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Observation of zebrafish embryonic and larval development at different doses of curcumin solution: the disparity between safe and toxic

Observação do desenvolvimento embrionário e larval de zebrafish em diferentes doses de solução de curcumina: a disparidade entre o seguro e tóxico

Observación del desarrollo embrionario y larvario del pez cebra a diferentes dosis de solución de curcumina: la disparidad entre seguro y tóxico

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Abstract
The aim of the study is to perform a fast and efficient toxicity test that demonstrates the safety or toxicity levels in the embryonic development of zebrafish exposed to different doses of curcumin. Embryotoxicity tests were performed according to the OECD guideline. A standard dose of 100 mg/kg curcumin per zebrafish embryo weight was determined. A stock solution was dissolved in dimethylsulfoxide and then used to perform a serial dilution in dechlorinated water to identify safe levels of curcumin concentration. The test was performed at concentrations of 0.6; 1.25; 2.50; 5; 6.25; 10; 12.5; 25; 50; 100 μM/mL, together with a control standard containing only dechlorinated water. After the start of exposure, the embryos were monitored with an optical microscope at 8, 24, 48, 72, and 96 hours post-exposure to identify possible changes and mortality. We observed that higher doses of curcumin solution influence the appearance of teratogenic characteristics such as eye malformation, retarded growth, yolk edema, crooked syrup, and short syrup. The results were also analyzed from the embryonic survival rate from the doses used, concluding that at higher concentrations, there was a significant difference in survival.
Keywords: Curcumin; Teratogenic characteristics; Embryo development; Toxicity; Food safety.

Resumo
O objetivo do estudo é realizar um teste de toxicidade rápido e eficiente que demonstre os níveis de segurança ou tóxicidade no desenvolvimento embrionário de zebrafish expostos em diferentes doses de curcumina. Os testes de embriotoxicidade foram realizados de acordo com a diretriz da OCDE. Foi determinada uma dose padrão de 100 mg/kg de curcumina por peso de embrião de zebrafish. Uma solução estoque foi dissolvida em dimetilsulfóxido e posteriormente utilizada para performar uma diluição seriada em água declorada para identificar os níveis seguros de concentração de curcumina. O teste foi realizado nas concentrações de 0,6; 1,25; 2,50; 5; 6,25; 10; 12,5; 25; 50; 100 μM/mL, juntamente com um padrão controle contendo apenas água desclorada. Após o início da exposição, os embriões foram monitorados com microscópio óptico nos períodos de 8, 24, 48, 72 e 96 horas pós-exposição para identificar possíveis alterações e mortalidade. Observamos que doses maiores de solução de curcumina influenciam no surgimento de características teratogênicas como má formação dos olhos, crescimento retardado, edema do yolk, calda torta, calda curta. Os resultados também foram analisados a partir da taxa de sobrevivência embrionária a partir das doses utilizadas, concluindo que em concentrações mais elevadas, houve uma diferença significativa na sobrevivência.
Palavras-chave: Curcumina; Características teratogênicas; Desenvolvimento embrionário; Segurança alimentar.

Resumen
El objetivo del estudio es realizar un test de toxicidad rápido y eficaz que demuestre los niveles de seguridad o toxicidad en el desarrollo embrionario del pez cebra expuesto a diferentes dosis de curcumina. Las pruebas de embriotoxicidad se realizaron de acuerdo con la directriz de la OCDE. Se determinó una dosis estándar de 100 mg/kg de curcumina por peso de embrión de pez cebra. Se disolvió una solución madre en dimetilsulfóxido y luego se usó para realizar una dilución en serie en agua sin cloro para identificar niveles seguros de concentración de curcumina. La prueba se realizó a concentraciones de 0,6; 1,25; 2,50; 5; 6,25; 10; 12,5; 25; 50; 100 µM/mL, junto con un estándar de control que contiene solo agua no clorada. Luego del inicio de la exposición, los embriones fueron monitoreados con un microscopio óptico a las 8, 24, 48, 72 y 96 horas post-exposición para identificar posibles cambios y mortalidad. Observamos que dosis más altas de solución de curcumina influyen en la aparición de características teratogénicas como malformación ocular, retraso en el crecimiento, edema de yema, jarabe torcido y jarabe corto. También se analizaron los resultados a partir de la tasa de supervivencia embrionaria de las dosis utilizadas, concluyendo que a mayores concentraciones hubo una diferencia significativa en la supervivencia.
Palabras clave: Curcumina; Características teratogénicas; desarrollo fetal; Seguridad alimenticia.

