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"EVALUACIÓN DEL EFECTO PROTECTOR DE LA *SPIRULINA* (*ARTHROSPIRA MAXIMA*) CONTRA LA TOXICIDAD INDUCIDA POR LA MEZCLA DICLOFENACO-CADMIO EN *XENOPUS LAEVIS*"

TESIS

QUE PARA OBTENER EL GRADO DE DOCTORA EN CIENCIAS Y TECNOLOGÍA FARMACÉUTICAS PRESENTA:

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1. RESUMEN

La ecofarmacovigilancia es la ciencia que se encarga de la detección, evaluación, comprensión y prevención de los efectos adversos de los productos farmacéuticos en el ambiente, dentro de estos se encuentran los antiinflamatorios no esteroideos (AINEs). En México son medicamentos de venta libre, por lo que son de los más vendidos y de uso más frecuente, en particular el diclofenaco (DCF). El cadmio (Cd) es uno de los metales encontrados en mayor cantidad en el ambiente debido a su amplio uso en distintos sectores industriales. Diversos estudios reportan la presencia de ambos contaminantes en cuerpos de agua en concentraciones que van de ng/L a µg/L, en sedimentos, agua superficial y subterránea; además, han demostrado que pueden inducir estrés oxidativo (EO) en diversas especies acuáticas; debido a que el EO está involucrado en el daño a proteínas, lípidos y ADN, puede afectar las señales de transducción, proliferación celular y la comunicación intercelular, lo que podría generar teratogénesis. Por otra parte, la Spirulina ha sido utilizada tradicionalmente como alimento en México y diversos estudios han demostrado que posee propiedades antioxidantes. Por lo anterior, en este trabajo se pretende evaluar la toxicidad inducida por DCF, Cd y la mezcla DCF-Cd en *Xenopus laevis*, para lo cual se realizó la evaluación de la teratogénesis mediante tres puntos principales: mortalidad, severidad de las malformaciones e inhibición del crecimiento, los cuales se utilizan para identificar compuestos teratógenos, así como la evaluación del EO a través de la determinación del grado de lipoperoxidación y la actividad de las enzimas antioxidantes: superóxido dismutasa y catalasa; además se evaluó el efecto protector de tres concentraciones de spirulina (2, 4, 10 mg/L) contra la teratogénesis y el EO inducido por estos contaminantes por sí solos y la mezcla en etapas tempranas del desarrollo de *Xenopus laevis.*

2. ABSTRACT

Ecopharmacovigilance is the science responsible for detecting, evaluating, understanding, and preventing the adverse effects of pharmaceuticals on the environment, within these kinds of compounds are nonsteroidal anti-inflammatory drugs (NSAIDs). In Mexico NSAIDs are over-the-counter medication, so they are one of the best-selling and most consumed drugs, particularly diclofenac (DCF). On the other hand, cadmium (Cd) is one of the metals detected in greater quantity in the environment due to its wide use in different industrial sectors. Several studies have reported the presence of both contaminants in bodies of water at concentrations ranging from ng/L to µg/L, in sediments, surface and groundwater, in addition, they have shown that they can induce oxidative stress (OS) in various aquatic species; because OS is involved in damage to proteins, lipids and DNA, it can affect transduction signals, cell proliferation, and intercellular communication, which could lead to teratogenesis. However, spirulina has been used as food in Mexico and other countries, some studies have shown that it has antioxidant properties. Therefore, this work aims to evaluate the toxicity induced by DCF, Cd and the DCF-Cd mixture in *Xenopus laevis*, for which the teratogenesis assessment was carried out by three main points: mortality, severity of malformations, and growth inhibition, which are implemented to identify teratogenic compounds, also the evaluation of OE was performed through the determination of lipid peroxidation level, and the activity of antioxidant enzymes: superoxide dismutase and catalase, in addition, the assessment of the protective effects of spirulina (2, 4, 10 mg/L) against teratogenesis and OS induced by DCF, Cd, and the mixture in early life stages of *Xenopus laevis* development was carried out.

CAPÍTULO I

3. INTRODUCCIÓN

En los últimos años ha existido una creciente preocupación por las concentraciones traza de los fármacos detectados en el ambiente y los efectos que estos pueden producir (Daughton, 2004; Daughton and Ternes, 1999; Halling-Sorensen et al., 1998). Después de la administración, algunos medicamentos se metabolizan, mientras que otros permanecen inalterados antes de ser excretados. Por lo tanto, una mezcla de fármacos y sus metabolitos alcanza el alcantarillado municipal y las plantas de tratamiento de aguas residuales (PTAR) (Kümmerer, 2001). Dependiendo de su polaridad, solubilidad en agua y persistencia, algunos de estos compuestos pueden no ser completamente eliminados o transformados durante el tratamiento de las PTAR y, por lo tanto, los fármacos inalterados y/o sus metabolitos pueden llegar a aguas superficiales. Estos productos también pueden entrar al ambiente a través de la eliminación de los medicamentos no utilizados y caducados, y de las emisiones de los procesos de fabricación (efluentes industriales) (Stackelberg et al., 2004). En el ambiente son considerados como contaminantes emergentes se definen como compuestos que no están regulados y que representan un riesgo para los ecosistemas acuáticos (Barceló, 2003; Deblonde et al., 2011).

Estos contaminantes incluyen a los antiinflamatorios no esteroideos (AINEs) los cuales son considerados uno de los grupos farmacoterapéuticos de mayor consumo mundial (Takagi et al., 2006), éstos cuentan con más de 70 millones de prescripciones anuales en Gran Bretaña, España y Japón. Además, son el sexto grupo más vendido en el mundo, con una producción anual de varias kilotoneladas. Son un grupo heterogéneo de medicamentos con actividad antiinflamatoria, analgésica y antipirética (Roberts J.L., 2001). Su mecanismo de acción se da a través de la inhibición del sistema de enzimas ciclooxigenasas, específicamente la COX-1 (constitutiva) y la COX-2, (inducida en el sitio de

inflamación), que son responsables de la conversión del ácido araquidónico en prostaglandinas (PGs) y tromboxanos (TBX), mediadores que intervienen en diferentes procesos homeostáticos en todo el organismo (Bacchi et al., 2012; Capone et al., 2003). Los fármacos más comunes dentro de este grupo farmacoterapéutico son el ácido acetilsalicílico (AAS), paracetamol (PAR), diclofenaco (DCF), ibuprofeno (IBP) y naproxeno (NPX). En México son fármacos de venta libre o OTC (por sus siglas en inglés: over the counter) y se consideran entre los medicamentos más utilizados, ya que solo en el año 2012 se reportaron ventas de medicamentos OTC por 1,840 mdd y se prevé que tengan una tasa media de crecimiento anual de 3.2% de 2013 al 2017 (Secretaría de Economía, 2013).

En el mundo, el DCF ha sido uno de los fármacos más estudiados y detectados en concentraciones que van desde ng/L hasta µg/L en diferentes ambientes acuáticos (Daughton and Ternes, 1999; Kolpin et al., 2002; Mezzelani et al., 2018; Santos et al., 2010). En México reportaron la presencia de diclofenaco en concentraciones que varían de 0.12 a 2.30 µg/L en efluentes del Valle de Mezquital (Siemens et al., 2008), de igual forma se reportaron concentraciones en un rango de 25 a 100 ng/L en agua superficial y de 11-5 ng/L en agua subterránea en efluentes del sistema Lerma-Cutzamala, el cual es uno de los más grandes sistemas en América latina (Félix-Cañedo et al., 2013). Por otro lado, se ha demostrado que estas concentraciones pueden producir efectos tóxicos en diferentes organismos acuáticos, entre los que se encuentran, afectaciones en la reproducción y el crecimiento, afectaciones en el comportamiento, y daño en enzimas antioxidantes en *Daphnia magna* (Du et al., 2016; Gómez-oliván et al., 2014; Nkoom et al., 2019), afecta tejidos, branquias y riñón de *Salmo trutta* (Hoeger et al., 2008), además estudios previos han reportado que induce estrés oxidativo y citogenotoxicidad en*, Hyalella azteca* y *Cyprinus carpio* (Gómez-oliván et al., 2014; Islas-Flores et al., 2013), también genera afectaciones en el desarrollo de diversos anfibios como *Xenopus laevis*, *Lithobates catesbeianus, Trachycephalus typhonius* y *Physalaemus albonotatus* (Cardoso-Vera et al., 2017; Chae et al., 2015; Peltzer et al., 2019).

Los metales pesados se consideran elementos potencialmente tóxicos dado que en concentraciones relativamente bajas afectan a los seres vivos y con el tiempo pueden acumularse en suelos donde pueden migrar hacia los cultivos y mantos acuíferos y de esta manera entrar a la cadena alimenticia; el cadmio (Cd) es uno de los metales encontrados en mayor cantidad en el ambiente debido a su amplio uso en distintos sectores industriales (Hayat et al., 2019; Järup and Åkesson, 2009) como la combustión de combustibles fósiles, fabricación de plásticos, pigmentos, entre otros productos. En México existen diferentes normas oficiales relacionadas con las concentraciones de metales pesados entre las que se encuentran las normas oficiales mexicanas 001, 002 y 004 de la Secretaría de medioambiente y recursos naturales (SEMARNAT, 2002, 1997, 1996) que manejan concentraciones permisibles para la emisión de Cd de 0.01 mg/L; sin embargo, existen reportes que indican que este metal se encuentra en concentraciones superiores a las establecidas por las normas oficiales en México (Pacheco et al., 2011; Vázquez-Sauceda et al., 2011), también se ha reportado que el Cd es capaz de inhibir la maduración de oocitos ya que interfiere con procesos de señalización que son de suma importancia en el periodo de organogénesis (Jorssen et al., 2015), esto debido a que su baja tasa de excreción permite que se bioacumule principalmente en hígado, riñón y órganos reproductivos, en donde afecta la ovulación, genera hemorragia y necrosis ovárica, además de fallas en el desarrollo embrionario (Slaby et al., 2017, 2016), por otra parte, debido a que el Cd es capaz de interactuar con los trasportadores de membrana ocasiona falla en la captación de calcio, hierro, zinc, cobre y manganeso, ya que se mimetiza con estos, lo que genera fallas en la captación de nutrientes, de igual forma tiene una alta afinidad por grupos fosfato, cistein e histidil, mismos que pertenecen a las cadenas terminales de diversas biomoléculas y al unirse a ellos genera fallas en distintos sistemas enzimáticos generando fallas en la función celular (Liu et al., 2018; Pizzi et al., 2017; Zhang and Reynolds, 2019).

La producción regulada de radicales libres (RL) y el mantenimiento de la homeostasis redox son esenciales para la salud fisiológica de los organismos. La generación de especies reactivas de oxígeno (EROs) es inducida por agentes internos y externos, tales como fagocitos, enzimas como el citocromo P450 monooxigenasa (CYP), radiaciones y productos químicos exógenos. De la misma manera, la generación de EROs se puede disminuir o revertir por diversas enzimas, llamadas antioxidantes, como son la superóxido dismutasa (SOD), catalasa (CAT) y glutatión reductasa (GR) (Kovacic and Somanathan, 2014). Las EROs endógenas sirven como un segundo mensajero en la transducción de señales y se cree que son importantes en el transporte de iones, la defensa inmunológica del huésped, la transcripción y la apoptosis de células (Dennery et al., 2007; Orrenius et al., 2007). Sin embargo, las EROs también pueden ser perjudiciales por su unión covalente o irreversible a macromoléculas celulares.

El EO, es un desequilibrio entre la generación de EROs y los mecanismos de defensa antioxidantes, causa la oxidación irreversible del ADN, proteínas y lípidos, lo que lleva a la inactivación de muchas enzimas y la muerte celular (Metcalfe and Alonso-Alvarez, 2010). Además, puede afectar la expresión génica al interferir con la actividad de factores de transcripción sensibles a redox y la transducción de señales mediante la oxidación de tioles (Dennery, 2007). Durante el período embrionario, esto puede dar lugar a defectos y retraso en el crecimiento, y en casos severos a la muerte (Sahambi and Hales, 2006; van Gelder et al., 2010; Wells et al., 2005). El desarrollo del embrión es especialmente susceptible a altos niveles de EROs debido a que tiene una defensa antioxidante débil, especialmente en etapas tempranas de la organogénesis(Hansen, 2006; Hansen and Harris, 2013), por lo que se ha propuesto que el EO esté implicado en la patogénesis de una amplia gama de defectos de nacimiento, incluyendo malformaciones esqueléticas, defectos de las extremidades, defectos del tubo neural y defectos cardiovasculares(Laforgia et al., 2018; Ryu et al., 2007).

Se define como teratogénesis a la alteración morfológica, bioquímica o funcional inducida durante el desarrollo embrionario, que es detectada durante la etapa

embrionaria, el nacimiento o con posterioridad (Conley and Richards, 2013). El ensayo de teratogénesis en embriones de rana Xenopus (FETAX por sus siglas en inglés, Frog Embryo Teratogenesis Assay- *Xenopus*) (American Society for Testing Materials, 2012) es una prueba para la detección de compuestos que pueden generar efectos teratogénicos o que tengan la capacidad de generar efectos tóxicos en el desarrollo, puede utilizarse con compuestos puros o mezclas complejas. La prueba emplea embriones de la rana con garras sudafricana *Xenopus laevis* en la etapa de blástula media durante el período de organogénesis. El potencial teratogénico del compuesto se determina tras el análisis de las observaciones de mortalidad y malformaciones en las larvas. Al mismo tiempo, esta prueba proporciona información sobre efectos embriotóxicos como la inhibición del crecimiento. Las primeras 96 horas del desarrollo embrionario de *Xenopus laevis* son similares a las primeras horas del desarrollo de muchos mamíferos, además, los embriones y larvas de ranas son considerados excelentes indicadores de la calidad del agua debido a su sensibilidad a compuestos químicos y su fácil mantenimiento (Greenhouse, 1976; Leconte and Mouche, 2013).

Por otra parte, la spirulina (*Arthrospira maxima*) es una cianobacteria fotosintética que se desarrolla en zonas tropicales y subtropicales, en cuerpos de agua que contienen elevadas concentraciones de carbonato y bicarbonato, se desarrolla más comúnmente en Estados Unidos, México, Asia y África. La spirulina también es una fuente importante de nutrientes, está compuesta en un 60% de contenido proteico, es baja en grasas, baja en calorías, libre de colesterol y además posee un elevado contenido de aminoácidos y fitonutrientes al igual que vitaminas y minerales (Soni et al., 2017), los primeros consumidores de esta alga como fuente alimenticia fueron las poblaciones mexicanas desde hace aproximadamente 400 años, era un alimento consumido por las poblaciones Maya y Tolteca, posteriormente se cultivó en el lago de Texcoco, además contiene diversos componentes que hacen de ella un agente antioxidante (Piñero Estrada, 2001); la ficocianina tiene la capacidad de capturar a los radicales libres y disminuir la producción de nitritos y la lipoperoxidación, también se ha reportado que las

ficocianinas son capaces de inhibir los factores proinflamatorios y la producción de prostaglandinas E2(Park et al., 2018; Romay et al., 2005; Wu et al., 2016); la spirulina también contiene beta carotenos mismos que poseen propiedades antinflamatorias y antioxidantes, además actúan como protectores ante la lipoperoxidación e inhiben la transcripción de citosinas inflamatorias (Park et al., 2018; Stahl and Sies, 2003). Los componentes de la spirulina actúan como neutralizadores de las ROS principalmente de los radicales hidroxilo, uno de los más dañinos, ya que es capaz de unirse covalentemente con lípidos, proteínas y ADN (Bhat and Madyastha, 2000).

4. JUSTIFICACIÓN

Debido a que la spirulina tiene un elevado contenido de moléculas antioxidantes, proteínas y nutrientes resulta de importancia realizar estudios para comprobar su eficacia como antioxidante en etapas tempranas del desarrollo, como es la organogénesis, ya que durante este periodo los embriones se encuentran en la fase más sensible de todo su ciclo de vida; *Xenopus laevis* es un organismo que se mantiene en un ambiente acuático durante las fases embrionarias y post-embrionarias y se encuentra frecuentemente expuestos a contaminantes de todo tipo como el DCF y el Cd de manera directa, y sin importar que las concentraciones de estos contaminantes en el ambiente sean bajas, estos organismos son capaces de evidenciar los daños producidos por ellos. Por lo anterior, en este proyecto se evaluó la teratogénesis inducida por DCF, Cd y la mezcla DCF-Cd en *Xenopus laevis* utilizando el ensayo FETAX mediante la evaluación de tres puntos: mortalidad, severidad de las malformaciones e inhibición del crecimiento, los cuales se utilizan para identificar compuestos teratógenos, además de realizar la evaluación del EO a través de la determinación del grado de lipoperoxidación y la actividad de las enzimas antioxidantes superóxido dismutasa y catalasa, finalmente se realizó la evaluación del efecto protector de la spirulina contra el daño inducido por DCF, Cd y la mezcla.

5. HIPÓTESIS

La spirulina disminuirá el estrés oxidativo, así como la mortalidad, severidad de malformaciones e inhibición del crecimiento inducido por DCF, Cd y la mezcla DCF-Cd en *Xenopus laevis*.

6. OBJETIVOS

6.1 Objetivo general

Evaluar el efecto protector de la spirulina contra la toxicidad inducida por DCF, Cd y la mezcla DCF-Cd en X*enopus laevis* utilizando el ensayo FETAX.

6.2 Objetivos específicos

Calcular el valor de concentración letal media (CL_{50}), la concentración efectiva media para malformaciones (CE_{50}), el índice de teratogénesis (IT), la concentración mínima para inhibir el crecimiento (CMIC) inducidas por DCF y Cd e identificar las malformaciones del desarrollo inducidas por DCF, Cd en X*enopus laevis.*

Evaluar la toxicidad subletal inducida por DCF, Cd y la mezcla DCF-Cd a través de la determinación del grado de lipoperoxidación, y la actividad de las enzimas antioxidantes: superóxido dismutasa y catalasa.

Demostrar el efecto protector de la spirulina (*Arthrospira maxima*) ante la incidencia en mortalidad y severidad de malformaciones, así como su efecto antioxidante contra DCF, Cd y la mezcla DCF-Cd en *Xenopus laevis.*

7. METODOLOGÍA

7.1 Organismos

El estudio se realizó en concordancia con los procedimientos de la guía estándar de la American Society for Testing Materials (ASTM (American Society for Testing Materials), 2012).

7.1.1 Especie

Los organismos se obtuvieron de un centro de acuacultura y biotecnología ubicado en el estado Querétaro. Para tener la seguridad de que las especies se encontraran aptas para su reproducción, se tomaron los siguientes criterios de selección: machos de *Xenopus laevis* de 7.5 a 10 cm de longitud (al menos 2 años) y hembras de 10 a 12.5 cm de longitud (al menos 3 años). Las hembras se identificaron por sus características físicas particulares, ya que son más grandes que los machos y tienen labios cloacales.

7.1.2 Mantenimiento

Los organismos se mantuvierón en un cuarto aislado, machos y hembras estuvierón separados en peceras de 60 L y 30 cm de altura, los lados de las peceras se cubrieron con plástico traslúcido para mantenerse opacos. La temperatura del agua se mantuvo en 21 ± 2ºC, con un fotoperiodo de 12 h luz/ 12 h oscuridad.

7.1.3 Dieta

Se alimentaron tres veces por semana *ad libitum* con *Chrisotoma sp.* de 0.5 +/- 0.3 cm de longitud, y con alimento comercial pellets Purina (NUPEC®).

7.1.4 Aclimatación

Para la aclimatación y mantenimiento de los adultos se utilizó agua natural. Los siguientes parámetros se determinaron una vez al mes: pH (entre 6.5 y 9), TOC (inferior a 10 mg/L), alcalinidad y dureza mediante la determinación de $CaCO_3$ (entre 16 y 400 mg/L).

7.2 Teratogénesis (Ensayo FETAX)

7.2.1 Reproducción

Se coloco una pareja de macho y hembra en un acuario de 40 L, equipado con una malla, de nylon, suspendida a 3 cm aproximadamente de la parte inferior en la que se depositaron los ovocitos, los lados del acuario se mantuvieron opacos, la temperatura del agua se mantuvo a 20 ± 2ºC, al igual que el fotoperiodo de 12 h luz/ 12 h oscuridad. Para la inducción del amplexus, la noche anterior al ensayo se aplicó una inyección subcutánea de 700 IU de hormona gonadotropina coriónica humana (hCG, disuelta en solución 0.9% de NaCl estéril) a las hembras y 300 UI a los machos en el saco doral-linfático, utilizando jeringas de 1 mL equipadas con agujas largas de calibre 26.

7.2.2 Selección de los huevos

A la mañana siguiente se revisó la puesta de los ovocitos, se extrajeron de la pecera utilizando la malla de nylon y se colocaron en un recipiente separado, posteriormente se limpiaron y se observaron utilizando un microscopio

espereoscopico (Zeiss Stemi 305) y se seleccionaron solamente aquellos que se encontraban en etapa de blástula media (Etapa 8).

7.2.3 Preparación del medio FETAX

El medio FETAX se preparó disolviendo 625 mg de NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4$ ● $2H_2O$ y 75 mg $MgSO_4$ por litro de agua destilada.

7.2.4 Preparación de las concentraciones madre y testigo

Para los grupos expuestos se utilizaron cinco concentraciones (1, 4, 16, 32 y 62.5 mg/L) de DCF y Cd por sí solos disueltos en medio FETAX, (el Cd funciono también como control positivo), el grupo testigo fue expuesto a medio FETAX únicamente. Todo el procedimiento se realizó bajo una campana de flujo laminar. Posteriormente se almaceno la solución madre en frascos ámbar, previamente lavados y etiquetados, el volumen mínimo de cada concentración final fue de 32 mL.

7.2.5 Tratamiento y siembra de los huevos

En la campana de flujo laminar, se colocaron 10 mL de cada concentración a evaluar de DCF y Cd en cajas Petri de 50 mm previamente marcadas. Adicionalmente se designó una caja con 10 mL de medio FETAX como control negativo. Con pinzas delgadas se recogieron los ovocitos que tenían una forma regular esférica y una división celular homogénea para lo cual se observó cada uno en el microscopio estereoscopio (Zeiss Stemi 305). Posteriormente, se colocaron 20 ovocitos en etapa de blástula media en cada caja Petri que contenía las diferentes concentraciones para los grupos expuestos o testigo. Finalmente,

los ovocitos expuestos se mantuvieron en una incubadora a una temperatura de 20 ± 2^{o}C durante 96 h, cada una de las pruebas se realizó por triplicado.

7.2.6 Monitoreo del cultivo

El medio FETAX de los grupos expuestos y testigo, se cambió diariamente bajo la campana de flujo laminar, para lo cual se añadieron 10 mL de cada solución preparada en nuevas cajas Petri estériles de 50 mm previamente marcadas las cuales se mantuvieron al menos 1 h 30 min a temperatura ambiente para asegurar que el medio FETAX estuviera a temperatura ambiente (20^{o}C) antes de añadir los oocytos. Cada 24 h se transferían las larvas vivas a nuevas placas de Petri con ayuda de un microscopio estereoscopio y pinzas finas de Moria. A las 48 y 72 h la transferencia de las larvas vivas se realizó con ayuda de una pipeta Pasteur de 2 mL. Se elaboro un informe diario con observaciones, número de larvas muertas y precipitados presentes en cada placa.

7.2.7 Examinación de las larvas

A las 96 h se comprobó que las larvas vivieran, en el caso contrario se informó en una hoja de parámetros de desarrollo, en la que se anotaron las malformaciones, además se registró la presencia de precipitados, las larvas muertas se removieron de las cajas de Petri, una vez realizado el registro de datos se procedió a la eutanasia de las larvas vivas, para lo cual se colocaron en una solución de MS-222 0,06% (dosis letal) en cajas Petri. Posteriormente se midieron todas las larvas desde la cabeza hasta el extremo de la cola utilizando el software ZEN Blue de Zeiss, y se reportó el valor en la hoja de parámetros de desarrollo. Se examino cada larva en el microscopio estereoscópico para identificar malformaciones de desarrollo, apoyándose en el Atlas de anomalías de Bantle (Bantle et al., 1991).

Después del examen, se eliminaron las larvas de acuerdo con las normas internas relativas a muestras biológicas.

7.3 Análisis de resultados

Una vez que se ha completado la prueba correspondiente al ensayo FETAX, se procedió al calculó de los parámetros y el análisis estadístico de los datos para lo que se utilizó el software Statgraphics Centurion XVI. Para la determinación de la concentración letal media (CL_{50}) y la concentración media de malformación (CE_{50}) se realizó un análisis probit con un valor de p <0.05. El índice teratogénico (IT) se determinó mediante la siguiente relación: IT = (CL_{50}) / (CE_{50}). La concentración mínima a la que se observan efectos (LOAEC) se determinó a través de una comparación múltiple de Dunett con valores $p<0.05$. Para determinar la concentración mínima que inhibe el crecimiento (CMIC) se compararon las medias de las mediciones de todas las larvas desde la cabeza a la cola usando un análisis de varianza de una vía (ANOVA) y prueba de múltiples rangos LSD Fisher, los valores se consideraron significativos a una $p<0.05$. Cada una de las pruebas se realizó por triplicado.

7.4 Evaluación del estrés oxidativo

Se prepararon los medios correspondientes a la LOAEC de cada contaminante (DCF, Cd y la mezcla DCF+CD) así como las mezclas con la spirulina en tres concentraciones (2, 4, 10 mg/L) y se siguió el procedimiento descrito del inciso 7.2.1 a 7.2.5; sin embargo para la evaluación del estrés oxidativo el período de exposición se extendió por 96 h más (es decir, una exposición de 192 h en total) con la finalidad de asegurar que las larvas cuenten con sistemas enzimáticos desarrollados. A las 192 h se pesaron las larvas de cada caja Petri y se homogenizaron mecánicamente en una proporción 1:4 (p/v) con solución

amortiguadora de fosfatos (pH 7.2), en frío (4^{o}C). Posteriormente se centrifugaron durante 30 min a 12,500 rpm a 4^{o}C, el sobrenadante fue separado y centrifugado a 2,500 rpm durante 15 min.

7.4.1 Determinación del grado de lipoperoxidación

Se determino mediante el método de Buege and Aust, 1978. En una celda de cuarzo se añadieron 500 µL del sobrenadante, se le adiciono 1 mL de solución reguladora tris-HCl 150 mM a pH 7.4 y se incubo a 37°C por 30 min. Se adicionaron 2 mL de la solución de ácido tiobarbitúrico al 0.38 % en ácido tricloroacético al 15 % y se incubo a 37 °C por 45 min. Posteriormente la muestra fue centrifugada a 12,500 rpm durante 15 min a -4 ºC y se determinó su absorbancia a 535 nm. Los resultados se expresaron en mM de malondialdehído /mg proteínas usando el coeficiente de extinción molar de 1.56 x 10^{5} $M^{-1}cm^{-1}$.

7.4.2 Determinación de la actividad superóxido dismutasa

La actividad de la SOD se determinó mediante el método de Misra and Fridovich, 1972. A 20 µL del sobrenadante se le adicionaron 150 µL de la solución amortiguadora de carbonatos (50 mM de carbonato de sodio y 0.1 mM de EDTA) a pH 10.2 y 100 µL de adrenalina 30 mM, y se determinó la absorbancia a 480 nm a los 30 seg y 5 min. La actividad de la SOD se determinó interpolando los datos en una curva tipo de SOD.

7.4.3 Determinación de la actividad de la catalasa

La actividad de la CAT se determinará mediante el método de Radi et al., 1991. A 20 µL del sobrenadante se le adiciono 1 mL de solución amortiguadora de aislamiento (0.3 M sucrosa, 1 mM EDTA, 5 mM HEPES y 5 mM KH_2PO_4) y 0.2 mL de la solución de H_2O_2 20 mM. Posteriormente se determinó la absorbancia a 240 nm, a un tiempo de 0 y 60 seg y se calculó la actividad de la CAT por minuto mediante el coeficiente de extinción molar del H_2O_2 (0.093 mM^{-1} cm^{-1}).

7.5 Análisis de resultados

Para los biomarcadores de estrés oxidativo se realizó un análisis ANOVA de una vía y una prueba de rangos múltiples LSD Fisher con una $P< 0.05$ utilizando el software Statgraphics Centurion XVI.

7.6 Evaluación de la mezcla DCF-Cd

Para la evaluación del efecto producido por la mezcla DCF-Cd, los ovocitos se expusieron a una mezcla de las concentraciones calculadas de LOAEC de DCF y Cd por sí solos, posteriormente se siguió el mismo procedimiento descrito en los apartados 7.2 y 7.4.

7.7 Evaluación del efecto protector de la Spirulina

Para la evaluación del efecto protector de la spirulina, los ovocitos se expusieron por triplicado a la LOAEC de DCF y Cd por sí solos y a la mezcla DCF-Cd; de igual forma los ovocitos se expusieron a las mezclas de cada contaminante con 3 concentraciones diferentes de spirulina, (como se muestra en la tabla 1) 2, 4 y 10 mg/L, posteriormente se siguió el mismo procedimiento descrito en los apartados 7.2 y 7.4 respectivamente. Para el análisis de los datos se utilizó un análisis

ANOVA de una vía y prueba LSD Fisher con una $P< 0.05$ utilizando el software Statgraphics Centurion XVI.

Tabla 1. Grupos de exposición para la evaluación del efecto protector de la spirulina ante la toxicidad de diclofenaco, cadmio y la mezcla DCF-Cd.

