments [26]. The standardized FETAX (Frog Embryo Teratogenesis Assay) is an acute (96-h) developmental screening assay conducted on *X. laevis* fertilized eggs that provide essential information about embryotoxicity and teratogenic malformations exerted by both single chemicals and composite environmental mixtures [87,93].

In previous studies, we found that exposure to Cd (Perez-Alvarez et al., 2021) and DCF (Perez-Alvarez et al., 2023) by themselves induced toxic effects in the early stages of *Xenopus laevis*; however, when we added Spirulina for 192 h, a significant decrease in mortality, frequency, and severity of malformations, as well as growth inhibition and oxidative damage was observed. Due to these results, it is essential to evaluate if Spirulina could have a protective effect on organisms exposed to mixtures of contaminants; and, therefore it can be considered a supplement to the amphibian diet that helps prevent or mitigate toxicity. The objective of this study was to evaluate the protective effect of Spirulina against the toxicity induced by the mixture of Cd and DCF in the early stages of the development of *Xenopus laevis* through the assessment of different endpoints, including mortality, malformations, growth inhibition, and oxidative damage and antioxidant activity biomarkers.

Table 1
Spirulina powder nutritional composition (by 100 g) (AEH Spiral Spring, Mexico).

Component	Amount	Component	Amount
Protein	65 g	**Zinc**	3 mg
Total lipids	6 g	**Beta carotenes**	201 mg
Polyunsaturated fat	6 g	**Tocopherol**	10 mg
Carbohydrates	16,4 g	**Thiamine**	3.5 mg
Fiber	8,3 g	**Riboflavin**	4 mg
Gama-linolenic acid	1 g	**Niacin**	14 mg
Alfa-linolenic acid	0.8 g	**Pantothenic acid**	100 μg
Sodium	900 mg	**Pyridoxine**	800 μg
Calcium	1 g	**Cobalamin**	250 μg
Phosphorus	800 mg	**Inositol**	64 mg
Magnesium	400 mg	**Phycocyanin**	15 mg
Iron	150 mg	**Chlorophyl**	1.1 mg
Potassium	1.4 g	**Carotenes**	370 μg

2. Materials and Methods

The procedures were carried out following the Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-*Xenopus* E-1439–12 80 (American Society for Testing Materials, 2019) and ethical protocols of care, use, and management of the species used in the testing of the Universidad Autonoma del Estado de México. The specifications mentioned in the corresponding Official Mexican Standards were also considered ([101], NOM-062-ZOO- 1999, Technical specifications for the production, care, and use of laboratory animals), and also complies with what is specified in Directive2010/63/UE.

2.1. Chemicals, reagents, and test solutions

All analytical grade reagents (> 99% purity), cadmium ($CdCl_2$), diclofenac, 3-amino-benzoic acid ethyl ester (MS-222), NaCl, $NaHCO_3$, KCl, $CaCl_2$, $CaSO_4{\cdot}2\,H_2O$, $MgSO_4$, were bought from Sigma-Aldrich (St. Louis, MO, USA). Spirulina (*Arthrospira maxima*) dried powder was bought from a local supplier (AEH Spiral Spring, Mexico); product characteristics can see in Table 1.

Composition of FETAX medium per liter of deionized water: 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 $MgSO_4$, final pH was 7.6–7.9.

For Cadmium 24.5 μg L^{-1} solution was prepared daily by dissolving $CdCl_2$ in FETAX medium; for diclofenac, a 149 μg L^{-1} solution was also prepared daily by dissolving diclofenac sodium salt in FETAX medium. Spirulina and cadmium-diclofenac mixtures were prepared by dissolving 2, 4, and 10 mg of Spirulina in a solution having 24.5 μg L^{-1} cadmium and 149 μg L− 1 diclofenac.

The concentration of cadmium (24.5 μg L^{-1}) was selected based on a previous study [89] and is the result of the construction of a concentration-response curve; for this purpose, cadmium was tested in 6 different concentrations (1, 4, 8, 16, 32 and 62.5 mg L^{-1}). The concentration selected for the mixture corresponds to the lowest test

Table 2
Test solutions.

Test solution	Composition
Control	FETAX medium
Spirulina (*Arthrospira maxima)*	10 mg L^{-1}
Cadmium	24.5 μg L^{-1} $CdCl_2$
Diclofenac	149 μg L^{-1} diclofenac sodium salt
Cd+DCF	24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac sodium salt
C+D+S 2	24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac sodium salt + 2 mg L^{-1} *Arthrospira maxima*
C+D+S 4	24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac sodium salt + 4 mg L^{-1} *Arthrospira maxima*
C+D+S 10	24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac sodium salt + 10 mg L^{-1} *Arthrospira maxima*

concentration with a statistically significant effect (0.5%, assumed to be the LOAEL) (San Juan Reyes et al., 2015). Diclofenac concentration (149 μg L^{-1}) was previously determined using the same method [90]. Spirulina (*Arthrospira maxima*) concentrations for the mixtures (2, 4, and 10 mg L−1) were selected based on a previous study [89], for the exposure of the control of Spirulina alone, we decided to use the highest concentration to visualize the maximum effect. Test solutions were prepared daily and were kept in darkness at 4 °C. The final composition of each test solution is as follows in Table 2.

2.2. Frog selection and husbandry

Xenopus laevis males and females three years old were obtained from the aquaculture center Aquanimals (Queretaro, Mexico). Frogs were housed in 60 L aquariums filled to 80% of their capacity with dechlorinated water at 21 ± 2 °C, pH 6.5–9, photoperiods 12 h light / 12 h darkness, total organic carbon < 10 mg L^{-1}, alkalinity, and hardness by Determination of $CaCO_3$ 16–400 mg L^{-1} and fed three times a week ad libitum with *Chrisotoma sp*. (0.5 ± 0.3 cm in length) and commercial food NUPEC pellets Purina®.