1. Introduction

The use of chemical compounds from natural sources, such as plants, to heal is called traditional medicine. The application of these active compounds is abundant in the elaboration of modern drugs, like quinine, derived from *Cinchona*, used to treat malaria, control fever, and detoxify the liver. Similarly, aspirin, derived from salicylic acid present in willow bark (*Salix*), has anti-inflammatory properties, fever control, reduces the risk of acute myocardial infart and prevents stroke (Corson & Crews, 2007) .

Curcumin is a phytochemical compound native to Asia, known for its intense pigment derived from saffron/ turmeric/ yellow ginger (*Curcuma longa*, in Hindi known as *Haldi*). Besides its prominent use in Indian cuisine as a condiment for flavor and conservation and as a natural dye, curcumin is used in traditional Chinese medicine (TCM) and Ayurveda (Traditional Indian Medicine) as a drug for treating stomach diseases, flatulence, dysentery, ulcer, jaundice, arthritis, sprains, wounds, acne, skin inflammation, eye inflammation, cancer, and Alzheimer's disease (Aggarwal & Sung, 2009).

The cyprinid teleost *zebrafish* (*Danio rerio*), popularly known in Portuguese as *Paulistinha* or *Zebrafish*, has emerged as a powerful model organism for studying disease mechanisms and understanding pathogen-host interactions invertebrates. Their larvae are tiny, only a few millimeters in length, and transparent. The embryogenesis occurs *ex vivo* and is complete in 3 days post-fertilization (dpf) (Murayama et al., 2006) allowing easy phenotyping. Each pair of fish produces approximately 100 to 300 embryos per week, and the cost for its maintenance is 1000 times smaller than for mice. Their transparency and easy genetic manipulation allow quantitative and high-resolution images to be taken, aiding the discovery of mechanisms underlying invasion and pathogen escape from a host (Ablain & Zon, 2013; Varela et al., 2017).

Z*ebrafish* has 25 pairs of chromosomes containing> 26,000 protein-encoding genes. The genetic homology of *zebrafish*, for mammals and humans, is relatively high, supporting the translational value of the *zebrafish* model. For example, anucleotide sequence of *zebrafish* gene shows approximately 70% homology to human genes, and 84% of the genes associated with human diseases have *zebrafish* homolog (Howe et al., 2013).

Zebrafish have become a prominent vertebrate model for the study of human diseases and have already contributed several examples of successful drug discovery, as well as drug tracking, target identification, pharmacology, and toxicology (Garcia et al., 2016; MacRae & Peterson, 2015). The importance of toxicity testing is to analyze and characterize the dose-dependent toxic effects and the degree of safety of a test substance (Savastano, 2008).

Toxicology may be the most prevalent use of *zebrafish*, with most major pharmaceutical companies using it for toxicological analysis to determine chemical safety and obtain regulatory approval for clinical trials. *zebrafish* is seen as an excellent model for the evaluation of toxicity and also for biosafety evaluation of pharmaceutical products (Liu et al., 2013).

Malformation assessment is one of the main tools to observe the adverse effects of plant extracts throughout the body.

Studies have shown that several plant compounds are toxic to different organs in a concentration-dependent manner, possibly causing death (Hudson et al., 2018). Because what differentiates the drug from the drug is the dose (Shin et al., 2010; Stumpf, 2006). To understand the level of toxicity of curcumin, we aimed to investigate the concentration-dependent malformation changes of curcumin and survival rates in the toxicity of bioactive compounds under direct exposure of zebrafish embryos. This methodology allows a preliminary trial to determine and understand the disparity between doses quickly and efficiently. Toxicity testing on zebrafish models is prevalently applied by industries, with a greater focus on pharmaceuticals, to determine the safety of compounds and obtain regulatory approval for clinical trials.