Grupo de exposición	**Tratamiento**
Control	Medio FETAX
Cadmio	0.04 mg/L $CdCl_2$
Cd+S 2	0.04 mg/L $CdCl_2$ + 2 mg/L *Arthrospira maxima*
Cd+S 4	0.04 mg/L $CdCl_2$ + 4 mg/L *Arthrospira maxima*
Cd+S 10	0.04 mg/L $CdCl_2$ + 10 mg/L *Arthrospira maxima*
Diclofenaco	0.14 mg/L diclofenaco
DCF+S 2	0.14 mg/L diclofenaco + 2 mg/L *Arthrospira maxima*
DCF+S 4	0.14 mg/L diclofenaco + 4 mg/L *Arthrospira maxima*
DCF+S 10	0.14 mg/L diclofenaco + 10 mg/L *Arthrospira maxima*
DCF+Cd	0.04 mg/L $CdCl_2$ + 0.14 mg/L diclofenaco
DCF+Cd+S 2	0.04 mg/L $CdCl_2$ + 0.14 mg/L

	diclofenaco + 2 mg/L *Arthrospira maxima*
DCF+Cd+S 4	0.04 mg/L $CdCl_2$ + 0.14 mg/L diclofenaco + 4 mg/L *Arthrospira maxima*
DCF+Cd+S 10	0.04 mg/L $CdCl_2$ + 0.14 mg/L diclofenaco + 10 mg/L *Arthrospira maxima*

Capítulo II

8. RESULTADOS

8.1 Articulo 1

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Protective effects of Spirulina (Arthrospira maxima) against toxicity induced by cadmium in Xenopus laevis

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Protective effects of Spirulina (Arthrospira maxima) against toxicity induced by cadmium in Xenopus laevis

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Abstract:	Spirulina (Arthrospira maxima) has been recognized as a superfood and nutraceutical by its high nutritional value, and the benefits of its consumption; it is an important source of lipids, proteins, vitamins, minerals, and antioxidants. It is known that Spirulina has positive effects on the toxicity induced by pharmaceuticals and metals. Heavy metals such as cadmium are frequently used in industrial activities, cadmium is continuously detected in water bodies and can generate adverse effects on aquatic organisms even at low concentrations. The aim of this study was to evaluate the protective effect of spirulina (Arthrospira maxima) against the toxic effects induced by cadmium, in the early life stages of Xenopus laevis. Twenty Xenopus laevis embryos were exposed to five different treatments on triplicate, control, cadmium (CdCl2 0.04 mg/L) and three spirulina mixtures Cd+S 1 (0.04 mg/L CdCl2 + 2 mg/L spirulina), Cd+S 2 (0.04 mg/L CdCl2 + 2 mg/L spirulina), Cd+S 3 (0.04 mg/L CdCl2 + 10 mg/L spirulina); after 96 h of exposure: Malformations, mortality and length were evaluated; also lipid peroxidation (LPX), superoxide dismutase (SOD) and catalase (CAT) were determined. All spirulina treatments decreased mortality from 34 to 50 %, malformations were reduced on incidence from 36 to 68 %. Treatment Cd+S 3 decreased growth inhibition significantly. Spirulina treatment Cd+S 2 decreased lipidic peroxidation and antioxidant activity; these results suggest that spirulina (Arthrospira maxima) can decrease the mortality, frequency of malformations, severity of malformations, growth inhibition, and oxidative damage induced by cadmium in Xenopus laevis embryos.
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Protective effects of Spirulina (*Arthrospira* 1 *maxima*) against toxicity induced by cadmium in *Xenopus laevis*

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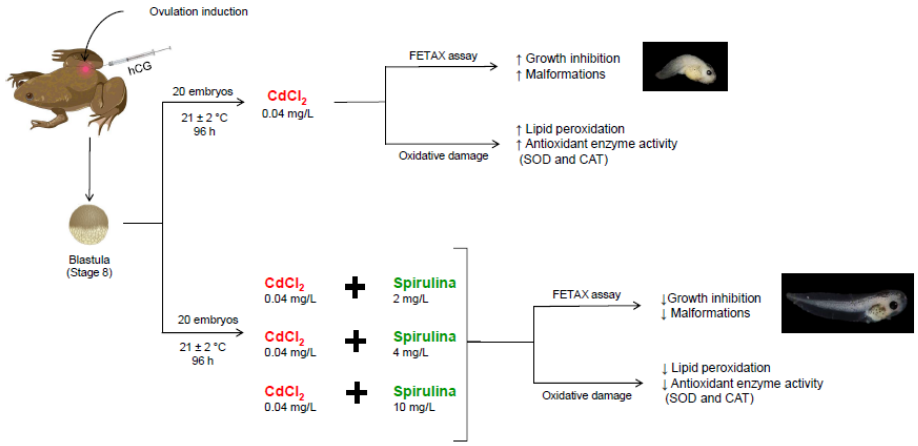
15 **Graphical abstract**



16

17 **Abstract**

18 Spirulina (*Arthrospira maxima*) has been recognized as a superfood and nutraceutical by its high
19 nutritional value, and the benefits of its consumption; it is an important source of lipids, proteins,
20 vitamins, minerals, and antioxidants. It is known that Spirulina has positive effects on the toxicity induced
21 by pharmaceuticals and metals. Heavy metals such as cadmium are frequently used in industrial activities,
22 cadmium is continuously detected in water bodies and can generate adverse effects on aquatic organisms
23 even at low concentrations. The aim of this study was to evaluate the protective effect of spirulina
24 (*Arthrospira maxima)* against the toxic effects induced by cadmium, in the early life stages of *Xenopus*
25 *laevis*.
26
27 Twenty *Xenopus laevis* embryos were exposed to five different treatments on triplicate, control, cadmium
28 ($CdCl_2$ 0.04 mg/L) and three spirulina mixtures Cd+S 1 (0.04 mg/L $CdCl_2$ + 2 mg/L spirulina), Cd+S 2
29 (0.04 mg/L $CdCl_2$ + 2 mg/L spirulina), Cd+S 3 (0.04 mg/L $CdCl_2$ + 10 mg/L spirulina); after 96 h of
30 exposure: Malformations, mortality and length were evaluated; also lipid peroxidation (LPX), superoxide
31 dismutase (SOD) and catalase (CAT) were determined.
32
33 All spirulina treatments decreased mortality from 34 to 50 %, malformations were reduced on incidence
34 from 36 to 68 %. Treatment Cd+S 3 decreased growth inhibition significantly. Spirulina treatment Cd+S
35 2 decreased lipidic peroxidation and antioxidant activity; these results suggest that spirulina (*Arthrospira*
36 *maxima*) can decrease the mortality, frequency of malformations, severity of malformations, growth
37 inhibition, and oxidative damage induced by cadmium in *Xenopus laevis* embryos.

38
39 **Keywords:** *Arthrospira maxima*, *Xenopus laevis*, cadmium, antioxidant, malformations, oxidative stress.

Introduction

Aquatic pollution has become one of the most important problems worldwide, water bodies are continuously over loaded with pollutants such as pesticides, pharmaceuticals, household and personal care products, industrial additives, heavy metals among others found in urban, hospital, and industrial wastes (Hayati et al., 2017). Heavy metals are released in to aquatic environments daily from atmospheric depositions, geologic processes or through anthropogenic activities (Authman, 2015). Metallic elements including iron, arsenic, lead and cadmium have shown to be toxic even at the low concentrations (Alhomaidan et al., 2015; Marin et al., 2015)

Cadmium (Cd) is one of the most toxic metals occurring in water bodies either naturally or as an anthropogenic pollutant arising from various sources, mainly industrial and agricultural activities (Järup and Åkesson, 2009) and has been detected in water in variable concentrations from 0.0001 to 0.0354 mg/L and in soils 0.019-0.143 mg/Kg (Rangkadilok et al., 2015; Salnikova et al., 2018) (Anatoly V. Skalny 2018, Nuchanart Rangkadilok 2014). Cadmium is a nonessential highly toxic metal for the aquatic biota with a weak hydrophilicity, high persistence, low rate of excretion and a strong bio accumulative behavior mainly in kidneys, liver and reproductive organs of many aquatic species (Farag et al., 2015; Hu et al., 2015).

Since cadmium shows a long environmental half-life, it has been related to severe deleterious effects in aquatic organisms and wild-life (Chan and Cheng, 2003). *In vitro* cadmium stimulates the inhibition of mitochondrial respiratory chain complexes triggering the increase of reactive oxygen species (ROS) (Wang et al., 2004) as well as the induction of DNA impair and apoptotic response in zebrafish embryos (Cambier et al., 2010; Chan and Cheng, 2003). This metal inhibits not only the DNA repairing system but also interfere with reduced glutathione (GSH) involved in the oxidative stress response and the redox cycling (Giaginis et al., 2006).

Mouchet et al., 2006 evaluated the potential toxic effects of Cd in *Xenopus laevis* larvae founding that at environmental concentrations 2, 10, 30 µg/L; this metal causes genotoxicity, increases production of metallothionein; and also it up-regulates *sod* cytoplasmic and mitochondrial genes, as well as mitochondrial metabolism (*coxI*) and detoxification (*mt1*), which is responsible for detoxification processes. Sharma and Patiño 2009 demonstrated that levels of Cd up to 860 µg/L delay metamorphosis in *Xenopus laevis* tadpoles. Cd promotes the production of free radicals via peroxide induction, enhancing oxidative cell biomarkers in *Meretrix meretrix* exposed to 1.5, 3, 6 and 12 mg/L (Xia et al., 2016). On the other hand, Wu *et al*. 2017 found that embryos of *Bufo gargarizans* exposed to 5 up to 500 µg L^{-1} of Cd shown an inhibition of growth and development, as well as an induction of morphological malformations mainly axial and fin flexures, abdominal edema and delayed growth. This study also shown that cadmium might have an endocrine-disrupting action in embryos as it was capable to change the mRA expressions of TRα, *Dio*2 and *Dio*3, genes related to developmental features.

Exogenous supplementation with antioxidant molecules, might have an essential role in the depletion of oxidative damage caused by heavy metals on mammals and aquatic organisms (Bashandy et al., 2016; Rodríguez-Sánchez et al., 2012; Szuroczki et al., 2016).

Spirulina (*Arthrospira maxima)* is an edible free-floating filamentous blue-green microalgae that belongs to the cyanobacteria class with photosynthetic activity (Deng and Chow, 2010; Kulshreshtha et al., 2008). It has multiple pharmacological and nutraceutical properties as it is highly rich in protein (ranges between 60-70%), γ-linoleic and linolenic acids, β-carotene and vitamins (Castro-García et al., 2018; Kulshreshtha et al., 2008; Mühling et al., 2005; Rodríguez-Sánchez et al., 2012). Phytochemicals and phycobiliproteins as chloropyll α, zeaxanthin, C-phycocianin, cryptoxanthin and allophycocianin (Yan *et al*., 2011; Kumar, Desai and Dwivedi, 2016) are also found in significant amounts in the *Arthrospira genus*. Due to the presence of bioactive compounds found in spirulina, multiple potential health benefits associated with consumption have been reported (Kumar, Desai and Dwivedi, 2016; de la Jara *et al*., 2018). Spirulina is known to have anti-inflammatory (Aladaileh et al., 2020; Deng and Chow, 2010; Joventino et al., 2012) , antioxidant (Abdelkhalek and Ghazy, 2014; Ferruzzi and Blakeslee, 2007; Nasirian et al., 2018; Rodríguez-Sánchez et al., 2012), neuroprotective (Lima et al., 2017; Pérez-Juárez et al., 2016), antimutagenic properties as well as properties for preventing the enhancement of oxidative stress and cellular damage induced by heavy metals (Castro-García et al., 2018; Rodríguez-Sánchez et al., 2012).

Studies carried out with *Arthrospira genus*, have evidenced the potential benefits of this microalgae for aquatic organisms exposed to environmental toxicants. Kilany *et al*. (2017) demonstrated the protective role of supplementary spirulina against oxidative stress induced by diazinon in Nile tilapia founding that

spirulina improved liver and kidney functions showing a significant enhancement of antioxidant activity. Sayed and Authman 2018 studied the potential ameliorative influence of *Spirulina platensis* (SP) in *Clarias gariepinus* exposed to sodium dodecyl sulfate (SDS), founding that the SP supplementation restored biochemical and genetical variations induced by the SDS including hepatic and renal dysfunctions, disruption in enzymatic and non-enzymatic antioxidants and apoptosis in erythrocytes among others. On the other hand, Mahmoud *et al*. (2018) demonstrated that feeding supplementation with Spirulina (*Arthrospira platensis*) improved growth performance, feed utilization, immune response and oxidative stress response in a trial performed with *Oreochromis niloticus* challenged with *Pseudomonas fluorescence*.

The presence of pollutants in the aquatic environment stimulates a variety of toxicity mechanisms including oxidative stress, known as an imbalance between prooxidant species and antioxidant defenses due to the overproduction of reactive oxygen species (ROS), and the depletion of protective intracellular mechanisms (Abdelkhalek and Ghazy, 2014; Ganesan et al., 2016). The quantification of biomarkers, has been extensively used to determine the effects of ROS in main biomolecules like lipids, proteins, and DNA in organisms exposed to environmental toxicants (Valavanidis et al., 2006)

The impact of oxidative stress on embryotoxic development and teratogenic effects has been well documented (Adeyemi, Da Cunha Martins and Barbosa, 2015; Ganesan *et al*., 2016; Wu *et al*., 2017;Sant *et al*., 2017; Timme-laragy *et al*., 2018). Redox status plays an essential role during important embryonic cellular processes including proliferation, differentiation, signaling and apoptosis (Dennery, 2007). ROS are crucial to the normal embryo development, cellular signaling, control of cellular mechanisms and as second messengers by regulating key transcription factors (Dennery, 2007; Wu et al., 2017a; Xia et al., 2016). Increase of ROS produces not only oxidative stress but also other toxicity mechanisms that may result in developmental alterations, functional abnormalities, congenital malformations and embryo death in aquatic organisms (Gilbert-Barness, 2010; Pašková, Hilscherová and Bláha, 2011).

Xenopus laevis African clawed frog is an important model organism for vertebrate development as it has been used as a suitable bioindicator due to its fully aquatic environment, short reproductive cycle and the throughout molecular and cellular characterization of its embryogenesis (Brausch et al., 2010; Cardoso-Vera et al., 2017; Pike et al., 2019; Robert and Ohta, 2009). The fertilization and development occurs externally allowing direct and easy observation during the embryogenesis, the embryo development takes place in a short period of time within 96 hpf, manipulations are simplified as the eggs have a relatively large size (Peuchen et al., 2016). The ecotoxicological importance of *Xenopus laevis* on the evaluation of embryotoxicity and teratogenic malformation using the FETAX assay has been well established (Bonfanti et al., 2004; Mouchet et al., 2006; Pérez-Alvarez et al., 2018). The frog embryo teratogenesis assay-*Xenopus* FETAX method, constitutes a valuable tool to evaluate the developmental toxicity hazards of pure chemical agents and mixtures as it determines developmental endpoints including lethality, morphologic alterations and minimum growth-inhibition concentration (Cardoso-Vera et al., 2017; Hu et al., 2015; Isidori et al., 2016). Amphibians are considered useful organisms for assessment of toxic effects exerted by a variety of pollutants (Hopkins, 2007).

In the present study, antioxidant effects of spirulina (*Arthrospira maxima*) were evaluated against cadmium-induced toxicity in early life stages of *Xenopus laevis*; throughout the evaluation of mortality, malformations, growth inhibition, lipoperoxidation, and antioxidant activity of superoxide dismutase and catalase.

1. Materials and Methods

1.2 Test substances

Cadmium chloride $CdCl_2$ (CAS# 10108-64-2, > 99% purity), 183.32 Da, Sigma-Aldrich (St. Louis, MO), *Arthrospira maxima* dried and as powder was purchased from a local supplier AEH Spiral Spring, Mexico. All other chemical reagents were acquired from Sigma-Aldrich (St. Louis, MO).

4

2.1 Test organisms

Adult *Xenopus leavis* were obtained from the aquaculture center ´Aquanimals´, located in Queretaro, Mexico. The selection criteria for males were 8 to 10 cm long and 2 years old, and for females 10 to 12.5

cm long and 3 years old. Females were identified by presence of cloacal labia and a larger size. *X. laevis* were acclimated in natural water, with the following conditions: pH 6.5 to 9, total organic carbon <10 mg/L, alkalinity and hardness by determination of $CaCO_3$ 16 to 400 mg/L; parameters were determined monthly. Organisms were kept in a light-protected room with photoperiods 12 h light /12 h darkness. Males and females stayed separate in 60 L fish tanks filled to 80% capacity, at 21 ± 3 °C. They were fed three times a week *ad libitum* with *Chrisotoma sp*. (0.5 ± 0.3 cm in length) and / or commercial food pellets NUTRIPEC® Purina.

1.3 FETAX assay

This study was carried out in accordance with the standard guide of the American Society for Testing Materials, ASTM (ASTM E1439 − 12 , 2012) All experiments were performed on triplicate.

1.3.1 FETAX medium

FETAX medium was prepared by dissolving 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4$ C_2H_2O and 75 mg $MgSO_4$ in a final volume of 1 L distilled water, final pH was 7.6 - 7.9. All reagents were purchased from Sigma-Aldrich (St. Louis, MO).

1.3.2 Breeding induction

One male and one female were placed in 40-L aquarium with a plastic mesh suspended roughly 3 cm over the bottom, into which oocytes could be laid. Aquarium sides were opaque and water temperature was 20 ± 2 °C, with a 12 h light / 12 h darkness photoperiod.
Human chorionic gonadotropin hormone (CHORAGON®, Ferring) was dissolved in a sterile NaCl 0.9% solution, subsequently males and females were administered in the dorsal lymph sac with 300 IU and 700 IU respectively, using a 1 mL hypodermic syringes fitted with long 26-gauge needles.

1.3.3 Embryo selection

Next day morning, fish tanks were inspected for oviposition. Fertilized oocytes were extracted from the tank using the nylon mesh, and sterile Pasteur pipettes and were placed in separate containers for examination under a Zeiss Stemi 305 stereoscopic microscope to select those in medium blastula stage (stage 8).

1.3.4 Preparation of cadmium, spirulina mixtures and control solutions

A stock solution of cadmium was prepared daily by dissolving 1 mg in 1 L of FETAX medium. Negative control was exposed to FETAX medium. Mixtures were prepared by dissolving 2, 4 and 10 mg of spirulina in a 0.04 mg/L cadmium solution. The entire procedure was done under a laminar flow hood. The stock solutions were stored in amber glass bottles at 4°C.

Cadmium concentration (0.04 mg/L) was selected based on a previous exposure described on point 2.3.5.1, spirulina (*Arthrospira maxima*) concentrations (2, 4 and 10 mg/L) were selected based on experimental procedures, due there are no concentrations reported to be used in embryonic stages of development of aquatic organisms or amphibians yet. Table 1 shows the exposed groups.

Table 1. Exposure groups

Exposure solution	Mixtures
Control	FETAX medium
Cadmium	0.04 mg/L of $CdCl_2$
Cd+S 1	0.04 mg/L of $CdCl_2$ plus 2 mg/L of *Arthrospira maxima*
Cd+S 2	0.04 mg/L of $CdCl_2$ plus 4 mg/L of *Arthrospira maxima*
Cd+S 3	0.04 mg/L of $CdCl_2$ plus 10 mg/L of *Arthrospira maxima*

Exposure solution
Mixtures
Control FETAX medium
Cadmium 0.04 mg/L of $CdCl_2$
Cd+S 1 0.04 mg/L of $CdCl_2$ plus 2 mg/L of *Arthrospira maxima*
Cd+S 2 0.04 mg/L of $CdCl_2$ plus 4 mg/L of *Arthrospira maxima*
Cd+S 3 0.04 mg/L of $CdCl_2$ plus 10 mg/L of *Arthrospira maxima*

1.3.5 Exposure

1.3.5.1 Cadmium

Under a laminar flow hood, 10 mL of each cadmium solution were added in previously labelled 50-mm petri dishes, embryos with spherical shape, homogeneous cell division, and in medium blastula stage (stage 8) were collected using dissecting forceps and a stereoscopic microscope, 20 oocytes were placed in each petri dish containing the different cadmium solutions for exposed (1, 4, 8, 16, 32, 62.5 mg/L) and control group (FETAX medium) all on triplicate, petri dishes were kept in the incubator at 21 ± 2 °C in the dark for 96 h.

1.3.5.2 Mixtures cadmium + spirulina (*Arthrospira maxima*)

Under a laminar flow hood, 10 mL of each solution were added in previously labelled 50-mm petri dishes. Embryos with spherical shape, homogeneous cell division and in medium blastula stage (stage 8) were collected using dissecting forceps and a stereoscopic microscope, 20 oocytes were placed in each petri dish containing different mixtures (Cd+S 1, Cd+S 2, Cd+S 3) and control group, all on triplicate; petri dishes were kept in the incubator at 21 ± 2 °C in the dark for 96 h.

1.3.6 Culture monitoring

Control, cadmium, and spirulina mixtures solutions were replaced daily under the laminar flow hood. To this end, 10 mL of each test concentration were added in new and previously labelled, sterile 50-mm Petri dishes which were maintained for 1 h 30 min at room temperature to ensure that the medium was at 20 ± 2°C before embryos were transferred. Each 24 h, live embryos were transferred to new petri dishes, using the stereoscopic microscope and Pasteur pipettes to separate them from the dead embryos. A daily record was taken, the number of dead embryos and precipitates (if any) in each culture were documented.

1.3.7 Examination of larvae

At 96 h of exposure larvae were checked for swimming, if not swimming, this was noted in a developmental parameter sheet. Precipitates (if any) were also registered, as well as the number of dead larvae.

X.laevis larvae were euthanized by placing them in a petri dish containing a 0.06% MS-222 solution (lethal dose). Each larva was then measured straight from head to tail using Zen Blue Zeiss software, values were registered in the developmental parameter sheet. Next, each larva was evaluated under the

microscope fitted with a Zeiss Axiocam 5s camera, to identify malformations in accordance to Atlas of Abnormalities (Bantle et al.,1991) and other resources. Cadmium LC_{50} and LOAEL were determined to perform the evaluation of spirulina and cadmium mixtures.

After examination, larvae were disposed of following institutional standards for the elimination of biological samples.

1.4 Oxidative stress evaluation

FETAX medium was prepared and embryos were exposed to the stated mixtures and control on triplicate, using the procedures described in sections 2.3.1 to 2.3.7. At 96 h, larvae in each plate were weighed and mechanically homogenized 1:4 (w/v) with 4 °C phosphate buffer solution (pH 7.2), then centrifuged at 2500 rpm for 15 min. All determinations were made on the supernatant.

1.4.1 Determination of lipid peroxidation (LPX)

Determination was made by the Buege and Aust, 1978 (1978) method.

1 mL Tris-HCl buffer solution (150 mM) pH 7.4 was added to 500 mL of supernatant. The resulting sample was incubated at 37 °C for 30 min, then 2mL of 0.38% thiobarbituric acid (TBA) (Fluka, Sigma-Aldrich, Toluca) in 15% TCA were added prior to incubation at 37 °C during 45 min. The samples were then centrifuged at 12,500 rpm and -4 °C for 15 min, subsequently absorbance was determined at 535 nm. Results were expressed as mM malondialdehyde (MDA)/mg protein, using the molar extinction coefficient (MEC) of 1.56 x 10_5/M/cm.

1.4.2 Determination of superoxide dismutase (SOD) activity

SOD activity was determined by Boehringer, 1987 method.

Samples were delipidated by the addition of 30 µL chloroform and 50 µL methanol to 100 µL of serum. The mixture was shaken for 1 min and centrifuged for 15 min at 6000 rpm, and the supernatant was kept. In a quartz cuvette, 50 µL of distilled water were mixed with 1.4mL Tris-HCl buffer solution (50 mM, pH 8.20) and 25 mL EDTA solution (1 mM), subsequently supplementing with 25 µL of a pyrogallol solution (0.124 mM). Absorbance was read at 0, 10 and 60 s, at 420 nm. The assay was performed on triplicate and the difference was taken as the optical density (OD) of the blank (DODblank), representing the difference in ODs for the non-inhibited reaction.
For each sample, 50 µL of supernatant were mixed in a quartz cuvette with 1.4 mL of Tris-HCl buffer solution and 25 mL of EDTA solution. Next, 25 µL pyrogallol was added and absorbance was read at 410 nm, at 0, 10 and 60 s. Results were derived by the following formula:
| [(Mean DODsample x 100)/(Mean DODblank)]-100|x 0.6
Where:
DODsample ¼ difference in optical densities in the sample
DODblank ¼ difference in optical densities in the blank

1.4.3 Determination of catalase (CAT) activity

CAT activity was determined by the Radi et al., 1991 method.

1 mL of isolation buffer solution [0.3M sucrose (Vetec, Sigma-Aldrich, St. Louis), 1mM EDTA, 5mM HEPES, 5mM KH_2PO_4 (Vetec)] was added to 20 µL of supernatant, plus 0.2 mL of 20mM H_2O_2 solution (Vetec). Absorbance was read at 240 nm, at 0 and 60 sec, and CAT activity per minute was estimated using the MEC of H_2O_2 (0.093 mM/cm).

2. Results

3.1 FETAX assay

Exposure to cadmium (1, 4, 8, 16, 32, 62.5 mg/L) mortality in 8, 16, 32, and 62.5 mg/L was 100%, medium lethal concentration (LC_{50}) was 4.275 mg/L (PROBIT analysis $p<0.05$), afterwards lowest adverse effect level (LOAEL) was calculated to be 0.04 mg/L.

3.1.1 Mortality and malformations

The mortality of *X. laevis* exposed to cadmium and spirulina mixtures Cd+S 1, Cd+S 2, Cd+S 3 is shown in figure 1. A reduction from 44.44 % up to 50% was observed in spirulina mixtures, the most effective mixtures were Cd+S 2 and Cd+S 3. Number of larvae with malformations is shown in figure 2. The three spirulina mixtures (Cd+S 1, Cd+S 2, Cd+S 3) reduced the incidence of malformations on 35.85 % to 67.93%. The main malformations observed (figure 3) were: Stunted embryos, gut miscoiling, rectum malformation, cardiac edema, abdominal edema, microcephaly, and axial malformations (bent tail, notochord, and fin), also spirulina mixtures (Cd+S 1, Cd+S 2, Cd+S 3) reduced the severity of malformations, Cd+S 3 was the most effective mixture in attenuating malformations as shown in figure 4. To summarize, Cd+S 3 reduced mortality, incidence on malformations, and severity of malformations, it is also important to highlight that all spirulina mixtures reduced damage in some proportion.

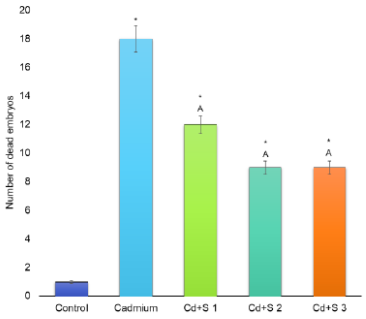


Fig.1 Number of *Xenopus laevis* dead embryos after 96h exposed to control, cadmium 0.04 mg/L, Cd+S 1 (cadmium 0.04 mg/L + spirulina 2 mg/L) Cd+S 2 (cadmium 0.04 mg/L + spirulina 4mg/L), Cd+S 3 (cadmium 0.04 mg/L + spirulina 10 mg/L), significant differences relative to: (*) control, (A) cadmium (One-way ANOVA and LSD Fisher, $p>0.05$).

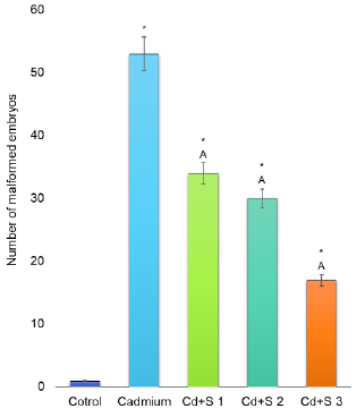


Fig.2 Number of *Xenopus laevis* embryos with malformations after 96h exposed to control, cadmium 0.04

mg/L, Cd+S 1 (cadmium 0.04 mg/L + spirulina 2 mg/L) Cd+S 2 (cadmium 0.04 mg/L + spirulina 4mg/L), Cd+S 3 (cadmium 0.04 mg/L + spirulina 10 mg/L), significant differences relative to: (*) control, (A) cadmium (One-way ANOVA and LSD Fisher, *p>0.05*).

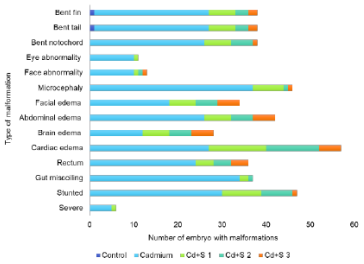


Fig.3 Histogram of frequency for malformations induced in *Xenopus laevis* embryos by the exposure to control, cadmium 0.04 mg/L, Cd+S 1 (cadmium 0.04 mg/L + spirulina 2 mg/L) Cd+S 2 (cadmium 0.04 mg/L + spirulina 4mg/L), Cd+S 3 (cadmium 0.04 mg/L + spirulina 10 mg/L) for 96 h.

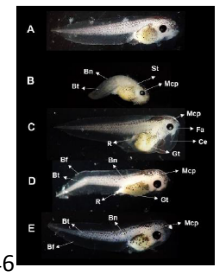


Fig.4 Most frequent and representative malformations in *X.laevis* embryos after 96 h of exposure to (A) control, (B) cadmium 0.04 mg/L, (C) Cd+S 1 (cadmium 0.04 mg/L + spirulina 2 mg/L) (D) Cd+S 2 (cadmium 0.04 mg/L + spirulina 4mg/L), (E) Cd+S 3 (cadmium 0.04 mg/L + spirulina 10 mg/L). Abbreviations: 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.

3.1.2 Growth inhibition

Head to tail measurements of larvae are shown in fig. 5. Spirulina mixtures reduced growth inhibition induced by cadmium, Cd+S 3 was the most effective mixture due to the length of larvae exposed to this mixture had a similar size than the control group according to statistical analysis (ANOVA, LSD Fisher *p<0.05*).

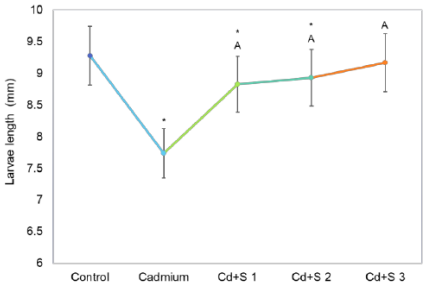


Fig.5 Head to tail measurement of *Xenopus laevis* larvae exposed to control, cadmium 0.04 mg/L, Cd+S 1 (cadmium 0.04 mg/L + spirulina 2 mg/L) Cd+S 2 (cadmium 0.04 mg/L + spirulina 4mg/L), Cd+S3 (cadmium 0.04 mg/L + spirulina 10 mg/L) for 96 h, significant differences relative to: (*) control (A) cadmium (One-way ANOVA and LSD Fisher, *P>0.05*).

2.2 Oxidative stress evaluation

2.2.1 Lipid peroxidation

Figure 6 shown lipid peroxidation levels, cadmium, and spirulina mixtures (Cd+S 1, Cd+S 2, Cd+S 3) increased lipid peroxidation levels compared to the control group, nonetheless, all spirulina mixtures reduced lipid peroxidation level compared to cadmium exposure. We observed that Cd+S 2 reduced lipid peroxidation levels effectively if compared to the other mixtures.

