

Universidade do Minho
Escola de Ciências

Luana Silva Magalhães

**Zebrafish embryos as a reliable in vivo model
to assess neurotoxicity induction or protection
by bioactive natural products**



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Zebrafish embryos as a reliable *in vivo* model to assess neurotoxicity induction or protection by bioactive natural products

Dissertation of master

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Work developed under supervision of

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and

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A todos, mais uma vez, e para sempre muito obrigada

STATEMENT OF INTEGRITY

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Embriões de peixe-zebra como modelo *in vivo* confiável para avaliar a indução de neurotoxicidade ou neuroproteção de bioativos de produtos naturais

Resumo

Os produtos naturais (ou de ocorrência natural) podem desempenhar um papel duplo no sistema nervoso central (SNC), dependendo do seu comportamento como agentes neurotóxicos ou neuroprotetores. A exposição a agentes neurotóxicos durante o desenvolvimento embrionário pode causar danos irreparáveis a curto e longo prazo aos animais vertebrados, incluindo seres humanos, pois podem afetar o SNC e a barreira hematoencefálica. A suscetibilidade relatada do SNC durante a embriogênese sugere que pode haver uma janela crítica de exposição durante o desenvolvimento do cérebro. Também foi argumentado que a estimulação precoce da vida através de “stressores” ambientais pode desencadear desvios genéticos ou epigenéticos com o potencial de induzir variações no desenvolvimento neurológico. A neuroproteção contra um insulto implica uma estratégia capaz de evitar possíveis danos neuronais no SNC que possam ocorrer, naturalmente ou não, como é o caso de lesões neurais típicas de doenças neurodegenerativas.

Algumas entidades químicas podem de fato ter efeitos opostos, dependendo do seu contexto. Metodologias otimizadas para avaliar adequadamente a sua bioatividade *in vivo* são cruciais. O desenvolvimento inicial do peixe-zebra surge como um modelo alternativo muito atraente para atingir esse objetivo.

A embriogênese do peixe-zebra é uma alternativa de baixo custo e altamente informativa para avaliar respostas precoces do desenvolvimento associadas à neurotoxicidade, dadas as suas múltiplas vantagens: fertilização *ex utero*, embriões transparentes permitindo observações em tempo real e desenvolvimento rápido. Além disso, possui características conservadas do desenvolvimento neural dos vertebrados, também o genoma do peixe-zebra foi totalmente sequenciado, possuindo cerca de 70% de correspondência com genes humanos, permitindo a modelagem de doenças genéticas humanas, fazendo assim deste um organismo modelo para avaliar a embrioneurotoxicidade *in vivo*.

No presente trabalho, será composto um modelo *in vivo* confiável usando embriões de peixe-zebra para avaliar se neurotoxinas naturais e agentes neuroprotetores são muito ou pouco ativos no SNC, e quais são os seus efeitos.

Palavras-Chave: peixe-zebra, neurotoxicidade e neuroproteção.

Zebrafish embryos as a reliable *in vivo* model to assess neurotoxicity induction or protection by bioactive natural products

Abstract

Natural (or naturally occurring) products may play a dual role in the central nervous system (CNS) depending on their behavior as neurotoxic or neuroprotective agents. Exposure to neurotoxic agents during embryonic development can cause irreparable short- and long-term damage to animal vertebrates, including humans, as it may affect the CNS and the immature blood brain barrier (BBB). The reported CNS susceptibility during embryogenesis suggests that there may be a critical window of exposure during brain development. It has also been argued that early life stimulation through environmental stressors can trigger genetic or epigenetic deviations with the potential to induce neurodevelopmental variations. Neuroprotection against an ongoing insult implies a strategy capable of avoiding possible neuronal damage in the CNS that may occur, naturally or not, such as is the case of neural lesions typically of neurodegenerative diseases.

Some chemical entities may in fact have opposite effects depending on their context. Optimized methodologies to properly evaluate their *in vivo* bioactivity are therefore crucial. Zebrafish early development emerges as a very attractive alternative model to accomplish such purpose.

Zebrafish embryogenesis provides a low-cost and highly informative to evaluate early developmental responses associated with neurotoxicity, given its multiple advantages: *ex utero* fertilization, transparent embryos that allow observations at real time and rapid development are among the main factors that facilitate its use in toxicity testing for a higher yield. In addition, zebrafish genome has been fully sequenced, possessing approximately 70% of genetic correspondence to human genes, allowing modeling of diseases derived from human genetics; and the conserved characteristics of neural development among vertebrates makes it a model organism to assess embryo neurotoxicity *in vivo*.

In the present work, a reliable *in vivo* developmental model will be designed by using zebrafish embryos to assess whether natural-derived neurotoxins and neuroprotector agents are very or poorly active in the CNS, and what their effects are.

Keywords: zebrafish, neurotoxicity and neuroprotection.

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List of Abbreviations

BBB	Blood brain barrier
CB	<i>Cystoseira baccata</i>
CNS	Central nervous system
CUR	Curcumin
DA	Domoic acid
d _{pf}	Days post-fertilization
ETOH	Ethanol
EXO-LIP	Curcumin-loaded liposomes
h _{pf}	Hours post-fertilization
HTA	Head trunk angle
KA	Kainic acid
L _T	Body length
MCs	Microcystins
OECD	Organization for economic co-operation and development
PBDEs	Polybrominated diphenyl monobutyl
ROS	Reactive oxygen species
ZET	Zebrafish embryotoxicity test

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General Introduction

The present work is based on validation of zebrafish as a reliable *in vivo* model to evaluate the induction or protection of neurotoxicity by bioactive natural products. For high throughput assessment of neurotoxicity and/or neuroprotective potential of different agents of natural origin, zebrafish (early life) was selected as organism model because of its similarity to mammals (Oliveira, et al, 2016), particularly at early life stages.

Zebrafish is one of the most used species in neuroprotection and neurotoxicity tests, as it permits to obtain insights from the neural development of the locomotor part of vertebrates (Oliveira, et al, 2016 and Chen, et al, 2012). As it can be seen in **Figure 1**, there are reasonable advantages to consider it for intermediate tests between *in vitro* cell models and more complex *in vivo* organisms such as small mammals (Rizzo, et al, 2013). Cell culture systems are suitable for an initial screening of the nanotoxicity, but these do not take into account the responses on the scale of organism as a whole, therefore results collected from such assays have as primary objective, the assessment of fundamental data to be used to define future tests on other systems (Oliveira, et al, 2016 and Rizzo, et al, 2013). A great advantage of zebrafish over mammal models is that it can provide swift and high throughput biological conceptual insights of vertebrate development, as neural related, given its external (*ex utero*) embryonic growth, small size and transparency of the embryos and real time monitoring of organogenesis at the end of gastrulation (Pereira, et al., 2019; Kimmel et al, 1995; Rodriguez-Fuentes, et al, 2015 and Panzica-Kelly, et al, 2015). Moreover, it is further well-recognized as a model to understand the neurodevelopment of vertebrates (Rafferty, et al, 2014).

The central nervous system (CNS) is sensitive to oxidative stress, which may result from an imbalance between the generation and elimination of reactive oxygen species (ROS) and nitrogen (Barros, et al, 2014 and Kumar et al, 2012). It is explained by the high metabolic rate of the organism, low concentration of glutathione, catalase and high concentrations of fatty acids (Barros, et al, 2014 and Kumar et al, 2012). Neurodegeneration is a process that occurs either as a consequence of native neuropathological conditions or as a result of the natural aging of the brain (Kumar et al, 2012), while neuroprotection refers to strategies and relative mechanisms capable of defending the CNS against acute neuronal lesions (e.g. stroke or trauma) and neurodegenerative disorders (e.g., Alzheimer's and Parkinson's) (Barros, et al, 2014).

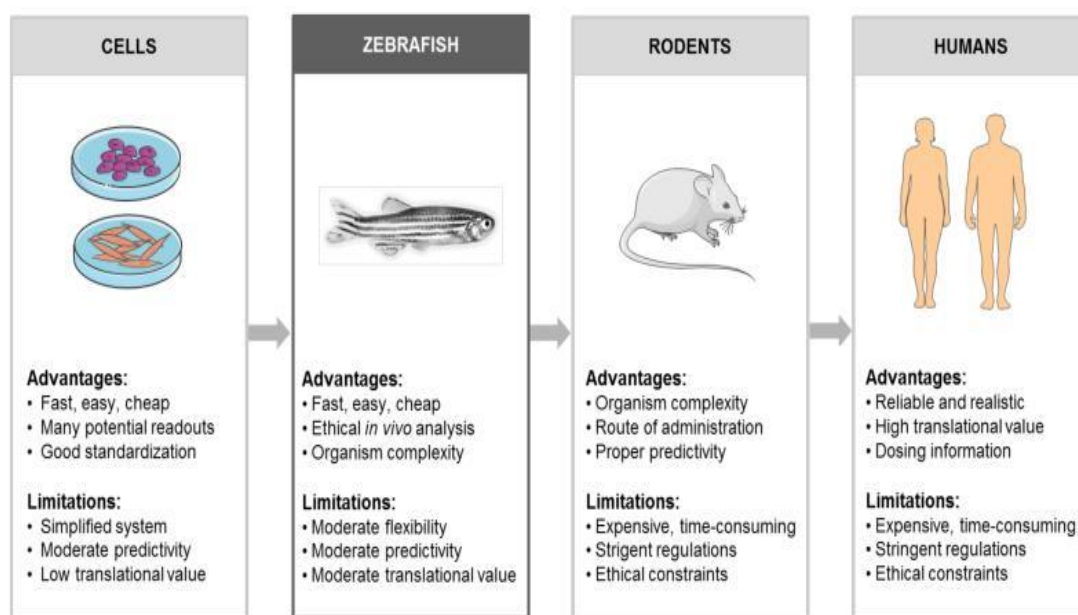


Figure 1: Sequence of the regular flow followed at the research trials, with the advantages and disadvantages of each model, from pre-clinical to clinical testing. De Rizzo et al., 2013.