2.3. Induction of ovulation, fertilization, and oocyte selection

One male and one female were placed in a 40 L aquarium adapted with a plastic mesh suspended 3 cm over the bottom to separate the embryos from the adult frogs at 20 ± 2 °C temperature and pH 6.5 – 9. Ovulation and fertilization were induced by a single injection of Human chorionic gonadotropin hormone (CHORAGON®, Ferring) in the dorsal lymph sac, 300 IU for male and 700 IU for female; afterward, the aquarium was inspected for oviposition. Oocytes were extracted from the aquarium with sterile Pasteur pipettes and placed in Petri dishes for examination with a Zeiss Stemi 305 stereoscopic microscope to select fertilized oocytes with a spherical shape, homogeneous cell division, and in a stage of blastula (stage 8–10). Before experiments, the de-jellying of embryos was carried out by gentle swirling for 2 min in a 2% w/v L-cysteine solution prepared in FETAX solution.

2.4. Exposure

Ten mL of each test solution (Table 2) were poured into sterile plastic Petri dishes (50 mm) to minimize bacterial contamination; twenty oocytes were collected and placed in each Petri dish via Pasteur pipettes using a stereoscopic microscope. Embryos were kept at 21 ± 2 °C for 96 h; each experiment was performed in triplicate. Two replicates were completed (American Society for Testing Materials, 2019).

Test solutions were replaced every 24 h under a laminar flow hood; at that exact moment, the embryos were inspected, and the dead larvae and residues (if any) were removed to avoid microbial growth that might kill the live embryos; these data were recorded. After 96 h of exposure, larvae were inspected to see if they were swimming, and malformations and dead larvae numbers were recorded in a developmental parameter sheet. Later, larvae were euthanized by placing them in a Petri dish 50 mm containing 10 mL of 0.06% MS-222 solution. Each larva was measured from head to tail using Zen Blue Zeiss software (if the embryo was curved or kinked, the measurement followed the contour of the embryo), and each value was registered to determine the minimum concentration to inhibit growth (MCIG). Larvae were observed and evaluated in the microscope fitted with a Zeiss Axiocam 5 s camera to identify malformations, according to Atlas of Abnormalities [16]. At the end of the assay, larvae were disposed of following institutional standards for eliminating biological residues.

2.5. Oxidative stress assessment

Assays were conducted as described previously (Sections 2.1 to 2.4). To determine lipid peroxidation, superoxide dismutase and catalase in *Xenopus laevis* embryos were exposed for 192 h to ensure the development of their antioxidant system. Embryos start feeding at stage 45 [84], and 24 h after fertilization, the activity of SOD and CAT are developed; after 48 h, GSH is activated, and the activity of other enzymes increases [99]. If the exposure time is extended further than stage 50, the alimentation must be enriched [54], which would involve a change in experimental conditions. For this reason, organisms were exposed for 192 h, or Nieuwkoop stage 57, to ensure they were feeding and developing their antioxidant enzymes. Larvae were weighed and homogenized with phosphate buffer (pH 7.2) at 4 °C, 1:4 (w/v). All samples were centrifuged at 2500 rpm for 15 min.

2.5.1. Determination of total protein

Total protein was determined by [20] method. 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]. Microtubes rest for 5 min in darkness; next, absorbance was read at 595 nm, and 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.

2.5.2. Determination of superoxide dismutase (SOD) activity

SOD activity was determined by [76] method. In a quartz cuvette were added 40 μL of supernatant, 260 μL carbonate buffer solution [50 mM sodium carbonate (Sigma-Aldrich, Saint Louis, MO, USA), 0.1 mM EDTA (Sigma-Aldrich, St. Louis, MO, USA)], pH 10.2, and 200 μL of adrenaline (30 mM, Sigma- Aldrich, St. Louis, MO, USA). Absorbance was measured at 480 nm after 30 s and 5 min. SOD activity was determined by the molar extinction coefficient 21 M cm-1, and results were expressed as IU SOD/mg of protein.

2.5.3. Determination of catalase (CAT) activity

[96] method were followed to determine CAT activity. 30 μL of supernatant, plus 420 μL of isolation buffer solution [0.3 M sucrose (Vetec, Sigma-Aldrich, St. Louis, MO, USA), 1 mM EDTA, 5 mM HEPES, 5 mM KH_2PO_4 (Sigma-Aldrich, St. Louis, MO, USA)] and 300 μL of 20 mM H_2O_2 solution (Sigma-Aldrich, St. Louis, MO, USA) were converted into a quartz cuvette. Absorbance was determined at 240 nm, at 0, and after 60 s, CAT activity was estimated using the MEC of H_2O_2 0.093 mM/cm.

2.5.4. Determination of lipid peroxidation (LPX)

Lipid peroxidation was determined by the method [24]. In a glass tube, 10 × 75 mm were added 50 μL of supernatant, 450 μL Tris-HCl buffer solution (150 mM) pH 7.4, and 1 mL of 0.38% thiobarbituric acid (TBA) (Fluka, Sigma-Aldrich, Toluca) in 15% TCA, later incubated at 37 °C for 45 min; absorbance was determined at 535 nm. Results were expressed as mM malondialdehyde (MDA)/mg protein using the molar extinction coefficient (MEC) 1.56 × 105/M/cm.