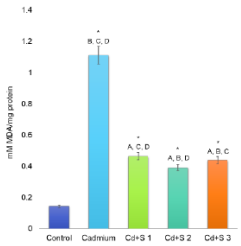
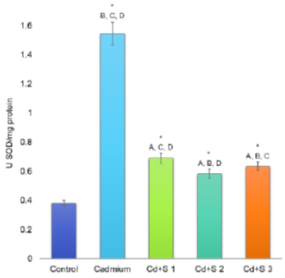


Fig. 6 LPX Lipid peroxidation in *Xenopus laevis* larvae exposed to control, cadmium 0.04 mg/L, Cd+S 1 (cadmium 0.04 mg/L + spirulina 2 mg/L) Cd+S 2 (cadmium 0.04 mg/L + spirulina 4mg/L), Cd+S 3 (cadmium 0.04 mg/L + spirulina 10 mg/L) for 96 h. Significant differences relative to: (*) control, (A) cadmium, (B) Cd+S 1, (C) Cd+S 2, (D) Cd+S 3. (One-way ANOVA and LSD Fisher, P>0.05).

2.2.2 SOD activity

SOD activity is shown in figure 7, we observed an increase in SOD activity induced by cadmium exposure compared to control, spirulina mixtures Cd+S 1, Cd+S 2, Cd+S 3 generated a decrease in SOD activity, the higher decrease was observed in Cd+S 2.



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b

Fig. 7 SOD Superoxide dismutase activity in *Xenopus laevis* larvae exposed to control, cadmium 0.04 mg/L, Cd+S 1 (cadmium 0.04 mg/L + spirulina 2 mg/L) Cd+S 2 (cadmium 0.04 mg/L + spirulina 4mg/L), Cd+S 3 (cadmium 0.04 mg/L + spirulina 10 mg/L) for 96 h. Significant differences relative to: (*) control, (A) cadmium, (B) Cd+S 1, (C) Cd+S 2, (D) Cd+S 3. (One-way ANOVA and LSD Fisher, P>0.05).

2.2.3 CAT activity

Figure 8 shown catalase activity with a similar tendency as SOD, spirulina mixtures Cd+S 1, Cd+S 2,

Cd+S 3 decreased CAT activity, but Cd+S 2 and Cd+S 3 shown the most effective reduction in CAT activity compared to cadmium.

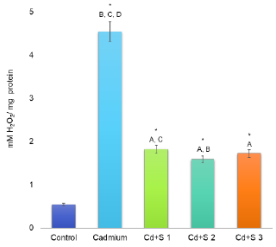

Fig.8 CAT Catalase activity in *Xenopus laevis* larvae exposed to control, cadmium 0.04 mg/L, Cd+S 1 (cadmium 0.04 mg/L + spirulina 2 mg/L) Cd+S 2 (cadmium 0.04 mg/L + spirulina 4mg/L), Cd+S 3 (cadmium 0.04 mg/L + spirulina 10 mg/L) for 96 h. Significant differences relative to: (*) control, (A) cadmium, (B) Cd+S 1, (C) Cd+S 2, (D) Cd+S 3. (One-way ANOVA and LSD Fisher, P>0.05).

3. Discussion

To determine if spirulina has a protective effect against the toxicity induced by cadmium in *Xenopus laevis* we performed the FETAX assay. The most relevant effects evaluated in our study were mortality, macroscopic malformations, and growth inhibition. Spirulina *(Arthrospira maxima)* reduced mortality, incidence, and severity of malformations, and enhance growth, spirulina also reduced oxidative damage induced by cadmium 0.04 mg/L in *Xenopus laevis*.

Mortality and malformations

The significant increase in mortality of *Xenopus laevis* larvae exposed to cadmium may be due cadmium prooxidant activity, the alteration in nutrients absorption (Zn, Mg, and Cu), and disturbances of meiotic

spindle morphogenesis (Slaby et al., 2017) this can lead to cell death; these effects were reported previously in *Xenopus laevis* exposed to cadmium (Sharma and Patiño, 2009). Groups exposed to spirulina Cd+S 1, Cd+S 2, Cd+S 3, had a reduction in mortality rate, the higher reduction was observed in Cd+S 3, with a decrease in mortality of 50%; the reduction in mortality may be due to the spirulina scavenge properties that generate protective effect against oxidative damage reducing ROS and NOS production, spirulina also has a chelating capacity to bind to heavy metals (Gelagutashvili, 2006), which can reduce the amount of cadmium-free to interact with biomolecules and reduce the disturbances in nutrient absorption, and cellular damage.

Main malformations observed in cadmium exposure were: Stunted embryos, gut miscoiling, rectum malformation, cardiac edema, abdominal edema, microcephaly, and axial malformations (bent tail, notochord, and fin); similar to those previously reported in *Xenopus laevis* (Sunderman et al., 1991) *Bufo gargarizans* (Wu et al., 2017b), *Danio rerio* (Cheng et al., 2000) and *Silurus soldatovi* (Zhang et al., 2012). Cadmium modifies apoptosis, cell cycle, stress and immune response (Liu et al., 2018), induce p53 phosphorylation, can replace Zn in the zinc-finger domain, and cause errors in DNA repair resulting in the accumulation of damaged DNA (Chen and Shaikh, 2009; Dally and Hartwig, 1997) and affect signaling processes, generates oxidative stress and redox potential alterations. As organogenesis is the most vulnerable stage of development and require signaling processes to regulate proliferation, and cell differentiation (Hayashi et al., 2018); cadmium can cause failure on embryonic development, thus generate changes in tissues function and structure and as a consequence diverse malformations (Laforgia et al., 2018). Spirulina mixtures Cd+S 1, Cd+S 2, Cd+S 3, generated a decrease on incidence and severity of malformations in larvae exposed to cadmium, Argüelles-Velázquez *et al*., 2013 also found a reduction in the frequency of malformations with the supplementation of spirulina in rats exposed to cadmium, this may be due to the effect of phycobiliproteins; which have anti-inflammatory activity (Khafaga and El-Sayed, 2018) by reducing inflammation, edema can be reduced, due to most of edema are produced by a chronic inflammatory process. Phycocyanin and β-carotenes inhibit the formation of proinflammatory

cytokines, thus suppressing the expression of cyclooxygenase II and the production of prostaglandin E2, which acts as an inflammatory mediator. Phycocyanin has antioxidant effects, can eliminate hydroxyl radicals responsible for oxidative damage (Bermejo et al., 2008), and reduce oxidative stress, the reduction in cellular damage can contribute to the reduction in the severity and incidence of malformations in *Xenopus laevis*.

Growth inhibition

Cadmium exposure decreased larvae length, this effect was also observed in previous studies, Wu et al., 2017b reported a reduction in total length induced by cadmium in *Buffo Garganzianis*, it may be due to cadmium has the ability to mimic other essential minerals, thus inhibits nutrient absorption (Goyer, 1995) and trigger failures in developmental processes, cadmium can generate alterations in calcium-dependent processes, which are highly important in early life stages (Borodinsky, 2017; Hayashi et al., 2018). The exposure to spirulina mixtures (Cd+S 1, Cd+S 2, Cd+S 3) shown beneficial effects in development, total body length increased compared to cadmium exposure, in mixture Cd+S 3 larvae had a similar size than larvae from the control group. This may be due to spirulina cell wall is porous and allows cadmium a free pass-thru, when cadmium gets to the intracellular compartment, chelating agents act binding to cadmium and neutralizing it (Bermejo et al., 2008), these chelating agents are often induced by heavy metals exposure (Knauer et al., 1998). Another important mediator is phycocyanin which can scavenge hydroxyl, alkoxy, and peroxyl radicals that may initiate the arachidonic acid cascade; phycocyanin also blocks the phosphorylation of p38 mitogen-active protein kinases, which regulate the synthesis of cytokines, including TNF- α and ILe1β (Khalil et al., 2017). Vitamins, proteins, and minerals of spirulina may also be involved in the enhance of development. Similar results have been reported previously in rabbits and rats exposed to lead (Pb) and spirulina mixtures, total size and weight of the organisms were diminished by Pb exposure, and after spirulina supplementation, weight and size increased significantly (Aladaileh et al., 2020).

Oxidative stress

Cadmium can generate alterations in mitochondrial processes such as electron transfer chain and energy generation (ATP), as mitochondria is a suitable source of oxygen when cadmium alters the activity of

complexes II (succinate: ubiquinone oxidoreductase) and III (ubiquinol: cytochrome C oxidoreductase), lead to an excessive ROS generation and hence oxidative damage (Gobe and Crane, 2010; Wang et al., 2004).

Cadmium exposure induced adverse effects in aquatic organisms previously (Avallone et al., 2015; Cambier et al., 2010; Chan and Cheng, 2003; Mouchet et al., 2006; Pizzi et al., 2017; Shirriff and Heikkila, 2017; Slaby et al., 2016; Thompson and Bannigan, 2008; Wu et al., 2017b, 2017a; Xia et al., 2016) many of this are related to oxidative stress. In this study cadmium induced an increase in lipid peroxidation, this may be attributed to an excessive production of ROS which interacted with cell membrane and triggered the formation of lipid-radicals, causing a loss of membrane function and hence cellular damage, nonetheless spirulina mixtures exposure (Cd+S 1, Cd+S 2, Cd+S3), achieved a reduction in lipid peroxidation levels, Cd+S 2 shown a reduction statistically significant compared to Cd+S 1 and Cd+S3; similar effects of decrease in lipid peroxidation with spirulina supplementation have been reported previously in *Oreochromis niloticus* exposed to deltamethrin,(Abdelkhalek et al., 2017); in rats exposed to sodium fluoride (Banji et al., 2013), Wistar rats exposed to methotrexate (Khafaga and El-Sayed, 2018), *Clarias gariepinus* exposed to sodium dodecyl sulfate (Sayed and Authman, 2018). Rabbits exposed to lead acetate (Aladaileh et al., 2019). Male rats exposed to sodium arsenite (Bashandy et al., 2016) and Wistar rats exposed to Cadmium (Karadeniz et al., 2009). As mentioned previously spirulina has the chelating ability to bind cadmium ions and inhibit Fenton reaction, it also can neutralize alkoxyl hydroxyl and peroxyl radicals (Wu et al., 2016), and if the lipid peroxidation process is inhibited in an early stage, damage can be reduced, vitamin E in spirulina can be protective against lipid peroxidation due it has a chroman ring on its structure that apports reductive effect, and can reduce peroxyl radicals into hydroperoxides which afterward can be degraded enzymatically (Miyazawa et al., 2019) carotenoids contained in spirulina also have antioxidant activity, can scavenge ROS and neutralize oxygen singlet (Stahl and Sies, 2003).

Antioxidant enzymatic activity (SOD and CAT) were also increased in cadmium exposure, an increase in enzymatic activity due cadmium exposure has been reported previously (Karadeniz et al., 2009; Wu et al.,

2017a; Xia et al., 2016). The increase in enzymatic activity can be the result of excessive production of reactive species, cadmium generates ROS and RNS, and increases superoxide ($O_2^{\cdot -}$) levels, SOD catalyzes a dismutation process, transforming $O_2^{\cdot -}$ into H_2O_2, which is later transformed by CAT and glutathione peroxidase into H_2O (Liu et al., 2018), these processes induce an increase in antioxidant enzyme activity to neutralize ROS and protect the cell from oxidative damage; however, groups exposed to spirulina Cd+S 1, Cd+S 2, Cd+S3 shown a decrease in both enzymatic activity, the higher decrease statistically significant in SOD activity was induced by Cd+S 2, whereas the higher decrease statistically significant in CAT activity was induced by Cd+S 3.

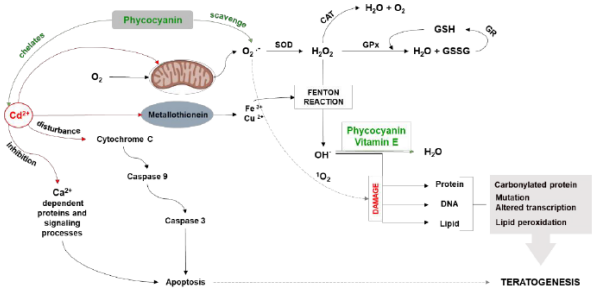

Fig.9 Proposed route through oxidative damage is minimized by spirulina due to the chelation of cadmium and scavenge of ROS and RNS induced in *Xenopus laevis* exposed to cadmium.

Spirulina can improve antioxidant activity and help to restore the ability to neutralize ROS and RNS, therefore reduce oxidative damage (fig.9). Other studies, where a supplementation with spirulina was performed report an increase in antioxidant enzymes (Abdelkhalek et al., 2017; Banji et al., 2013; Karadeniz et al., 2009; Sayed and Authman, 2018) contrary to the results obtained in this work, this may be due other studies are focused on an adult organism, or in embryos in the gestational process (mammals), in early life stages some recovery responses are not fully developed, this may contribute to a different response against damage.

4. Conclusions

Cadmium can generate diverse alterations, induce an increase in ROS and RNS, inhibit nutrients absorption, generate disturbances in some signaling processes, hence cellular damage and malformations. On the other hand, spirulina can scavenge highly toxic radicals such as hydroxyl, peroxyl, and superoxide radical, also spirulina can chelate cadmium, inhibiting damage in cellular components, reducing malformations, growth inhibition and oxidative damage. Spirulina (*Arthrospira maxima*) shown protective effects against cadmium-induced toxicity in *Xenopus laevis*, the higher beneficial effects were observed in 4 and 10 mg/L; nonetheless 2mg/L also shown significant positive effects, oxidative damage was reduced, mortality and malformation rate decrease, as well as the severity of malformations and growth inhibition, spirulina can be considered as a diet complement for amphibians to prevent toxicity induced by metals in early life stages, further studies focused on the effects of spirulina in amphibians and aquatic organisms are recommended.

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8.2 ARTICULO 2

Environmental Toxicology and Pharmacology

Spirulina (Arthrospira maxima) helps decrease oxidative damage and abnormalities induced by diclofenac in Xenopus laevis at early life stages

--Manuscript Draft--

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Abstract:	• Diclofenac increased mortality, abnormalities, and oxidative damage • Diclofenac reduced larvae total body size • Spirulina reduced the incidence and severity of abnormalities • Spirulina enhanced growth in diclofenac exposure • Spirulina decreased oxidative damage induced by diclofenac
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Spirulina (*Arthrospira maxima*) helps decrease oxidative damage and abnormalities induced by diclofenac in *Xenopus laevis* at early life stages

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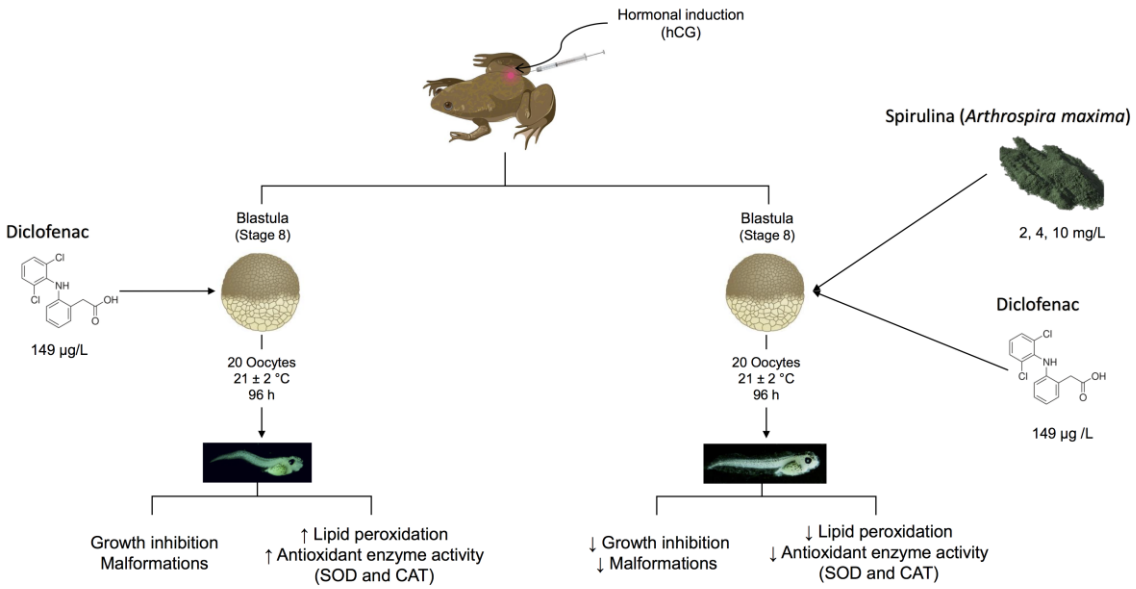
Highlights

- Diclofenac increased mortality, abnormalities, and oxidative damage
- Diclofenac reduced larvae total body size
- Spirulina reduced the incidence and severity of abnormalities
- Spirulina enhanced growth in diclofenac exposure
- Spirulina decreased oxidative damage induced by diclofenac

Graphical abstract



Abstract

Diclofenac (DCF) is a highly consumed and eliminated pharmaceutical worldwide, is constantly detected in the environment, mainly in water, and is resistant to conventional degradation processes; due its occurrence and toxic environmental effects was included in the European Union watch list of the water framework, but in Mexico there are not regulations for this kind of compounds yet. Therefore, the aim of this study was to evaluate the protective effect and antioxidant activity of spirulina (*Arthrospira maxima*) against toxicity induced by DCF in *Xenopus laevis* at early life stages. For which *Xenopus laevis* oocytes were selected and exposed in medium blastula stage for 96 h to three different mixtures: DCF+S 2 (149 µgL-1 DCF plus 2 mg L-1 spirulina), DCF+S 4 (0.149 µgL-1 DCF plus 4 mgL-1 spirulina), DCF+S 10 (0.149 µgL-1 DCF plus 10 mgL-1 spirulina), another oocytes were also exposed to diclofenac 0.149 µgL-1 and a control group. To assess spirulina effects the mortality and malformations rate, growth, lipid peroxidation and

antioxidant enzymatic activity (superoxide dismutase and catalase) were determined. The results shown that spirulina 4 and 10 mgL-1 achieved a reduction of 80% in DCF induced mortality, also malformations were reduced in severity and frequency, the abnormalities mainly observed were: eye malformation, tail, notochord, intestine and rectum, also all spirulina exposure groups shown an increase in total body size compared to DCF exposure. Regarding oxidative damage the groups exposed to the mixture with spirulina decreased lipid peroxidation level, and diminished antioxidant activity. The results showed that spirulina reduced damage induced by DCF in *Xenopus laevis* at early life stages, and decreases mortality, frequency and severity of abnormalities, growth inhibition and oxidative damage. Further research is needed to assess benefic effects of spirulina against toxicity induced by pharmaceuticals in early life stages of development.

Keywords: Pharmaceuticals, Diclofenac, Spirulina, Teratogenesis, Oxidative stress, *Xenopus laevis*.

1. Introduction

In the last decades, aquatic environment pollution has become a global issue, since it has been demonstrated that domestic and industrial discharges can cause detrimental effects, even at trace concentrations, mainly on aquatic organisms. Among the compounds that have been identified as environmental pollutants are the emerging contaminants (a group of unregulated contaminants present in different water matrices for decades ago), such as personal care compounds, synthetic hormones, steroids, endocrine disrupting chemicals and pharmaceuticals products, among others, which represent only a small fraction of the total chemical pollution (Stefanakis and Becker, 2015).

Pharmaceutical products are compounds that have been used extensively in human and veterinary medicine (Ajima et al., 2015), and it has been estimated that hundreds of tons of them are dispensed and consumed worldwide, annually (Fekadu et al., 2019), due to its physic-chemical properties are considered an important class of emerging environmental pollutants representing a threat to aquatic organisms and environmental health (Fekadu et al., 2019; Hanif et al., 2020; Melvin, 2016), between these, the nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used, not only in Mexico, but throughout the world (Gómez-Oliván et al., 2014; Hanif et al., 2020). Being the diclofenac (DCF, a member of the phenylacetic acid class, with analgesic, anti-inflammatory, and antipyretic effects) since its introduction in 1973, one of the most prescribed worldwide due to its effectiveness in treating a variety of acute and chronic pain and inflammatory conditions (Altman et al., 2015; Bickley et al., 2017; Cunha et al., 2017; Gan, 2010; Ulubay et al., 2018). As a member of NSAIDs, DCF exerts its action by the inhibition of prostaglandin synthesis, it binds to both COX-1 and COX-2 and inhibits the conversion of arachidonic acid into pro-inflammatory prostaglandins by means of chelation nonetheless, contrary to the action of many NSAIDs, diclofenac inhibits cyclooxygenase COX-2 enzyme with greater potency than it does with COX-1, making it able to inhibit tumor angiogenesis. Several researches indicate that DCF activity

goes beyond COX inhibition, including multimodal and novel mechanisms of action (Altman et al., 2015; Gan, 2010; Ulubay et al., 2018).

Because of its ubiquitous nature in the aquatic environment, mainly in surface water such as rivers, lake canals, estuaries and seas, and its frequent occurrence in groundwater and wastewater effluent, and due to its potential toxicity reported over the last 20 years, causing deleterious changes in aquatic biota such as fish and mussels, DCF was included in the First Watch List of the EU Water Framework Directive (Bio and Nunes, 2020; Bonnefille et al., 2018; Hanif et al., 2020; Lonappan et al., 2016; Sathishkumar et al., 2020).

DCF has been detected in several aquatic matrices, in concentrations ranging from ngL^{-1} to μgL^{-1}. For example, in surface water, has been found at different concentrations < 100 ngL^{-1} (Yang et al., 2018) (Kermia et al., 2016), to 364 ngL^{-1} (de Sousa et al., 2018), 419 ngL^{-1} (Branchet et al., 2019) and 7.76 μgL^{-1} (González-Alonso et al., 2017); in groundwater, the reported concentrations varying from <10 ngL^{-1} (Yang et al., 2018) (Sharma et al., 2019) to 48.1 ngL^{-1} (Jindal et al., 2015), 518 ngL^{-1} (Branchet et al., 2019) and 2.77 μgL^{-1} (Kapelewska et al., 2018). In drinking water samples, DCF has been found from <10 ngL^{-1} (Rodil et al., 2012) (Tröger et al., 2018) to 16 - 18 ngL^{-1} (Simazaki et al., 2015) (Carmona et al., 2014); while in seawater, it has been found at 0.021, 48 and 11.6 ngL^{-1} concentrations (Brumovský et al., 2017) (Kallenborn et al., 2018) and (Bayen et al., 2013), respectively, and 10.2 and 31.9 ngL^{-1} (Ali et al., 2017) (Biel-Maeso et al., 2018); and in municipal wastewater inffluent/effluent at concentrations from <500 ngL^{-1} (Lindholm-Lehto et al., 2016; Liu et al., 2017; Wilkinson et al., 2016) to 812 ngL^{-1} (Česen et al., 2019) and 2.5 μgL^{-1} (Chiffre et al., 2016).

Other matrices include soil, where DCF has been detected in concentrations from 0.3 - 0.35 $mgkg^{-1}$ (Christou et al., 2017; Corada-Fernández et al., 2015), 257 $mgkg^{-1}$ (Ashfaq et al., 2017) and 0.2 ngg^{-1} (Grossberger et al., 2014); in sediments from 3.95, 6.8, 10.6 and 13.88 ngg^{-1} (de Sousa et al., 2018), (Čelić et al., 2019), (Peng et al., 2017) and (Omar et al., 2018), respectively; in suspended

solid where it has been detected in concentrations ranging from 119 ngg^{-1} (Wilkinson et al., 2017) to 1.3 mgg^{-1} (de Sousa et al., 2018); in sewage sludge in concentrations from <1 ngg^{-1} (Yan et al., 2014) to >10 ngg^{-1} (Stasinakis et al., 2013; Xue et al., 2010) even at 35.3 $mgKg^{-1}$ (Martín et al., 2015) and 4968 $mgKg^{-1}$ (Ashfaq et al., 2017) and finally, DCF has been detected in leachate at different concentrations such as 40 and 613.3 ngL^{-1} (Rodríguez-Navas et al., 2013) and (Lu et al., 2016), respectively and 108.34 mgL^{-1} (Kapelewska et al., 2018).

On the other hand, it has also been reported that DCF is capable of causing toxic effects, even at trace concentrations, not only for human health, but also for aquatic organisms, including significant inhibition of AChE (as a biomarker of neuronal regulation) in *Daphnia magna* (at 0.08–18.4 ngL^{-1}) (Oliveira et al., 2015), significant decrease in growth observed in *Danio rerio* (at 30, and 60 mgL^{-1}) (Praskova et al., 2014), alterations in hematological parameters including higher mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV) and white blood cell (WBC), with significantly lower haemoglobin (Hb), haematocrit, red blood cell (RBC) and mean corpuscular haemoglobin (MCH) in *Clarias gariepinus* (at 1.57, 3.14 and 6.28 mgL^{-1}) (Ajima et al., 2015), observed modulating genes associated with kidney repair and regeneration in *Pimephales promelas* (at 25 μgL^{-1}) (Bickley et al., 2017), a reduced larval developmental growth and several morphological abnormalities such as altered body axis, and organ and visceral abnormalities including cardiac hypoplasia and malrotated guts in both *Trachycephalus typhonius* and *Physalaemus albonotatus* (at 125 and 250 μgL^{-1}) (Peltzer et al., 2019), and effects on hatching rate, development rate, survival, growth and histopathological changes in both *Rainbow trout* and *Danio rerio* (at concentrations ranging from 3.2 to 1000 μgL^{-1}) (Memmert et al., 2013).

Also, due to its biotransformation leading the formation of reactive metabolites and reactive oxygen species (ROS) can induce oxidative stress and damage in diverse biomolecules (Cunha et al., 2017;

Nava-Álvarez et al., 2014; Saucedo-Vence et al., 2015), it has been highlighted that relevant environmental concentrations may result in negative effects, mainly at chronic exposure, in aquatic and marine organisms (Almeida et al., 2020; Bonnefille et al., 2018; Lonappan et al., 2016), the main toxic effects reported by several working groups all around the world including increasing of oxidative stress biomarkers such as lipid peroxidation (LPX), hydroperoxides content (HPC), protein carbonyl content (PCC), and the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferases (GSTs), and glutathione-peroxidase (GPx) activities, teratogenic effects and malformations in tail and notochord, edema and stunted growth and *Xenopus laevis* and *Lithobates castesbeianus* at 1, 4, 8, 16, 32 and 62.5mgL-1 (Cardoso-Vera et al., 2017), *Daphnia magna* at 0.08–18.4 ngL^{-1} (Oliveira et al., 2015) and 2.9 mgL^{-1} (Gómez-Oliván et al., 2014), *Danio rerio* at 5, 15, 30, and 60 mgL^{-1} (Praskova et al., 2014) and 0.5, 5, 50 and 500 mgL^{-1} (Bio and Nunes, 2020), and *Cyprinus carpio* at 7.098 mgL^{-1} (Islas-Flores et al., 2013) and 70.98 mgL^{-1} (Saucedo-Vence et al., 2015).

Spirulina (S) is a unicellular blue-green cyanobacterium microalga, for many years has been used as an alimentary supplement due its high nutritional value, wide medicinal properties and pharmacological activity. Spirulina is a good source of proteins, vitamins, fatty acids, and amino acids, also is a powerful stimulant of immune system, as many experiments in organisms shown an increase in phagocytic and natural killer activity (Qureshi and Ali, 1996), enhancement of IL-1 and activation of antibodies (Hayashi et al., 1994). Other properties as antiviral, anticancer, hipoglycemic activity and antihyperlipidemic were proved in different organisms (Karadeniz et al., 2009; Khan et al., 2006; Kulshreshtha et al., 2008; Parikh et al., 2001). The antioxidant and protective effects of SP have been demonstrated against several toxicants as mercury (Sharma et al., 2007), D-galactosamine (Al-Qahtani and Binobead, 2019), acetaminophen (Lu et al., 2010), copper (James et al., 2009), CCL_4 (Gad et al., 2011), beta-cypermetrin (Zhang et al., 2018). Enzymes as glutathione, glutathione peroxidase, glutathione reductase, and glutathione S-transferase were

significantly improved in some organisms including rabbits (Kim et al., 2010), rats (Afkhami-Ardakani et al., 2018), hamster (Muga and Chao, 2014), fish like C*yprinus carpio* (Toughan et al., 2018), *Nile Tilapia* (Abdelkhalek et al., 2017; Sayed et al., 2015) and *Danio rerio* (Rajasekar et al., 2019). However, there is no evidence yet of beneficial effects on *Xenopus laevis*. Therefore, the aim of this study was to evaluate protective effects of spirulina (*Arthrospira maxima*) against mortality, malformations, growth inhibition, and oxidative damage of induced by diclofenac in *Xenopus laevis* at early life stages.

2. Materials and Methods

2.1 Chemicals and reagents

All the chemical reagents used were analytical grade (> 99% purity), diclofenac, 3-amino-benzoic acid ethylester (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 contraire is indicated herein. Spirulina (*Arthrospira maxima*) dried powder was purchased from a local supplier (AEH Spiral Spring, Mexico).

2.2 Frog selection and husbandry

Adult *Xenopus laevis* males and females were obtained from the aquaculture center Aquanimals, located in state of Queretaro, Mexico. The selection criteria for males were 8 to 10 cm long and 2 years old, and for females 10 to 12.5 cm long and 3 years old. 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% of its capacity with dechlorinated water, following conditions were kept and monitored: temperature 21 ± 2 ° C, pH 6.5 to 9, photoperiods 12 h light / 12 h darkness, total organic carbon <10 mgL^{-1}, alkalinity and hardness by determination of $CaCO_3$ 16 to 400 mgL^{-1}, parameters were determined monthly. Frogs were fed three times a week ad libitum with *Chrisotoma sp*. (0.5 ± 0.3 cm in length) and / or commercial food NUPEC pellets Purina.

All procedures were performed in accordance with the 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 (NOM-062-ZOO- 1999, Technical specifications for the production, care, and use of laboratory animals).

2.3 FETAX assay

This study was carried out in accordance with the American Society for Testing Materials Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-*Xenopu*s E-1439-12 (American Society for Testing Materials, 2012).

2.3.1 FETAX medium and test solutions

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, final pH was 7.6 - 7.9. All reagents were purchased from Sigma-Aldrich (St. Louis, MO).