Some of the most effective toxins and bioactives having potential as neuroprotectors or neurodisruptors are found among plants and aquatic environments. In fact, aquatic ecosystems encompass two-thirds of earth surface and are assumed as the cradle of primitive molecules that boosted the evolution to more complex life-forms (Grant, et al, 2010; Montaser R, et al, 2011). To resist adversity in such a competitive environment, some organisms developed complex defense strategies relying on production of unique chemical entities (Montaser R, et al, 2011). Once released, these compounds are rapidly diluted, therefore need to be potent to be effective. Several marine bioactive metabolites sharing mechanisms of action with other derived drugs are proven to be the most potent among their classes (Montaser R, et al, 2011).

Among them are some freshwater neurotoxins such as microcystins (MCs) released by cyanobacteria *Microcystis* sp. during harmful algae blooms. MCs are known to disrupt zebrafish neuromotor coordination at the early stages of life (Wu et al, 2016). MCs inhibit essential amino acids, such as serine and threonine, protein phosphatase and the production of ROS, a deficiency that causes cytoskeletal obliteration leading to hepatic apoptosis, necrosis and hemorrhage, and which mechanism is still not well understood (Li et al., 2011). In the literature, data can be found further demonstrating that MCs may accumulate and cause damage to the kidneys and heart of zebrafish; it has also been disclosed their potential to cause developmental malformations during embryogenesis and atypical hatching (Li, et al. 2011 and Wu, et al., 2016). Moreover, concern exists about a possible multiple organ toxicity effect (Li, 2016, Wu, 2016 and Zhao, et al, 2015).

Another potent neurotoxic agent found at aquatic environment is domoic acid (DA). It is a neurotoxic amino acid produced by marine algae *Pseudonitzschia* ssp. (Dizer, et al, 2001; Grant, et al, 2010 and Mos, 2000), which toxic flowering is increasingly frequent (Grant, et al, 2010). DA enters marine food chain through filter feeding animals as marine mollusks (Mos, 2000). Several neurological symptoms have been reported in marine mussels and otters, dolphins and sea lions, as in humans. DA toxicity is mainly exerted by gastrointestinal imbalance (e.g. nausea, vomiting and gastroenteritis) and neurological symptoms (e.g. confusion, disorientation and short-term memory loss) (Dizer, et al, 2001; Grant, et al, 2010 and Mos, 2000).

Kainic acid (KA) is also a natural neurotoxin produced by marine algae. It acts as neuroexcitatory amino acid agonists that activate the glutamate receptor (Zheng, et al, 2011). In the mammalian nervous system, glutamate is an excitatory neurotransmitter (Mussulini, et al., 2018), that under overexcitation can cause brain damage (Mussulini, et al., 2018). KA is responsible for an excitotoxic syndrome leading to neuronal damage and thus associated to several neuropathological disorders. KA can even induce a status epilepticus (SE), which leads to prolonged epileptic seizures (Castell, et al, 2014; Ojo, et al., 2019 and Mussulin, et al., 2018).

Apart from aquatic species, diverse phytochemicals present the so-called “medicinal” status having the potential to play a vital role in maintaining the chemical balance of the brain, as it influences the function of the receptors of the main inhibitory neurotransmitters (Kumar, et al, 2012). In the last decades, several plants have been reported as promising agents against cognitive disorders, cardiovascular diseases, cancer and neurodegenerative effects (Kumar *et al*, 2012). It is believed that there are phytochemicals present in vegetables and fruits competent to reduce the risk of developing neuronal dysfunction (Kumar *et al*, 2012; Selva, 2008, Lobo et al, 2010). Mechanisms of phytotherapies are not yet well described, but some are believed to have an anti-inflammatory and/or anti-oxygenating effect (Kumar *et al*, 2012).

1.2 Zebrafish

1.2.1 Species characteristics

Danio rerio Hamilton (1822), or zebrafish, is a small vertebrate fish (**Figure 2**). In the adult phase, it is approximately 3-4 cm long (Marques, et al., 2019 and Scheneidr, 2009). It is an oviparous species (Spence, et al., 2008). It has a varied diet, mainly including zooplankton, but also feeding on fish and invertebrate's eggs (Spence, et al., 2007). Zebrafish adapts well to different environmental circumstances (Lawrence, 2007 and Silva, 2010), supporting considerably large

ranges of temperatures and pH (Spence et al, 2008). It is native to southern Asia, with a distribution on freshwater ecosystems of India, Bangladesh, Nepal and Pakistan, regions having a monsoon climate (rainy and dry season). Zebrafish are found in standing water, slow or brook edges (Uechi, et al., 2019; Schneider, 2009 and Hamilton, 2011).



Figure 2: Zebrafish adults. **(A)** female, **(B)** male. Adapted from OECD, 2006.

In the embryonic and larval phases, zebrafish are optically transparent, which makes possible to easily analyze its development during these phases (**Figure 3**). Inversely, during the juvenile and adult phases the formation of blue colored bands along the trunk occur (Panzica-Kelly, et al, 2015). Males (**Figure 2A**) are usually yellowish next to the caudal fin and present a more flat abdomen than females (**Figure 2B**) which exhibit a more silver coloration along trunk and have a rounder belly (Singh et. al, 2015). Females lay about 200 to 300 eggs every 2 to 3 days (Oliveira et al, 2016 and Muniand 2018). Embryos develop rapidly outside the maternal body (*ex utero*), with some organs already being formed on the first day (Muniand, 2018; Oliveira, et al, 2016 and Panzica-Kelly, et al, 2015).



Figure 3: Zebrafish embryonic development. **(A)** Embryo at 8 hours post-fertilization **(B)** Embryo at 32 hours post-fertilization **(C)** Hatched larvae at 56 hours post-fertilization.

In recent years, the urgency to reduce the use of animals in embryotoxicity tests has increased (Panzica-Kelly, et al, 2015). Zebrafish embryos have emerged as an alternative model due to its numerous advantages (Tavares, et al, 2013 and Panzica-Kelly, et al, 2015). It is currently considered a leading model organism for acute aquatic toxicity screening (Panzica-Kelly, et al, 2015). Embryos of zebrafish have been used to test toxicity of environmental chemicals and

pollutants affecting human health (Li et al, 2011; Panzinca-Kelly, et al, 2015; Rodriguez-Fuentes, et al, 2015), as polybrominated diphenyl ethers (PBDEs), herbicides (2,4 dichlorophenoxyacetic acid), drugs and heavy metals (Chen, et al, 2012; Gaared, et al, 2019; Nishimura, et al, 2015; and Wang, et al, 2019).

1.2.1.1 Life cycle

Zebrafish has a short generation time of approximately 3 to 4 months (Lawrence, 2007 and Spence, et al., 2008), from fertilization to adult age (**Figure 4**). When the eggs are fertilized, their size in diameter is about 0.70 mm (Spence, et al, 2008). Embryonic development is rapid and in 36 hours post fertilization (h_{pf}) the main organs are already fully developed (Spence, et al, 2008; Lawrence, 2007 and Kimmel, 1995). Between 48 and 72 h_{pf} hatching occurs, a process that is intertwined with chorion ply and spontaneous movements (muscle activity) (Spence, et al, 2008). At 72 h_{pf} , larvae begin to demonstrate a free-swimming behavior (Kimmel, 1995 and Spence, et al, 2008), as after hatching the process of inflating the swim bladders occurs. Zebrafish larvae yolk is completely absorbed up to 7 days post fertilization (d_{pf}), during which time the digestive system is fully developed and functional (Kimmel, et al., 1995 and Spence, et al, 2008).

The maturation of zebrafish is more related to its size than the age, as when the animals reach about 23 mm these are already sexually mature (Lawrence, 2007), or may already be playing sexual traits, what usually happens having 3 or 4 months of age (Lawrence, 2007 and Kimmel et al., 1995), and therefore can be used in breeding experiments. During the first 3 months the growth rate is rapid, but over time it decreases to zero, at 18 months of age (Lawrence, 2007).

In the laboratory, zebrafish live for nearly 4 to 5 years (Lawrence, 2007), but after 2 years of age, muscular disorders and spinal curvatures become common (Lawrence, 2007 and Spence, et al, 2008).

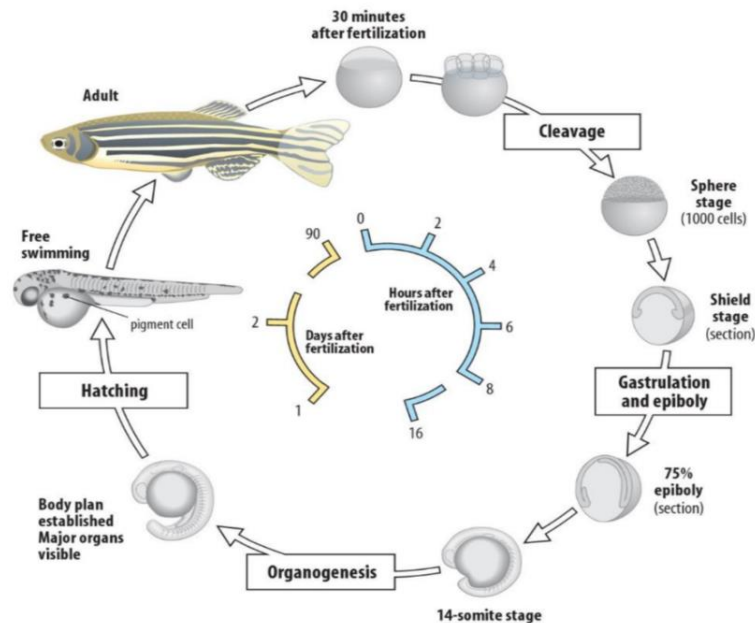


Figure 4: Life cycle of zebrafish (Lawrence, 2007).

1.2.1.2 Reproduction

In nature, zebrafish show an annual reproductive behavior (Spence, et al., 2008). The spawning starts just before the period of the monsoon because it is when there is a greater availability of food and the temperature is close to 34 °C (Spence, et al., 2008). Studies indicate that females exposed to male pheromones present a satisfactory spawn and that embryos are more viable than those from females who do not have this chemical stimulus from males (Lawrence, 2007 and Spence, et al., 2008).