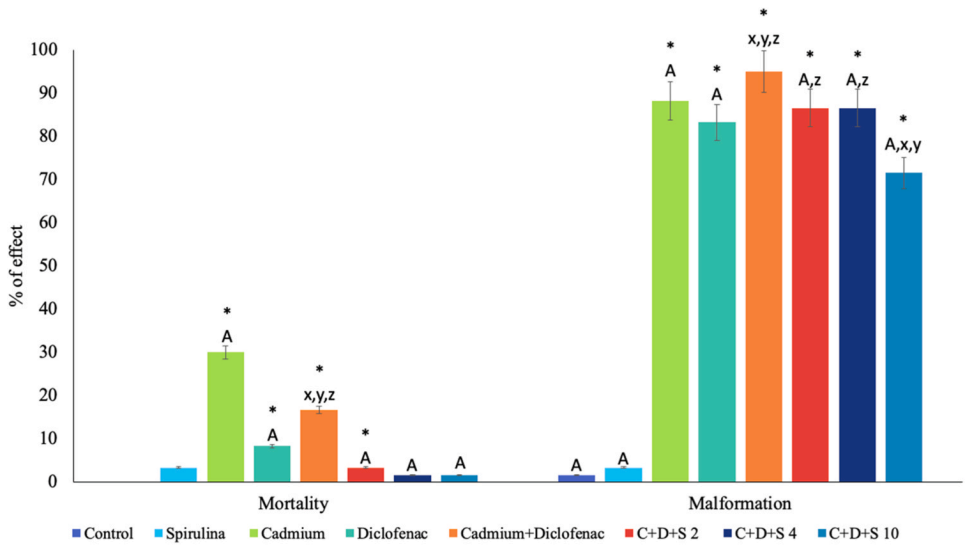

Fig. 1. Number of dead and malformed *Xenopus laevis* larvae after 96 h exposed to: control, spirulina 10 mg L^{-1}, cadmium 24.5 μg L^{-1}, Diclofenac 149 μg L^{-1}, Cd+DCF (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac), C+D+S 2 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac + 2 mg L^{-1} *Arthrospira maxima*), C+D+S 4 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac+ 4 mg L^{-1} *Arthrospira maxima*), C+D+S 10 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac + 10 mg L^{-1} *Arthrospira maxima*). Significant differences compared to (*) control, (A) Cd+DCF, (x) C+D+S 2, (y) C+D+S 4, (z) C+D+S 10 (One-way ANOVA and Fisher's test, $P < 0.05$).

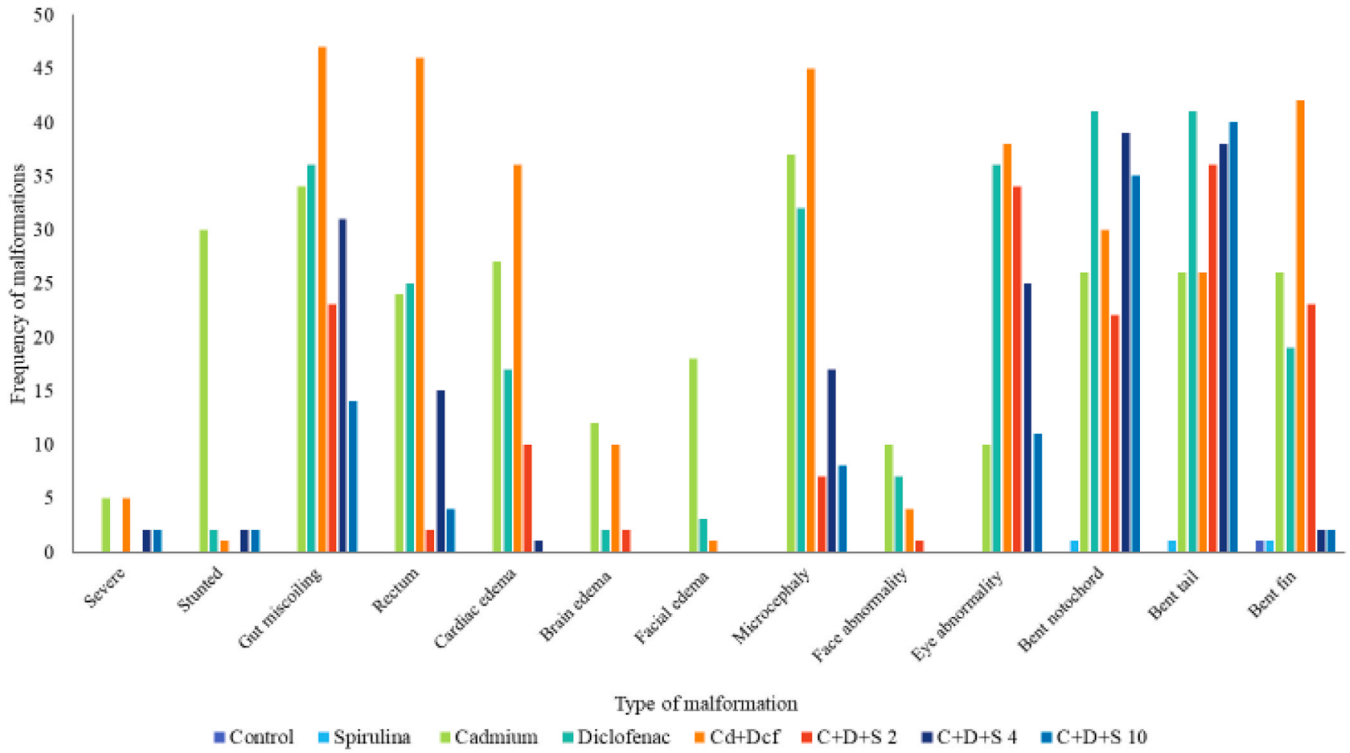

Fig. 2. Histogram of frequency for malformations induced in *Xenopus laevis* larvae after 96 h exposed to control, spirulina 10 mg L^{-1}, cadmium 24.5 μg L^{-1}, Diclofenac 149 μg L^{-1}, Cd+DCF (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac), C+D+S 2 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac + 2 mg L^{-1} *Arthrospira maxima*), C+D+S 4 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac+ 4 mg L^{-1} *Arthrospira maxima*), C+D+S 10 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac + 10 mg L^{-1} *Arthrospira maxima*).

2.6. Statistical analysis

All results were analyzed using the software Stat Graphics Centurion XIX. The results were expressed as the mean of three experiments performed under the same conditions. Shapiro-Wilk and Kolmogorov-Smirnov tests were performed to examine data distribution. Each larva was measured from head-to-tail in order to determine the differences in growth, and the mean values were compared by one-way analysis (ANOVA) and Fisher's multiple comparisons ($p < 0.05$). Lipid peroxidation and enzymatic activity (SOD and CAT) were analyzed by one-way analysis of variance (ANOVA) and Fisher's multiple comparisons ($p < 0.05$).