2. Materials and Methods

2.1 Preparation of curcumin and aliquot solution

Curcumin was obtained commercially from the brand NEON (CAS: 458-37-7), 69.80% purity, MM: 368 g/ ml. Other reagents used were of analytical grade.

2.2 Obtaining the embryos

Zebrafish embryo obtaining was performed at "PeixeGen" Research Laboratory of the State University of Maringa (UEM).

Zebrafish adults (<18 months old) of wild type AB strain line (International Z*ebrafish* Resource Center, OR) were kept separated by sex in a glass aquarium with oxygenation and, at 28 ºC under standard conditions of temperature, pH, and light (14 h light/ 10h dark). Feeding was done with commercial feed two times a day. In addition, did maintenance of water exchange and sanitization of the aquariums was done every two days.

A ratio of 2 males to 1 female was established for breeding. The fish were placed in a spawning box at the beginning of the light cycle for a period of 60 to 90 minutes for the collection of embryos 0 hpf (hours post-fertilization). After acquiring the sources, the adult fish were separated by sex and returned to their respective aquariums. The verification of the unfertilized or irregular eggs was established through the visualization under light microscope Motic BA310E, MOTICAM 5.0 MP camera and separated from those with normal development and classified according to Kimmel (Kimmel et al., 1995).

The experiments were carried out according to the Brazilian Commission for Animal Experimentation (COBEA) laws. Also, the works-protocols were approved by the Animal Use Ethics Committee of the University Center UniCesumar (CEUA - 4.2/2020).

2.3 Curcumin toxicity test on zebrafish embryos

Zebrafish embryotoxicity tests were performed following OECD guideline; No. 236: Acute Fish Toxicity Test (FET) with adaptations. A standard dose of 100 mg/ kg of curcumin per embryo weight of zebrafish (3.8 mg) was established (Lubbad et al., 2009). Based on this theory, an aliquot diluted in dimethylsulfoxide (DMSO 0.01%) was prepared, which was used to create a serial dilution in dechlorinated water to identify and establish safe levels of curcumin concentration by the embryo. These were run in 24-well plates with five embryos per well (20 embryos/concentration) and stored in the chamber at 28°C.

In triplicate, they were submitted to concentrations of 0.6; 1.25; 2.50; 5; 6.25; 10;12.5; 25; 50; 100 μM/ mL, along with a control containing only dechlorinated water.

After the beginning of the exposition, the embryos were monitored with a Motic BA310E light microscope, MOTICAM 5.0 MP camera at periods of 8, 24, 48, 72, and 96 hours post-exposure (hpe) to identify mortality (embryonic coagulation).

At the end of the experiment (96 hpe), the larvae were anesthetized with Eugenol(Biodynamic) and analyzed for the

presence of teratogenic characteristics, including malformations in eyes, head, shortened tail, curved tail, pericardium edema, yolk sac edema, retarded growth and absence of hatching according to (Kimmel et al., 1995). All larvae that presented teratogenic characteristics were photographed with a Leica EZ4D magnifying glass.