Also at the laboratory environment, the reproduction of zebrafish is interconnected with photoperiod (Spence, et al., 2008), so it can reproduce throughout the year. Its sexual behavior begins at the onset of light and ends about an hour later (Spence, et al., 2008). For mating, males pursue the females poking their flanks with the muzzle. Sexual mates swim side by side for oocytes and sperm release (Lawrence, 2007 and Spence, et al., 2008). This behavior lasts throughout the playback process, but the activities are most active in the first 30 min of the event (Spence, et al., 2008).

1.2.1.3 Embryonic development

The embryonic development of zebrafish is fully described (Kimmel, et al., 1995). Seven periods were staged: zygote, cleavage, blastula, gastrula, segmentation, pharyngula and hatching (Figure 5). As zygote, the egg is fertilized and first cell formation occurs (Figure 5A) (Kimmel, et

al., 1995). From 0.75 h_{pf} to 2.25 h_{pf} its cleavage occurs (**Figure 5B**) and rapidly cell cycles take place synchronously. As result, the initial single cell is divided in 64 cells (Kimmel, et al., 1995). In blastula (**Figure 5C-D**), a phase transition occurs at the 128 cell stage and epibolic arc at 30% is formed (Kimmel et al., 1995). At gastrula (**Figure 5E**) the generation of germ layers occurs, also the primary structures of the head and the epibolic process become complete, besides the tail begins to be formed (Kimmel et al., 1995). With completed epiboly, the embryo enters on segmentation phase (**Figure 5F-G**), with the formation of the primary organs, somite and morphogenetic movements being more intense, as also the first spontaneous movements arise (Kimmel et al. 1995). At 24 h_{pf} (**Figure 5H**) the nervous system is being developed and extended, the brain is at this period divided into five lobes. Another developing system at this stage is the circulatory, with the heart having its first beating. Also, the pectoral fins are developed (Kimmel et al., 1995). From 48 h_{pf} onwards, embryos start extruding from their chorions, at the so called hatching period (**Figure 5I**). As zebrafish embryos continue to develop, the jaws and gills are formed (Kimmel et al., 1995). At 72 h_{pf} (**Figure 5J**), newly hatched larvae continue to grow rapidly and start to exhibit free swimming (Kimmel et al., 1995).

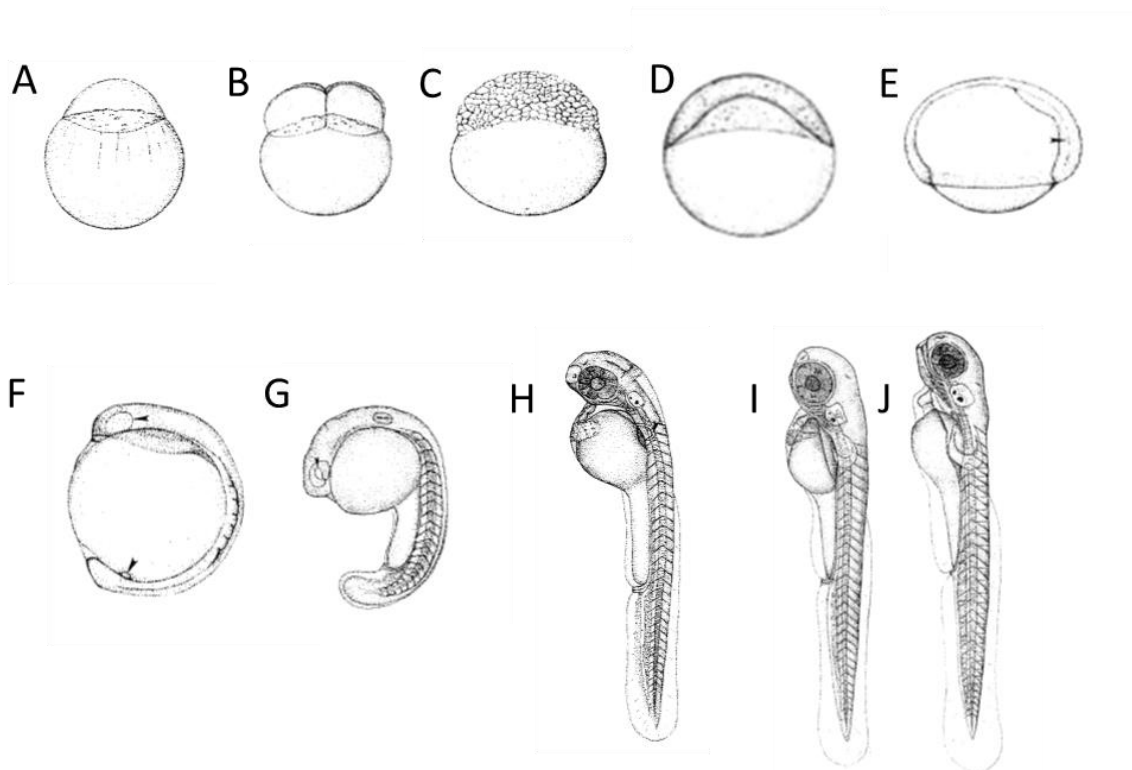


Figure 5: Stages of zebrafish embryonic development. (A) Zygote; (B) Cleavage; (C) Blastula (512 cell); (D) Blastula (30% epiboly); (E) Gastrula (75% epiboly); (F) Segmentation (6-somites); (G) Segmentation (21 somites); (H) Pharyngula; (I) Hatching and (J) Early. Adapted from Kimmel, et al 1995.

1.2.2 Zebrafish as a model organism

In recent decades, zebrafish has been used as a model organism alternative in numerous studies (Lawrence, 2017; Maes, et al, 2012 and Xi, et al, 2011), including neurological tests as it has a neurodevelopmental anatomy homologous to that of humans (Cassar et al., 2017), so it fills the gap between *in vitro* cell culture trials and *in vivo* testing in small mammal models (Cassar, et al., 2017).

Its embryonic development and organs functionality are similar to those of humans (Santoriello, et al, 2012). Besides, the genome of zebrafish is fully sequenced since 2011, presenting a high similarity with human genetics (Howe, et al, 2013; Lam, et al, 2019 and Santoriello, et al, 2012), having 70% similarity to human genes and 82% human disease-causing proteins (Lucini, et al., 2018 and Miller, et al., 2018). The general physiology of zebrafish is also similar to that of humans, composing therefore an optimal model to study metabolic, hematopoietic, cardiovascular, muscle, eye and central nervous system disorders (Lucini, et al., 2018 and Santoriello, et al., 2012). Zebrafish sensory pathways also have general homology with those of humans (Miller, et al., 2018). It is furthermore important to note that many laboratories have shown good agreement in studies using zebrafish as model for toxicity assessment (Lam, et al, 2019; Panzinca-Kelly, et al, 2015 and Rodriguez-Fuentes, et al, 2015), including neuro-associated disorders (Chen, et al, 2012; Santoriello, et al, 2012 and Xi, et al, 2011).

1.2.3 Modeling human brain diseases and disorders in zebrafish

Zebrafish is a well-recognized vertebrate model species to some human diseases as those of neurological origin, given that its genetics, physiology and anatomy are similar to mammals (Oliveira, et al, 2015), including endothelial cells, immune responses and blood brain barrier (BBB) (Oliveira et al, 2015 and Liu et al, 2018). Also, the functionality of its BBB is complete at 3 d_{pf} (Teixidó, et al, 2009). Between zebrafish and humans, the fundamental mechanism of neural development is preserved (Miller, et al., 2018). Mammals and zebrafish also have the same three-part brain structure: telencephalon, mesencephalon and rhombencephalon (Miller, et al., 2018), so between the human brain and that of the zebrafish there are some differences, but the general organization is similar. Some regions of the telencephalon in the zebrafish are in fact homologous to the striate in humans (Nishimura, et al, 2015 and Xi, et al, 2011). There is also homology in neurotransmitter pathways and functions between the zebrafish brain and that of mammals (Lucini, et al, 2018).

Unlike mammals, zebrafish has a high capacity of regeneration, which is an important feature for the exploration of neuroprotection mechanisms, neurogenesis and functional integration of the neural cells at earlier life stages (Fortana et al, 2018). Zebrafish is commonly used to investigate oxidative stress (Barros et al, 2014). Studies have shown that this event is involved in the toxicity of multiple developing organs, as the premature embryonic brain, causing neurotoxicity (Wang et al., 2018). Considering the rapid embryonic development of zebrafish (Gaared, et al, 2019), the conservation of metabolic pathways and basic cellular processes among vertebrates (Gaared, et al, 2019), its embryogenesis composes indeed a robust and adequate “time-window” of development to monitor and measure oxidative stress toxicity.

1.2.4. Zebrafish embryotoxicity test (ZET)

In 2006, the Organization for Economic Co-operation and Development (OECD) published a document highlighting some of the most important endpoints to consider on acute toxicological testing using zebrafish embryogenesis as a multi-responsive platform. Since then, the protocol known as “zebrafish embryotoxicity test” (ZET) has been applied and slightly adjusted to screen the safety profile of various pharmaceutical drugs, industrial-produced chemicals, toxins and nano-sized particles and materials (Chen et al, 2012; Marslin et al, 2016; Wu et al, 2016).

The Europe Council guidelines concerning experimental animal protection allow zebrafish embryos to be used up to the moment of free living, i.e. up to 120 h_{pf} (Directive 2010/63/EU), corresponding to the end of its embryonic development (Oliveira et al, 2016). Therefore, the present study does not need a specific license for its realization.

ZET protocol represents a fast and competent “tool” to assess vertebrate-specific developmental pathways and acute toxicity for example, evaluating whether nanotransporters are affected by toxic agents, screening for chemicals affecting morphogenesis and motor neurons (Mute-kone, et al, 2012; Oliveira et al, 2016 and Raldúa et al, 2012).