3. Results

Fig. 1 shows the % of mortality and % of organisms with malformation induced by control, Spirulina, cadmium, diclofenac, Cd+DCF, C+D+S 2, C+D+S 4, C+D+S 10. In the groups exposed to the mixtures with Spirulina (C+D+S 2, C+D+S 4, C+D+S 10), the mortality was reduced by 80–90%, and malformations were reduced in 12–24% compared to the Cd-DCF mixture. The mixture with 10 mg L^{-1} of Spirulina had a minor malformation rate compared with the other

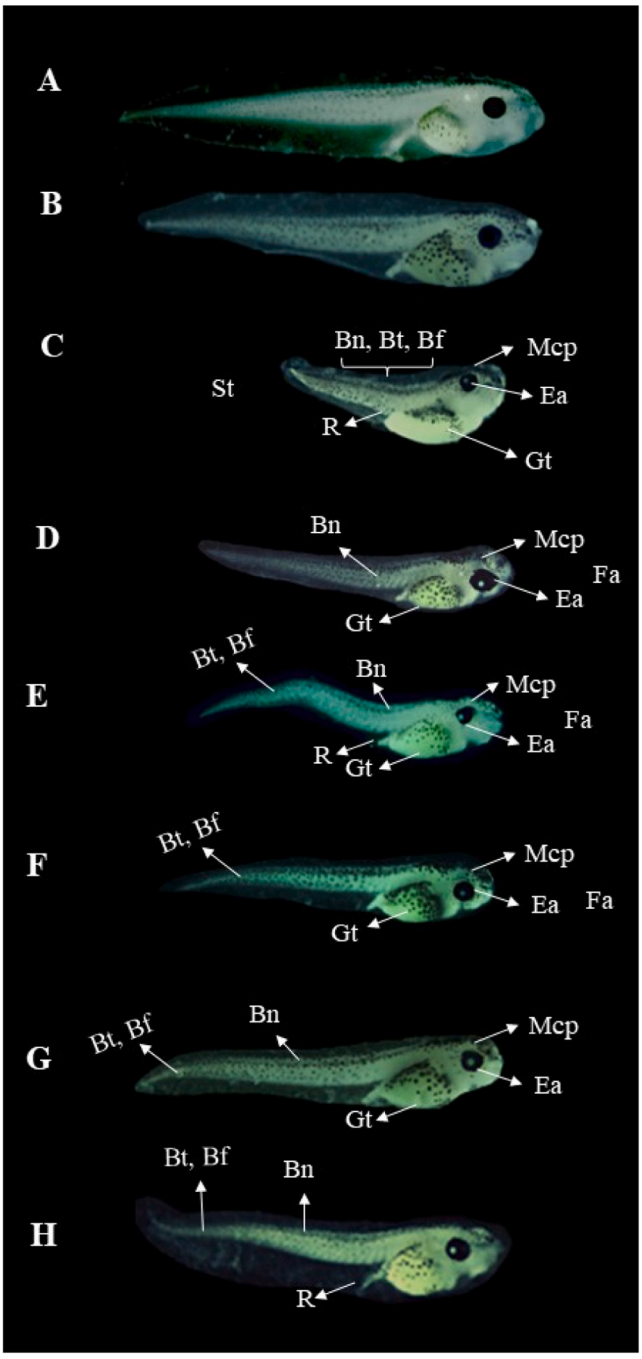

Fig. 3. Recurrent and distinctive malformations in *X. laevis* larvae after 96 h of exposure to (A) control, (B) spirulina 10 mg L^{-1}, (C) cadmium 24.5 μg L^{-1}, (D) Diclofenac 149 μg L^{-1}, (E) Cd+DCF (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac), (F) C+D+S 2 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac + 2 mg L^{-1} *Arthrospira maxima*), (G) C+D+S 4 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac+ 4 mg L^{-1} *Arthrospira maxima*), (H) C+D+S 10 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac + 10 mg L^{-1} *Arthrospira maxima*) Abbreviations: St: stunted, Gt: gut miscoiling, R: rectum, Mcp: microcephaly, Fa: face abnormality, Ea: eye abnormality, Bn: bent notochord, Bt: bent tail, Bf: bent fin, Ce: cardiac edema.

concentrations ($p < 0.05$ ANOVA Fisher's test).

Fig. 2 shows the incidence of embryos with malformation. The most frequent were gut malformation, rectum malformation, cardiac edema, microcephaly, eye malformation, and axial malformation (bent notochord, bent tail, bent fin). The frequency of malformations in mixtures with Spirulina (C+D+S 2, C+D+S 4, C+D+S 10) was lower than the group exposed to Cd+DCF, the mixtures with concentrations of 4 and 10 mg L^{-1} of Spirulina reduced the frequency of the malformations in more significant proportion.

On the other hand, as seen in Fig. 3, even though the embryos exposed to the Spirulina mixture presented malformations, these were not as severe as those induced by the contaminants alone or the Cd-DCF mixture. Although the ASTM E-1439–12 80 guide [12] does not include the severity of malformations, authors such as Hu et al. [53] have reported a classification based on phenotypic characteristics that allow a qualitative evaluation of the effects induced by contaminants.

Total body size measures of *X.laevis* are shown in Fig. 4; as can be seen, all the mixtures with Spirulina showed a significant enhancement in the head-to-tail size of the larvae compared to the mixture Cd-DCF (ANOVA, Fisher's test $p < 0.05$).

Fig. 5 (A) shows the antioxidant activity of SOD; there is an increase in exposure to Cd + DCF, the exposure to C+D+S 2, C+D+S 4, and C+D+S 10 generated a decrease in the activity of this enzyme, with the most significant decrease was observed in the groups exposed to C+D+S 10 (One-way ANOVA and Fisher's test, $P < 0.05$). Fig. 5 (B) shows the activity of catalase with a similar trend to SOD activity; an increase in enzyme activity was observed in the groups exposed to CD+DCF, nonetheless in groups exposed to C+D+S 2, C+D+S 4, C+D+S 10 CAT activity decreased, the reduction in activity was more significant in the group exposed to C+D+S 10 One-way ANOVA and Fisher's test, $P < 0.05$). Fig. 5 (C) shows the Determination of lipid peroxidation through the quantification of malondialdehyde in *X. laevis*; there is a significant increase in exposure to CD+DCF; on the other hand, there is a reduction statistically significant in the groups exposed to C+D+S 2, C+D+S 4, C+D+S 10 (One-way ANOVA and Fisher's test, $P < 0.05$).