2.4 Statistical analysis

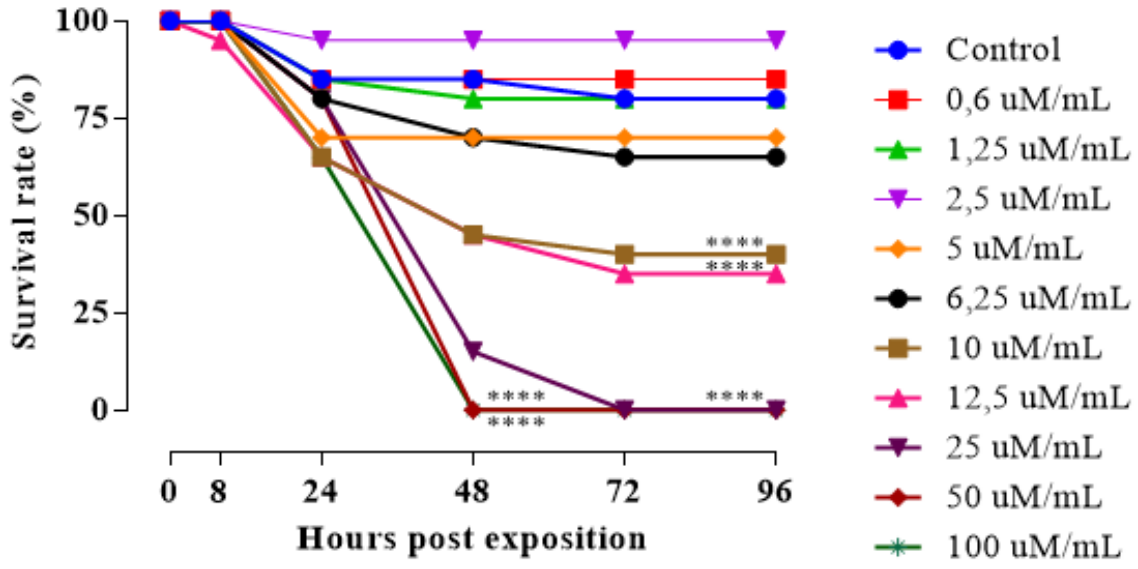
Statistical analysis was performed using *GraphPad Prism software* (GraphPad 6 Software, California, USA) to analyze variance by ANOVA using the Dunnett test. A log-rank test was used to calculate statistical differences in survival of the different experimental groups.

3. Results

3.1 Survival Rate

The effect of curcumin on the survival rate of *zebrafish* embryos was analyzed during periods of 8, 24, 48, 72 and 96 *hpe*, where it wasobserved the occurrence of coagulated eggs and non-hatched eggs. Following the control group, embryos in concentrations of 0.6, 1.25, and 