2 Natural bioactives with potential to affect vertebrate's nervous system development

2.1 Aquatic neurotoxins and pollutants

There are chemicals and natural compounds that can cause damage to the nervous system of vertebrates, as neurotoxins. Usually, these cause an oxidative stress at the CNS (Barros, et al, 2014), and may increase the production of ROS which in turn can cause damage to DNA

(Rodriguez-Fuentes, et al, 2015) and other biomolecules. Changes on antioxidant levels can contribute to the occurrence of degenerative or anomalies in embryos development and eventually inducing cell death (Rodriguez-Fuentes et al, 2015 and Barros, et al, 2014). Antioxidants defenses are enzymatic or non-enzymatic compounds that decrease the concentration of ROS (Barros, et al, 2014). A neuronal damage induced by ROS will activate the glial cells at the site of this lesion (Barros, et al., 2014). The immunologic system will then release mediators of glial cells, a mechanism that can thus be considered that of a putative neurotoxin (Barros, et al, 2014).

In recent years, there has been a higher interest in cyanotoxins worldwide (Li, et al, 2011 and Wu, et al, 2016), with MCs being the most studied because of their higher distribution and significant higher toxicity (Wu, et al, 2016). Their mechanism of action is not well-known, usually being associated with hepatotoxicity, kidney and heart damage (Wu et al, 2016). Studies with zebrafish indicate that there is a possibility of transferring accumulated maternal MCs for the progeny at a very early stage of development at which embryos are highly sensitive (Chen, et al, 2012; Li et al, 2011 and Wu, et al, 2016). In view of the highly conserved embryonic developmental period of vertebrates, it is particularly crucial to investigate the potential effects of MCs exposure at this early stage of life, as it can act as sentinel for later development (Chen et al, 2012; Li et al, 2011). There are other toxic effects described at literature related to zebrafish, such as organs malformations having adulterated functions, atypical hatching and developmental delays (Li, et al, 2011 and Maes, et al, 2012).

In the aquatic ecosystem there are other relevant chemicals, as man-produced environmental pollutants, with potential to impact neuro-sourced developmental features of vertebrates, as polybrominated diphenyl monobutyl (PBDEs), that was already found in animal tissues, including humans, being reported to have a more concentrated exposure at tissues of juvenile animals (Chen, et al, 2012). It has already been shown that PBDEs may cause alterations at the genetic expression of proteins involved in the synapse and in the formation of axons (Chen, et al, 2012). Affected cellular and molecular mechanisms include neuronal viability, as through apoptosis and oxidative stress (Chen, et al, 2012).

KA is a neurotoxin extracted from seaweed (Zheng, et al., 2011), being a neuroexcitatory agonist of amino acids that act on glutamate receptor activation (Castell, et al., 2014 and Zheng, et al., 2011). In mammals, the glutathetic system is responsible for the main excitatory neurotransmitter of the CNS (Mussulini, et al., 2018) and its overexcitation, negatively affects brain physiology (Mussulini, et al., 2018). KA can induce neuropathological features (Ojo, et al., 2019), and studies in mice demonstrated that a systemic ministration of KA can cause neuronal death and induce spontaneous seizures (Cassar et al., 2017 and Ojo, et al., 2019).

2.2 Phytochemicals

In recent years, there has been a greater interest in herbal medicines and related phytochemicals because of their high efficiency with little or no side effects (Kumar *et al*, 2012; Marslin *et al*, 2016). It is worth remembering that aqueous solubility is critical for the production of a drug (Marslin *et al*, 2016), and some of these are highly lipophilic, therefore nanotechnological solutions are commonly applied to tune their low water solubility and make the most of their interesting features. A probable alternative therapy is algae green biosynthesis of gold nanoparticles (called green products) that represents an ecological and cost-effective advantage, higher than chemical products (N. Gonzalez-Ballesteros, *et al*, 2016).

Curcuma longa is a vegetable compound found in Chinese medicinal plants (Xu, *et al*, 2007 and Monroe *et al*, 2018). This plant has a natural polyphenolic compound called curcumin, which has been reported as having anti-inflammatory, antioxidant, antibacterial, antiviral and hyperthermal effects. It has also been shown to exert neuroprotection and anticancer effects (Marslin *et al*, 2016 and Xu, *et al*, 2007). Curcumin (CUR) has also been demonstrated to exert neuroprotection and ability to eliminate ROS and anticancer effects (Marslin *et al*, 2016; Monroe, *et al*, 2018 and Xu, *et al*, 2007). Despite its bioactivity, CUR presents low solubility and bioavailability, mal absorption and rapid metabolic rates in mammals (Marslin *et al*, 2017 and Monroe, *et al*, 2018). Nevertheless, these problems can be solved by encapsulating bioactive compounds or mixtures into nanoparticles. It has been already demonstrated that polymeric nanoparticles entrapping plant compounds have a meaningful purpose to increase bioavailability and retention time of phytotherapeutics (Marslin, *et al*, 2017).

In last decades, algae-derived bioactives have gained a greater interest for applications in food, biomedical and pharmaceutical industries (Sousa, *et al*, 2017; Otero, *et al*, 2018 and Vizetto-Duarte C, *et al.*, 2016). Algae bioactives are known to present anti-inflammatory, antioxidant, cytotoxic, antifungal, anti-bacterial, anticancer and antibiotic properties (Chan, *et al*, 2002 and N. Gonzalez-Ballesteros, *et al*, 2016). Brown seaweed of the genus *Cystoseira*, found in Mediterranean sea or in the adjacent shores of the Atlantic sea (Jegou, *et al*, 2010 and Sousa *et al*, 2017), have other bioactivities: antiprotozoal, enzyme inhibitory and antiproliferative (Souza, *et al.*, 2017), and studies indicate that its extract can be used with neuroprotective agents (Custodio, *et al.*, 2016; N. Gonzalez-Ballesteros, *et al*, 2016 and Sousa, *et al.*, 2017).

3 Aim

This work aims to create a reliable consistent developmental model to assess neurotoxicity induced or protected by natural-sourced bioactive products, using zebrafish embryogenesis as a biological tool. It is expected to obtain a consistent protocol that can be used and extended to different studies modeling human brain-associated diseases and disorders, and eventually using other vertebrate species

4 Material and Methods

4.1 Zebrafish

4.1.1 Zebrafish maintenance and husbandry

For animal well-being, various maintenance activities were executed daily such as feeding, monitoring of freshwater physicochemical properties and aquaria sanitation. Aquaria set described (Figure 6) was supplied by a UV lamp sterilized filtering and dechlorinated freshwater circuit.

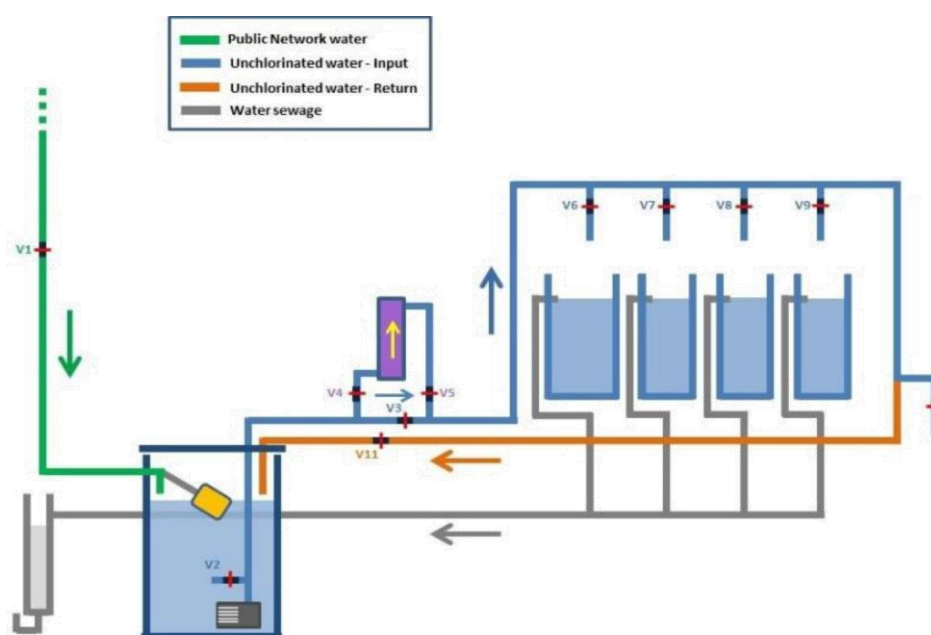


Figure 6 Freshwater aquaria system layout

4.1.2 Parental zebrafish feeding

A good nutrition of the zebrafish breeding stock is essential for their reproductive fitness with high viable egg spawning rate.

Animals were fed three times a day, with two different types of food, namely: solid food as commercial Nutrafin Max flakes; or live food, as nauplii of brine shrimp *Artemia* sp. cultured in laboratory. Particularly live food is of major importance, because of its higher nutritional value. As result, it was usually given as the first meal of the day, or as the last on pre-spawning days. Moreover, live food acts as stimulus factor for fish to "hunt" it, tempting to their predatory instinct. At the weekend, parental fish were just fed with a single type of food (solid flakes) by an automatic feeder, twice a day.

4.1.3 Stock aquarium maintenance

Stock aquarium (**Figure 7**), with a capacity of 100 L, was used to house zebrafish adults used as breeding specimens (F0, parental). At this, optimal freshwater conditions were daily verified and ensured, as follows: temperature of about $25.0 \pm 2.0^\circ\text{C}$; pH of 7.40 ± 5.0 ; chlorine at zero level, as well as nitrates and nitrites. Tank was renewed (and filtered, by activated carbon filtering) 50% daily automatically by peristaltic pumps, under an open circulation system. Fecal waste though was removed twice a day manually, using hand nets. Besides, aeration was ensured by air stones placed at the aquarium corners.



Figure 7 Stock aquarium

4.1.4 Breeding aquarium

Breeding system is composed of an aquarium with capacity of about 30 L, equipped with a spawning cage (**Figure 8**), presenting at its bottom a net, so the breeding fish have no direct contact with the embryos, avoiding cannibalism behavior by parental animals. To mimic natural habitat and induce spawning, marbles were added to the bottom of the cage (Westerfield, 2000 and Spence, et al., 2008). Zebrafish females tend to rub their genital papilla on these, which serves as substrate supporting the releasing of oocytes to the freshwater. In turn, males capture this behavior as a signal to release their sperm, and *ex utero* fertilization occurs (Spence, et al., 2008). In order to secure a stable aquatic system for reproduction, it was always assembled in the mornings, and used in late afternoons.