4. Discussion

All biological systems are continuously exposed to a mixture of potentially dangerous chemical cocktails whose deleterious effects can be higher than the arithmetic sum of the individual responses [74]. Despite this, most ecotoxicology research and chemical regulation focus only on the hazard and exposure assessment of individual substances, and the problem of chemical mixtures in the environment is largely ignored [14], which leads to a lack of information about its possible effect on the environment and possible treatment or remediation. Among the most frequently found contaminants in aquatic environments are metals and pharmaceuticals. Cadmium is widely used in industrial processes, and its toxic effects have been widely described [42]. On the other hand, diclofenac is one of the most consumed pharmaceuticals and is also constantly reported in the environment. Both are contaminants constantly detected in water bodies, so these are expected to be present together. Also, both are hard to eliminate in water treatment plants [10]; there is evidence that metals can form complexes with pharmaceuticals which can alter the behavior of pharmaceuticals in the environment [31] and the toxicity in aquatic organisms; for these reasons it is important to carry out studies where the possible toxic effect of this kind of mixtures is evaluated; in addition, assess possible alternatives that help to minimize the possible damage induced by these mixtures.

Fig. 1 shows a significant increase in mortality of *Xenopus laevis* exposed to DCF and Cd + DCF mixture. Therefore the Cd exposure induced the highest significant mortality ($P < 0.05$); It can also be observed that the Cd-DCF mixture induces higher mortality than DCF by itself but less than Cd. On the one hand, this may be due to the prooxidant activity of Cd; it induces changes in the absorption of nutrients (Zn, Mg, and Cu) and alterations in morphogenesis and mitotic spindle [110]. On the other hand, DCF damages the mitochondria due to the high production of ROS, generates intermediate radicals, which later modify the synthesis of superoxide radicals, and induces failures in mitochondrial permeability, thus reducing ATP concentration; it also generates the production of ROS, and inhibits ATP phosphorylation; in

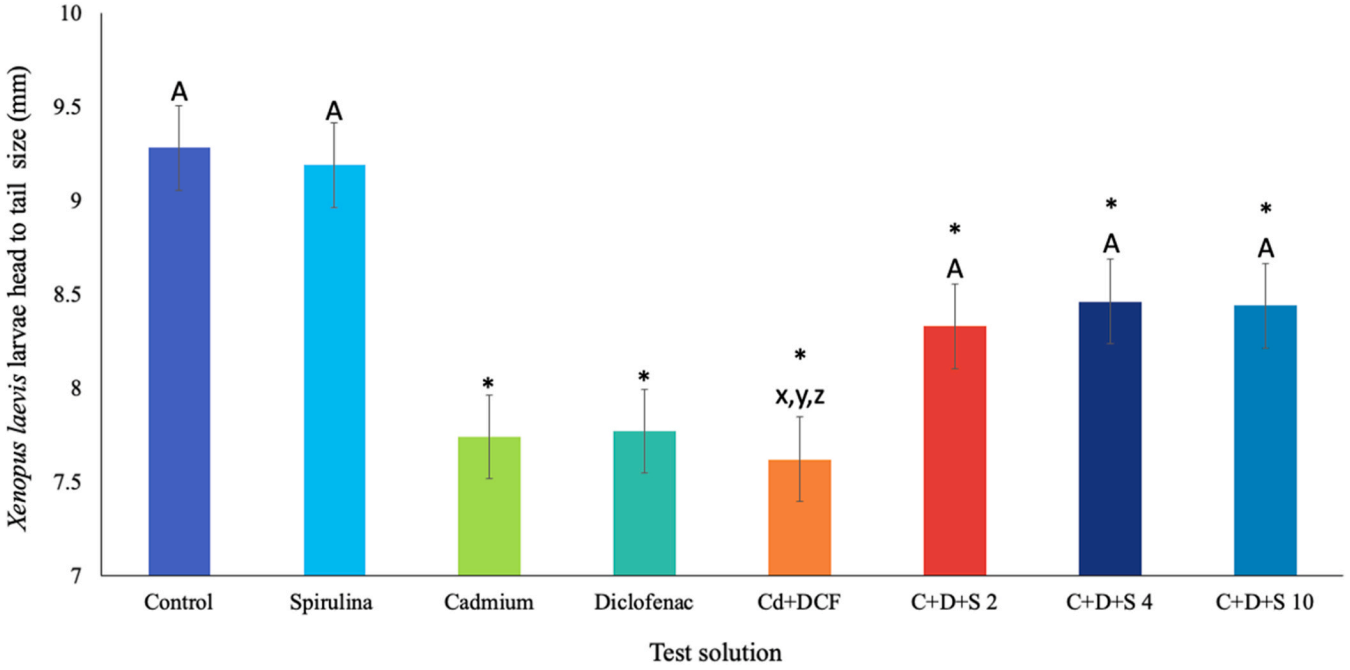

Fig. 4. Head to tail size of *Xenopus laevis* larvae exposed to: control, spirulina 10 mg L^{-1}, cadmium 24.5 μg L^{-1}, Diclofenac 149 μg L^{-1}, Cd+DCF (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac), C+D+S 2 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac + 2 mg L^{-1} *Arthrospira maxima*), C+D+S 4 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac+ 4 mg L^{-1} *Arthrospira maxima*), C+D+S 10 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac + 10 mg L^{-1} *Arthrospira maxima*). Significant differences compared to: (*) control, (A) Cd+DCF, (x) C+D+S 2, (y) C+D+S 4, (z) C+D+S 10 (One-way ANOVA and Fisher's test, $P < 0.05$).