2.5 µM/ mL had a survival rate above 75% at 96 hpe. After 8 *hpe*, there was a decrease in survival of all trials, including control. Between the 24 and 48 *hpe* periods, embryos exposed to concentrations greater than 6.25 µM/ mL presented values below 50% survival, remaining around 45% for concentrations of 10 and 12.5 µM/ mL, lower than 25% for the concentration of 25 µM/ mL and 0% for the concentrations of 50 and 100 µM/ mL. At 72 hpe the 25 µM/ mL assay reached 0% survival (Figure 1).

Figure 1 – A Survival rate of *zebrafish* embryos after exposure to curcumin solution.



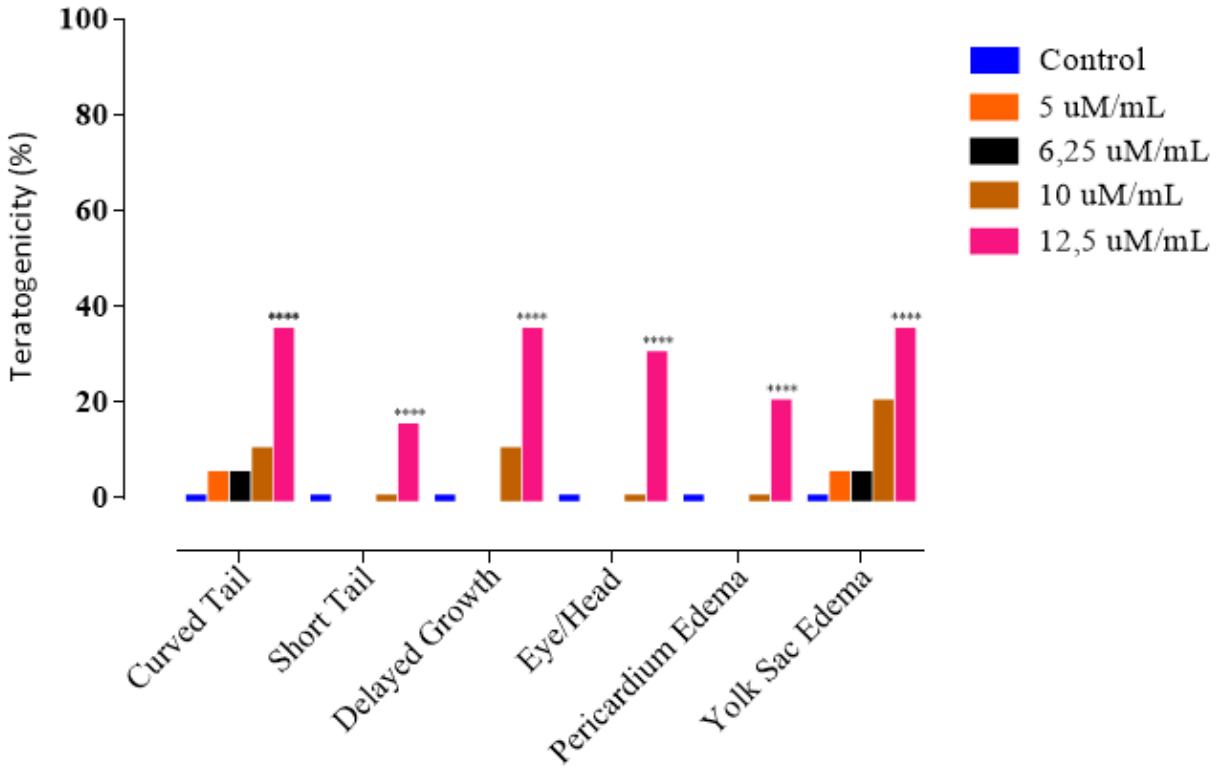
Source: Authors.