For diclofenac exposure a stock solution was prepared daily by dissolving 1 g in 1 L of FETAX medium, later diclofenac solutions were prepared having final concentration of 1, 4, 8, 16, 32 and

62.5 mgL^{-1} for LC_{50} and LOAEL determination. Spirulina and diclofenac mixtures were prepared by dissolving 2, 4 and 10 mg of spirulina in a 149 μgL^{-1} diclofenac solution. The entire procedure was done under a laminar flow hood.

Concentration of diclofenac 149 μgL^{-1} in mixtures was previously determined to be LOAEL in *Xenopus laevis* and spirulina 2, 4 and 10 mgL^{-1} were selected based on previous internal laboratory experiments, due there is no spirulina concentration reported to be tested in embryonic stages of development of amphibians yet.

Final test solutions for the evaluation of spirulina protective effects were as follows in Table 1. New solutions were prepared daily to avoid degradation and were kept protected from the light at 4°C.

Table 1. Exposure groups

Test solution	Composition
Control	FETAX medium
Diclofenac	149 $\mu gL^{-1}$of diclofenac
DCF+S 2	149 μgL^{-1} of diclofenac plus 2 mgL^{-1} of *Arthrospira maxima*
DCF+S 4	149 $\mu gL^{-1}$of diclofenac plus 4 mgL^{-1} of *Arthrospira maxima*
DCF+S 10	149 μgL^{-1} of diclofenac plus 10 mgL^{-1} of *Arthrospira maxima*

2.3.2 Ovulation induction and fertilization

The night before assay was performed one male and one female were placed in a 40 L aquarium adapted with a plastic mesh suspended 3cm over the bottom, to separate the embryos form the adult frogs, opaque sides, temperature was maintained at 21± 2 °C and pH was monitored to be 6.5 – 9.

Ovulation and spermatogenesis were induced through the administration of Human chorionic gonadotropin hormone (hCG) (CHORAGON®, Ferring) in the dorsal lymph sac, using a 1 mL hypodermic syringes fitted with long 26-gauge needles, hCG was previously dissolved with a NaCl 0.9% sterile solution, later males were administered with 300 IU and females with 700 IU.

2.3.3 Oocytes selection

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

2.3.4 Exposure

2.3.4.1 Diclofenac

For diclofenac exposure, 10 mL of each solution (1, 4, 8, 16, 32, 62.5 mgL^{-1}) were verted in previously labeled Petri dishes 50 mm under a laminar flow hood, twenty oocytes were collected and placed in each petri dish using Pasteur pipettes and a stereoscopic microscope, also a control group exposed to FETAX medium was placed at same conditions 21 ± 2 °C for 96 h, all experiments were performed on triplicate.

2.3.4.2 Diclofenac and spirulina (*Arthrospira maxima*) mixtures

As mentioned previously (2.3.4.1), 10 mL of each solution (Diclofenac, D+S 2, D+S 4, D+S 10) were verted in previously labeled Petri dishes 50 mm under a laminar flow hood, twenty oocytes were collected and placed in each petri dish using Pasteur pipettes and a stereoscopic microscope,

also a control group exposed to FETAX medium was placed at same conditions 21 ± 2 °C for 96 h, all experiments were performed on triplicate.

2.3.5 Culture monitoring

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

2.3.6 Examination of larvae

At 96 h of exposure larvae were checked for swimming, if not swimming, this was noted in a developmental parameter sheet used to record malformations present in larvae. Precipitates (if any) were also recorded, as well as the number of dead larvae.

After 96 h larvae were euthanized by placing them in a petri dish 50 mm containing a 0.06% MS-222 solution (lethal dose).

Each larva was measured from head to tail using Zen Blue Zeiss software, values were registered to determine minimum concentration to inhibit growth (MCIG), also each larva was observed and evaluated in the microscope fitted with a Zeiss Axiocam 5s camera, to identify developmental abnormalities in accordance to Atlas of Abnormalities (Bantle et al.,1991), and other resources. After examination, larvae were disposed of following institutional standards for the elimination of biological samples.

2.4 Oxidative damage assessment

Test solutions were prepared and organisms were exposed as mention previously in section 2.3, after 96 h larvae were weighted and homogenized with a phosphate buffer solution (pH 7.2) 4°C in a 1:4 (w/v) proportion, later samples were centrifuged at 2500 rpm for 15 min.

2.4.1 Determination of lipid peroxidation (LPX)

Lipid peroxidation level was determined by the Buege and Aust, 1978 method. 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 were added in a glass tube 10x75 mm, then incubated at 37°C for 45 min, and later absorbance was determined at 535 nm. Results were expressed as mM malondialdehyde (MDA)/mg protein using the molar extinction coefficient (MEC) 1.56 x 105/M/cm.

2.4.2 Determination of superoxide dismutase (SOD) activity

SOD activity was determined by Misra and Fridovich, 1972 method. 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) were added to a quartz cuvette, absorbance was measured at 480nm at 30 seconds and 5 minutes, SOD activity was determined with the molar extinction coefficient 21 M/cm, and results were expressed as IU SOD/mg of protein.

2.4.3 Determination of catalase (CAT) activity

CAT activity was determined by the Radi et al., 1991 method. 30 µL of supernatant were verted into a quartz cuvette plus 420 µL of isolation buffer solution [0.3M sucrose (Vetec, Sigma-Aldrich, St. Louis, MO, USA), 1mM EDTA, 5mM HEPES, 5mM KH_2PO_4 (Sigma-Aldrich, St. Louis, MO, USA)] and 300 µL of 20mM H_2O_2 solution (Sigma-Aldrich, St. Louis, MO, USA) were added. Absorbance was read at 240 nm, at 0 and 60 s, and CAT activity was estimated using the MEC of H_2O_2 0.093 mM/cm.

2.5 Statistical analysis

The data were analyzed using the software Statgraphics Centurion XVI. All the results were expressed as the mean of three experiments performed under the same conditions. To calculate the values of medium lethal concentration (LC_{50}) we performed a PROBIT analysis ($p<0.05$), 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 by one-way analysis (ANOVA), and Fisher's multiple comparison ($p<0.05$). Lipid peroxidation and enzymatic activity (SOD and CAT) were analyzed by one-way analysis of variance (ANOVA) and Fisher's multiple comparison ($p<0.05$).

3. Results

3.1 FETAX assay

Medium lethal concentration (LC_{50}) 14.905 mgL^{-1} was determined (PROBIT analysis $p<0.05$), lowest adverse effect level (LOAEL) was also calculated 149 μgL^{-1}.

3.1.1 Mortality and malformations

Mortality and malformation data are shown in figure 1, mortality was reduced in spirulina treated embryos from 60 up to 80%, the highest reduction in mortality was on D+S 4 and D+S10 both groups shown no statistical differences regarding to control, but a significant reduction compared to diclofenac exposure was observed, malformations were reduced in 6 to 16% of incidence, D+S 4 demonstrated to have the higher reduction on abnormalities incidence. The most frequent malformations observed were bent tail, bent notochord, gut and rectum malformation, eye abnormalities, microcephaly, and cardiac edema (figure 2), all of spirulina mixtures achieved a significant reduction on severity of malformations as shown in figure 3.

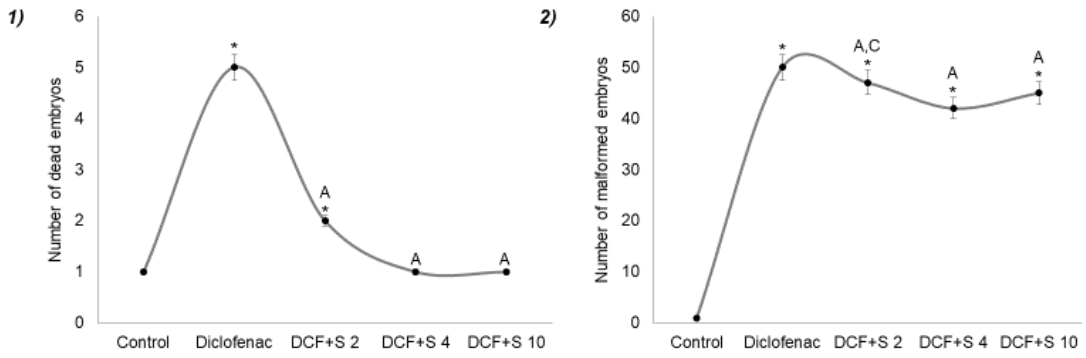


Fig. 1 Total number of dead embryos of *Xenopus laevis* after exposure 96 h to control, diclofenac, DCF+S 2 (diclofenac 149 μgL^{-1}+ spirulina 2 mgL^{-1}), DCF+S 4 (diclofenac 149 μgL^{-1} + spirulina 4 mgL^{-1}), DCF+S 10 (diclofenac 149 μgL^{-1}+ spirulina 10 mgL^{-1}) are presented in number 1), number of *Xenopus leavis* embryos with malformation are shown in *2)*, after exposure 96 h to control, diclofenac, DCF+S 2 (diclofenac 149 μgL^{-1}+ spirulina 2 mgL^{-1}), DCF+S 4 (diclofenac 149 μgL^{-1}+ spirulina 4 mgL^{-1}), DCF+S 10 (diclofenac 149 μgL^{-1}+ spirulina 10 mgL^{-1}). Significant differences relative to: (*) control, (A) diclofenac, (B) DCF+S 2, (C) DCF+S 4, (D) DCF+S 10 (One-way ANOVA and LSD Fisher, $p<0.05$).

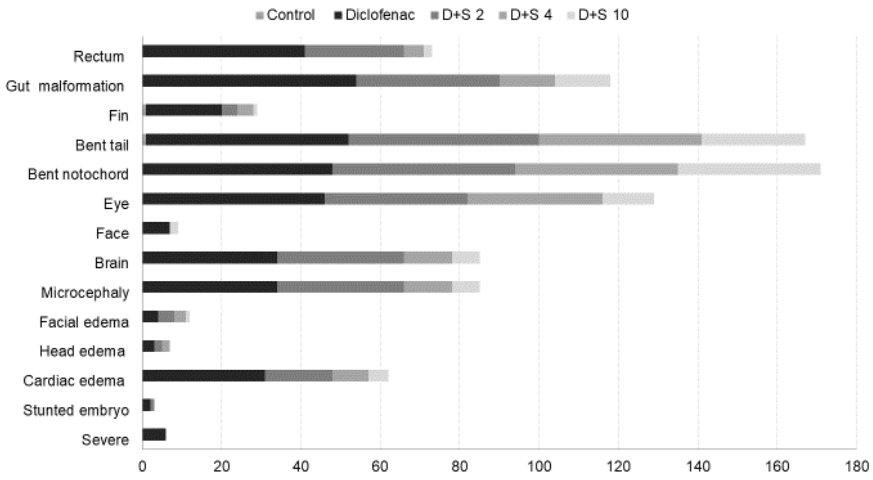


Fig. 2 Frequency histogram for malformations induced in *Xenopus laevis* embryos after 96 h exposure to control, diclofenac, DCF+S 2 (diclofenac 149 μgL^{-1}+ spirulina 2 mgL^{-1}), DCF+S 4 (diclofenac 149 μgL^{-1}+ spirulina 4 mgL^{-1}), DCF+S 10 (diclofenac 149 μgL^{-1}+ spirulina 10 mgL^{-1}).

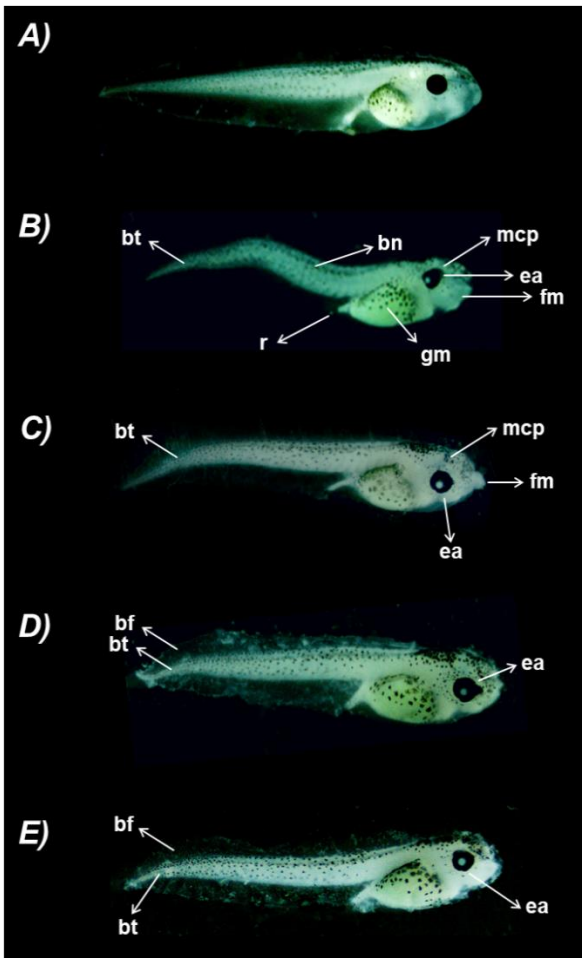


Fig. 3 Representative, and most frequent malformations observed in *Xenopus laevis* exposed for 96 h to A) control, B) diclofenac, C) DCF+S 2 (diclofenac 149 μgL^{-1}+ spirulina 2 mgL^{-1}), DCF+S 4 (diclofenac 149 μgL^{-1}+ spirulina 4 mgL^{-1}), DCF+S 10 (diclofenac 149 μgL^{-1}+ spirulina 10 mgL^{-1}).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

3.1.2 Growth inhibition

Diclofenac exposure induced a reduction in total body size of the larvae, spirulina mixtures increased total body size and enhanced growth, head to tail measurements are shown in figure 4.

Larvae exposed to D+S 4 and D+S 10 had similar total body size as control, nonetheless, D+S 10 was the most effective in reducing growth inhibition induced by diclofenac.

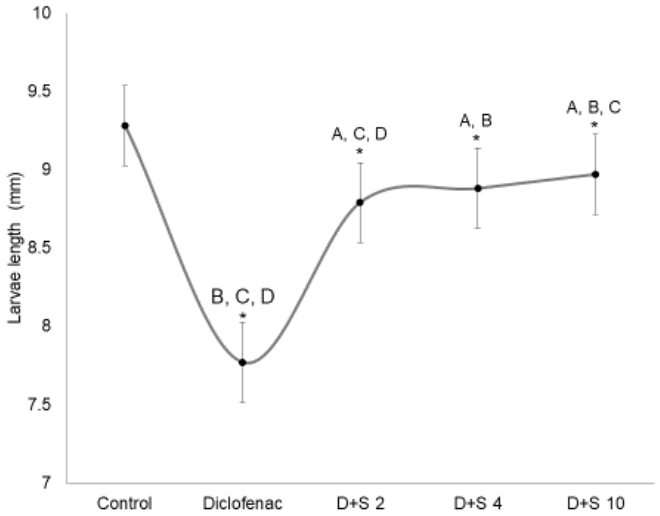


Fig. 4 *Xenopus laevis* larvae total body length head to tail after 96 h exposure to control, diclofenac, DCF+S 2 (diclofenac 149 μgL^{-1} + spirulina 2 mgL^{-1}), DCF+S 4 (diclofenac 149 μgL^{-1} + spirulina 4 mgL^{-1}), DCF+S 10 (diclofenac 149 μgL^{-1}+ spirulina 10 mgL^{-1}). Significant differences relative to: (*) control, (A) diclofenac, (B) DCF+S 2, (C) DCF+S 4, (D) DCF+S 10 (One-way ANOVA and LSD Fisher, $p<0.05$).

3.2 Oxidative damage

3.2.1 Lipid peroxidation

Lipid peroxidation data are shown in figure 5, diclofenac induced a significant increase in MDA levels compared to control, on the other hand, all spirulina mixtures demonstrated a significant

reduction in lipid peroxidation compared to diclofenac, D+S 10 was the mixture that achieved the higher reduction.

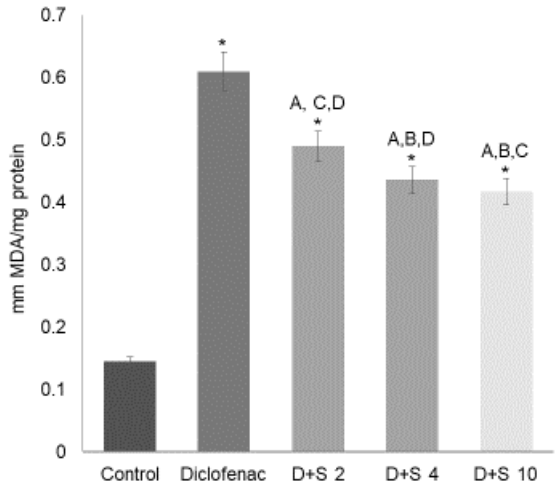


Fig. 5 Lipid peroxidation level assessed in *Xenopus laevis* after 96 h exposure to control, diclofenac, DCF+S 2 (diclofenac 149 μgL^{-1} + spirulina 2 mgL^{-1}), DCF+S 4 (diclofenac 149 μgL^{-1} + spirulina 4 mgL^{-1}), DCF+S 10 (diclofenac 149 μgL^{-1} + spirulina 10 mgL^{-1}). Significant differences relative to: (*) control, (A) diclofenac, (B) DCF+S 2, (C) DCF+S 4, (D) DCF+S 10 (One-way ANOVA and LSD Fisher, $p<0.05$).

3.2.2 Superoxide dismutase activity (SOD)

Figure 7 shown SOD activity, diclofenac exposure induced an increase compared to control, spirulina mixtures achieved a significant reduction in SOD activity, the most effective decrease is observed in D+S 10, this mixture have similar SOD activity levels than control group.

3.2.3 Catalase activity (CAT)

Catalase activity (fig 7) was also increased by diclofenac, however, all spirulina mixtures achieved a reduction in CAT levels compared to diclofenac, the mixture with most effective reduction in CAT levels was D+S 10, this mixture achieved similar values to control.

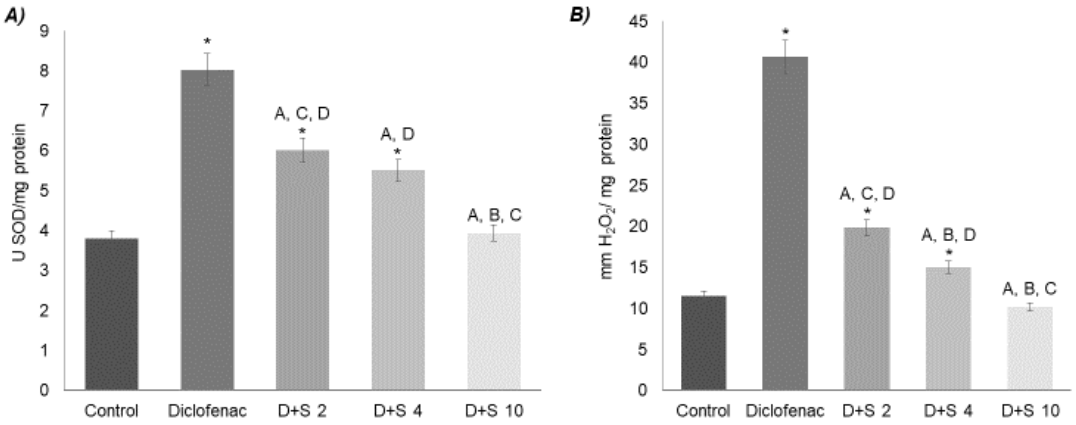


Fig. 6 Antioxidant enzymes evaluated in *Xenopus laevis* larvae exposed to control, diclofenac, DCF+S 2 (diclofenac 149 μgL^{-1} + spirulina 2 mgL^{-1}), DCF+S 4 (diclofenac 149 μgL^{-1} + spirulina 4 mgL^{-1}), DCF+S 10 (diclofenac 149 μgL^{-1} + spirulina 10 mgL^{-1}). Significant differences relative to: (*) control, (A) diclofenac, (B) DCF+S 2, (C) DCF+S 4, (D) DCF+S 10 (One-way ANOVA and LSD Fisher, $p<0.05$).

4. Discussion

It is well known that pharmaceuticals have been determined in aquatic environments, and because these products are biologically active and are constantly verted to water, they represent a risk for organisms and human health. Diclofenac is a widely consumed pharmaceutical that belong to the NSAIDs, unfortunately, it has been determined in many aquatic environments in concentrations

ranging from ngL^{-1} -μgL^{-1} (Lonappan et al., 2016; Miarov et al., 2020), even though diclofenac has a relatively short half-life in water (8 days) (Tixier et al., 2003) its constant elimination can be a problem due to most of the common remotion processes in wastewater treatment plants are ineffective to eliminate these kind of compounds (Petrie et al., 2015), and their transformation-remotion rates can be compensated by their constant introduction to the environment.

In México studies about diclofenac concentrations in water are scarce, nonetheless, it was determined in the influent of a wastewater treatment plant in Ciudad Juarez, giving as a result a concentration of 160 parts per billion (Bernadac Villegas et al., 2019). Unfortunately, Mexico does not have any regulation yet that stipulates the maximum permissible limit of emission of pharmaceutical products to aquatic environment, thus can lead to the generation of adverse in aquatic organisms induced by pharmaceutical products; we have to explore some alternatives in order to reduce the toxic effects of pharmaceuticals in aquatic organisms, that is why in this work we tested the beneficial properties of spirulina against diclofenac toxicity.

In this study, as in others previously reported (Chae et al., 2015) (Cardoso-Vera et al., 2017), diclofenac induced mortality (Figure 1) and malformations (Figure 2 and 3), growth inhibition (Figure 4), and was also able to induce oxidative damage in *Xenopus laevis* in early life stages (Figures 5 and 6), this may be due to it can induce oxidative stress, increases the generation of radical species, and activates pro-apoptotic factors, all these induce cellular damage and death. Different studies have proved that diclofenac induce toxic effects at relatively low concentrations in aquatic organisms, it is teratogenic and embryotoxic, induces malformations (axial, edema, intestine, heart, head, eye), and growth inhibition, in *X.laevis* (Chae et al., 2015), growth restriction and malformations in viscera and skeleton, variations in acetylcholinesterase and glutathione S transferase levels, neurotoxic and cardiotoxic damage in *T. typhonius* and *P. albonotatus* (Peltzer et al., 2019), increases mortality in embryos, variations in weight and size, increases glutathione S transferase, and reduces glutathione reductase in *Cryprinus carpio* (Stepanova et al., 2013),

increases the antioxidant activity of glutathione S transferase and ATP binding cassette transporters and increases lipid peroxidation in *Danio rerio* larvae and adults (Penha et al., 2021), induces oxidative stress and increases the level of reactive oxygen species associated with cytotoxicity in *Daphnia magna* (Ajima et al., 2021; Gómez-Oliván et al., 2014). Also, previous research have reported that can cause toxic effects due to it can damage mitochondria by the increase in production of reactive oxygen species; and can generate quinones and semiquinones when it goes thru redox cycle, the resulting radicals induces failure in mitochondrial permeability, reduce ATP level, and stimulate the liberation of proapoptotic factors like cytochrome C which activates caspase 9 and caspase 3, and generates cellular death (Ghosh et al., 2016; Jung et al., 2020; Ramachandran et al., 2018).

Spirulina (*Arthrospira maxima*) is a unicellular microalga with a high content of nutrients, proteins, minerals antioxidants, and other compounds biologically active (Soni et al., 2017; Wu et al., 2016). Some of the properties of spirulina reported are antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective, and neuroprotective, (Abu-Taweel et al., 2019; Aladaileh et al., 2019; Khafaga and El-Sayed, 2018; Kumar et al., 2016; Liang et al., 2020; Nasirian et al., 2018; Pestana et al., 2020; Slaby et al., 2017); also can reduce the levels of proinflammatory interleukins, and reduce inflammation (Abu-Taweel et al., 2019). Besides that the protective effects of spirulina in different organisms have been reported (Abdelkhalek et al., 2017; Aladaileh et al., 2020; Argüelles-velázquez et al., 2013; Banji et al., 2013; Bashandy et al., 2016; El-Tantawy, 2016; Hedayatirad et al., 2020; Khafaga and El-Sayed, 2018; Khalil et al., 2017; Peter S et al., 2017).

Our results showed that exposure to spirulina (*Arthrospira maxima*) 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 spirulina concentrations of 4 and 10 mgL^{-1}, nonetheless, all concentrations of spirulina tested shown positive effects against toxicity induced by diclofenac. These beneficial effects have been

reported previously, Rajbanshi et al. (2016) reported that spirulina was able to restore enzyme levels and reduced histomorphologic damage in the liver and kidney of Wistar albino rats exposed to diclofenac. The reduction of toxic effects induced by diclofenac may be due to the activity of spirulina components (Figure 7). Phycocyanin are well known for their antioxidant capacity particularly phycocyanin C (PC), which is a water-soluble biliprotein that have a chromophore group (phycocyanobilin), PC can scavenge some reactive oxygen species: hydroxyl, alkoxy, and peroxyl (Park et al., 2018), it also can inhibit lipid peroxidation at early stages, PC can scavenge alkoxy radical (which propagates lipid peroxidation), thus avoids the structural membrane damage (Bhat and Madyastha, 2000; Romay et al., 2005).

Besides, spirulina has other components that also inhibit lipid peroxidation, carotenoids can neutralize oxygen singlet and peroxyl radicals, also inhibits the production of prostaglandin E2 and nitric oxide thru the suppression of inflammatory mediators (Deng and Chow, 2010; Schafer et al., 2002), another component with important activity is tocopherol, due to it can interact with superoxide anion, hydroxyl and hydroperoxyl radicals when tocopherol interacts with peroxyl radicals it forms non-radical species which are less reactive, thus tocopherol reduces the lipidic peroxidation process and reduces membrane damage (Miyazawa et al., 2019; Moradi et al., 2019). There are some interactions between non-enzymatic antioxidants, for example, carotenoids can regenerate tocopherol from its radical form (tocopheroxyl), the resulting carotenoid radical would be restored afterward by vitamin c, this kind of interactions can also neutralize reactive nitrogen species, and reduce oxidative damage (Choi et al., 2004; Ryan et al., 2010).

The beneficial properties mentioned previously may be involved in the reduction of oxidative stress induced by diclofenac, and as oxidative stress is also related to teratogenesis, the decrease in reactive radical species can offset the generation of malformations and its frequency, as seen in figures 2 and 3, where spirulina achieved a significant restorative effect mainly at 4 and 10 mgL^{-1}.

On the other hand, spirulina has high nutritional value, contains carbohydrates, proteins, minerals, and vitamins, this may contribute to enhance the growth and development of *X. laevis* exposed to diclofenac (Figure 4); the total body size of embryos exposed to spirulina mixtures was similar to the control group, comparable results have been reported previously in other organisms (Abdelkhalek et al., 2017; Pestana et al., 2020).

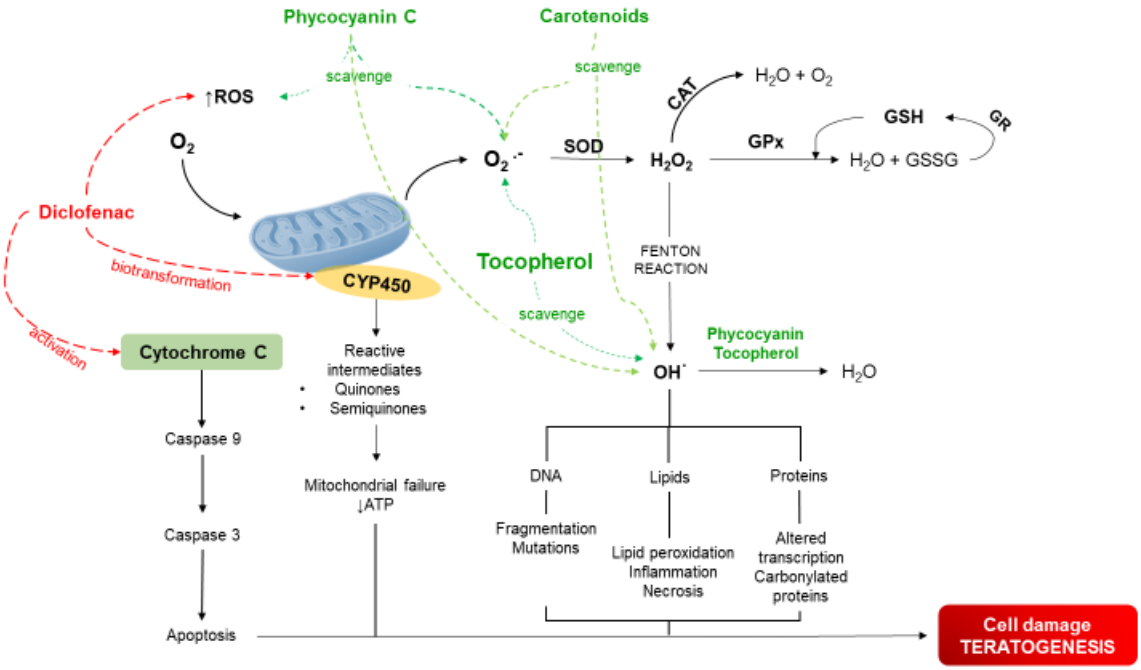


Fig.7 The proposed mechanism of damage reduction due to spirulina in *X.laevis* exposed to diclofenac. The main scavenge routes of spirulina by some of its components.

5. Conclusions

As mentioned above, diclofenac is a pharmaceutical that has been found in different concentrations in bodies of water around the world, and there have even been reports of its presence in drinking water, also due to its physicochemical properties as well as its mechanism of action has been able to induce different toxic effects in organisms that come into contact with it. Because of this, it is

necessary to continue researching about compounds that could help reduce or remedy these effects. In this work, diclofenac induces toxic effects in *Xenopus laevis* in early life stages, oxidative damage, malformations, and growth inhibition, nonetheless, *Spirulina* (*Artrhospira maxima*) reduced the damage effectively mainly at concentrations of 4 and 10 mgL^{-1}, due to spirulina composition it may be consider as part of the diet for aquatic organisms, thus may protect organisms from toxicity induced by pharmaceutical products such as diclofenac, further studies focused in potential protective effects of spirulina against other pharmaceutical products, metals and emerging pollutants are highly recommended.