Figure 8: Breeding system assembled

4.1.5 Hatching aquarium

When successful reproduction occurs, with about 1000 embryos, the surplus is incubated in a 5 L aquarium, under the same physical-chemical conditions of the stock aquarium. These small tanks are placed inside another aquarium that serves as water bath for fish development, designated as hatching aquarium (**Figure 9**). At this, dead embryos were verified and removed daily, to prevent microbiological cross-contamination. Besides, the freshwater was renewed daily to ensure optimal oxygenation.

Another function of this aquarium, is to serve as incubator of *Artemia* sp. Brine shrimp is cultured in artificial saltwater at 35 ppm, $28.0 \pm 1.0^{\circ}\text{C}$ in a tapered cylindrical container at the bottom. Dry cysts were always left for hatching at this for 24 to 48 h. While the first were used to feed developing larvae, the last were used to feed zebrafish adults, as referred at Section 2.1.2.

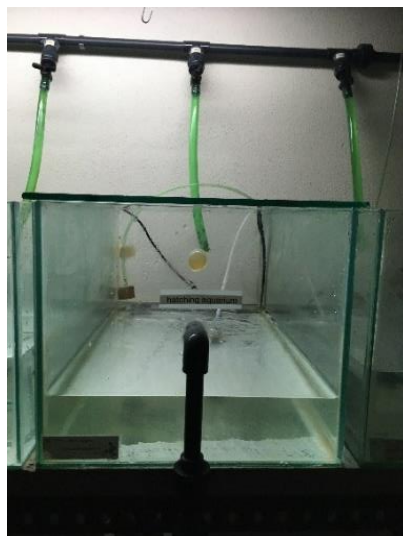


Figure 9: Hatching aquarium

4.1.6 Microplates incubation

The so designated “microplates aquarium” (**Figure 10**) was adapted to allow incubation of microplates containing developing embryos, treated during ZET assays, as it has a foam support platform that permits these to be partially immersed in acclimated freshwater at $28.0 \pm 1.0^\circ\text{C}$. This is an essential requirement of ZET protocol that was ensured from the beginning (2 h_{pf}) to the end (80 h_{pf}) of the exposure tests.



Figure 10: Aquarium form microplates incubator

4.1.7 Photoperiod

Zebrafish is a photoperiodic animal (Spence, et al., 2008), so the periods of light and dark should be regular and constant, without interference or disturbance, especially on pre-breeding days. When fish are isolated, the dark period serves to accommodate the animals in the new environment, which is at the breeding aquarium, and when the lights turn on, the zebrafish begins its mating behavior, which ends with a spawning event. At the NANODANIO laboratory, a timer was set to 12 h light and 12 h darkness.

4.1.8 ZET protocol

The development of the work requires the isolation of adult fish, which can be done once or twice during the week.

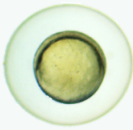
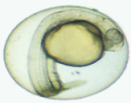
The protocol begins with the parental fish incubation at the breeding cage at the end of the day, using a sex ratio of 3 females and 5 males. The next day, 1 h after the onset of the light period, zebrafish embryos were collected, and viable embryos were separated from the dead under a stereo microscope, as the last are transparent, while unviable eggs are opaque and white. At 2 h_{pf}, the embryos were incubated in 24-well plates, each with a final volume of 2 mL, renewed daily until the end of the test. Dead embryos were always removed. Test conditions and defined controls had 4 replicates, each well representing one replicate with 10 embryos. Embryos were exposed to a given concentration of the tested chemicals or nanoparticulated material. There are two important factors not to induce embryo death, namely, temperature and pH. Optimal environmental temperature for zebrafish embryos is $28.0 \pm 1.0^{\circ}\text{C}$, and pH is 7.40 (Lawrence, 2007). For the ZET assay to be reliable, the mortality rate must be below 20%, therefore, HEPES E3 buffer was used as experimental control, as it demonstrated a previous enhanced survival of zebrafish embryos, superior to the only freshwater or ultrapure water (data not shown). It has been selected because, as a saline solution, it offers the best compromise between ensuring the optimum pH for zebrafish embryos and avoiding sublethal effects throughout development.

4.2 Analyses of toxicological endpoints

Data collection during ZET required microscopic observations at four different endpoints. At these, several embryonic developmental characteristics were evaluated. Such characteristics considered at each h_{pf} are listed at **Table 1**.

The parameters previously referred are particularly important since these correlate with vital processes coordinating neuro-motor dependent events (Oliveira et al., 2016). Morphometric measurements of the egg, yolk, eye, pupil and body length (L_t) were performed to assess phenotypical abnormalities on zebrafish embryonic development.

Table 1: Characteristics observed at each endpoint of zebrafish embryonic development.

8 h _{pf}	32 h _{pf}	56 h _{pf}	80 h _{pf}
			
Mortality Malformations Developmental delay Epiboly	Mortality Malformations Cardiac frequency Spontaneous movements Head trunk angle Pupil/Eye Yolk/Egg	Mortality Malformations Cardiac frequency Hatching Body length (LT)	Mortality Free-swimming

4.2.1. 8 hours post fertilization

At 8 h_{pf}, epibolic arc was measured (**Figure 11**). Kimmel *et al.* 1995 described that at this developmental stage, zebrafish eggs epiboly corresponds to about 75%.

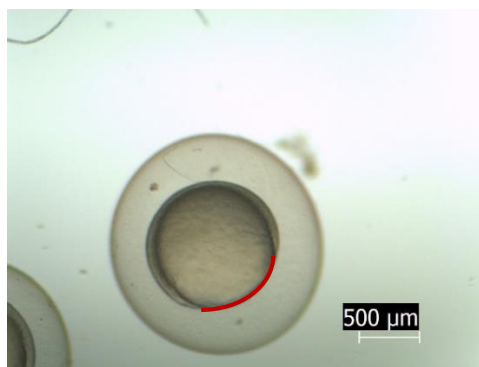


Figure 11 Embryos at 8 h_{pf}, show the epiboly arc in red

4.2.2. 32 hours post fertilization

At 32 h_{pf} most organs are already formed, and it is possible to measure the head-trunk angle (HTA) to detect a possible atypical development (**Figure 12**). It is described that at this embryonic phase, HTA is around 90° (Kimmel *et al.*, 1995), so any value above or below this angle may translates toxicity. Cardiac frequency was also evaluated at this endpoint, since at this age zebrafish embryonic heart should be fully formed and functional (Kimmel *et al.*, 1995). The cardiac

frequency was recorded for 10 s on 10 embryos *per* test condition, to evaluate whether there were signals of induced cardiotoxicity or not. The eyes and pupils are also already formed and their volumes were measured in order to verify normal development.

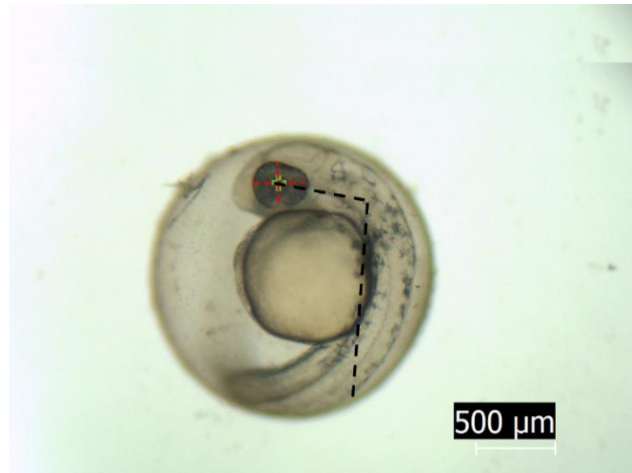


Figure 12: Embryo at 32 h^{pf}, show the measurements of HTA in black, pupil in green and eye in red.

Spontaneous movements (SM) are defined as involuntary erratic motion patterns exerted by zebrafish embryos still enclosed at their chorions (Kimmel *et al.*, 1995). Thus, an excess or scarcity of these movements may be indicative of possible defects and disorders at zebrafish embryonic nervous system. So, the number of SM was also recorded.

4.2.3. 56 hours post fertilization

It is expected that at this stage about 50% of the embryos at the control group are hatched, meaning that these should be out of their chorions, entering to a larval phase (Kimmel, et al., 1995). If this does not verify, it may indicate that the development of the embryos is delayed.

Cardiac frequency is also evaluated at this stage. Additionally, L_T, yolk volume and yolk extension were further analyzed. It is expected that the yolk volume decreases along embryo development, since it is a process that requires energy, and the yolk act as reserve (and therefore, as energetic source) providing important nutrients, as lipids (Kimmel, et al., 1995 and Lawrence, 2007). As developing larva cannot feed otherwise at this stage, malformations at the yolk extension can be used to flag for toxic signals. L_T further provides an indication of normal development.

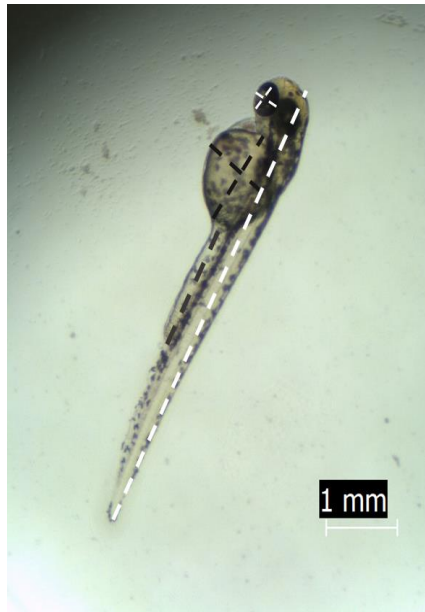


Figure 13: Larva of zebrafish at 56 h^{pf}, show the measurements of eye in white, yolk volume in black, yolk extension in black and L¹ in white.

4.2.4. 80 hours post fertilization

At this endpoint, zebrafish larvae are fully developed and already have free swimming to search for food and get around (Kimmel, et al., 1995 and Lawrence, 2007). Given that it is triggered by a mature neuronal substrate, its absence or disorder can serve as benchmark of induced neurotoxicity. Mortality rate was also assessed. Zebrafish lethality was analyzed as a cumulative rate of dead embryos, at the end of embryonic development.