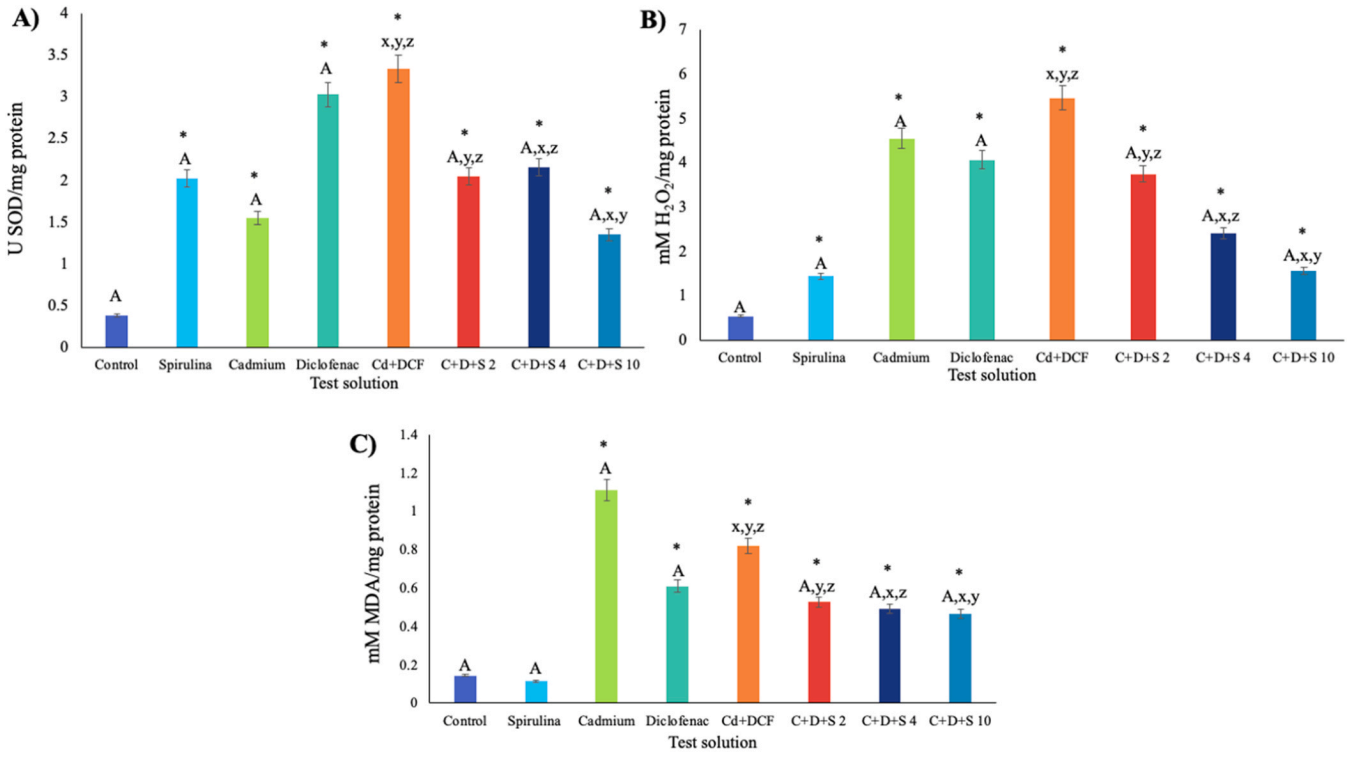

Fig. 5. (A) Superoxide dismutase activity, (B) Catalase activity, (C) Lipid peroxidation (level of malondialdehyde). A, B, and C were determined in *Xenopus laevis* exposed to: control, spirulina 10 mg L^{-1}, cadmium 24.5 μg L^{-1}, Diclofenac 149 μg L^{-1}, Cd+DCF (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac), C+D+S 2 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac + 2 mg L^{-1} *Arthrospira maxima*), C+D+S 4 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac+ 4 mg L^{-1} *Arthrospira maxima*), C+D+S 10 (24.5 μg L^{-1} $CdCl_2$ + 149 μg L^{-1} diclofenac + 10 mg L^{-1} *Arthrospira maxima)*. Significant differences compared to (*) control, (A) Cd+DCF,(x) C+D+S 2, (y) C+D+S 4, (z) C+D+S 10 (One-way ANOVA and Fisher's test, $P < 0.05$).

addition to the damage it generates in mitochondria, diclofenac interferes in the release of lysosomal enzymes of cytochrome C and the release of proapoptotic factors such as protease activating factor 1 which activates the precursor of caspase 9 that subsequently activates caspase 3 and generates cell death [43,56,98]. The reduction in mortality in groups exposed to mixtures with Spirulina may be due to the neutralization of ROS; Spirulina has protective effects against oxidative damage by reducing the activity of ROS and NOS. Also, Spirulina has chelating action. It binds to heavy metals [41], which can reduce the amount of free cadmium by preventing its interaction with biomolecules.

In addition to the above, it can also be observed (Fig. 1) that the Cd-DCF mixture induced a more significant number of embryos with malformations than the contaminants alone, so it could be inferred that the mixture is embryotoxic. Malformations with high incidence (Fig. 2) have been previously reported, cadmium exposure in *Xenopus laevis* [89,117] *Bufo gargarizans* [122], *Danio rerio* [33] and *Silurus soldatovi* [128], and in organisms exposed to diclofenac, *Xenopus laevis* [28,90] other amphibians [88], *Cyprinnus carpio*, [115]. These toxic effects may be due to cadmium modifying some processes such as apoptosis, cell cycle, stress, and immune response [66], inducing phosphorylation of p53, can replace Zn and cause errors in DNA repair which result in the accumulation of damaged DNA [32,38], and affects signaling processes, generates oxidative stress and alterations in redox potential. On the other hand, it has been shown that diclofenac modifies the mechanisms of cell signaling Wnt (Wnt3, Wnt8) and Gata 4, which is vital in the early stages of development since they are essential in body patterns and cell proliferation[29,88]. Since organogenesis is the most vulnerable stage and requires signaling processes to regulate cell proliferation and differentiation [52], cadmium and diclofenac can cause alterations in these processes and embryonic development, generating changes in the function and structure of tissues and, therefore, malformation [63].