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8.3 CAPÍTULO DE REVISIÓN 1

Non-Steroidal Anti-Inflammatory Drugs in Water

Emerging Contaminants and Ecological Impact

Volume Editor: Leobardo Manuel Gómez-Oliván

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Contents

Teratogenesis and Embryotoxicity Induced by Non-steroidal Anti-Inflammatory Drugs in Aquatic Organisms

Itzayana Pérez-Alvarez, Hariz Islas-Flores, Leobardo Manuel Gómez-Oliván, and Octavio Dublán García

Contents

Abstract The continuous elimination of pharmaceutical products to water sources has become a worldwide problem and has been getting considerable attention due to the effects that this compounds have induced in aquatic organisms, specifically non-steroidal anti-inflammatory drugs (NSAIDs), one of the most representative group of medications and the most consumed around the world, highlighting the teratogenic and embryotoxic effects induced by NSAIDs on early life stages of different organisms being this the most vulnerable stages in development; the main representants of NSAID group (diclofenac, ibuprofen, naproxen, ketoprofen, paracetamol, acetylsalicylic acid) have induced adverse embryonic effects, which can be consider for the development of strategies for an appropriate disposal of pharmaceutical residues, as well as establish maximum permissible limits for its emission to the environment.

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1 Introduction

The presence of pharmaceutical products in the environment has become one of the main causes of concern worldwide; since the 1970s, interest in the determination of organic substances of these drugs in the environment began [1]; however, it was until the 1990s when analytical methodologies allowed the detection of this kind of products at concentration in the order of μg/L. Pharmaceuticals have been considered as emerging pollutant substances of diverse origin and nature that do not have an established environmental regulation but may be candidates for a future regulation depending on the data of the effects they generate on health and incidence; another important feature is that they do not need to persist in the environment to generate negative effects, because their high rates of transformation can be compensated by their constant introduction in to the environment, and also data regarding their impact on the environment and health risks are scarce [2–4].

The main sources of pharmaceuticals to bodies of water are derived from anthropogenic activities such as effluents from wastewater treatment plants, effluents from hospital, domestic activities (including pharmaceuticals and their metabolic products of phases I and II in feces and urine, waste and inappropriate disposal of expired pharmaceuticals), and effluents from industrial activities as well as livestock activities [5–8]. So that, the increase in the use and consumption has led to the continuous elimination of these products or their transformation and biotransformation products towards the aquatic environment [9], and due to are substances that were designed with the purpose of having a biological effect, either preventing or treating a disease, their presence in the environment can generate various acute and chronic adverse or toxic effects in non-target organisms.

Non-steroidal anti-inflammatory drugs are a group of pharmaceuticals that have analgesic, antipyretic, and anti-inflammatory activity; they are usually weak acids that owe their pharmacological activity to the inhibition of the enzymatic systems of cyclooxygenases (COX) 1 and 2 (Fig. 1); COX-1 catalyzes the conversion of prostaglandins and thromboxane A2 responsible for controlling the mucosal barrier in the gastrointestinal tract, renal homeostasis, and platelet aggregation among other physiological functions, while COX-2 is induced in inflammatory cells as a response to stimuli [11]. Its therapeutic uses are diverse, and some of them are over-the-counter drugs and are available to consumers without medical prescription; so they are positioned as one of the highest consumption groups worldwide [6], the most common ones are diclofenac, ibuprofen, naproxen, ketoprofen, acetaminophen, acetylsalicylic acid, and indomethacin; main characteristics of NSAIDs are described on Fig. 2.

NSAIDs have demonstrated their ability to generate toxic effects in different organisms inducing oxidative stress alterations on growth, development, and energy storage on *Limnodynastes peronii* [13], also oxidative stress in the brain, gill, liver, and blood of *Cyprinus carpio*, increasing lipoperoxidation and enzymatic activity

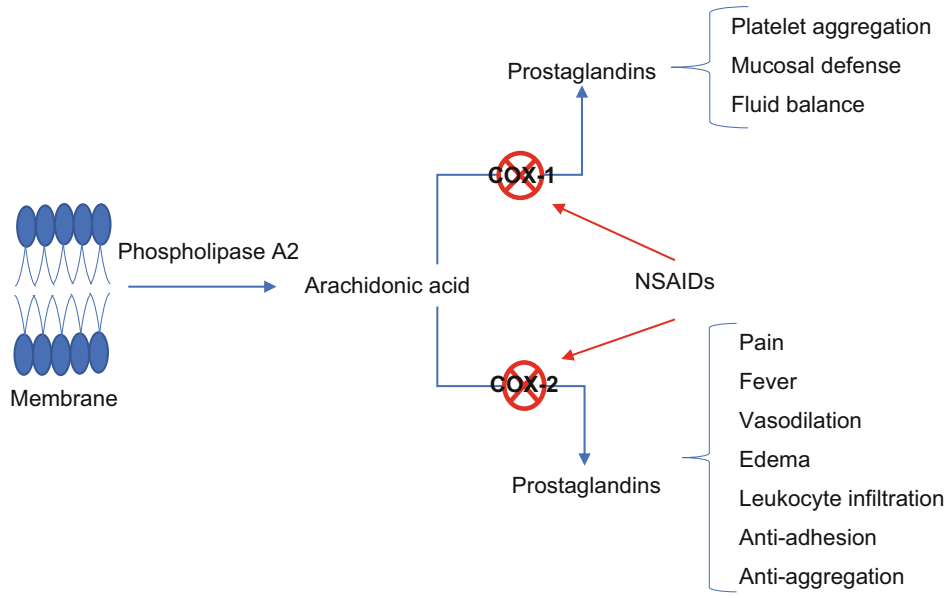

Fig. 1 General NSAIDs mechanism of action [10]

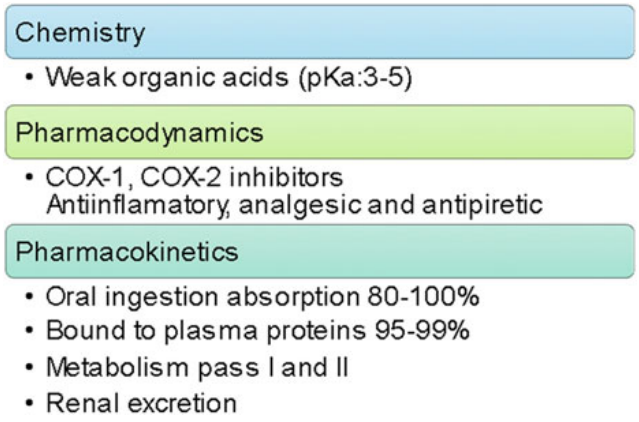

Fig. 2 Main characteristics of NSAIDs [12]

[14], increased oxidative damage and lipid catabolism in *Dreissena piolymorpha* [15], cytotoxicity and genotoxicity in *Daphnia magna* [16], stunting or growth inhibition on *Xenopus laevis* and *Lithobates catesbeianus* [17], and adverse effects on reproduction and offspring in *Danio rerio* [18] to mention some; however, to evidence this effects, bioassays must be done.

Biological methods or bioassays have been used for the determination of toxic on early life stages; these tests assess acute toxicity of chemicals or effluents to embryo and early life stages with lethality as the main endpoint [19].

Bioassays that focus on the evaluation of the development are important to identify if a substance or mixture of them can generate alterations in development;

these effects can be subtle or severe and can be manifested during embryonic development or subsequently throughout the life of the organisms. For the most part, embryotoxicity and teratogenicity studies focus on the development of mammals; however, contaminants such as pharmaceuticals manage to reach the bodies of water and come into contact with aquatic organisms; therefore, toxicity tests in early development stages of aquatic organisms are important since these organisms have its entire life cycle in aquatic environments and are frequently exposed to multiple stressors; this kind of studies can be useful for the identification and prioritization of development toxic substances [20].

Aquatic organisms are more sensitive during early stages of development; this may be because organisms in early stages of development have highly permeable membranes as well as different rates of absorption distribution and detoxification. Immature detoxification mechanisms can increase sensitivity to toxic agents, due to diverse physiological, morphological, and biochemical characteristics; since in the early life stages these responses are underdeveloped or have not yet fully developed, this contributes to a greater sensitivity compared to adult organisms [21].

Teratogens can affect morphogenesis, development, differentiation, and cell death; generate failures in cell interactions and cell movement; and affect cellular processes and different tissues; this can generate abnormalities and necrosis and can cause birth defects [22]. The effects on the development occur due to different mechanisms, depending on the teratogen agent will be the mechanism of action, and there may be more than one of them involved in the generation of adverse effects; there are some mechanisms described that can cause developmental alterations, some are disruptions in the central nervous system, modifications to DNA, enzymatic inhibition, hormonal alterations, cell membranes disruption, proteins or cellular organelles disturbances, and oxidative stress; Fig. 3 illustrates briefly how in one way NSAIDs can cause alterations in development and therefore teratogenesis [24, 25].

Since the toxicity of NSAIDs has been proven, special attention has been paid to the study of the possible toxic effects that these can generate in early development, in aquatic organisms; therefore some embryotoxic and teratogenic effects reported are described below.

1.1 Diclofenac

Diclofenac is one of the most widely used non-steroidal anti-inflammatory pharmaceuticals worldwide [26] and has been frequently detected in surface waters and effluents from wastewater treatment plants in concentrations in order of μg/L [27]. Some adverse effects caused by diclofenac have been reported previously, and herein are described some effects detected in early life stages of aquatic organisms.

The exposure of two Argentina native amphibians *Trachycephalus typhonius* and *Physalaemus albonotatus* to diclofenac at concentrations ranging from 125 to 4,000 μg/L for 96 h resulted in an LC_{50} of 2,828.43 μg/L and 2,462.29 μg/L,

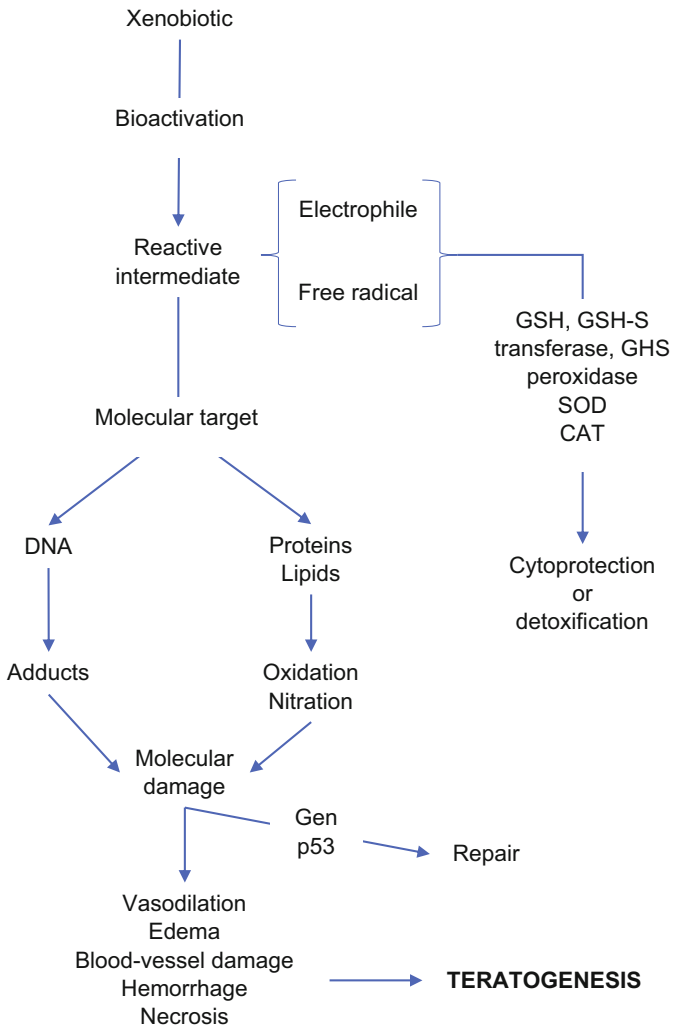

Fig. 3 Possible mechanisms by which NSAIDs can induce teratogenesis [23]

respectively; statistically significant differences were found in the size of the larvae with respect to the control group in both species, in addition to a small size and emaciation. Regarding the development and growth, there were also differences between the larvae; *T. typhonius* had a smaller size and lower degree of development than *P. albonotatus*; meanwhile, when evaluating the malformations for *T. typhonius*, the following were identified, absence of chondrocranium structures, absence of left hyobranchial skeletons, microcardia, increased gallbladder, and asymmetric pattern of gut, whereas for *P. albonotatus*, abdominal edema and altered axis, bilateral external body asymmetry, swollen body, absence of chondrocranium structures, partial hyobranchial skeleton, microcardia, and asymmetric pattern of gut were identified; microcardia was also observed in both species, and in

P. albonotatus, heart rhythm alterations were detected compared to the control group. Finally, a teratogenic index of 22.62 for *T. typhonius* and 19.69 for *P. albonotatus* was obtained, which indicate that diclofenac is a teratogenic agent to both species. Enzymatic activity of acetylcholinesterase and glutathione S transferase was also affected in both species showing different behaviors according to the concentrations tested; at low concentrations (125 μg/L), enzymes were inhibited, meanwhile at high concentrations, they were induced (2,000 μg/L); swimming behavior followed the same trend; at lower concentrations, a lower frequency was observed in the swim and less activity, while at higher concentrations, the frequency of activity and swimming was higher. These results show that diclofenac is a teratogenic drug for *Trachycephalus typhonius* and *Physalaemus albonotatus* triggering effects on embryogenesis and larval development; diclofenac is able to interfere with different biological functions affecting processes such as growth and development as well as generating abnormalities in different organs [28].

When *Mytilus galloprovincialis* were exposed to diclofenac at concentrations of 1 and 10 μg/L, the percentage of malformed embryos was approximately 30%; the malformations with the highest incidence were convex shell hinges, mineralization failures, transcription effects of several genes involved in biomineralization, biotransformation, antioxidant defense, and apoptosis; this demonstrates that diclofenac is capable to induce effects on the development of *Mytilus galloprovincialis* [29].

Danio rerio embryos were exposed to diclofenac at concentrations of 1.01, 3.38, 10.13, and 15.2 μM for 4 days; highest concentrations reached the maximum mortality effect at the fourth day of exposure, manifesting several abnormalities, mainly axial malformations and pericardial edema; abnormalities increased in severity as the concentration increased; at lower concentrations, malformations observed were shorter body length; smaller eye; muscle degeneration; lack of liver, intestine, and circulation; pericardial and body edema; and abnormal pigmentation. Diclofenac has the ability to be absorbed through non-covalent junctions and easily interact with embryos and thus generates developmental damage resulting in malformations such as curvature of the trunk and tail, as well as failure to regulate certain genes; such failures can lead to alterations in cardiogenic differentiation, which can generate pericardial edema as well as failures in the nervous system [30].

The exposure of *Salmo trutta* embryos to diclofenac at concentrations 0.1, 0.5, 1, 10, and 100 μg/L showed no toxic effects, and statistical differences to the control group were determined after the morality, hatching, development, or heart rhythm test through the embryonic development of *Salmo trutta* when it is exposed to these concentrations [31].

Danio rerio was exposed to diclofenac at 3.8, 7.5, and 15 mg/L; different malformations were observed, and the most recurrent were pericardial and yolk sac edemas and restricted systemic circulation; at 15 mg/L, a decrease in heart rate and a 100% inhibition of hatching were observed; at 3.8 mg/L, no severe effects were observed, and the hatching rate was not affected, nor were behavioral or in the swimming activity effects [32]. In another research, *Danio rerio* was exposed to 1, 20, 100, 500, 1,000, and 2,000 μg/L of diclofenac diluted with DMSO; no significant effects on the development of this organism were observed, even though

at concentrations of 1,000 and 2,000 μg/L; a decrease in the hatching rate was observed; however, the development was not affected, and no malformations were observed, nor significant adverse effects in early life stages, nor substantial changes were detected in stress proteins [33].

The evaluation 0.01, 0.05, 0.1, 0.5, 1 μg/L of diclofenac using *Mytilus galloprovincialis* as a bioindicator, changes in the larval development were observed, from the lowest concentration of barley (0.01 μg/L). Several malformations were observed, as well as deformations of the dorsal margin line in the shell of D-larvae; the LOAEC determined value was 0.01 μg/L. Diclofenac can seriously disturb the development of mollusks in the larval state at concentrations as low as 0.01 μg/L, without showing effects at higher concentrations. This research shows that bivalves are sensitive to environmentally relevant concentrations of diclofenac demonstrating the capacity of this pharmaceutical of generating irregularities in the formation of the shell [34].

Xenopus laevis embryos were exposed at diclofenac 1, 4, 16, 32, and 64 mg/L; the frequency of malformations increased from 16 mg/L and higher concentrations; at 24 h of exposure, 100% mortality was generated in embryos exposed to 64 mg/L. LC_{50} of 30.32 mg/L and MC_{50} of 12.25 mg/L were obtained, and a teratogenic index of 2.64 was determined, demonstrating that diclofenac has teratogenic potential, and as the degree of mortality increases, the degree of malformations increases as well. The most commonly observed malformations were axis, gut, heart, head, and eye abnormalities as well as blistering (edema); as the diclofenac concentration increased, larval length decreased; during stage 36, malformations observed were cardiac and intestinal. Effects on gene expression were generated; these failures indicate that the damage caused by diclofenac may be related with some proteins; it also generated neurological development failures [35]. The exposure of *Xenopus laevis* and *Lithobates catesbeianus* to 1, 4, 8, 16, 32, and 62.5 mg/L diclofenac resulted in a LC_{50} for *X. laevis* of 12.11 mg/L and LC_{50} 9.56 mg/L for *L. catesbeianus*; the highest concentration (62.5 mg/L, 100%) of mortality was reached in both organisms; all concentrations generated a decrease in the larvae size in both organisms; *X. laevis* was more sensitive than *L. catesbeianus*. The teratogenic index for *X. laevis* was 3.5 and for *L. catesbeianus* was 4.2. The most frequently observed malformations were axial malformations in the tail and notochord, edema, and hypopigmentation. Thus, this drug is a teratogenic agent for *Xenopus laevis* and *Lithobates catesbeianus* [17].

1.2 Ibuprofen

It is the third over-the-counter anti-inflammatory drug with the highest consumption worldwide, which is why it has been constantly detected in many bodies of water, rivers, and wastewaters, and the concentrations in which it has been detected are in the range of ng/L–μg/L [36, 37].

The exposure of adult organisms of *Danio rerio* to ibuprofen 1 μg/L induced alterations in reproduction, decreased the number of spawned eggs, 10 μg/L

exposure, as well decreased hatching rate of the progeny; this pharmaceutical is able to increase the mortality, decline maturation of sperm and gametogenesis, and produce developmental abnormalities; the most observed abnormalities due to ibuprofen exposure were cardiac edema and spinal malformations, and exposed embryos were also manifested [18].

Danio rerio embryos were exposed to ibuprofen 1 and 5 μg/L, mortality increases, and a decrease in swimming was identified, as well as failures in the response to stimuli. At 10 and 50 μg/L, ibuprofen increased mortality to 50%, and developmental damage was manifested; the hatching rate was also affected, and the most frequent malformations observed were failure in the organization of tail bud, optic vesicle, brain, and somites, as well as a decrease in body weight, size, and heart rate. At 100 μg/L, ibuprofen reached the highest degree of mortality to 57%, several malformations were observed, cardiac abnormalities could be seen visibly, and the most frequent malformations were cardiac edema, smaller size, absence of movement, and absence of response to external stimuli [38].

Ibuprofen concentrations of 0.01, 0.1, 1, 10, 100, and 1,000 μg/L were tested in *Mytilus galloprovincialis*; the exposure generated a dose-dependent behavior in terms of embryonic development; at 100 and 1,000 μg/L, several malformations were generated, the main one was convex hinge shells, and a LOAEC of 100 μg/L was obtained; this pharmaceutical can adversely affect embryonic development of bivalves at higher concentration than those detected in the environment [34].

Rana catesbeiana embryos were exposed to ibuprofen for 96 h; the first step was to obtain the premetamorphic LC_{50}, and it was 41.5 mg/L; then the specimens were subsequently exposed to 15 μg/L which generated effects on hepatic transcriptome and altered the levels of RNA in the liver; a reduction in the production of prostaglandins leads to effects in the metabolic state of this amphibian in the larval and post-embryonic stages; also, this pharmaceutical can act as an endocrine disruptor, disrupting the activity of certain genes that are involved in the metamorphosis processes of *Rana catesbeiana* [39].

After the exposure to ibuprofen LC_{50}, 56.7 mg/L, EC_{50} 39.9 mg/L, CMIC 30 mg/L, and IT 1.4 were obtained; the main abnormality observed was thoracic edema, and these results suggest that ibuprofen is a teratogenic pharmaceutical for *X. laevis* [40].

1.3 Naproxen

Naproxen is one of the anti-inflammatory drugs most commonly detected in bodies of water, wastewater treatment plants, rivers, and surface water in concentrations ranging from ng/L to μg/L [41, 42].

Danio rerio larvae were exposed to naproxen 0.1, 1, 10, and 100 μg/L, to subsequently evaluate the bioconcentration and evaluate effects such as thyroid disruption, as well as the mechanisms involved in the metabolism of this drug in zebrafish. At 0.1 and 1 μg/L, naproxen did not induced significant toxic effects on mortality compared to control group; however, at 10 μg/L, a 5% decrease in survival

was observed and at 100 μg/L a decrease of 7.5%; likewise at these concentrations (10 and 100 μg/L), there was a decrease in the larvae size and a reduction in the general weight. Regarding bioconcentration, values of 2052.69 ng/g were determined. Naproxen can generate a decrease in the growth of zebrafish and affect early life development remarkably [43].

Cyprinus carpio exposed to naproxen 10, 50, 100, and 200 μg/L showed a delay in embryo hatching; a mortality of 24% was determined, and growth was delayed. Some abnormalities were observed, and mainly pigmentation failure was observed; at 6-day postfertilization, in addition to malformations, gill cells were affected, alterations in the larval growth were generated, there was also a reduction in the body weight in all treated groups, the enzymatic activity was evaluated, and glutathione reductase activity declined; likewise variations were observed in glutathione transferase levels at 100 and 200 μg/L; finally the LOEC concentration was determined to be 10 μg/L [44].

The exposure of *Danio rerio* to naproxen at 0, 10, 20, 50, 75, 100, 125, 150, 175, 200, and 240 mg/L for 120 h induced a LC_{50} of 115.2 mg/L; for embryos at 96 h, a LC_{50} 147.6 mg/L was obtained, and a decrease in the hatching rate of the embryos was observed at 240 mg/L and also generated the greatest delay in embryonic hatching. The heart rate was decreased as the pharmaceutical concentration increased, and it was significantly inhibited at concentrations of 100 and 125 mg/L. The most frequently observed abnormalities were pericardial edema, yolk sac edema, and hemagglutination, weak pigmentation, hemorrhage, yolk condensation, and trunk abnormalities including without somites, tail not detached, axial malformation, and tail twisting. The most frequent sublethal effect was pericardial edema at a concentration of 20 mg/L; in addition, this malformation aggravated according to the increase in naproxen concentration, and also exposure to this pharmaceutical induced liver damage during the larval stage [45].

1.4 Ketoprofen

It is one of the first-line anti-inflammatory drugs for the treatment of several diseases, and its consumption for human and veterinary use is high; its production in Taiwan reached 7.9 kilotons in 2006 [46]. It has been detected in many bodies of water, surface water, groundwater, wastewater, and drinking water and has also been detected in solid atmospheres around the world [47].

Danio rerio embryos were exposed at 1, 10, and 100 μg/L of ketoprofen through 96 h; several malformations were observed in all concentrations tested, and the most relevant ones were edema, spinal curvature, slow heartbeat, an elongation of the heart, yolk sac edema, pericardial edema, and delayed hatching. After 48 h, a high mortality rate was observed and a decrease in the heart rate at 10 and 100 μg/L, whereas at 96 h, heart rate decreased significantly in comparison with the control group. According to these results, it is possible that ketoprofen produces abnormalities in the pericardium that cause alterations in heart rate and blood flow [48].

1.5 Celecoxib

Celecoxib is a non-steroidal anti-inflammatory drug that selectively inhibits COX-2 and is used as an anti-inflammatory and analgesic used to treat rheumatic diseases [49].

Xenopus laevis frog embryos were exposed to celecoxib, and LC_{50} of 8.99 mg/L, EC_{50} of 5.8 mg/L, and an IT of 1.54 were obtained; mortality and malformations increased according to the increase of celecoxib concentration. This pharmaceutical generated several malformations being the most frequent in the intestine, edema, hemorrhage, and abnormalities in the heart and blood vessels; mainly affected systems were cardiovascular due to the induction of effects on vascular cutting during development, which culminated in hemorrhage and edema, and digestive system due to its important effects. According to the results obtained, celecoxib is a teratogen pharmaceutical for this species [50].

1.6 Paracetamol

Paracetamol is a commonly used pharmaceutical; it is ubiquitous in the natural environment, and it easily accumulates in the aquatic environment; paracetamol has been detected in surface waters, sewage, and drinking water worldwide [51].

Daphnia magna exposure to paracetamol induced a significant increase in toxicity, with a dose-dependent behavior. The exposure of *Scenedesmus subspicatus* algae gave as a result an impaired growth of 50% at 134 mg/L of paracetamol. There were not significant effects in the exposure of *Brachydanio fish* embryos even at concentrations of 1 g/L [52].

In the damage assessment of paracetamol in *Xenopus laevis*, the following parameters were obtained, an LC_{50} of 191.1 mg/L, EC_{50} of 143.3 mg/L, and an IT 1.3. Based on the results obtained, paracetamol can be classified as an agent with low teratogenic potential; however, it can generate malformations in the absence of mortality. Malformations observed were intestinal, craniofacial, cardiac, pericardial, and ophthalmic edema [53]. Another research reports that no statistically significant differences were observed regarding the control group; however, all embryos showed malformations such as tail bending edemas and abnormalities in bowel curl; as for growth, no differences were found regarding the control group [40].

1.7 Acetylsalicylic Acid

It is a frequently used anti-inflammatory that is detected in the environment and contributes to environmental pollution and has been detected in surface waters at concentrations up to 340 ng/L [54].

Daphnia magna exposure to acetylsalicylic acid gave as a result an effective concentration for malformations EC_{50} of 118 mg/L, and for *Brachydanio fish*, the biological response to acetylsalicylic acid showed a more sensitive behavior, with an LC_{50} of 37 mg/L and a pulse reduction at 50 mg/L [52].

Cyprinus carpio was exposed to 0.004, 0.04, 0.4, 4, and 20 mg/L of acetylsalicylic acid; the hatching rate was significantly higher in the exposed embryo group compared to the control in concentrations of 0.004, 0.04, and 0.4 mg/L. In terms of mortality, it remained lower than 17% in the exposed groups as well as in the control group; a reduction in development was observed after day 6 of exposure to 20 mg/L and on day 13 in groups exposed to 0.004, 0.04, and 0.4 mg/L; while at the end of the experiment, a stimulation was observed in the development of organisms exposed to 0.004, 0.04, and 0.4 mg/L compared to the control group; in contrast, at 20 mg/L, a diminution in development was observed. Numerous abnormalities in development were observed axial hyperpigmentation and/or lateral curvature of the spine as well as dermal alterations and an increase in mucous cells to name some; also there was a decrease in body weight at 20 mg/L; however, as the concentration of pharmaceutical was minor, body weight was increased. With regard to the oxidative stress tests, an increase in lipoperoxidation and a decrease in the activity of antioxidant enzymes CAT, GPX, and GR were determined; finally a LOEC of 0.004 mg/L was obtained, and at this concentration, histopathological damage was observed [55].

2 Conclusions

Pharmaceutical products are substances that were designed to have a biological effect, to either prevent or treat a disease; however, their constant use and elimination towards the aquatic environment during several years have generated an alarming problem; due to its effects on most aquatic organisms still unknown, on the other hand, scientific groups have special attention in the study of the effects that pharmaceuticals can generate in different organisms. NSAIDs are the most consumed and eliminated group of drugs worldwide, and numerous effects have been evidenced in aquatic organisms and in the environment; however, research in early stages of development are scarce; nevertheless some research showed toxic effects that these pharmaceuticals can generate at environmentally relevant concentrations in early stages of development; this stage of life is important because it is the stage in which organisms are more susceptible to damage. More studies related to drug toxicity in organisms at early stages of development are necessary since it is the most critical period of development because embryos are at the topmost in cell division and differentiation and in the process of tissue and organ formation.

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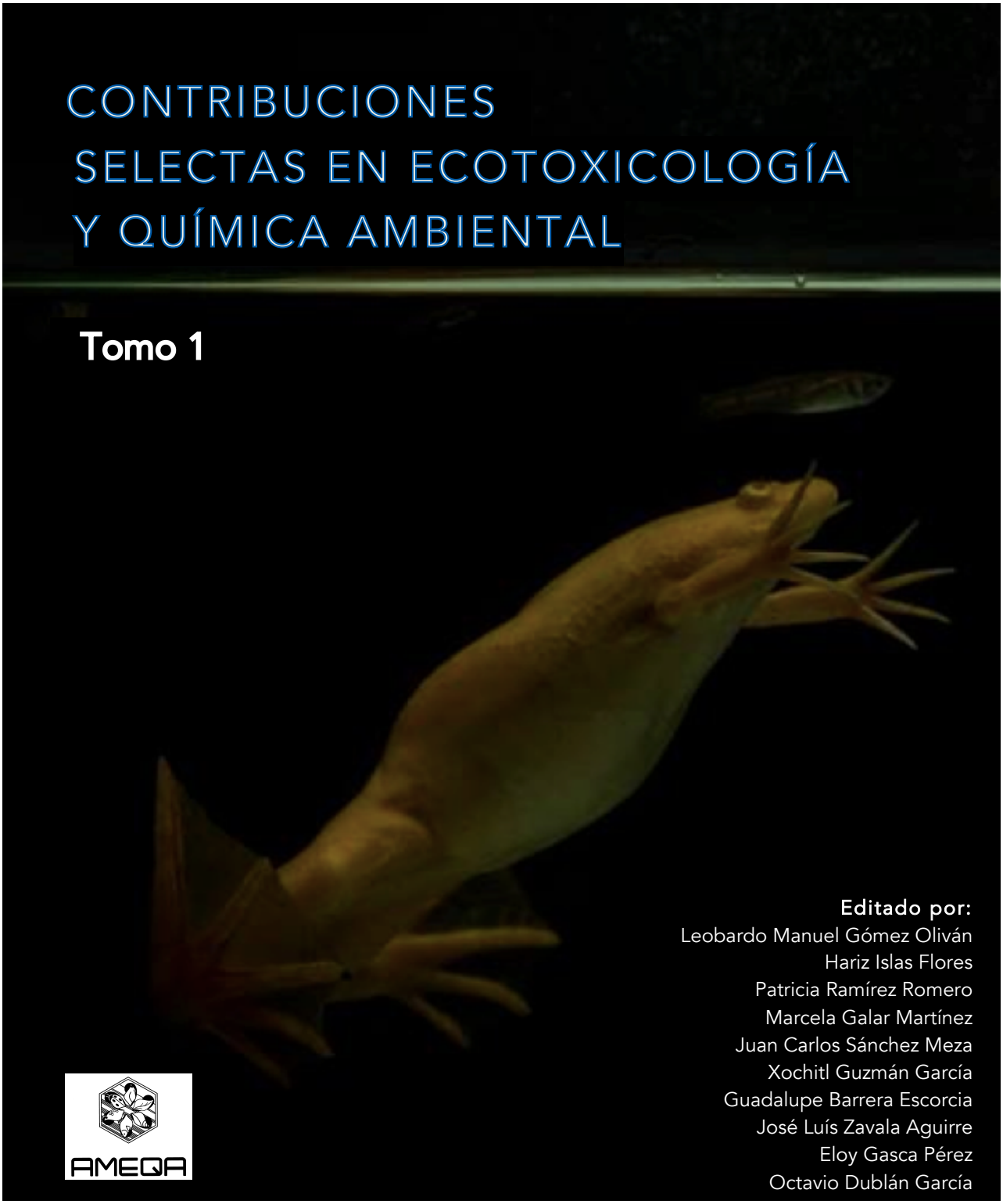
8.4 CAPÍTULO DE REVISIÓN 2

CONTRIBUCIONES SELECTAS EN ECOTOXICOLOGÍA Y QUÍMICA AMBIENTAL

Tomo 1



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José Luís Zavala Aguirre
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Foto de Portada por Leopoldo I. Flores. 2020.