4.3 Sampling and storage for genotype analysis

At the end of ZET assay, zebrafish embryos were collected and storage for future complementary genotype analysis, as follows: the microplate with the tested embryos was placed at 4 °C for 30 min to anesthetize them, after which were transferred to a labeled microtube; next, test medium was aspirated and discarded into a suitable recipient. After this procedure, 500 µL of RNAlater solution was added. Samples should be stored at 4 °C for 48 h to allow RNAlater solution penetration on the tissues. For long term storage, samples were then stored at -80 °C.

4.4. Preparation of solutions and nanoparticles for the ZET assay

4.4.1 Solvent

Zebrafish embryos exposed to ethanol (EtOH) were evaluated by DCFH-DA, Oil red O and Acridine Orange to assess ROS, apoptosis and cell membrane permeability (Chan, et al., 2002). Test concentrations were 0.25% and 0.50% of EtOH. In the ZET protocol, it was necessary to consider the removal of the embryonic chorion, as previous reports demonstrated that the fluorescence of the probes was retained in this porous protein membrane, as can be observed in **Figure 14**, the protocol was as follows: zebrafish embryos were incubated at 24 h_{pf} in 96-wells plates, lined with 2% agarose, placing at each well just 1 egg in 250 µL of a given concentration of EtOH. 10 embryos were considered *per* test condition, and two replicates were defined. At 24 h_{pf} the chorion of the embryos were removed via enzymatic protocol coupled with mechanical agitation. After dechorionation, viable zebrafish embryos were incubated with EtOH. At 32 and 80 h_{pf} (**Figure 15**), one embryo from each test condition was taken for fluorescence microscopy analysis. Such specific timepoints were selected because at 32 h_{pf} embryonic development is advanced, with the more organs formed and functioning, and at 80 h_{pf} embryonic development is completed and specimens are already considered at larvae stage.

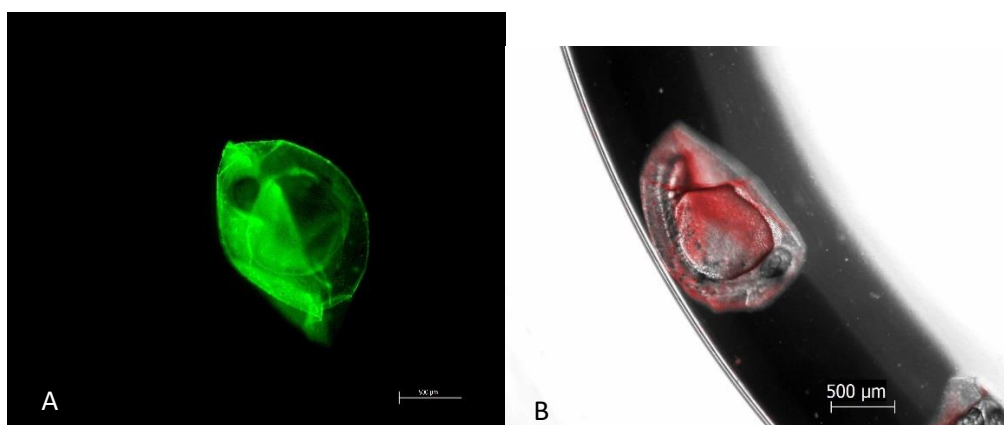


Figure 14: Embryos at 32 h_{pf} exposed to the probes. (A) embryo exposure in DCFH-DA and (B) embryo exposure in Acridine Orange.

Probes used required a specific labeling protocol. For Acridine Orange and DCFH-DA, 5µg.mL⁻¹ and 5 µM of probe, respectively, were prepared in DMSO, and incubated for 20 min at 28.0±1.0°C. After three washes with PBS 1x, 150 µL of Killik-OCT was added and incubated for 30 min under anesthetic protocol at 4°C. Photographic records were then performed under a fluorescence microscope. For Oil Red O, after anesthetized, zebrafish embryos were incubated for

30 min at 4 °C. Following this period, these were fixed using 2% PFA in PBS 1x, and incubated overnight at 4 °C in dark. Next day, fixation buffer were discarded, and the embryos were washed (3x) with PBS 1x, after which 60% of isopropanol was added to incubate for 30 min. After this step, 0.3% of Oil Red O in 60% isopropanol was added and incubated for 60 min. At last, after two washes were performed, 200 µL of 60% of isopropanol was added on top of the treated embryos for fluorescence microscopy.

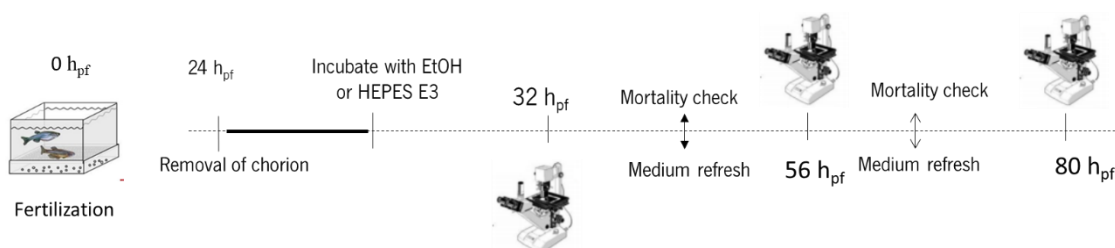


Figure 15: Scheme of ZET assay for EtOH. Adapted from Msc thesis authored by Ana Teixeira, CBMA, at University of Minho (Braga, Portugal) .

4.4.2 Extracts of macroalga

In this study, it was evaluated the toxicity of gold nanoparticles produced by green synthesis using two different vegetal extracts having anti-inflammatory, antioxidant, antifungal and antibacterial bioactivities. ZET protocol was followed to incubate zebrafish embryos at 2 h_{pf} at 80 h_{pf} (**Figure 16**). At this, the following concentrations were tested: for CB extracts: 0.003125, 0.00625, 0.0125, 0.03125 and 0.0625g/mL; CT extracts: 0.00625, 0.003125 and 0.03125 g/mL.

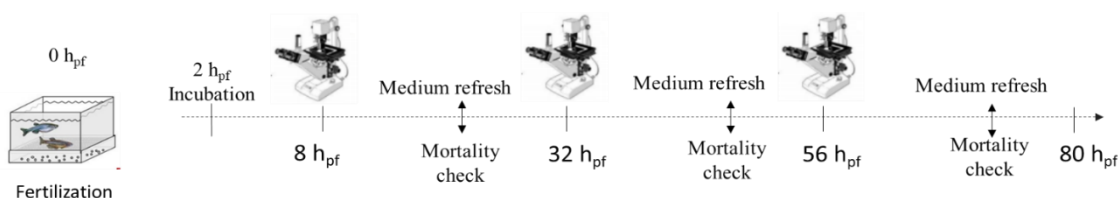


Figure 16: Scheme of ZET assay for CB extracts and nanoparticles. Adapted from Msc thesis authored by Ana Teixeira, CBMA, at University of Minho (Braga, Portugal) .

4.4.3 Free-curcumin and curcumin-loaded liposomes

Curcumin (CUR) has neuroprotective and anticancer effects (Marslin, et al., 2017), however as free compound, it has low solubility and bioavailability (Monroe, et al., 2018). Several examples are described substantiating that encapsulation of plant compounds into nanoparticles may increase their bioavailability and solubility (e.g. Marslin, et al., 2017). Aligned with this, in this work CUR was encapsulated into liposomes and its effects as free compound and loaded into these nanoscale vehicles were evaluated in zebrafish embryos via ZET assay (**Figure 17**). At this, the

following nominal concentrations were tested: free-curcumin 8 and 20 μM , curcumin-loaded liposomes 8 μM and 20 μM with or without DODAP, and empty liposomes 40 and 200 μM with or without DODAP. Incubation media were renewed daily. Dead embryos were removed frequently to avoid cross contamination.

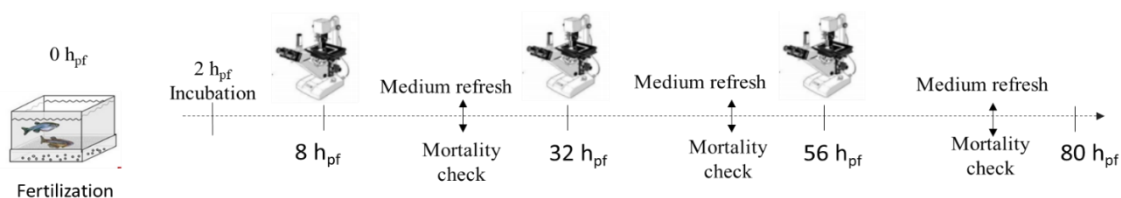


Figure 17: Scheme of ZET assay for free-curcumin and curcumin-loaded liposomes. Adapted from Msc thesis authored by Ana Teixeira, CBMA, at University of Minho (Braga, Portugal) .

4.4.4 Microcystin and free-curcumin and curcumin-loaded liposomes

Microcystin (MC) is a naturally occurring neurotoxin, described to induce malformations in zebrafish embryos (Li, et al, 2011), which can be genetically transmitted from parents to children (Wu, et al., 2016).

In this work, ZET assay was applied to test the neuroprotective effect of free-curcumin and curcumin-loaded liposomes (Exo-Lip) in embryos post-exposed to MC (**Figure 18**). In this case, the protocol was adjusted as follows: zebrafish embryos, with and without the chorionic membrane, were exposed in 96-well plates, each well incubating just one embryo, and each test condition having two replicates, totaling 10 embryos per test condition. For this assay, before exposure to the neurotoxin at the nominal concentration of 4 mg/mL, the following neuroprotective agents were (pre-)incubated: free-curcumin 20 μM and curcumin-loaded liposomes with or without DODAP 20 μM .