In groups exposed to mixtures with Spirulina, the incidence, and severity of malformations decreased (Fig. 3); It is also observed that the concentration of 10 mg L^{-1} of Spirulina was the most effective in reducing mortality, incidence, and severity of malformation in *X. laevis* induced by exposure to Cd, DCF, and Cd+DCF. Similar effects were described by Argüelles-velázquez et al., [11] in rats exposed to cadmium; they also found a reduction in the frequency of malformations when rats were supplemented with Spirulina; this may be due to the effect of phycobiliproteins; which have anti-inflammatory activity [58] and decrease proinflammatory interleukins, tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1 β) and interleukin six (IL-6). Abu-Taweel et al., ($year$) [2] By reducing inflammation, edema can be reduced because edema may be the result of chronic inflammatory processes. Phycocyanin and β-carotenes inhibit the formation of proinflammatory cytokines, thus suppressing the expression of cyclooxygenase II and the production of prostaglandin E2, which act as an inflammatory mediator, also has antioxidant effects, can neutralize hydroxyl radicals responsible for oxidative damage [18]. Reducing cell damage may reduce the severity and incidence of malformations in *Xenopus laevis.* Regarding DCF, the beneficial effect of Spirulina has also been reported; it can reduce diclofenac-induced toxicity; in Wistar albino rats, diclofenac significantly increased the levels of liver enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), cell damage, impair liver and kidney functions, and generated a significant reduction in the activity of antioxidant enzymes superoxide dismutase, catalase, glutathione S transferase, and glutathione peroxidase; however, Spirulina normalized enzyme levels and liver function, and mitigated histomorphological damage in the kidney [92,97].

The ability of a material to inhibit embryonic growth is often the most sensitive indicator of developmental toxicity (American Society for Testing Materials, 2019). As shown in Fig. 4, the Cd-DCF mixture significantly reduced the larvae size compared to the control and the contaminants themselves ($P < 0.05$). Growth inhibition was observed in previous studies; [122] reported a body size reduction in *Buffo garganzianis* exposed to cadmium; this may be because cadmium can mimic other essential minerals, inhibit its absorption [45] and alters developmental processes; as well diclofenac induces the overproduction of ROS, affects mitochondrial functions, increases lipoperoxidation levels and generates cell damage, decreases mitochondrial respiration and ATP levels, which can lead to mitochondrial fragmentation [56], these can disrupt cell signaling and proliferation mechanisms, which end up in a decrease in embryonic development.

On the other hand, The mixtures with Spirulina benefit the growth of the larvae, although they did not reach the size of the controls. It may be because the cell wall of Spirulina is porous and allows cadmium to pass freely through it; when cadmium reaches the intracellular compartment, phytochelatins bind to Cd and neutralize it [18], phytochelatins oligomers synthesized from glutathione through phytochelatin synthase, and are usually induced by the exposure to heavy metals [60]. Another essential mediator is phycocyanin which can remove hydroxyl, alkoxy, and peroxyl radicals; phycocyanin also blocks the phosphorylation of mitogen-active protein kinases p38, which regulates cytokine synthesis [59]. Spirulina's vitamins, proteins, and minerals may be involved in improving development. Similar results have been reported previously, where the size and total weight of organisms decreased upon exposure to toxic agents, and after supplementation with Spirulina, weight, and size increased significantly [1,3,80,91]. It is important to note that the nutritional content of Spirulina (carbohydrates, proteins, minerals, vitamins) can contribute to an improvement in the development *of X. laevis*.

Regarding oxidative damage, Fig. 5 (C) shows the Determination of lipoperoxidation through the quantification of malondialdehyde in *X. laevis*; there is a significant increase of this biomarker in exposure to Cd, DCF and CD+DCF; similar results have been reported previously in *Chironomus riparius* larvae, the mixture Cd+DCF may induce oxidative damage by the decrease of glutathione transferase and glutathione reductase and hence an increase of ROS production ending up in oxidative stress [125]. It is also important to note that cadmium can form complexes with diclofenac and decrease the pharmacological effect of diclofenac due to the blockade of one more functional group with biological activity [118]; this interaction could stop Cd from being available to interact with biomolecules and thus reduces the damage caused by Cd to some extent; this may explain why lipid peroxidation in the group exposed Cd+DCF was lower than in the group exposed only to Cd; however, exposure to these xenobiotics in mixture eases their bioaccumulation [124] which could increase its long-term toxicity.

In the groups exposed to the mixtures with Spirulina, there is a decrease of MDA; it can be observed that the concentration of 10 mg L-1 was the one that produced the greatest significant reduction ($P < 0.05$) compared to the other two concentrations; probably because Spirulina have cysteine and methionine, these amino acids are involved in glutathione synthesis [23], previous studies have reported that Spirulina increases the level of GSH in mice [72]; the increment in GSH by Spirulina may decrease the amount of ROS and subsequently ameliorate the lipid peroxidation. Other study reports a decrease in lipid peroxidation after supplementation with Spirulina in organisms such as *Oreochromis niloticus* exposed to deltamethrin [1]; in rats exposed to sodium fluoride [15], in Wistar rats exposed to methotrexate [58], *Clarias gariepinus* exposed to sodium dodecyl sulfate [105], in rabbits exposed to lead acetate [6] in male rats exposed to sodium arsenite [17], Wistar rats exposed to cadmium [57], and in albino Wistar rats exposed to diclofenac [92,97].

Regarding the activity of antioxidant enzymes, Fig. 5(A) shows the SOD activity; It can be observed that there is a significant increase ($P < 0.05$) in the groups exposed to Cd, DCF, and Cd + DCF mixture compared to the control group; on the other hand, Fig. 5 (B) shows the activity of catalase with a similar trend to SOD activity; an increase in enzyme activity was observed in the groups exposed to Cd, DCF, and CD+DCF; this behavior may be the result of excessive production of reactive species ROS and RNS; the increase in the production of these