Embryos of *zebrafish* at 0 h (n= 5 per well) were exposed in a 24-well plate containing different concentrations of curcumin (Control, 0.6, 1.25, 2.5, 5, 6.25, 10, 12.5, 25, 50, 100 µM) and kept at 28° C for evaluation of survival rate at 96 hours post-exposure (hpe). Asterisks represent significant difference, P <0.05 **** compared to control.

3.2 Teratogenic Development

Concentrations of 5 and 6.25 µM/ mL resulted in a discrete number of deformities, below 10%, showing no significant difference compared to the control group. Concomitantly, the 10 µM/ mL concentration presented slightly higher values of deformations, resulting in 20% yolk sac edema, however, with no significant difference. Expressing exacerbated values, the concentration of 12.5 µM/ mL developed a greater deformity index, reaching 35% for curved tail, delayed growth, and yolk sac edema, where all deformities pointed out in this concentration obtained a significant difference when compared to the control group (Figure 2).

Figure 2 - Identification of different teratogenic characteristics in *zebrafish* larvae in different curcumin concentrations.

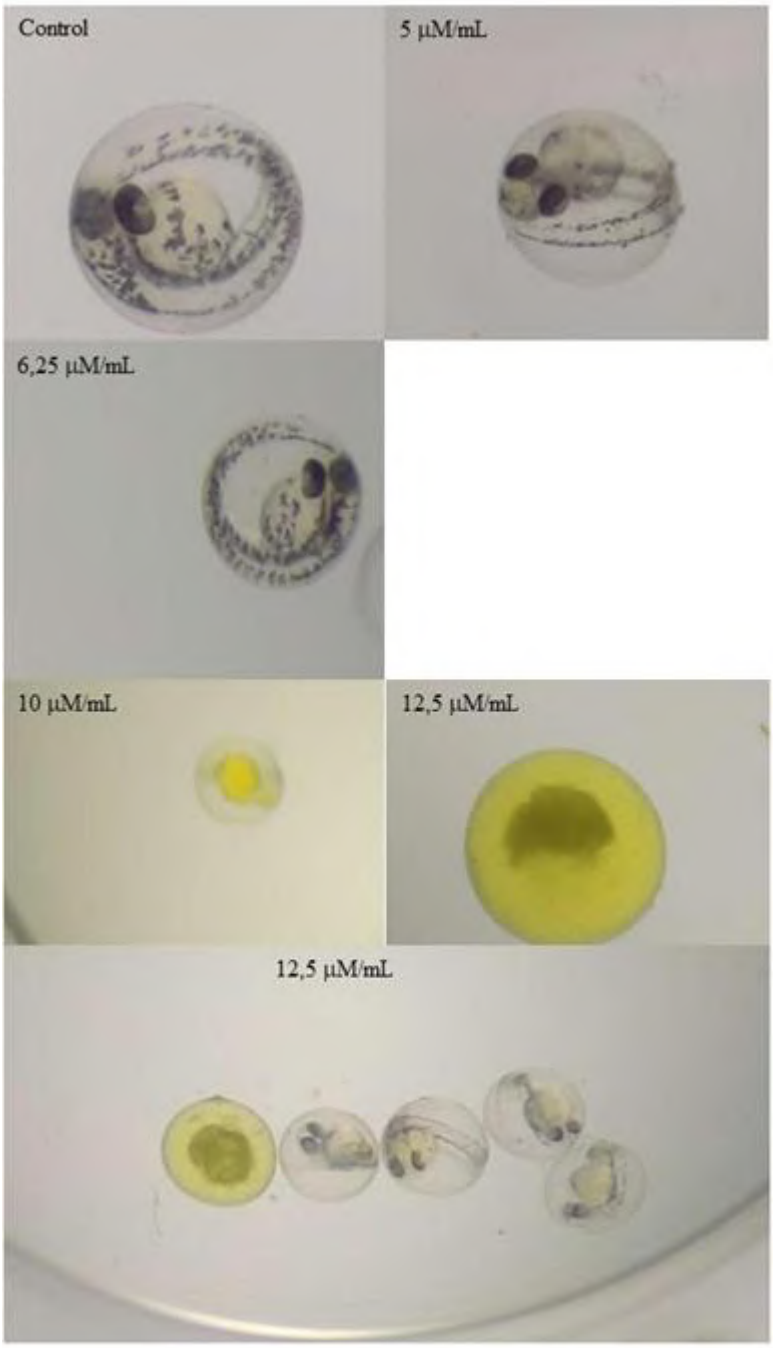


Source: Authors.

Morphological changes related to larval development of *zebrafish* exposed to varying concentrations of curcumin, 5; 6.25; 10; 12.5 µM/ mL after 96 hours of continuous exposure. Asterisks represent a significant difference between treatment and control group (determined by Dunnett's test, P <0.05 ****).

Embryos exposed in curcumin solution showed a yellowish color inside it, proving the passage of the compound through the chorion of the sources, reaching the embryonic development cells and later affecting them. As the concentration increased, the absorption of the blend increased, which could lead to embryonic coagulation, which occurred more frequently at concentrations of 10 and 12.5 µM / mL, as well as the onset of teratogenic characteristics (Figure 3).

Figure 3 - Influence of the exposure of embryos in curcumin solution.