At the chorionated assay (**Figure 18A**), the incubation with above-mentioned neuroprotective agents lasted from 2 to 32 h_{pf}. At 24 h_{pf}, zebrafish embryos were washed three times with HEPES E3 and then exposed to the neurotoxin until 24 h_{pf}. At the dechorionated assay (**Figure 18B**), the incubation with the neuroprotective agents lasted from 32 to 56 h_{pf}. At 56 h_{pf}, zebrafish embryos were washed three times with HEPES E3 and then exposed to the neurotoxin until 80 h_{pf}. An “only MC” procedure control was further considered at both ZET assays.

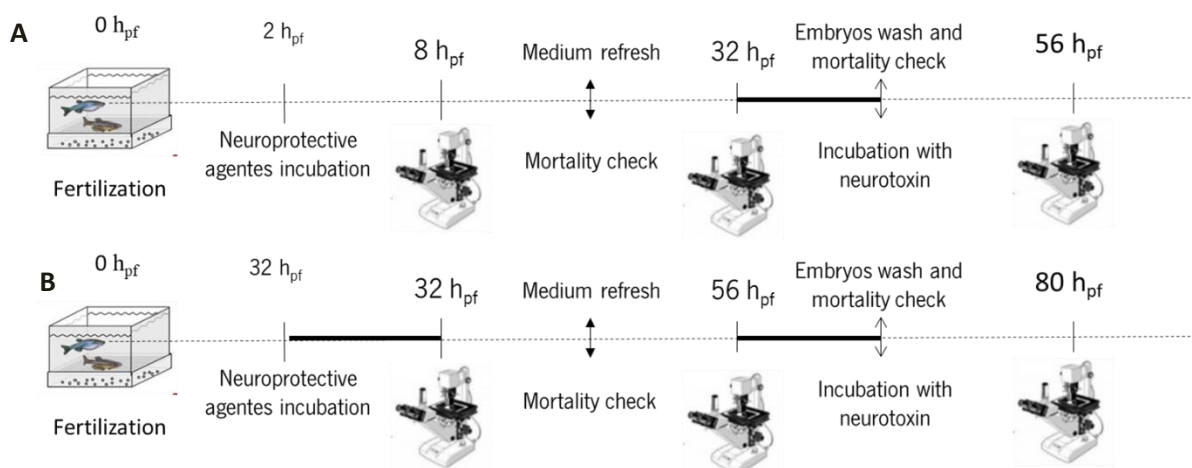


Figure 18: Scheme of ZET assay to evaluate the neuroprotective effect of free-curcumin and curcumin-loaded liposomes after exposure to microcystin, following A chorionated or B -dechorionated protocols. Adapted from Msc thesis authored by Ana Teixeira, CBMA, at University of Minho (Braga, Portugal) .

4.4.5 Kainic acid and free-curcumin and curcumin-loaded liposomes

KA is a natural occurring neurotoxin produced by some seaweed (Zheng, et al., 2011). At this work, it was used as positive control for zebrafish embryonic neurodevelopment disruption (Castell, et al., 2014; Ojo, et al., 2019). At ZET assay, zebrafish embryos were incubated in 24-well plates from 2 to 80 h_{pf} (**Figure 19**), at nominal concentrations of 2, 4 and 6 mg/kg of KA.

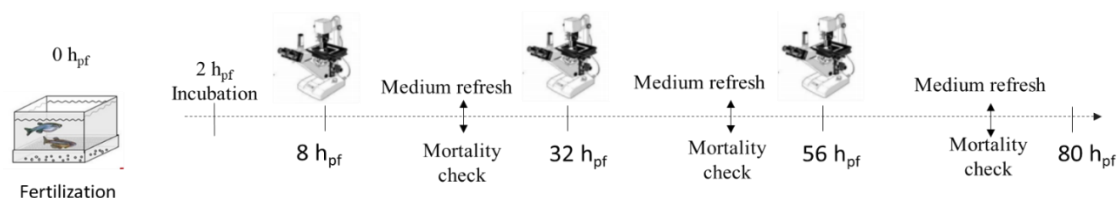


Figure 19 Scheme of ZET assay for KA. Adapted from Msc thesis authored by Ana Teixeira, CBMA, at University of Minho (Braga, Portugal) .

A ZET assay was performed to evaluate the neuroprotective effect of free-curcumin and curcumin-loaded liposomes before embryos come into contact with KA (**Figure 20**). The protocol was adjusted, namely: zebrafish embryos were incubated in 24 wells plates, from 2 h_{pf} to 32 h_{pf}, at the following nominal concentrations: free-curcumin 8 or 20 μ M, and curcumin-loaded liposomes at 8 and 20 μ M with or without DODAP. At 32 h_{pf}, zebrafish embryos exposed to the above-mentioned neuroprotective agents were washed three times with HEPES E3, and then incubated

with 6 mg/kg of KA until 80 h_{pf}. An “only KA” methodologic control control was further considered at this ZET assay.

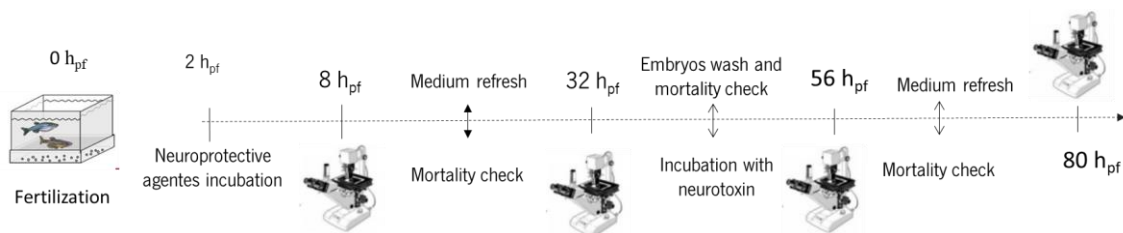


Figure 20: Scheme of ZET assay to evaluate the neuroprotective effect of free-curcumin and curcumin-loaded liposomes after exposure to KA. Adapted from Msc thesis authored by Ana Teixeira, CBMA, at University of Minho (Braga, Portugal) .

4.5 Statistical Analysis

Images were acquired under a Leica microscope using a Las EZ 3.4 software (<https://www.leica-microsystems.com>), and analyzed via ImageJ. Statistical analyzes were performed using STATISTICA software (StatSoft v.10), and graphs were performed using GraphPad Prism (GraphPad Inc v.6). One-way ANOVA was used for the analysis of zebrafish embryonic development for the following parameters: percentage of epiboly (8 h_{pf}), HTA (32 h_{pf}) and free swimming (80 h_{pf}). Two-way ANOVA was used for cardiac frequency at 32 h_{pf} and 56 h_{pf}. ANCOVA test was used to analyze yolk volume at 8 h_{pf} and 32 h_{pf} (egg volume was used as co-variable), and pupil surface at 32 h_{pf} (eye surface was used as co-variable). For Post-hoc comparisons, Newman-Keuls test was used. $P \leq 0.05$ was statistically considered as significant.

5 Results and discussion

5.1 Ethanol

To identify putative interference on zebrafish development of the solvents used to prepare the extracts, the embryos were initially incubated from 2 to 80 h_{pf} with 0.75% EtOH. When performing protocols to assess cellular ROS, apoptosis and cell membrane permeability using fluorescent probes, microscopy images revealed that these were retained by chorion (see **Figure 14**) that acted as barrier that impeded these to transpose it. To surmount this situation, a chorion removal protocol optimized by our research group was applied (unpublished data). So, before exposing the embryos to EtOH, dechorionation was performed at 24 h_{pf}, as embryos were already at pharyngula period. Besides, previous studies by our research group (unpublished data) further allowed to demonstrate that the removal of this membrane did not greatly affect the embryos at this timepoint.

The first parameter analyzed was survival rate at 80 h_{pf}, because in this team the survival rate can be evaluated throughout the trial, statistically not significant ($\chi^2=0.0109170$ $P=0.994556$) but the survival rate is higher in the condition: control and EtOH 0.5% (96%), and in the 0.25% EtOH (95%) condition is lower, as observed in **Figure 21**.

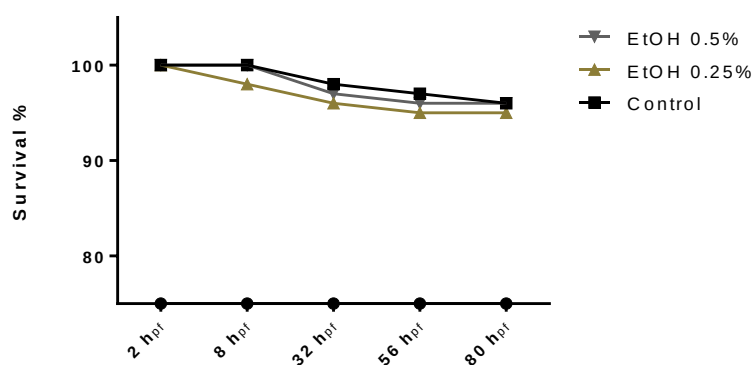


Figure 21: Effect of ethanol (EtOH) (0.25% and 0.5%) exposure on zebrafish embryo survival rate along 80 h_{pf} assay

At 32 h_{pf}, the parameters spontaneous movements, pupil surface and cardiac frequency were analyzed. Statistically, there is no significance for spontaneous movements ($\chi^2=0.954$ $P=0.620620$), as shown in **Figure 22A**, but there are differences between conditions, control (82.68%) and 0.25% EtOH (71%), thus indicating that 0.25% EtOH may have a negative effect on the embryo's motor development. For the parameter HTA show that statistically there is significance ($P < 0.05$),

as shown in figure 22B. At 56 h_{pf}, statistically there is no significance as to yolk extension ($P=0,055745$), and no statistical significance between conditions (figure 22C).