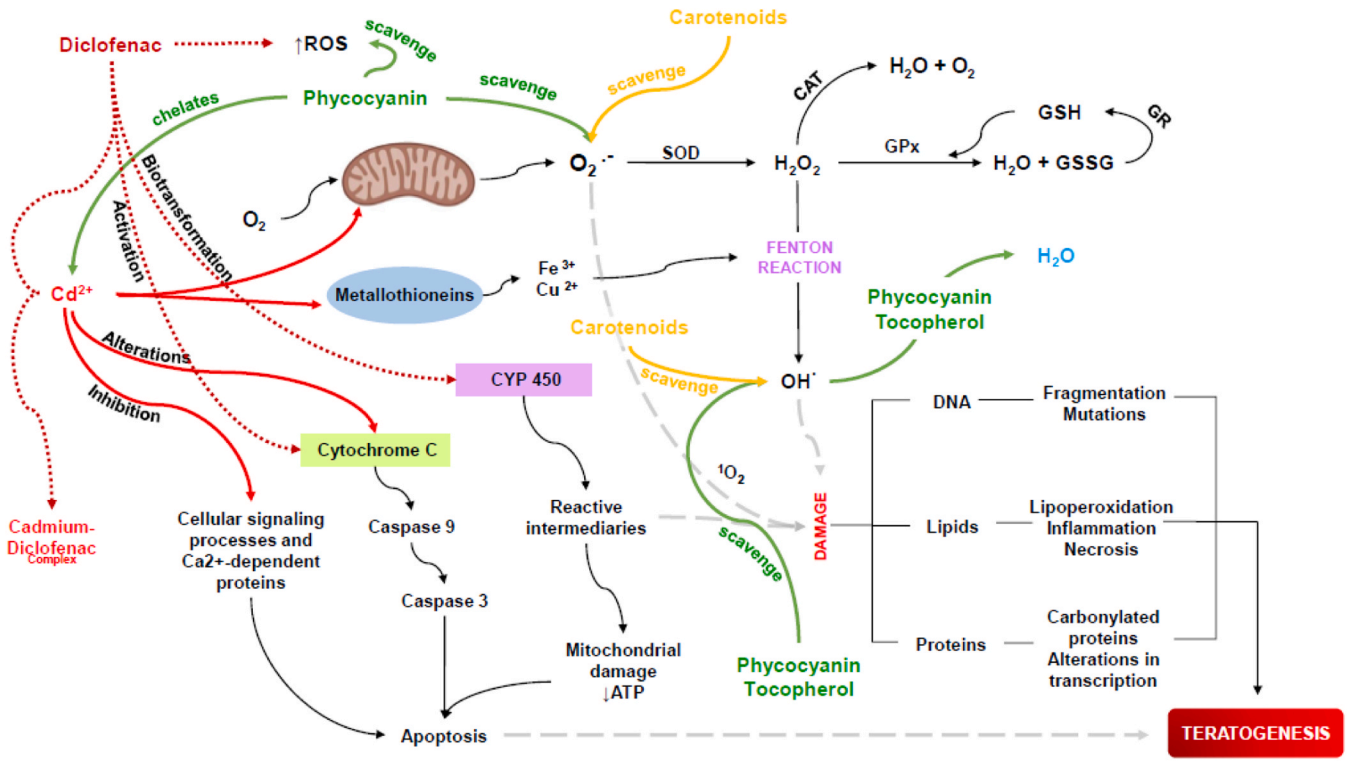

Fig. 6. Proposed mechanism of damage reduction by Spirulina in *Xenopus laevis* exposed to cadmium, diclofenac and its mixture.

species usually increases the levels of SOD since its function is to catalyze the process of dismutation, transforming $O_2^{\cdot-}$ in H_2O_2; likewise, the increases in CAT may be due to this enzyme is responsible for transforming H_2O_2 into H_2O [66], the increase in the activity of the enzymes has the purpose of neutralize ROS and protect the cell from oxidative damage. Also, this has been previously reported; Cd has been widely described as a pro-oxidant and generates adverse effects in aquatic organisms, most of which are related to the generation of oxidative stress [108,111,120–123,13,25,30,73,81]. On the other hand, diclofenac is a pharmaceutical that generates variations in the levels of acetylcholinesterase and glutathione S transferase [88]; it has increased the activity of glutathione S transferase and reduced glutathione reductase in *Cyprinus carpio* [115], in *Daphnia magna* induced oxidative stress and increased the production of reactive oxygen species associated with cytotoxic damage [44].

Regarding the activity of Spirulina, the exposure to mixtures with Spirulina generated a decrease in the activity of SOD enzyme; the most significant decrease was observed in the groups exposed to 4 and 10 mg L^{-1}; also, CAT activity decreased, while the reduction in activity was more significant in the groups exposed to the mixture of 10 mg L^{-1}. These results may be due to Spirulina has a chelating capacity. It binds to cadmium ions in such a way that it manages to inhibit the Fenton reaction, also neutralizes the radicals alkoxyl hydroxyl and peroxyl (Wu et al., 2016), inhibits the process of lipid peroxidation at an early stage, and reduces damage. Another critical component in Spirulina is tocopherol; it protects against lipid peroxidation because it has a chroman-ring in its structure that provides a reducing effect and can reduce peroxyl radicals to hydroperoxides which can be enzymatically degraded [77,79]. On the other hand, the carotenes present in Spirulina are also able to prevent lipoperoxidation processes, those triggered by singlet oxygen and peroxyl radicals; carotenes also can inhibit the production of prostaglandin E2 and nitric oxide through suppression of inflammatory mediators [106,114,39]. Carotenes cooperate in the antioxidant activity by regenerating tocopherol from its radical tocopherol; the resulting carotenoid radical could subsequently be restored by vitamin C; this sort of interaction can neutralize RNS and therefore reduce oxidative damage [34,100].

5. Conclusions

Fig. 6 represents the proposed route of oxidative damage induced by exposure to Cd and DCF and how this damage may be minimized through chelation and neutralization of ROS by Spirulina and its components in *Xenopus laevis*. As shown previously, the Cd + DCF mixture can alter *Xenopus laevis* development through different pathways, mainly oxidative stress. However, the damage was reduced in mixtures with Spirulina (*Arthrospira maxima*). It may be to the nutritional rate of Spirulina and due to the components named previously (phycocyanin, tocopherol, and carotenoids, to name some). All the mixtures with Spirulina showed beneficial effects; nonetheless, mixtures with concentrations of 4 and 10 mg L^{-1} were the most effective damage reduction. We suggest that Spirulina can be considered as a diet complement for amphibians to prevent toxicity induced by mixtures of contaminants in early life stages; further studies focused on the effects of Spirulina in amphibians and other aquatic organisms are recommended.

Declaration of Competing Interest

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.

Data Availability

Data will be made available on request.

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Scientists EDOMEX 2022".

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