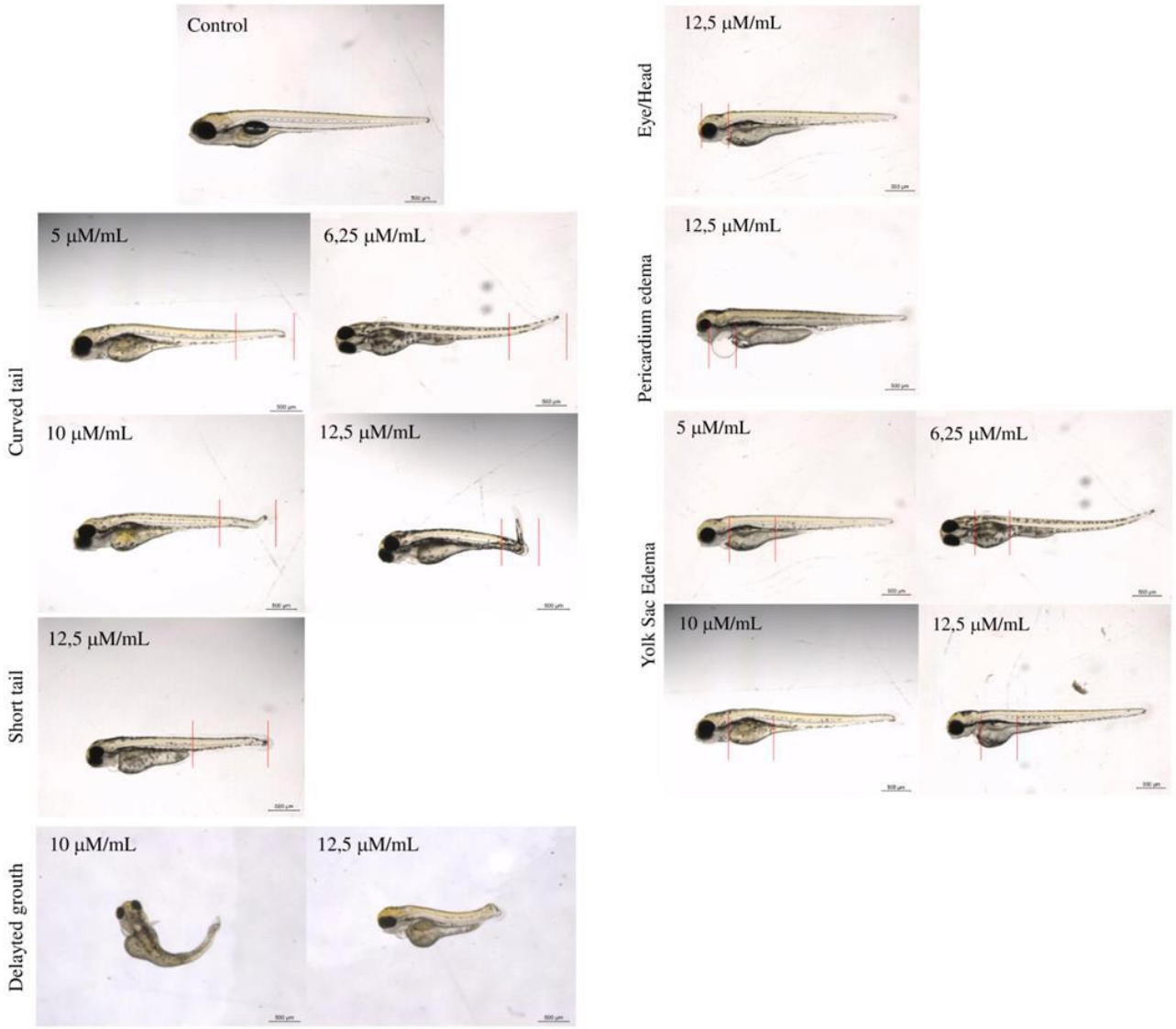
Source: Authors.

Roots exposed to different concentrations of curcumin presented similar developments. At concentrations of 5 and 6.25 µM / mL the development occurred corresponding to the control. In contrast, the 10 and 12.5 µM / mL groups had a more full absorption, causing the coagulation or the beginning of the teratogenic development.

Deformities were found in larvae after 96 hours of continuous exposure. The concentrations of 5 and 6.25 µM/ mL of curcumin expressed discrete changes in larvae development, presenting crooked tails and pericardial edema. In addition, more severe and incisive characteristics were found in the 10 and 12.5 µM/ mL concentrations of curcumin, manifesting malformations such as the short or deformed tail, delayed growth, eyeand head issues, pericardial edema, and yolk sac edema (Figure 4). Higher concentrationsof 25, 50, and 100 µM/ mL curcumin did not corroborate the manifestation of teratogeniccharacteristics because

these doses were highly toxic, providing no additional data for this analysis.

Figure 4 - Development of teratogenic characteristics in larvae of *zebrafish* 96 hpe to curcumin solution.



Source: Authors.

The concentrations of 5 and 6.25 µM/ mL of curcumin resulted in a curved tail and edema of the yolk sac. At the 10 µM/ mL, curved tail, delayed growth and edema of the yolk sac were observed. Finally,

12.5 µM/ mL presented all deformities, including the short tail, eye and head malformation, and pericardial edema. The scale bar is 500 µM. The red traces highlight the deformities found.