Capítulo 8

EVALUACIÓN DE LA TOXICIDAD DE CONTAMINANTES AMBIENTALES MEDIANTE EL ENSAYO FETAX

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Resumen

El ensayo FETAX (ensayo de teratogénesis de embriones de rana-*Xenopus*) es una prueba útil para evaluar la toxicidad de sustancias puras, mezclas complejas y sedimentos, durante etapas tempranas del desarrollo de la rana *Xenopus laevis*, esta prueba se centra en la organogénesis, proceso más sensible durante el desarrollo de todos los organismos debido a la falta de madurez de los sistemas de defensa. Este ensayo cuenta con la opción de agregar un sistema de activación metabólica que simula los procesos de biotransformación que se llevan a cabo durante el desarrollo embrionario de los mamíferos, ha sido utilizado en diversos estudios toxicológicos para la evaluación de contaminantes presentes en el medio ambiente debido a sus diversas ventajas: exposición durante tiempos cortos (96h), costos relativamente accesibles y reproducibilidad entre otras. El principal objetivo de esta revisión bibliográfica es destacar la utilidad y flexibilidad que ha demostrado tener este ensayo, además de su aplicación en la evaluación del riesgo ambiental de varias sustancias.

Palabras clave: FETAX, *Xenopus laevis*, teratogénesis.

1. Introducción

Desde hace aproximadamente 50 años existe una demanda creciente de diversos recursos materiales, mismos que han originado la necesidad de fabricar productos nuevos para satisfacer las necesidades de la población, lo que da como resultado problemas de contaminación. Los cuerpos de agua son uno de los recursos más afectados debido a la presencia de varios productos provenientes de procesos industriales, descargas domésticas y hospitalarias, los cuales resultan ser tóxicos para el medio ambiente y pueden generar efectos adversos en varios organismos (Dumont, Bantle, & Linder, 2003).

Para evaluar los efectos de dichos contaminantes en el ambiente, se utilizan varias herramientas para la identificación, cuantificación y evaluación del riesgo (Committee on Methods for Acute Toxicity Tests with Aquatic Organisms, 1975), los cuales han contribuido a que a lo largo de la historia se desarrollen algunos programas de protección ambiental y manejo adecuado de sustancias tóxicas. Entre estas herramientas se encuentra el uso de bioensayos, que generalmente evalúan los efectos adversos generados en una especie, bajo condiciones controladas en laboratorio y cuyo objetivo principal es mostrar los efectos tóxicos de una sustancia o mezcla de sustancias. Un bioensayo utilizado durante las últimas décadas es el ensayo FETAX, el cual tiene el potencial de evaluar distintos tipos de muestras, evidenciado efectos adversos generados en la fase de organogénesis.

1.1 Ensayo FETAX: historia y desarrollo

Durante 1970-1980, se desarrollaron diferentes tecnologías en todo el mundo, razón que propició la búsqueda de recursos energéticos; Estados Unidos fue uno de los países más productivos. El principal objetivo de las industrias era obtener recursos energéticos para cumplir con los requerimientos industriales para manufactura de diversos productos, la provisión de servicios e incluso cubrir necesidades domésticas, dicha situación llevó a la generación de una variedad de contaminantes, llamando la atención de muchas organizaciones de investigación ambiental, entre las que se encontraba la Agencia de Protección Ambiental (EPA) (Bergman, 1985). En ese momento, ya se habían desarrollado algunos ensayos biológicos para la evaluación del riesgo ambiental, entre ellos la prueba de Ames para evaluar la mutagenicidad (Ames & McCann, 1975) y la de carcinogenicidad (Hsie *et al.,* 1978), el cultivo celular y las pruebas de toxicología acuática en organismos como *Daphnia magna* y *Daphnia pulicaria* (Biesinger & Christensen,

1972), también algunos ensayos en peces como *Pimephales promelas*, pero ninguno de ellos fue útil para evaluar la toxicidad durante el desarrollo embrionario.

James Dumont y sus colaboradores se centraron en el desarrollo de una prueba de toxicidad aguda en la cual emplearon embriones de anfibios como organismos bioindicadores; este proyecto fue diseñado y llevado a cabo en el laboratorio nacional de Oak Ridge en Tennessee, y su principal objetivo fue evaluar la toxicidad del carbón y los productos de conversión de aceite y combustibles. Con dicho estudio comenzó el desarrollo del ensayo FETAX, mismo que se publicó en 1983. Después de la estandarización de la prueba, sugirieron utilizarla para la evaluación de diferentes tipos de muestras (suelo, agua, efluentes, sedimentos, etc.) (Bruner *et al.,* 1998) como una herramienta para la evaluación del riesgo ambiental (Bantle & Sabourin, 1991).

Las primeras pruebas se llevaron a cabo siguiendo la metodología propuesta por Dumont en 1980, centrándose en el estudio de los efluentes y productos de desecho provenientes de industrias productoras de combustibles, que en su mayoría eran mezclas de diferentes sustancias orgánicas e inorgánicas en rangos de concentración variable. El primer estudio que se llevó a cabo duró 96 horas, utilizando agua ácida proveniente de los procesos de gasificación de carbono para obtener combustible (Schultz *et al.,* 1982), para lo cual se seleccionaron 50 ovocitos para cada concentración probada, con 4 réplicas. Los resultados obtenidos revelaron efectos dependientes de la concentración en términos de mortalidad, desarrollo anormal, malformaciones e inhibición del crecimiento (Dumont & Schultz, 1980).

Estos estudios se propagaron y replicaron para la evaluación de varias sustancias y productos provenientes de diferentes procesos industriales; los resultados observados en cada prueba fueron totalmente diferentes en términos de los parámetros evaluados y llevaron a la siguiente proposición: "Cada sustancia o mezcla causará efectos característicos y propios en los embriones expuestos, en caso de usar sustancias que tengan un mecanismo de acción similar los resultados podrían ser similares" (Rogers y Kavlock, 1996). Una vez que se realizaron las pruebas en mezclas y extractos, se procedió a realizar ensayos de compuestos puros, que se realizaron utilizando sustancias que previamente se clasificaron como compuestos no teratógenos en mamíferos. Se probaron un total de 41 sustancias, 85% de ellas resultaron teratogénicas para *Xenopus laevis* evidenciando respuestas similares a las ya observadas en mamíferos, lo que sugirió que los mecanismos de acción son similares entre estos y los anfibios, principalmente en el desarrollo embrionario. Después de que se realizaron estas pruebas, se inició el proceso de estandarización (*American Society for Testing Materials*, 2012; Bantle *et al.,* 1994).

Durante dicho proceso se especificaron los requisitos mínimos necesarios para llevar a cabo este ensayo: la duración de la prueba sería de 96 horas clasificándola como una prueba de toxicidad aguda, los embriones deberían seleccionarse en la fase de blástula media a gástrula temprana, y se utilizarían 20-25 embriones para cada concentración probada, por triplicado, manteniendo una temperatura de 22±2 °C. Además se debería usar una solución de cultivo estandarizada; aunado a la determinación de cinco parámetros finales: mortalidad evaluación diaria durante 96 h mediante la concentración letal media CL_{50}, el número de embriones con malformaciones, así como su tipo a través de la concentración efectiva para malformaciones CE_{50}, el índice teratogénico obtenido del cociente de la mortalidad y las malformaciones ($IT = CL_{50} / CE_{50}$) y la concentración mínima para inhibir el crecimiento misma que se obtiene de la medición de las larvas de la cabeza a la cola.

Posteriormente, la *American Society for Testing Materials* (ASTM) publicó el protocolo FETAX, que especifica los detalles para complementar dicho ensayo, así mismo, se publicó un atlas de anormalidades en la década de 1990; y se realizaron estudios interlaboratorio para corroborar la confiabilidad y repetibilidad de la prueba (Bantle *et al.,* 1994). Los 8 laboratorios que participaron inicialmente colaboraron realizando la misma prueba, hicieron mejoras a la metodología, modificaciones que fueron publicadas en 1998. Además se realizó el estudio empleando otras especies de anfibios y se agregó un sistema de activación metabólica (Fort *et al.,* 1998) para simular los procesos de embriogénesis que se llevan a cabo en organismos mamíferos y se estableció el uso de un agente tóxico de referencia para proporcionar una mayor confiabilidad al ensayo. Finalmente, el ensayo FETAX se confirmó como una herramienta confiable, repetible y útil para predecir la toxicidad de una sustancia o mezcla tóxica para el desarrollo embrionario de *Xenopus laevis* (Bantle *et al.,* 1999).

Desde entonces se han realizado varias pruebas, para evaluar mezclas complejas (Dumont *et al.,* 1983), sustancias puras y suelos; además se ha utilizado para la evaluación de sistemas de tratamiento de aguas residuales, con el propósito de estimar la calidad de los procesos y el agua que fluye hacia los cuerpos de agua, así como para evaluar los efectos del riesgo ecológico en los organismos acuáticos, principalmente anfibios.

Los estudios ecotoxicológicos emplean en su mayoría organismos juveniles o adultos. Los estudios enfocados en etapas tempranas del desarrollo son escasos; a pesar de que durante la organogénesis los organismos son muy sensibles y el daño puede permanecer durante el resto de su vida o inclusive causar la muerte; por lo anterior ha sido importante la evaluación de diversos contaminantes presentes en el

ambiente utilizando el ensayo FETAX, algunos de los cuales incluyen la utilización de embriones de *Xenopus laevis* expuestos a aguas provenientes de minas (Dawson, McCormick, & Bantle, 1985), industria y plantas de tratamiento de aguas residuales, así como también sedimentos que contienen metales (Herkovits *et al.,* 1997) y plaguicidas (Yu *et al.,* 2013), sustancias altamente persistentes en el ambiente. Desde la década de 1980 hasta el año 2000 se estimó que habían sido evaluados 40 compuestos heterocíclicos, 29 amidas e hidrazidas, 24 ácidos fenólicos y ácidos carboxílicos, 22 alcoholes, 20 sales, 45 productos farmacéuticos, 17 compuestos de síntesis química, 13 pesticidas, 11 aditivos alimentarios y 7 colorantes. A continuación se mencionan algunos estudios en los cuales se ha utilizado este ensayo para la evaluación de distintos tipos de contaminantes.

1.2 Evaluación de plaguicidas

Los plaguicidas pueden definirse como un grupo de compuestos con un rango amplio de actividad, pueden utilizarse como herbicidas, fungicidas, nematicidas, reguladores de crecimiento de plantas entre otros, y pueden clasificarse por su modo de acción o por su naturaleza química (Arias-Estévez *et al.,* 2008; McKnight *et al.,* 2015), a pesar de su amplio uso, también son conocidos sus efectos negativos sobre organismos silvestres y seres humanos, y siguen siendo tema de interés para realizar diversos trabajos de investigación, entre los cuales el ensayo FETAX ha sido una herramienta útil de la evaluación de toxicidad en etapas tempranas del desarrollo. A continuación se muestran algunos estudios en los cuales se demuestran los potenciales efectos tóxicos de este tipo de sustancias.

a. α-cipermetrina

Es un plaguicida piretroide que actúa por contacto e ingestión sobre el sistema nervioso central y periférico de lepidópteros, hemípteros y otros órdenes de importancia agrícola; al realizar la evaluación de este compuesto en *Xenopus laevis* expuesto durante 96 h se obtuvo una CL_{50} de 30.6 µg/L, los embriones sufrieron malformaciones axiales, así como alteraciones en su comportamiento, espasmos y convulsiones. En larvas expuestas a este compuesto durante 96 h se obtuvo una CL_{50} de 6.9 µg/L, indujo cambios en el comportamiento, hipersensibilidad, espasmos, convulsiones y alteraciones en el nado al igual que malformaciones axiales (Yu *et al.,* 2013).

b. Endosulfán

Es un plaguicida organoclorado utilizado como insecticida cuyo uso es restringido ya que es capaz de generar diversas afectaciones en el humano,

principalmente en el sistema endocrino, inmunológico y neurológico además de causar daño severo al hígado (*Agency for Toxic Substances & Disease Registry*, 2011). Generó una CL_{50} de 1266 µg/L, ocasionó retraso en el crecimiento, letargia y malformaciones axiales severas, así como hemorragia en cola y aleta durante la etapa embrionaria. Al exponer a los organismos en su etapa larvaria, se obtuvo una CL_{50} de 121µg/L, de igual manera se presentaron alteraciones en el comportamiento, alteraciones en la prueba de nado e inactividad (Yu *et al.,* 2013).

c. Malatión

Es un plaguicida organofosforado utilizado para el control de insectos, cumple su función actuando por contacto, inhalación e inclusive ha sido utilizado para el control de piojos en humanos. Al igual que otros plaguicidas afecta el sistema nervioso, puede generar problemas respiratorios y gastrointestinales *(Agency for Toxic Substances and Disease Registry*, 2003). Al exponer a embriones de *Xenopus laevis* se obtuvo una CL_{50} de 5396 µg/L, de igual manera se observó un incremento en la mortalidad durante las últimas horas de exposición y generó malformaciones a las concentraciones más elevadas probadas en un periodo de 72h. En larvas expuestas a malatión se obtuvo una CL_{50} de 6756 µg/L, se identificaron malformaciones axiales y anormalidades en el nado (Yu *et al.,* 2013).

Al comparar los tres plaguicidas (α-cipermetrina, endosulfán, malatión) las malformaciones más frecuentes observadas tanto en embriones como en larvas fueron en intestino, malformaciones axiales y edema; de igual manera fueron capaces de inducir una significativa inhibición del crecimiento; además las alteraciones en el comportamiento fueron comunes ante la exposición a plaguicidas ya que actúan como disruptores de los canales de sodio dependientes de voltaje que se encuentran en las membranas de las células nerviosas, de igual manera la sobrestimulación de músculo debida a la inhibición de la acetil colinesterasa causa doblamiento de la cola de embriones y larvas (Yu *et al*., 2013).

d. Clorpirifós

Es un plaguicida organofosforado utilizado como insecticida y para controlar plagas de nematodos, con anterioridad fue utilizado en las viviendas para el control de plagas, sin embargo sus efectos tóxicos han generado una reducción en su uso pues genera neurotoxicidad (Whitney *et al.,* 1995), desordenes inmunológicos y malformaciones. Fue capaz de causar efectos en el comportamiento asociados a un daño neurológico. Las principales alteraciones generadas en *Xenopus laevis* por este plaguicida tras 120 h de exposición corresponden a defectos neuromusculares espasmos, temblores parálisis y fallas al nadar, efectos que siguieron un comportamiento concentración-dependiente. Además dieron lugar a

malformaciones en intestino y alteraciones axiales, daños en somitas y daños a nivel de tejido en miocitos y miotomas, estos efectos pueden terminar en distrofia muscular y comprometer el desarrollo de los organismos (Bonfanti *et al.,* 2004).

e. Ácido giberelico

Es un fitorregulador de crecimiento de acción hormonal que estimula y regula el desarrollo de las plantas (*University of Hertfordshire*, 2018), al exponer a *Xenopus laevis* a este compuesto durante 96 h se obtuvo una CL_{50} de 1117.5 mg/L y un IT de 1.69, se mostró un comportamiento concentración-dependiente en cuanto a la incidencia de malformaciones en los embriones tales como edema, doblamiento de cola, microftalmia y microcefalia (Pekmezekmek *et al.,* 2013).

f. Triadimefon y Triadimenol

Son derivados de triazoles utilizados como agentes antifúngicos, interfieren con la biosíntesis de esteroides como lanosterol y ergosterol que constituyen en mayor medida la pared fúngica y por esta razón son utilizados ampliamente en la agricultura *(United States Environmental Protection Agency*, 2016). Al evaluar estos compuestos durante un periodo de 96 h utilizando el ensayo FETAX y como bioindicador a *Xenopus laevis*, el triadimefon resultó con un IT mayor a 3 que lo categoriza como un agente teratógeno; el cual ocasionalmente generó acortamiento de la parte anterior de la cabeza y una ligera protrusión de la mandíbula no móvil. Los elementos mandibulares como articulaciones no fueron evidentes, las vesículas cerebrales estaban presentes pero anormalmente dobladas en dirección ventral como consecuencia de anormalidades craneofaciales severas. En el caso del triadimenol las malformaciones a nivel mandibular fueron menos severas; los defectos en la mandíbula y parte dorsal-anterior de la cabeza al igual que edema y reducción en tamaño se observaron ocasionalmente para ambos compuestos (Groppelli *et al.,* 2005).

g. Atrazina (2-cloro-4-etilamino-6-isopropilamino-s-triazina) y 2,4-D (ácidodiclorofenoxiacético),

Son herbicidas que han sido detectados en aguas profundas y superficiales, debido a su uso frecuente que permite la entrada de manera directa o indirecta al medio ambiente (Pimentel & Levitan, 1986). Al exponer a embriones de *Xenopus laevis* durante 96 h a estos herbicidas se obtuvieron los siguientes resultados: para Atrazina CL_{50} de 100 mg/L, CE_{50} 33 mg/L, IT 3, mientras que para 2,4-D se obtuvo una CL_{50} de 254 mg/L, CE_{50} de 245 mg/L y un IT de 1.04; lo que indica que ambos herbicidas poseen bajo riesgo teratógeno, debido a que los efectos embriotóxicos y teratogénicos ocurrieron a concentraciones elevadas (Morgan, 1996).

h. Glifosfato

Es uno de los herbicidas más utilizados a nivel mundial en campos de cultivo y en el sector doméstico (Benbrook, 2016). Es un compuesto lipofílico y su tiempo de vida media es de 2 a 14 días, además ha sido detectado en concentraciones de 0.7 mg/L en campos de cultivo(Peruzzo, Porta, & Ronco, 2008). Debido la exposición a este compuesto durante 96 h se produjeron anormalidades como edema, alteraciones craneofaciales, microftalmia, estrechamiento de ojos y malformaciones severas a concentraciones elevadas. Se obtuvo una CL_{50} de 24,78 mg/L, CE_{50} de 7.8 mg/L, además se obtuvo una concentración mínima inhibitoria del crecimiento (CMIC) de 5 mg/L además de un IT de 3.4, mismo que indica que la sustancia es altamente teratogénica, evidenciando el potencial tóxico de este compuesto en *Xenopus laevis* (Bonfanti *et al.,* 2018).

1.2 Metales pesados

a. Cromo

Es un metal ampliamente distribuido a nivel mundial y en ambientes acuáticos puede ser detectado en concentraciones traza, sin embargo, es capaz de provocar efectos tóxicos, específicamente el cromo (VI) que es un mutágeno cuya actividad toxica ha sido demostrada en bioensayos realizados empleando bacterias y mamíferos. Por otra parte es capaz de causar malformaciones esqueléticas y alta mortalidad en embriones de ratón (Trivedi *et al.,* 1989).

Al exponer a embriones de rana *Xenopus laevis* a cromo (VI) durante 96 h se obtuvo una CL_{50} de 890 µM, una CE_{50} de 260 µM y un IT de 3.42, ubicando al cromo (VI) como un metal que posee un alto riesgo teratogénico. Generó diversas malformaciones en intestino, cola y aleta además de edema múltiple acompañado de un severo retraso en el crecimiento.

Se evidenció un proceso de bioacumulación, mismo que variaba de acuerdo con la fase de desarrollo del organismo. Durante las primeras fases el cromo se concentra principalmente en la capa protectora del embrión, posteriormente su concentración permanece en la larva, los órganos en los que se observó mayor concentración de este metal fueron abdomen, cabeza y cola (Bosisio *et al.,* 2009).

b. Níquel

Es un metal ampliamente utilizado en la industria para la producción de baterías, recubrimientos anticorrosivos, obtenido en procesos de minería, utilizado para galvanoplastia entre otras actividades (Raval, Shah, & Shah, 2016), razón por la

cual es eliminado de manera constante al ambiente en donde puede llegar a generar daños. Durante la exposición de *Xenopus laevis* a Ni^{2+} durante 101 h generó daños principalmente en ojo, a concentraciones de 4.5 µmol/L, los daños manifestados fueron microftalmia, hipopigmentación focal, y quistes en el plexo coroideo y la retina, habiendo daño también en el iris, engrosamiento del nervio óptico, desplazamiento lateral y/o dorsal de los ojos y daño craneofacial. La severidad de las malformaciones así como su incidencia siguió una relación concentración-dependiente (Hauptman *et al.,* 1993).

c. Cadmio

El cadmio es un metal ampliamente utilizado en procesos industriales, por lo que se encuentra ampliamente distribuido en el ambiente. Es un metal no esencial y los principales órganos afectados por la exposición son el hígado y el riñón; puede entrar en contacto con diversos organismos a través del consumo de agua o alimentos contaminados (Okorie *et al.,* 2014).

Ante la exposición de *Xenopus laevis* a este metal durante 96 h se obtuvieron una CL_{50} de 32 µmol/L, CE_{50} de 3.7 µmol/L, CMIC de 18 µmol/L y un IT de 8.6, lo que lo ubica como un agente teratógeno. Las malformaciones encontradas siguieron un comportamiento concentración-dependiente y fueron principalmente en intestino, notocorda y aleta, displasia facial, anormalidades en ojo y cardiomegalia (Sunderman, Plowman, & Hopfer, 1991). Adicionalmente se ha detectado que este metal es capaz de generar problemas en el proceso de eclosión de embriones a concentraciones bajas (0.6 ppm) además de generar un disminución de crecimiento larvario (Haywood *et al.,* 2016).

d. Cobalto

Es un metal utilizado principalmente en la manufactura de aleaciones, por lo que se encuentra en diversos ambientes. Es capaz de producir daños a nivel reproductivo, asma alveolitis entre otras patologías (Domingo, 1989). Por la exposición de *Xenopus laevis* a $CoCl_2$ durante 96 h se logró obtener la CL_{50} de 10.4 mM/L, la CE_{50} de 25 µM/L, y un IT de 416. Se observó un patrón concentración-dependiente en la incidencia y severidad de malformaciones, el intestino fue el órgano mayormente afectado. También se hallaron malformaciones oculares como microftalmia y disminución en la distancia interocular, axiales en notocorda y cola, displacía craneofacial y malformaciones cardiacas (Plowman *et al.,* 1991).

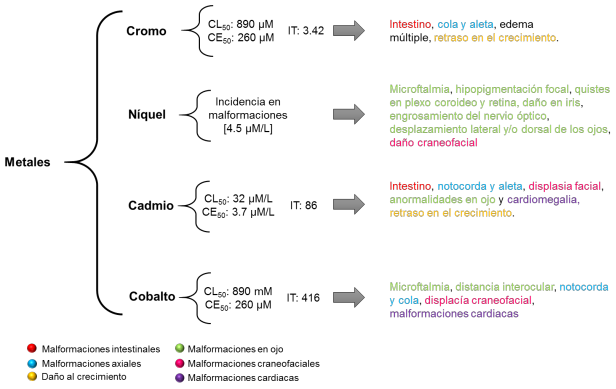

Fig. 1 Efectos tóxicos generados por metales en embriones de *Xenopus laevis.* Fuente: elaboración propia.

En la figura 1 se muestran en resumen los efectos tóxicos generados por metales en *Xenopus laevis*, es evidente que todos poseen elevado potencial teratogénico. Las afectaciones que comparten estos metales son: intestinales, oculares, axiales, craneofaciales, cardiacas y disminución del crecimiento. El hecho de que exhiban los mismos efectos tóxicos puede deberse a que su toxicidad se da por mecanismos similares; son capaces de bioacumularse, inhibir procesos fisiológicos, reproductivos y causar daño histológico.

1.3 Aditivos alimenticios

a. Ácido cítrico (E330)

Está presente en altas concentraciones en frutas como limón y limas, es un conservador natural y se utiliza como saborizante, tiene diversos usos en la industria alimenticia, cosmética e incluso textil (Nica & Woinaroschy, 2010). Se consideraba un compuesto no tóxico, sin embargo en diversos estudios se ha demostrado que a largo plazo puede generar efectos adversos en diversos organismos principalmente mamíferos. En *Xenopus laevis* causó diversas malformaciones en intestino, malformaciones oculares como microftalmia, en riñón, edema y anormalidades en las somitas, a través del ensayo FETAX se obtuvo una CL_{50} de 0.0124 g/L, sin embargo, las malformaciones fueron poco frecuentes razón por la cual no se obtuvo un valor de CE_{50} e IT, por otro lado, la CMIC fue de 0.010 g/L (Boğa-Pekmezekmek *et al.*, 2013).

1.4 Fármacos

a. Paracetamol

Es un antiinflamatorio no esteroideo de amplio uso y consumo a nivel mundial. Tiene intermediarios altamente electrofílicos que son capaces de unirse covalentemente a macromoléculas nucleofílicas de importancia biológica y generar daño (Nunes *et al.,* 2014). Al realizar la evaluación de daño se obtuvieron los siguientes parámetros, una CL_{50} de 191.1 mg/L, CE_{50} de 143.3 mg/L y un IT 1.3. Con base en los resultados obtenidos el paracetamol se puede clasificar como un agente con bajo potencial teratógeno, sin embargo, es capaz de generar malformaciones en ausencia de mortalidad. Las malformaciones observadas fueron en intestino, craneofaciales, cardiacas, edema pericárdico y oftálmico (Fort *et al.,* 1992). En otro estudio se encontró que no se observaron diferencias estadísticamente significativas con respecto al grupo testigo, sin embargo, todos los embriones mostraron malformaciones como doblamiento de la cola edemas y anormalidades en el enrollamiento del intestino, en cuanto al crecimiento no se encontraron diferencias con respecto al grupo testigo (Sean *et al.,* 2006).

b. 2 fenoxietanol (Etilen glicol monofenil éter)

Es un agente ampliamente recomendado para generar una inducción a la anestesia así como inmovilización por periodos cortos de tiempo en peces y anfibios (Ross & Ross, 2008). Al realizar la evaluación obtuvieron una CL_{50} de 588 mg/L, CE_{50} de 384 mg/L, CMIC de 300 mg/L; las malformaciones más frecuentes fueron edema severo y malformaciones axiales, el IT fue 1.69, clasificando así al fenoxietanol como un agente con potencial teratógeno para *Xenopus laevis* (Vrskova & Modra, 2012).

c. Eugenol

Es en un 80-90% la sustancia activa del aceite de clavo, mismo que es utilizado como anestésico debido a su baja incidencia en cuanto a reacciones adversas en peces y anfibios (Ke *et al.,* 2018). Al exponer embriones de *Xenopus laevis* a este compuesto se obtuvo una CL_{50} de 21.60 mg/L, acompañada de una CE_{50} de 35.71 mg/L. Las alteraciones más frecuentes fueron malformaciones axiales como doblamiento, malformación de intestino, microftalmia y edema. El valor obtenido para la CMIC fue de 20 mg/L finalmente el IT fue 0.61; los valores obtenidos ante la exposición a eugenol evidencian que no representa riesgo teratogénico para *Xenopus laevis* (Vrskova & Modra, 2012).

d. Ritodrina

Es un fármaco utilizado como tocolítico para la prevención de parto pretermino. Su mecanismo de acción se basa principalmente en la relajación del musculo liso uterino (Aronson, 2016). Al realizar la evaluación de riesgo teratogénico se obtuvo una CL_{50} de 28.571 mg/L, debido a la baja frecuencia de malformaciones no se obtuvo la CE_{50}; sin embargo, las malformaciones que se presentaron incluyeron problemas en intestino, microftalmia, malformaciones en las somitas y edema. Se obtuvo una CMIC de 12 mg/L; sin IT por lo que puede asumirse que la ritodrina es un agente con bajo potencial teratogénico para *Xenopus laevis* (Boğa - Pekmezekmek *et al.,* 2015).

e. Nifedipino

Es un fármaco bloqueador de los canales de calcio. Inhibe el influjo de calcio, regularmente utilizado para tratar hipertensión aguda. Suele relajar todos los músculos lisos incluyendo aquellos en el útero, inhibiendo por tanto la labor de parto y las contracciones uterinas (Reid y Struthers, 1983). Tras la exposición de *Xenopus laevis* a este fármaco se obtuvieron los siguientes resultados: una CL_{50} de 0.606 µg/L, CE_{50} 0.006 µg/L, siendo las malformaciones más frecuentes en cola, quistes axiales, esqueléticas y dérmicas además de la notable inhibición del crecimiento con una CMIC de 0.0001 µg/L, e IT de 101. Debido al valor tan elevado de este parámetro y correlacionándolo con los protocolos para realizar el ensayo FETAX el nifedipino se ubica como un agente con alto potencial teratogénico para esta especie (Boğa-Pekmezekmek *et al.,* 2015).

f. Celecoxib

Es un antiinflamatorio no esteroidal que inhibe selectivamente la COX-2 y se utiliza como antiinflamatorio y analgésico (Puljak *et al.,* 2017). Al exponer embriones de rana *Xenopus laevis* se obtuvo una CL_{50} de 8.99 mg/L, una CE_{50} de 5.8 mg/L y un IT de 1.54, que ubica a este fármaco como teratógeno para esta especie; generando diversas malformaciones siendo las más frecuentes en intestino, edema, hemorragia y anormalidades en el corazón y vasos sanguíneos. El rango de mortalidad y malformaciones se incrementó de acuerdo con la concentración. Los sistemas principalmente afectados fueron el cardiovascular debido a la inducción de efectos en la pared vascular durante el desarrollo, que culminó en hemorragia y edema, además de afectaciones importantes en el sistema digestivo (Yoon *et al.,* 2018).

g. Dihidroartemisina (DHA)

Es un sesquiterpeno de tipo lactona obtenido de *Artemisa annua*. Sus principales derivados semisinteticos son: artesunato, arteméter y dihidroartemisina, que son utilizados como agentes antimalaria (Price, 2000). Este fármaco indujo diversos efectos tóxicos en larvas de *Xenopus laevis* expuestas durante 24 y 78 h, generando fallas cardiacas y una depleción de glóbulos rojos primitivos, sin embargo los glóbulos rojos definitivos no se vieron severamente afectados y posteriormente se recuperaron. No se observaron áreas necróticas, pero si presentaron defectos cardiacos. La artemisina puede inducir embriotoxicidad interviniendo en los procesos metabólicos de los glóbulos rojos primitivos activos, al finalizar el estudio se observó un ritmo cardiaco normal al igual que un desarrollo y crecimiento normal (Longo *et al.,* 2008).

h. Citostáticos

5-flourouracilo, capecitabina, cisplatino, etoposido, e imatinib.
Al exponer a *Xenopus laevis* a estos fármacos ninguno indujo mortalidad estadísticamente significativa con respecto al grupo testigo en concentraciones de 0.01 mg/L a 50 mg/L (dependiendo del fármaco), el crecimiento embrionario tampoco se vio afectado. El único fármaco con el cual se obtuvo la CE_{50} fue el 5 fluorouracilo con un valor de 15.18 mg/L; mientras que para el resto de los fármacos obtuvo la concentración más baja a la cual se observan efectos adversos (LOAEC), para capecitabina 20 mg/L, 5-Fluorouracilo 50 mg/L y finalmente etoposido 30 mg/L. Sin embargo indujeron frecuencias elevadas en cuanto a malformaciones, mismas que incrementaban en incidencia conforme se aumentaba la concentración de fármaco, lo que indica que tienen efectos teratogénicos. Las más frecuentes fueron: edema abdominal, doblamiento axial, daño en cabeza ojos y corazón.