4. Discussion

Curcumin is a compound with high biological activity by mechanisms that are not yet fully understood. It has antioxidant and anti-inflammatory activity (Almeida et al., 2018) and is also used to treat diseases such as cancer and diabetes (Aggarwal & Sung, 2009). Thus, they are widely used as nutritional supplements for culinary and health promotion due to their natural

compounds (Ismail et al., 2017). In animals, a dose of curcumin between 100-200 mg per kilogram of weight has anti-inflammatory properties (Mohapatra, 2019). In human cancer patients, the daily tolerance dose of curcumin was 8 g (Cheng et al., 2000).

However, their compounds can be toxic. Due to this, their toxicity should be measured to ensure sufficient safety for human health (Falcão et al., 2018). Studies involving toxicity analysis have been carried out for the development of new therapies in pharmaceutical science (Guengerich & MacDonald, 2007). The therapeutic capacity of curcumin is widely discussed, exhibiting distinct toxic profiles, varying according to the concentration of the compound, model organism, and methodology used.

As already mentioned in this study, the use of zebrafish embryos for compound toxicity testing is prevalently applied by the pharmaceutical industry, aiming to identify compounds' safety levels and obtain regulatory approval for clinical trials. For this, our team emphasizes the importance of knowing in advance the origin of the adult fish used to obtain the embryos, knowing that zebrafish aged from two years start with the aging process, when cognitive changes are observed, increased senescence, less prolificity, decreased generative capacity (Kishi et al., 2009; Ruhl et al., 2016).

In this study, we found that high concentrations of curcumin negatively affect the survival and embryonic development of *zebrafish*, as observed in past issues (Wu et al., 2007). Higher concentrations resulted in the appearance of the curved tail, yolk sac edema, pericardial edema, and retarded growth, confirming that some concentrations may play partial or total lethal action on embryonic development and trigger teratogenic characteristics.

The concentrations of 10, 12.5; 25; 50, and 100 μM/ mL resulted in low survival rates, corroborating that high concentrations of curcumin have adverse effects. From a bit of attention, the substance starts to present defects that contribute to the unwanted formation of free radicals; this imbalance irretrievably results in oxidative stress, resulting in a decrease in cell viability (Farrugia & Balzan, 2012; Mendonça et al., 2009).

The curcumin has 10 times greater antioxidant activity than vitamin E, which, depending on the dose or concentration, time of action, and tissue/ cell, can cause opposite effects, increasing oxidative stress to DNA, resulting in lethality and erroneous tissue development (Shishodia et al., 2005; Wells et al., 2009). Thus, reducing cell viability due to oxidative stress from high concentrations of curcumin influences in embryonic development of zebrafish, which is confirmed by low survival and teratogenic characteristics in 96 hours of exposure to curcumin.

Tests for the carcinogenicity of chemicals and toxicology, checking the behavior, loss of function, and occurrence of tumors in zebrafish are performed regularly. These tumors are similar in humans, including neural sheath neoplasia, liver tumors, seminoma, and sarcomas (Lieschke & Currie, 2007). However, in 1842 Vogel used curcumin as a pure compound (Alvarez & Láiz, 2013). Because of its lower adverse effects, when compared to synthetic drugs, its application as a drug for health maintenance and prevention of human diseases such as anxiety, Alzheimer's, diabetes, cardiovascular disease, and cancer has been widely used (Li et al., 2020).

Toxicological assays using zebrafish embryos are comparable to those found with other animal models, indicating reliability in the studies. Besides the advantage of the transparent zebrafish embryo, which allows daily visualization and recording of the development of structures, it has rapid growth and lower cost than other models (Falcão et al., 2018). Following demand from many countries to reduce the number of animals in experiments. Using the zebrafish toxicity methodology is feasible for approaching curcumin as herbal medicine, establishing the possibility of identifying safe dose/concentration levels for the development of new drugs and new assays.

5. Conclusion

For a long time, curcumin has been used as a nutraceutical compound to maintain human health since its significant influence in Asian culture and later spread throughout the continents. In summary, our results confirm that high concentrations of curcumin may cause a wide range of adverse consequences in embryonic development of *zebrafish*, including levels of partial or total lethality, as well as severe or mild teratogenic characteristics, according to the concentration applied in 96 hours, satisfying the need to identify safe compound concentrations for any therapeutic purpose previously.

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