Los anti metabolitos de 5-fluorouracilo y su profármaco capecitabina inhiben la síntesis de timidina vía timidilato sintasa, lo que puede determinar la activación de algunas vías moleculares que producen el edema abdominal. Se ha comunicado que el 5-fluorouracilo y su mecanismo de toxicidad crónica/subletal está relacionado con alteraciones en procesos de metabolismo primario, división celular y señalización celular, así como funciones del sistema inmune y desarrollo, genera cambios histopatológicos en el hígado y riñón en pez cebra (Kovacs *et al.,* 2016). *Xenopus laevis* es sensible a fármacos antineoplásicos, ya que estos son capaces de inducir malformaciones complejas (Isidori *et al.,* 2016).

i. Inhibidores selectivos de la receptación de serotonina (ISRS).

Su mecanismo de acción se basa en la inhibición de la receptación de 5-HT de manera potente y selectiva, bloqueando las bombas de transporte de serotonina en el espacio sináptico, por lo que se incrementan los niveles de serotonina. Su principal actividad es la antidepresiva (Schloss & Williams, 1998). Se muestran los resultados obtenidos de la exposición de *Xenopus laevis* a 3 ISRS distintos ver Tabla 1.

Tabla 1. Datos de toxicidad en embriones de *Xenopus laevis* expuestas a ISRS durante 96 h.

ISRS	CL_{50} (mg/L)	CE_{50} (mg/L)	CMIC (mg/L)	IT	Malformaciones
Fluoxetina	7.5	4.9	4	1.5	Cola, faciales, lóbulo óptico y edema en cabeza
Paroxetina	5.12	4.1	3.0	1.2	Doblamiento en cola y malformaciones en intestino
Sertralina	3.9	3.3	2	1.2	Doblamiento en cola y edema torácico

Relacionando los resultados mostrados con los criterios establecidos en el protocolo para el ensayo FETAX, los ISRS poseen bajo riesgo teratogénico, sin embargo, poseen elevada letalidad (Richards & Cole, 2006b).

j. Atorvastatina

Es un regulador lipídico y su mecanismo de acción se basa en la inhibición de la enzima HMG CoA reductasa, misma que impide la síntesis del colesterol en el hígado (Von Keutz & Schluter, 1998). Posterior a la exposición de *Xenopus laevis* a atorvastatina durante 96 h se obtuvo una CL_{50} de 38.6 mg/L, CE_{50} de 23.1 mg/L, CMIC de 30 mg/L y un IT de 1.6. La malformación más frecuente fue enrollamiento anormal de intestino.

k. Ibuprofeno

Es uno de los antiinflamatorios no esteroideos más utilizados a nivel mundial, su mecanismo de acción se basa en la inhibición del sistema enzimático ciclooxigenasa, mismo que se encarga de la conversión de ácido araquidónico en prostaglandinas (Roberts, 2001). Las prostaglandinas se encargan de regular diversas funciones fisiológicas que en los anfibios están involucradas en la liberación de neurotransmisores y el transporte de agua e iones a través de la membrana celular. Los resultados obtenidos ante la exposición de *Xenopus laevis* durante 96 h a ibuprofeno fueron los siguientes: CL_{50} 56.7 mg/L, CE_{50} 39.9 mg/L, CMIC 30 mg/L, IT 1.4. La principal malformación observada fue edema torácico, de acuerdo con los criterios establecidos en el protocolo FETAX este fármaco no es teratogénico para *Xenopus laevis* (Richards & Cole, 2006).

l. Ciprofloxacino y levofloxacino

Son compuestos de tipo antibiótico que pertenecen al grupo de las fluoroquinolonas. Su mecanismos de acción se basa en la inhibición de la subunidad A de la ADN girasa en las bacterias, una topoisomerasa que se encarga de cortar y sellar las cadenas de ADN durante la replicación; sin esta enzima el ADN no podría ser replicado, además las quinolonas inhiben la relajación de la estructura del ADN en su forma empacada, lo que incrementa la ruptura de la cadena. No generaron diferencias estadísticamente significativas con respecto al grupo testigo en cuanto a mortalidad o malformaciones en un rango de concentraciones de 1.0 mg/L hasta 100 mg/L durante un periodo de exposición de 96 h (Richards & Cole, 2006).

m. Cafeína

Es altamente consumida a nivel mundial, actúa en el organismo como un antagonista de los receptores de adenosina, por tanto contrarresta los efectos sedantes de la adenosina en el sistema nervioso central, además de incrementar los niveles de norepinefrina y la actividad neuronal en ciertas regiones del cerebro. Se encuentra en algunas plantas, ha sido adicionada a los alimentos y bebidas desde hace algunos años, actúa como un estimulante del sistema nervioso central (EFSA, 2005). Los embriones expuestos a este fármaco presentaron hipopigmentación pero no se observaron otras anormalidades (Richards & Cole, 2006). Sin embargo en otro estudio después de 96 h de exposición se encontró una CL_{50} de 0.48 mg/mL, CE_{50} de 0.12 mg/mL y CMIC de 0.05 mg/mL. Las malformaciones más frecuentes se presentaron en intestino, defectos craneofaciales, microftalmia, microencefalia, edema visceral y cráneo facial, boca, hemorragia y acortamiento musculo-esquelético. Como se ha mencionado en algunos estudios al agregar un sistema de activación metabólico a este ensayo incrementó el potencial embriotóxico de la

cafeína, sin embargo al someter a los metabolitos de la cafeína al ensayo FETAX ninguno de ellos mostró actividad teratogénica mayor que el compuesto original (Fort *et al.,* 1998).

1.5. Misceláneos

a. Nicotina y cotinina

La nicotina es un compuesto que se encuentra en el tabaco, es capaz de atravesar la barrera hemato-encefálica y unirse a los receptores colinérgicos (Drug and Therapeutics Bulletin, 2014). Su principal producto de biotransformación es la cotinina, misma que es útil para la determinación de exposición al tabaco. Los resultados obtenidos ante la exposición de *Xenopus laevis* durante 96 h a estos dos compuestos dieron como resultado una CL_{50} de 141 mg/L, CE_{50} de 0.45 mg/L y CMIC de 0.625 mg/L, acompañada de diversas malformaciones como doblamiento de cuerpo, malformaciones en mandíbula e hiperplasia branquial, microcefalia, intestino, inflamación cardiaca acompañada de edema y mocroftalmia; mientras que para la cotinina se obtuvo una CL_{50} de 4.290 mg/L, CE_{50} de 740 mg/L y CMIC 250 mg/L. Las malformaciones más frecuentes fueron acortamiento esquelético lateral y ventral y anormalidades en intestino, microcefalia malformaciones en mandíbula y boca, ojo y edema, además de malformaciones cardiacas (Dawson *et al.,* 1988).

b. 4-bromobenceno.

Es un compuesto utilizado como agente hepatotóxico, se sabe que su potencial tóxico se debe a la unión covalente que forma con compuestos hepáticos de importancia, generando lipoperoxidación, disfunción mitocondrial y fallas en la homeostasis de calcio (Yoshioka *et al.,* 2017). Al probar su potencial teratógeno en embriones de rana *Xenopus laevis* y durante 96 h de exposición los resultados fueron los siguientes: CL_{50} 2,800 mg/L, CE_{50} 280 mg/L, CMIC 500 mg/L, y un IT de 10, acompañado de malformaciones en intestino, defectos craneofaciales y edema oftálmico, principalmente. La bioactivación de este compuesto aumenta significativamente su potencial tóxico. El 4-bromobenceno tiene un potencial teratógeno significativo, sin embargo al ser activado metabólicamente se incrementa notablemente su potencial embriotóxico, es capaz de generar efectos nefrotóxicos y hepatotóxicos. Después de su biotransformacion a bromofenol y bromohidroxiquinona, el 4 bromobenceno es capaz de unirse covalentemente al Ca-ATPasa presente en el retículo endoplásmico y a la membrana celular, lo que da lugar a una disminución en la expulsión de calcio desde la célula lo que lleva a fallas en la homeostasis celular (Fort *et al.,*1996).

c. Compuestos de tipo retinoico.

Las cianobacterias son capaces de producir diversos compuestos de tipo biológico, mismos que han sido poco caracterizados. Sin embargo, algunos pueden generar efectos tóxicos, entre ellos los retinoides, entre los que se incluyen el retinol, retinal y ácido retinoico, mismos que han demostrado ser capaces de generar efectos adversos en humanos en concentraciones elevadas, sin embargo una deficiencia de los mismos puede generar efectos teratogénicos (Collins & Mao, 1999).

Al exponer embriones de rana *Xenopus laevis* a exudados de *Cylindrospermopsis raciborskii* y *Microcystis aeruginosa*, se obtuvieron los siguientes resultados: LOEC para malformaciones de 2.5 µg/L en el caso de equivalentes de retinol y 5 µg/L para ácidos retinoicos. Los exudados de las dos cianobacterias produjeron diversas malformaciones en cola, intestino y ojo además de interferencia en el crecimiento de los embriones. Para todos los ácidos retinoicos se obtuvo una CL_{50} de 20 µg/L y CE_{50} de 11.9 µg/L. Este estudio confirmó la capacidad de ciertas cianobacterias para producir y liberar compuestos de tipo retinoico al ambiente, mismos que pueden llegar a generar efectos adversos en el desarrollo de anfibios (Smutná *et al.*, 2017).

d. Nanopartículas de plata recubiertas con polietilenimina

Las nanopartículas de plata han sido hasta ahora de las más utilizadas como agentes antimicrobianos, se comercializan dos tipos distintos de recubrimientos, los que están cargados negativamente como el citrato y los que están cargados positivamente como la polietilenimina.

Al realizar el ensayo FETAX utilizando nanopartículas de plata con un recubrimiento de polietilenimina exponiendo a *Xeopus laevis* durante 96 h se encontró una CL_{50} de 0.385 mg/L, CE_{50} 0.240 mg/L con lo que se obtuvo un IT de 1.60, mismo que ubica a las nanopartículas de plata con recubrimiento de polietileimina como agente con potencial teratógeno, las principales malformaciones observadas fueron craneofaciales, cardiacas, abdominales e intestinales, debido a la falta de plegamiento en el intestino, fue el órgano de mayor interés en este estudio. Los iones de plata pudieron retrasar el periodo de morfogénesis del epitelio intestinal afectando el tiempo de elongación de las células endodérmicas y generando un mal doblamiento de este órgano. Con los resultado obtenidos se evidenció que este tipo de nanopartículas son capaces de cruzar la barrera del epitelio intestinal y generar efectos tóxicos en *Xenopus laevis*. Es de importancia destacar que dependiendo el tipo de recubrimiento que posean las

nanopartículas pueden tener un mayor o menor potencial teratógeno (Colombo *et al.*, 2017).

e. Efectos agudos de nanomateriales Fe_2O_3, TiO_2, ZnO y CuO.

Actualmente, los nanomateriales se utilizan para generar innovaciones científicas en ingeniería, química, e incluso medicina. Los nanomateriales que contienen óxidos de metales en su estructura pueden producir diversos efectos tóxicos en organismos acuáticos, como afectaciones en funciones mitocondriales, anormalidades y mortalidad.

Se examinaron los efectos de nanomateriales que contenian Fe_2O_3, TiO_2, ZnO y CuO durante un periodo de 96 h; no se observaron cambios en cuanto a mortalidad en los embriones expuestos, sin embargo, se observaron diversas malformaciones gastrointestinales y espinales principalmente. A continuación se menciona la CE_{50} para cada nanomaterial probado: ZnO 10.3 mg/L, CuO, Fe_2O_3 y TiO_2 >1000mg/L, mientras que la concentración mínima inhibitoria del crecimiento se obtuvieron los siguientes resultados: ZnO y CuO 10mg/L, Fe_2O_3 y TiO_2 1000mg/L. Con estos resultados puede concluirse que algunos nanomateriales son capaces de generar efectos negativos en fases tempranas del desarrollo de anfibios como *Xenopus laevis*, sin embargo, los estudios realizados en cuanto a este tipo de materiales son escasos (Nations *et al.*, 2011).

f. Colorantes textiles

Rojo astrazon (FBL), azul astrazon (FGRl), rojo remazol (RR), azul turquesa ramazol (G-A), rojo cibacron (FN-R).

Aproximadamente de un 10 a un 15 % de los colorantes son eliminados en los efluentes de industrias textiles (Sumathi *et al.*, 2001). Tras la exposición de *Xenopus laevis* a distintos colorantes durante 96 h (FBL, FGRl, RR, G-A, FN-R) el valor de CL_{50} más bajo fue de 4.73 mg/L por la exposición a rojo de astrazon, la toxicidad del resto de los colorantes textiles en el siguiente orden FGRL>G-A>FBL>FN-R>FN-3G>RR, siendo FGRL el que posee mayor toxicidad y RR el que posee la menor. El colorante azul astrazon fue el compuesto más teratogénico de los 6 probados. Las malformaciones identificadas fueron microftalmia, edema en cabeza y en saco vitelino, malformaciones en ojo, ciclopia, microcefalia, despigmentación y doblamiento de la cola. Más de una de éstas se presentó en los embriones que fueron expuestos a los colorantes en concentraciones elevadas; el IT más alto obtenido correspondió a azul de astrazon y el más bajo a rojo remazol; los radios de malformaciones fueron concentración-dependinetes con un 100% de malformaciones en las concentraciones más elevadas de cada colorante. El edema

fue la malformación más frecuente y específicamente el de cabeza fue el más común. El grado de inhibición del crecimiento también mostro una tendencia concentración-dependiente en los 6 colorantes probados.

Todos los colorantes utilizados en este estudio mostraron efectos tóxicos y/o teratogénicos; además los valores de IT indican que deben ser considerados como sustancias tóxicas para el desarrollo de anfibios. Los colorantes a bajas concentraciones pueden generar fallas en la comunicación celular y por tanto resultar en la aparición de malformaciones en ojos y edema en cabeza, así como doblamiento de cola, mientras que a concentraciones más elevadas inducen microcefalia, microftalmia, despigmentación y daño ocular (Birhanli & Ozmen, 2005).

g. Lixiviados de colillas de cigarrillo

Las colillas de cigarrillo poseen impacto tóxico en el ambiente debido a su persistencia y su composición química. La mayoría de sus compuestos se lixivian hacia ambientes acuáticos, contaminando sistemas y poniendo en riesgo a los organismos acuáticos (Slaughter *et al.,* 2011). Al exponer a embriones de *Xenopus laevis* a lixiviados de colillas de cigarrillo común (RCB), cigarrillo mentolado (MCB) y cigarrillo electrónico (ECB) durante 96 h, se obtuvieron los resultados mostrados en la Tabla 2.

Tabla 2. Datos de toxicidad en embriones de *Xenopus laevis* expuestas a lixiviados de colilla de cigarrillo (RCB, MCB, ECB) durante 96 h.

Lixiviados de colillas de cigarrillo	CL_{50} (Colillas/L)	CE_{50} (Colillas/L)	IT	CMIC (Colillas/L)	Malformaciones
Cigarrillo común (RCB)	0.68	0.34	1.95	0.25	Notocorda, enrollamiento anormal de intestino, malformaciones faciales, restricción del crecimiento
Cigarrillo mentolado (MCB)	1.140	0.30	3.77	0.5	Edema, intestino, corazón, craneofaciales
Cigarrillo electrónico (ECB)	26.8	15.58	1.72	10	Cola, edema leve, mal doblamiento en intestino

Los lixiviados de cigarrillo común y de cigarrillo mentolado demostraron poseer riesgo teratogénico para *Xenopus laevis* y organismos acuáticos. Los lixiviados de cigarrillo mentolado mostraron poseer mayor toxicidad posiblemente debido a la interacción de sus componentes con el mentol; por otra parte, los lixiviados de cigarrillo electrónico son 10 veces menos tóxicos que los cigarrillos tradicionales, sin embargo, aún poseen un ligero riesgo teratogénico (Parker & Rayburn, 2017).

2. Conclusiones

Desde su implementación, el ensayo FETAX ha demostrado ser una herramienta útil para la evaluación del riesgo tóxico de diversas sustancias durante fases vulnerables del desarrollo de anfibios, es posible obtener información en cuanto a la mortalidad, malformaciones e inhibición del crecimiento producidos por el xenobiótico de interés, dichos parámetros son importantes en estudios toxicológicos. Por otra parte, este ensayo posee diversas ventajas:

- Debido a las características anatómicas del organismo modelo, es posible observar las fases del desarrollo embrionario fácilmente, con un microscopio estereoscópico.
- Consta de 96 horas de duración.
- Es relativamente sencillo de llevar a cabo, siempre y cuando se tomen en cuenta los puntos críticos establecidos en el protocolo (ASTM E 1439-12)
- Es económico comparado con otros ensayos.
- Además de la evaluación de compuestos puros, permite evaluar mezclas complejas poco caracterizadas y sedimentos.

Este ensayo es una herramienta útil para evidenciar efectos tóxicos generados por xenobióticos pertenecientes a diversas clases en anfibios, así como para tomar medidas en cuanto al desarrollo de metodologías para la detección de contaminantes en cuerpos de agua y métodos de remoción de los mismos.

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9 ANEXOS

9.2 Resultados y discusión mezcla Cd+DCF+S

9.2.1 Ensayo FETAX

9.2.1.1 CL_{50} y LOAEL

Los resultados correspondientes a la exposición de *X.laevis* a cadmio y diclofenaco se muestran en la tabla 2. En la exposición a cadmio (1, 4, 8, 16, 32, 62.5 mg/L) los grupos de exposición de 8, 16, 32, 62.5 mg/L generaron una mortalidad del 100%; la concentración letal media (CL_{50}) calculada fue de 4.275 mg/L (Análisis PROBIT $p < 0.05$), la concentración más baja que genera efectos adversos (LOAEL) 0.04 mg/L, mientras que en la exposición a diclofenaco (1, 4, 8, 16, 32, 62.5 mg/L) se obtuvo una CL_{50} de 14.90 mg/L y una LOAEL de 0.149 (todos los experimentos se realizaron por triplicado).

Tabla 2. Mortalidad de embriones de *Xenopus laevis* expuestos a cadmio y diclofenaco durante 96 h.

Concentración (mg/L)	Organismos expuestos	Organismos muertos (cadmio)	Organismos muertos (diclofenaco)
1	60	18	25
4		25	26
8		60	28
16			28
32			30
62.5			57
CL_{50}		4.27	14.90
LOAEL		0.042	0.149

9.2.1.2 Mortalidad y malformaciones

La figura 1 muestra los datos de mortalidad obtenidos por la exposición de *X. laevis* a Cd, Cd+S, DCF, DCF+S, Cd+DCF, Cd+DCF+S. En los grupos expuestos a las mezclas con spirulina se observa una reducción en la mortalidad que va desde 44.44% hasta un 80%; los tratamientos más efectivos fueron 4 y 10 mg/L de spirulina, sin embargo, la mayor disminución de la mortalidad se dio en los grupos expuestos a 10 mg/L con diferencias estadísticamente significativas (ANOVA una vía, LSD Fisher $p<0.05$).

El aumento significativo en la mortalidad de *Xenopus laevis* expuestos a Cd, DCF y Cd+DCF, puede deberse a la actividad prooxidante que tiene cadmio, la alteración en la absorción de nutrientes (Zn, Mg y Cu) y las alteraciones de la morfogénesis y del huso mitótico (Slaby et al., 2017), esto puede conducir a la muerte celular. Por otra parte el diclofenaco posee un mecanismo de toxicidad a través del cual puede producir daño en la mitocondria debido a la producción de ROS, mediante el ciclo redox puede generar radicales intermediarios como son las quinonas y semiquinonas, esto pueden inducir fallas en la permeabilidad mitocondrial, el potencial transmembranal y pueden generar una reducción de ATP; el diclofenaco también puede generar la liberación de factores proapoptoticos que contribuye a la activación de caspasa 3 y 9 y genera muerte celular (Ghosh et al., 2016; Jung et al., 2020; Ramachandran et al., 2018).

La disminución de la mortalidad en los grupos expuestos a las mezclas con spirulina puede deberse a las propiedades de neutralización que tiene, ya que debido a ellas se genera un efecto protector contra el daño oxidativo, al reducir la actividad de ROS y NOS La spirulina también tiene capacidad quelante, logra unirse a metales pesados (Gelagutashvili, 2006), lo que puede reducir la cantidad de cadmio libre impidiéndole interactuar con las biomoléculas.

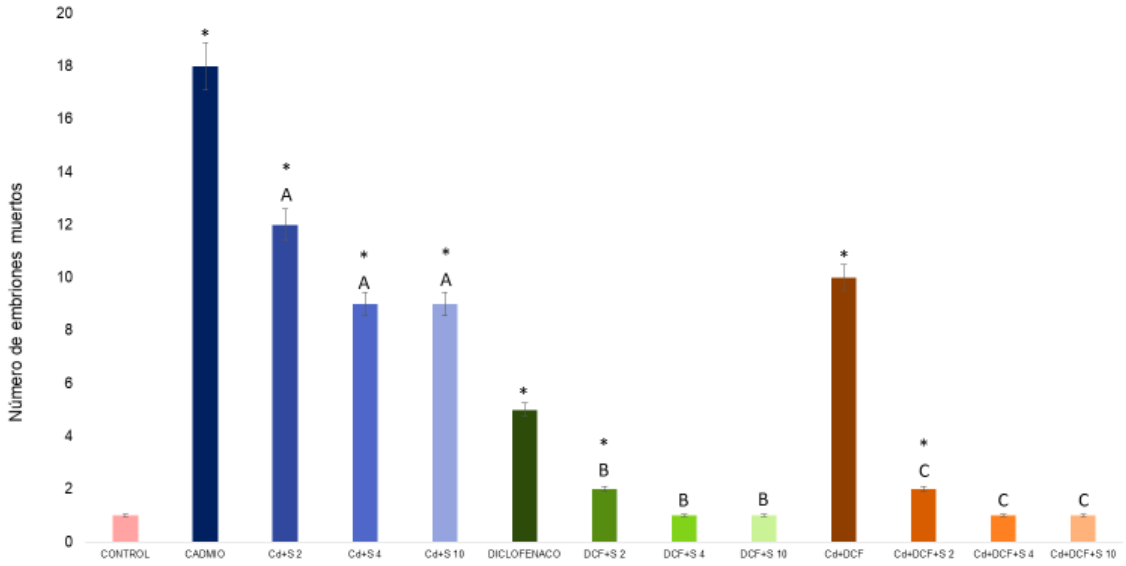

Figura 1. Número de embriones de *Xenopus laevis* muertos después de 96 horas de exposición a: control, Cd, Cd + S 2, Cd + S 4, Cd + S 10,DCF, DCF + S 2, DCF + S 4, DCF + S 10, Cd+DCF, Cd+DCF+S 2, Cd+DCF+S 4, Cd+DCF+S 10, diferencias significativas con respecto a: (*) control, (A) Cd, (B) DCF, (C) Cd+DCF (ANOVA una vía y LSD Fisher, $p< 0.05$).

En la figura 2 se muestra el número de embriones con malformaciones, este parámetro siguió una tendencia similar a la que se presentó en la mortalidad; la frecuencia de anormalidades en los grupos expuestos a spirulina se redujo significativamente en un 10% a 67.93%. Las principales malformaciones observadas (figura 3) fueron enanismo, mal enrollamiento intestinal, malformaciones del recto, edema cardíaco, edema abdominal, microcefalia y malformaciones axiales (cola doblada, notocorda y aleta dobladas y/o curvadas); este tipo de malformaciones ya se han reportado previamente en organismos expuestos a cadmio, en *Xenopus laevis* (Sunderman et al., 1991) *Bufo gargarizans* (Wu et al., 2017a), *Danio rerio* (Cheng et al., 2000) y *Silurus soldatovi* (Zhang et al., 2012), y en organismos expuestos a diclofenaco como *Xenopus laevis* (Chae et al., 2015) otros anfibios anfibios (Peltzer et al., 2019), *Cyprinnus carpio*, (Stepanova et al., 2013). Sin embargo, en los grupos expuestos a spirulina se redujo la severidad de las malformaciones con una tendencia concentración-

dependiente, siendo 10 mg/L la concentración más efectiva como se muestra en la figura 4.

Este tipo de efectos pueden deberse a que el cadmio modifica procesos como la apoptosis, el ciclo celular, el estrés y la respuesta inmune (Liu et al., 2018), induce la fosforilación de p53, puede reemplazar al Zn y causa errores en la reparación del ADN que resultan en la acumulación de ADN dañado (Chen y Shaikh, 2009; Dally y Hartwig, 1997) y afecta los procesos de señalización, genera estrés oxidativo y alteraciones del potencial redox. Por otra parte se ha demostrado que el diclofenaco es capaz de generar afectaciones en los mecanismos de señalización celular Wnt (Wnt3,Wnt8) y Gata 4, importantes en etapas tempranas del desarrollo ya que son esenciales para el establecimiento de patrones corporales y proliferación celular (Chan et al., 2001; Peltzer et al., 2019). Dado que la organogénesis es la etapa de desarrollo en la que los organismos se encuentran más vulnerables y requiere de procesos de señalización para regular la proliferación y la diferenciación celular (Hayashi et al., 2018); el cadmio y el diclofenaco pueden provocar fallas en el desarrollo embrionario, generando cambios en la función y estructura de los tejidos y como consecuencia diversas malformaciones (Laforgia et al., 2018).

En los grupos expuestos a las mezclas con spirulina, se observó una disminución en la incidencia y severidad de malformaciones, efectos similare fueron descritos por Argüelles-velázquez et al., 2013 en larvas expuestas a cadmio, también encontraron una reducción en la frecuencia de malformaciones con la suplementación de spirulina y en ratas expuestas a cadmio; esto puede deberse al efecto de las ficobiliproteínas; que tienen actividad antiinflamatoria (Khafaga y El-Sayed, 2018) es capaz de disminuir los niveles de interleucinas proinflamatorias y por tanto reducir inflamación (Abu-Taweel et al., 2019) y al reducir la inflamación, se puede reducir el edema, debido a que la mayoría de estos son producidos por un proceso inflamatorio crónico. Las ficocianinas y los β-carotenos inhiben la formación de citocinas proinflamatorias, suprimiendo así la expresión de ciclooxigenasa II y la producción de prostaglandina E2, que actúa como

mediadores inflamatorios, también tiene efectos antioxidantes, puede neutralizar los radicales hidroxilos responsables del daño oxidativo(Bermejo et al., 2008), la reducción del daño celular puede contribuir a la reducción de la severidad e incidencia de malformaciones en *Xenopus laevis*.

La spirulina también ha demostrado tener efectos benéficos capaces de contrarrestar la toxicidad inducida por diclofenaco; en ratas Wistar albinas el diclofenaco incremento significativamente los niveles de enzimas hepáticas, daño celular, lesiones y afectaciones en las funciones hepáticas y renales, además de generar una reducción significativa de la actividad de enzimas antioxidantes superóxido dismutasa, catalasa, glutation S tranferasa y glutatión peroxidasa,sin embargo, la spirulina logro normalizar los niveles enzimáticos y función hepática, y mitigo los daños histomorfologicos en tejido renal (Peter S et al., 2017; Rajbanshi et al., 2016).

Las mezclas que contenían 10 mg/L de spirulina fueron la más efectivas para reducir la mortalidad, incidencia en malformaciones y gravedad de malformaciones en embriones de *X. laevis* en comparación, con la exposición a cd, DCF, y Cd+DCF, también es importante destacar que todos los tratamientos con spirulina disminuyeron el daño en cierta proporción.

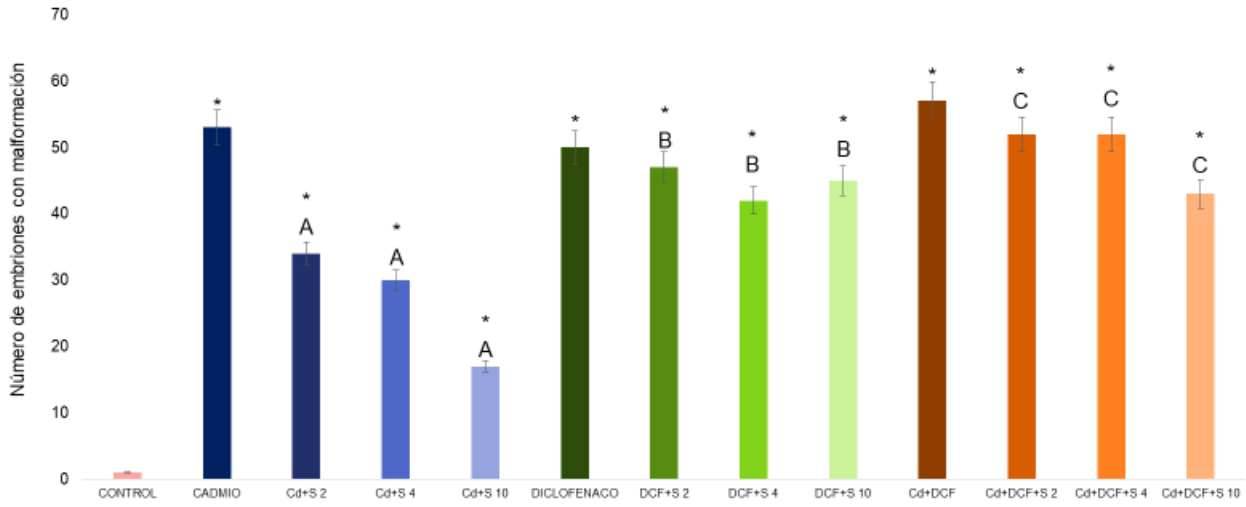

Figura 2. Número de embriones de *Xenopus laevis* con malformaciones después de 96 horas de exposición a: Cd, Cd + S 2, Cd + S 4, Cd + S 10,DCF, DCF + S 2, DCF + S 4, DCF + S 10, Cd+DCF, Cd+DCF+S 2, Cd+DCF+S 4, Cd+DCF+S 10, diferencias significativas con respecto a: (*) control, (A) Cd, (B) DCF, (C) Cd+DCF (ANOVA una vía y LSD Fisher, $p< 0.05$).

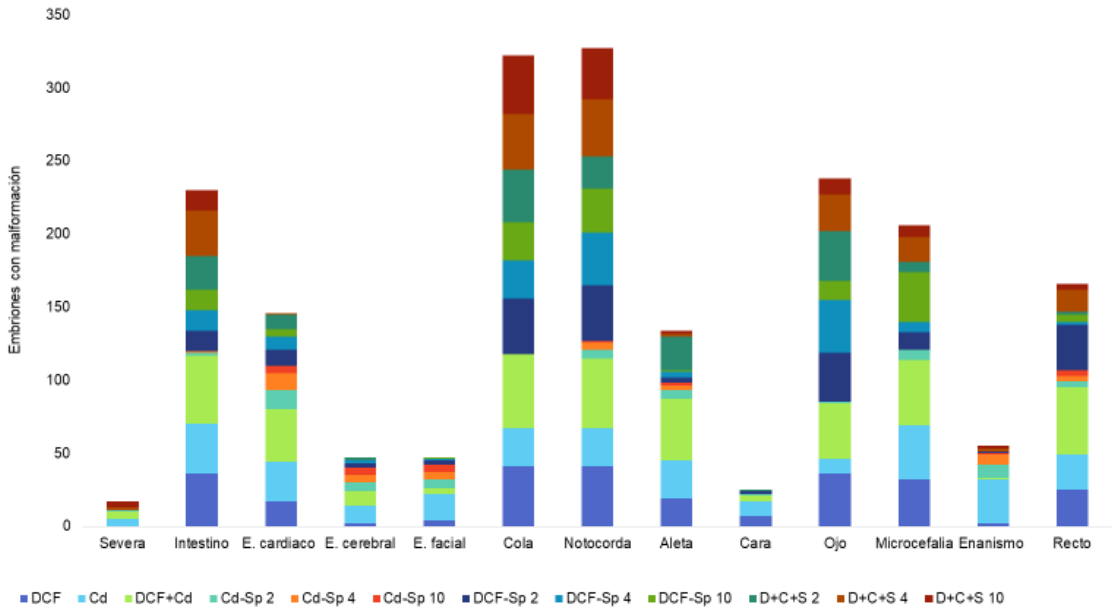

Figura 3.Histograma de frecuencia de malformaciones inducidas en *Xenopus laevis* expuestas a: Cd, Cd + S 2, Cd + S 4, Cd + S 10,DCF, DCF + S 2, DCF + S 4, DCF + S 10, Cd+DCF, Cd+DCF+S 2, Cd+DCF+S 4, Cd+DCF+S 10 durante 96h.

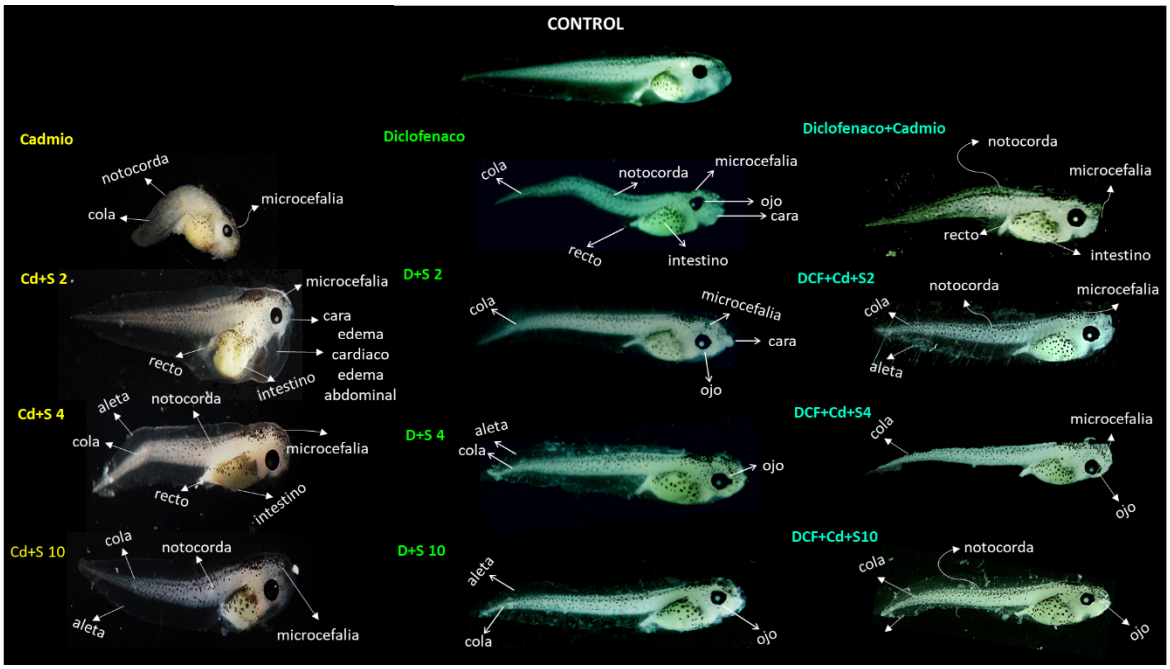

Figura 4. Malformaciones más frecuentes y representativas en *X.laevis* después de 96 h de exposición a: Cd, Cd + S 2, Cd + S 4, Cd + S 10,DCF, DCF + S 2, DCF + S 4, DCF + S 10, Cd+DCF, Cd+DCF+S 2, Cd+DCF+S 4, Cd+DCF+S 10.

9.2.1.3 Inhibición del crecimiento

Las medidas de longitud de cabeza a la cola de los organismos se muestran en la fig.5, se encontraron diferencias significativas en todos los tratamientos con spirulina, se redujo la inhibición del crecimiento inducida por Cd, y DCF así como la mezcla Cd+DCF, siendo las mezclas con 10mg/L de spirulina las que mostraron mayor efecto benefico en cuanto al desarrollo, de acuerdo con el análisis estadístico (ANOVA, LSD Fisher $p < 0.05$).

La disminución en la longitud de las larvas se observó en estudios anteriores, Wu et al., 2017b informaron una reducción en el tamaño inducida por el cadmio en *Buffo Garganzianis*, esto puede deberse a que el cadmio tiene la capacidad de imitar otros minerales esenciales, por lo tanto inhibe la absorción de los mismos (Goyer, 1995) y desencadena fallas en los procesos de desarrollo, a su vez la sobreproducción de ROS inducida por diclofenaco afecta las funciones mitocondriales , incrementa los niveles de lipoperoxidación y genera daño celular,

disminuye la respiración mitocnodrial y los niveles de ATP, lo que puede llevar a una fragmentación mitocondrial (Jung et al., 2020), estos efectos pueden interrumpir los mecanismos de señalización y proliferación celular, lo que puede generar una diminución en el desarrollo embrionario.

La exposición a mezclas de spirulina mostraron efectos beneficios en el desarrollo, la longitud total aumentó en comparación con la exposición a Cd, DCF y la mezcla. Esto puede deberse a que la pared celular de la spirulina es porosa y permite que el cadmio pase libremente, cuando el cadmio llega al compartimento intracelular, los agentes quelantes actúan uniéndose al él y neutralizándolo (Bermejo et al., 2008), estos agentes quelantes suelen ser inducidos por la exposición a metales pesados (Knauer et al., 1998). Un mediador importante es la ficocianina que puede eliminar radicales hidroxilo, alcoxi y peroxilo; la ficocianina también bloquea la fosforilación de las proteínas quinasas activas en mitógeno p38, que regulan la síntesis de citocinas, (Khalil et al., 2017). Las vitaminas, proteínas y minerales de la spirulina también pueden participar en la mejora del desarrollo. Se han reportado resultados similares previamente en donde el tamaño y peso total de los organismos disminuyeron ante la exposición a agentes tóxicos y después de la suplementación con spirulina, el peso y el tamaño aumentaron significativamente, (Abdelkhalek et al., 2017; Adel et al., 2016; Mostafa et al., 2020; Pestana et al., 2020).

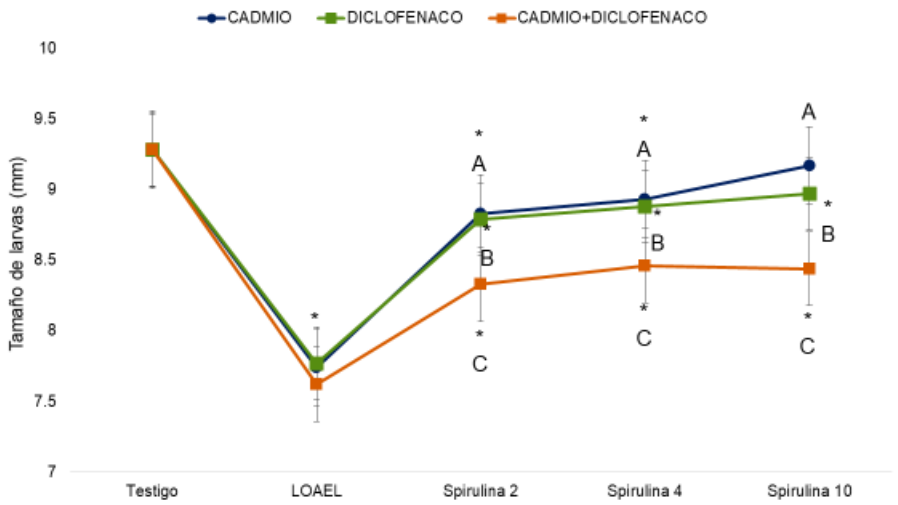

Figura 5. Medición de cabeza a cola de *Xenopus laevis* expuestas a Cd, Cd + S 2, Cd + S 4, Cd + S 10,DCF, DCF + S 2, DCF + S 4, DCF + S 10, Cd+DCF, Cd+DCF+S 2, Cd+DCF+S 4, Cd+DCF+S 10 durante 96 h, diferencias significativas con respecto a: (*) control (A) Cd (B) DCF, (C) Cd+DCF (ANOVA una via y LSD Fisher, *p<0.05*).

9.2.2 Estrés oxidativo

9.2.2.1 Determinación del grado de lipoperoxidación

En la Figura 6 se muestran los datos de la determinación de lipoperoxidación, se observó un incremento significativo en la exposición a los agentes tóxicos Cd, DCF y la mezcla CD+DCF, sin embargo, en los grupos expuestos a los tratamientos con las mezclas de spirulina se logró una disminución de este biomarcador, las concentraciones de spirulina que mostraron la mayor disminución fueron 4 y 10 mg/L de acuerdo con el análisis estadístico.

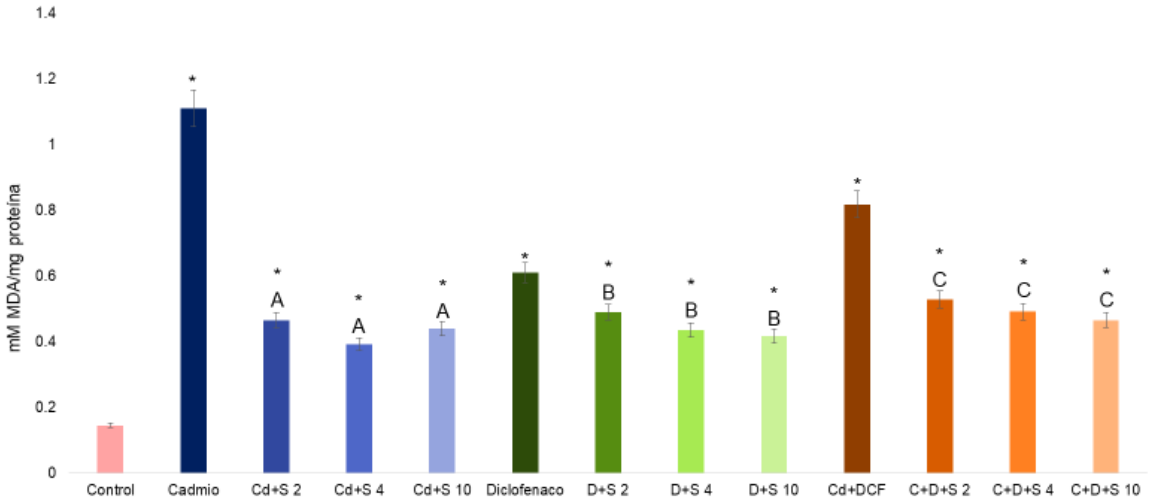

Figura 6.Grado de lipoperoxidación en larvas de *Xenopus laevis* expuestas a: control, Cd, Cd + S 2, Cd + S 4, Cd + S 10,DCF, DCF + S 2, DCF + S 4, DCF + S 10, Cd+DCF, Cd+DCF+S 2, Cd+DCF+S 4, Cd+DCF+S 10 durante 96 h. Diferencias significativas con: (*) control, (A) Cd, (B) DCF, (C) Cd+DCF, (ANOVA una vía y LSD Fisher, $p< 0,05$).

9.2.2.2 Actividad antioxidante superóxido dismutasa (SOD)

La actividad antioxidante SOD se muestra en la figura 7, se incrementó en la exposición a Cd, DCF y la mezcla Cd+DCF, en comparación con el grupo control, los tratamientos con spirulina generaron una disminución en la actividad de esta enzima, la mayor disminución se vio en los grupos expuestos a 4 y10 mg/L de spirulina.

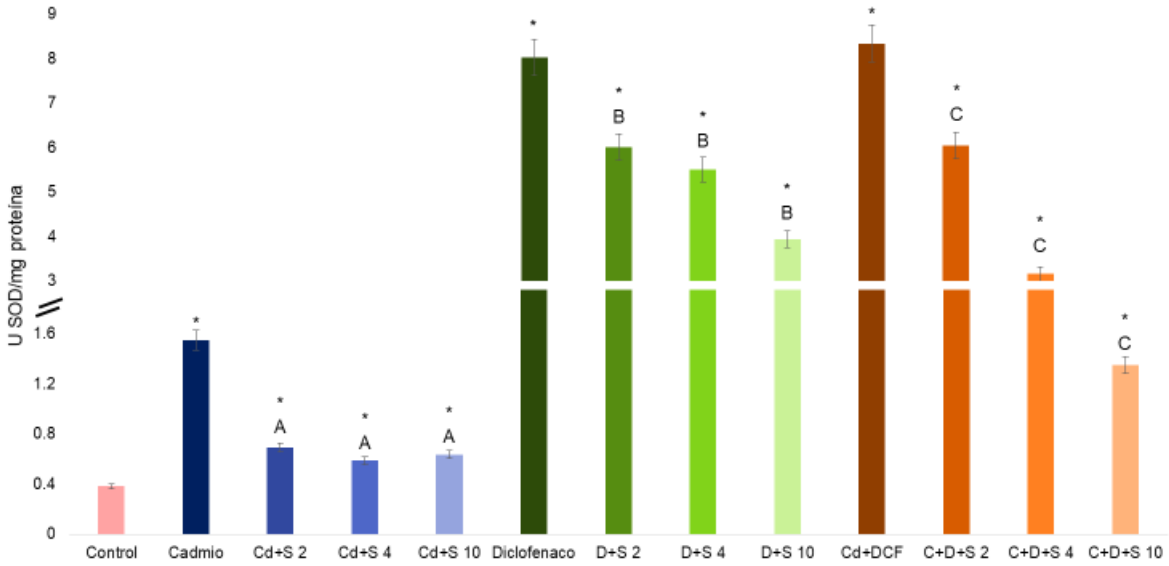

Figura 7. Actividad superóxido dismutasa (SOD) en larvas de *Xenopus laevis* expuestas a: control, Cd, Cd + S 2, Cd + S 4, Cd + S 10,DCF, DCF + S 2, DCF + S 4, DCF + S 10, Cd+DCF, Cd+DCF+S 2, Cd+DCF+S 4, Cd+DCF+S 10, diferencias significativas con respecto a: (*) control, (A) Cd, (B) DCF, (C) Cd+DCF (ANOVA una vía y LSD Fisher, $p< 0.05$).

9.2.2.3 Actividad antioxidante catalasa (CAT)

La figura 8 muestra la actividad de catalasa con una tendencia similar a la evidenciada en la determinación de la actividad SOD, se observó un incremento en la actividad enzimática en los grupos expuestos a Cd, DCF, CD+DCF, en los grupos expuestos a las mezclas con spirulina, los niveles de actividad CAT se vieron disminuidos, la reducción de actividad fue mayor en los grupos que tenían mezclas con spirulina 4 y 10 mg/L ,sin diferencias estadísticamente significativas entre ambos tratamientos en la exposición a cadmio.

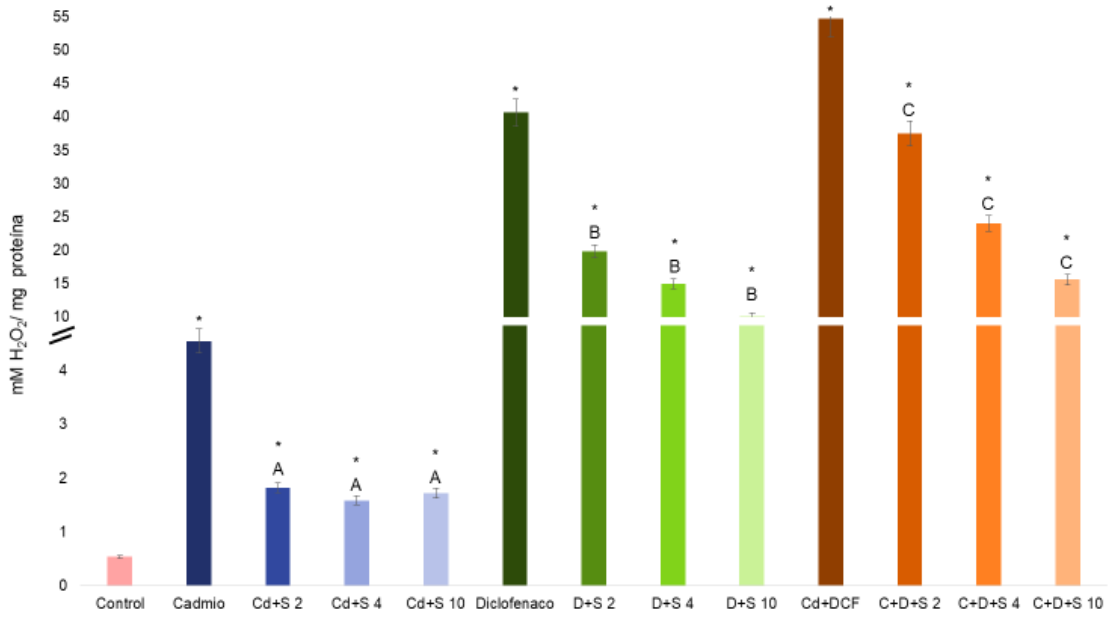

Figura 8. Actividad de catalasa (CAT) en larvas de *Xenopus laevis* expuestas a: control, Cd, Cd + S 2, Cd + S 4, Cd + S 10,DCF, DCF + S 2, DCF + S 4, DCF + S 10, Cd+DCF, Cd+DCF+S 2, Cd+DCF+S 4, Cd+DCF+S 10, diferencias significativas con respecto a: (*) control, (A) Cd, (B) DCF, (C) Cd+DCF (ANOVA una vía y LSD Fisher, $p< 0.05$).

Se conocen bien los efectos prooxidantes del cadmio, así como su capacidad para generar efectos adversos en organismos acuáticos (Avallone et al., 2015; Cambier et al., 2010; Chan y Cheng, 2003; Mouchet et al., 2006; Pizzi et al., 2017; Shirriff y Heikkila, 2017; Slaby et al., 2016; Thompson y Bannigan, 2008; Wu et al., 2017b, 2017a; Xia et al., 2016) la mayoría de estos están relacionados con la generación de estrés oxidativo. Por otra parte, el diclofenaco es un fármaco capaz de generar variaciones en los niveles de acetil colinesterasa y glutatión S transferasa (Peltzer et al., 2019), incremento en la actividad de la glutatión S transferasa, una reducción en la glutatión reductasa en *Cyprinnus carpio* (Stepanova et al., 2013), en *Daphnia magna* indujo estrés oxidativo e incremento la producción de especies reactivas de oxígeno asociadas con daño citotóxico (Gómez-Oliván et al., 2014). Por lo cual ambos xenobióticos tienen la, capacidad de generar estrés oxidativo en organismos acuáticos.

Los cambios observados en los grupos expuestos a Cd, DCF y la mezcla Cd+DCF, fueron principalmente incrementos en la actividad enzimática, dichos incrementos puede ser el resultado de una producción excesiva de especies reactivas ROS y RNS, el aumento en la producción de estas especies suele aumentar los niveles de SOD ya que su función es catalizar un proceso de dismutación, transformando $O_2^{\cdot -}$ en H_2O_2, de igual forma los incrementos en CAT pueden deberse a que esta enzimas es la encargada de transformar al en H_2O (Liu et al., 2018), el aumento en la actividad de las enzimas antioxidantes tiene el objetivo de neutralizar las ROS y proteger a la célula del daño oxidativo; sin embargo, en este estudio los grupos expuestos a mezclas con spirulina disminuyeron la actividad enzimática, la mayor disminución estadísticamente significativa en la actividad SOD fue inducida por las mezclas que contenían 4 y 10 mg/L, mientras que la mayor disminución estadísticamente significativa en la actividad CAT fue inducida por las mezclas con 10 mg/L.

Se han descrito previamente efectos similares en cuanto a la disminución de la peroxidación lipídica en la suplementación con spirulina en organismos como *Oreochromis niloticus* expuesto a deltametrina (Abdelkhalek et al., 2017); en ratas expuestas a fluoruro de sodio (Banji et al., 2013), en ratas Wistar expuestas a metotrexato (Khafaga and El-Sayed, 2018), *Clarias gariepinus* expuestas a dodecil sulfato de sodio (Sayed and Authman, 2018), en conejos expuestos a acetato de plomo (Aladaileh et al., 2020) en ratas macho expuestas a arsenito de sodio (Bashandy et al., 2016), y ratas Wistar expuestas a cadmio (Karadeniz et al., 2009), en ratas Wistar albinas expuestas a diclofenaco (Peter S et al., 2017; Rajbanshi et al., 2016).

Como se ha mencionado previamente la spirulina tiene capacidad quelante y se une a los iones de cadmio de tal forma logra inhibir la reacción de Fenton, también puede neutralizar los radicales alcoxil hidroxilo y peroxilo (Wu et al., 2016), puede inhibir el proceso de peroxidación de lípidos en una etapa temprana y el daño puede reducirse. Otro componente importante es el tocoferol, este puede proteger contra la peroxidación de lípidos debido a que tiene un anillo de cromano en su

estructura mismo que aporta un efecto reductor y puede reducir los radicales peroxilo a hidroperóxidos que luego pueden degradarse enzimáticamente (Miyazawa et al., 2019; Moradi et al., 2019). Por otro lado los carotenos presentes en la spirulina también son capaces de impedir los procesos de lipoperoxidación, principalmente aquellos desencadenados por el oxígeno singulete y radicales peroxilo; los carotenos también tienen la capacidad de inhibir la producción de prostaglandina E2 y óxido nítrico a través de la supresión de los mediadores inflamatorios (Deng and Chow, 2010; Schafer et al., 2002; Stahl and Sies, 2003).

También se han descrito las interacciones que tienen los antioxidantes de tipo no enzimático entre sí, la participación que tienen los carotenos como adyuvantes en la actividad antioxidante regenerando al tocoferol de su formal radicalaria tocoperoxil, el carotenoide radicalario resultante posteriormente podría ser restaurado por la vitamina C, este tipo de interacciones también son capaces de neutralizar especies reactivas de nitrógeno y por tanto reducir el daño oxidativo (Choi et al., 2004; Ryan et al., 2010). Además, es importante resaltar que el contenido nutrimental que posee la spirulina (carbohidratos, proteínas, minerales, vitaminas) puede contribuir a una mejora en el desarrollo de los organismos.

También se ha reportado que el camdio es capaz de formar complejos con el diclofenaco, uno de los efectos de esta interacción es la disminución del efecto farmacológico debido al bloqueo de uno mas grupos funcionales con actividad biológica del diclofenaco (Tabrizi et al., 2014), este tipo de interacción puede prevenir que el cadmio se encuentre disponible para interactuar con biomoléculas y de esta manera reduce el daño causado por cadmio en cierta medida, puede ser que esta interacción sea la razón por la que el grado de lipoperoxidación en el grupo expuesto a la mezcla Cd+DCF fuera menor que el observado en el grupo expuesto a Cd, sin embargo, también se ha demostrado que la exposición a estos tóxicos de manera conjunta facilita su bioacumulación (Xie et al., 2020) lo que podría incrementar su toxicidad a largo plazo.

En la **figura 9** se ilustra la ruta propuesta a través de la cual se da el daño oxidativo por la exposición a Cd y DCF, y como este daño es minimizado por la

spirulina y sus componentes, a través de los procesos de quelación del cadmio y la eliminación de ROS en *Xenopus laevis* expuesto a cadmio y diclofenaco.

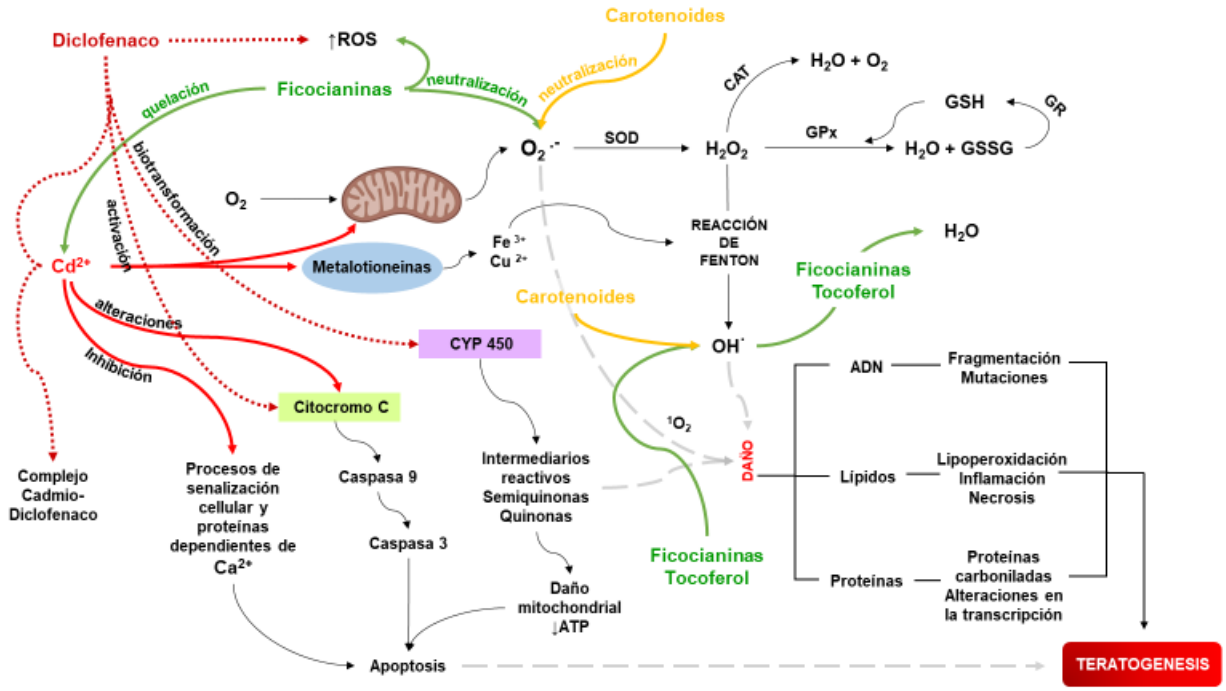

Figura 9. Propuesta del mecanismo a través del cual la spirulina logra reducir el daño inducido por el daño oxidativo inducido por cadmio, diclofenaco y la mezcla en *Xenopus laevis*.

10 CONCLUSIONES

Tanto el cadmio como el diclofenaco son capaces de generar daño a través de distintas vías, principalmente estrés oxidativo, en este trabajo se evidenciaron los efectos tóxicos generados por estos contaminantes, sin embargo, también se comprobó la capacidad que tiene la spirulina para disminuir el daño mediado a través de estrés oxidativo, además de que su aporte nutrimental ayuda a mejorar el crecimiento, en este trabajo las concentraciones de 4 y 10 mg/L de spirulina mostraron tener mejores efectos, sin embargo, es importante destacar que 2 mg/L, también demostró efectos benéficos en cuanto a la disminución de daño.

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