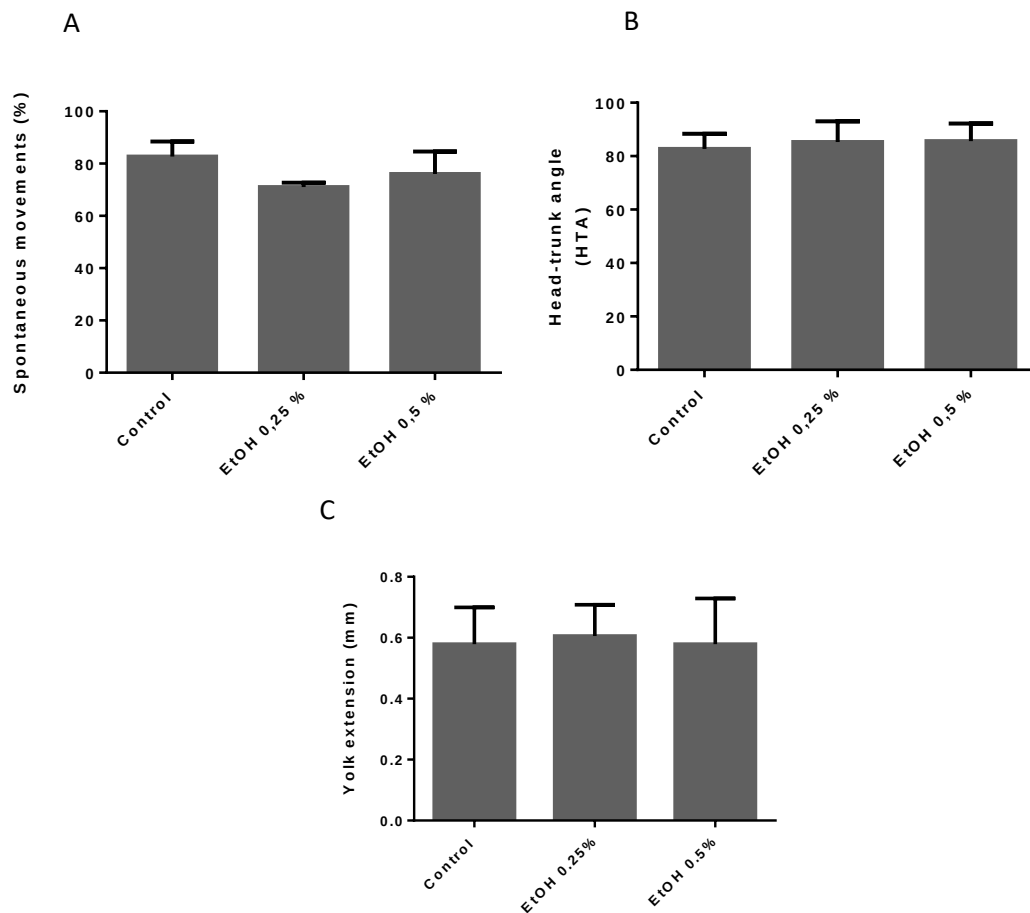


Figure 22 The effects the of exposure of ethanol (EtOH (0.25% and 0.5%) exposure on.(A) spontaneous movements at 32 h_{pf},.(B) head-trunk angle (HTA), and (C) yolk extension at 56 h_{pf}.

The cardiac frequency was analyzed at 32 and statistically significant ($P<0.05$), but all conditions present statistical differences in cardiac frequency at timepoints, and it is evident that at 56 h_{pf} this difference increases if bought with control, indicating that EtOH has a negative effect on the development of the cardiovascular system, causing a decreased cardiac frequency in the embryos (**Figure 23A**). Finally, the last parameter analyzed was free swimming at 80 h_{pf}, because in this team normally developing embryos are capable of free swimming. Statistically not significant ($\chi^2= 4.8370$ and $P=0.08905$), but with differences in swimming rate between conditions, control (81.39%), 0.25% EtOH (63.61%) and 0.5% EtOH (56.67%) (**Figure 23B**), indicating that EtOH causes a developmental delay, resulting in embryos being free-swimming.

In the present test, it can be observed that with the removal of the chorion, it does not affect the development of the embryo, which only observed developmental delays in embryos

exposed to EtOH, even in low condition, because with the removal of chorion. Embryos have lost their primary physical barrier, and there was no selectivity in what would be in contact with the embryo, and under these conditions EtOH may have a negative effect on embryonic development, but is not toxic enough to cause significant delays in embryonic development.

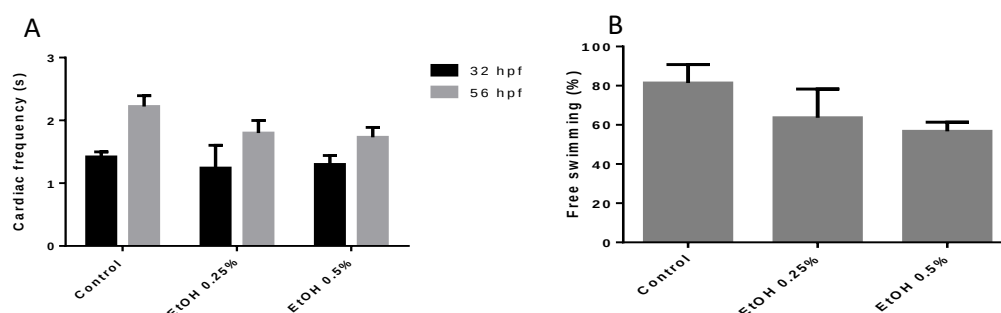


Figure 23: The effects of ethanol (EtOH) (0.25 and 0.5%) exposure on zebrafish. (A) cardiac frequency at 32 and 56 h_{pf}, and (B) 80 h_{pf} on free swimming at 80 h_{pf}.

5.2 Establishment of a neurotoxicity model

5.2.1 Microcystin

Cyanotoxins in recent decades had a higher occurrence in waters of consumption and recreation (Li, et al., 2011 and Wu, et al., 2016). From all of these the most studied is microcystin (Wu, et al., 2016), it is important to study its toxicity. According to the literature (Che, et al., 2018; Li, et al., 2011; Qi, et al., 2016; Tzima, et al., 2017 and Wu, at el., 2016), from 2 mg/mL of microcystin there are evidences of toxicity and according to (Qi, et al., 2016) the condition 4 mg/mL of microcystin is toxic. For these reasons, the assays were started with 4 mg/mL microcystin.

Survival was analyzed at 80 h_{pf}, and for the chorion test, there was statistical significance ($\chi^2 = 0.5882$ and $P < 0.05$), as show in **Figure 24A**, for the without chorion test, statistically no significant ($\chi^2 = 0.00$ and $P = 1.00$), as show in **Figure 24B** the survival is lower in test with chorion from condition microcystin.

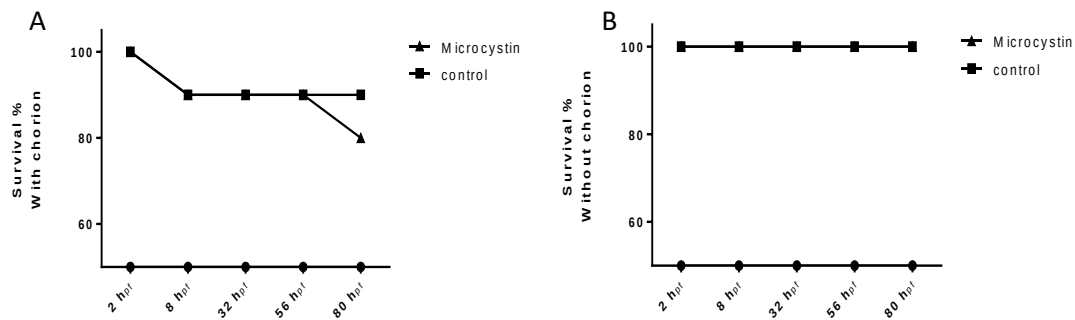


Figure 24 Effect of microcystin exposure on zebrafish embryos survival rate along 80 h^{pf} of (A) chorionated and (B) dechorionated zebrafish embryos. assay

At 32 h^{pf} the analyzed parameter is the spontaneous movements the test with chorion feature statistical significant ($\chi^2=16.666$ $P<0.05$), as observed in **Figure 25A**, from test without chorion statistically no has significant ($\chi^2=0.0$ and $P=1$), as observed in **Figure 25B**. The results of test with chorion, it is observed that microcystin has a spontaneous movement rate lower than the control, and in assay without chorion the movement rate is similar between control and microcystin, indicating that with chorion microcystin does not cause delays.

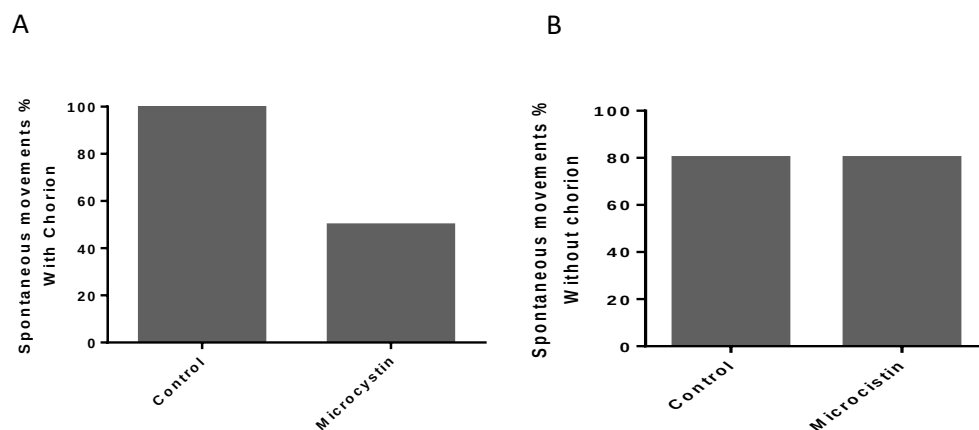


Figure 25: The effects of microcystin exposure on spontaneous movements at (32 h^{pf}) of (A) chorionated and (B) dechorionated zebrafish embryos.

Cardiac frequency is analyzed at 32 and 56 h^{pf}, the statistical chorion test has no significance ($P=0.547936$), but when analyzing cardiac frequency there are differences between the condition and microcystin, embryos exposed to microcystin had their frequency. decreased over time, as shown in **Figure 26A**. In the test without corium, there was no statistical significance ($P=0.38170091$), and cardiac frequency analysis shows differences between the two conditions, with embryos exposed to microcystin and cardiac frequency decreasing over time, as shown in

Figure 26B. Thus for the two trials with or without chorion the microcystin embryos have delays in the cardiovascular system.

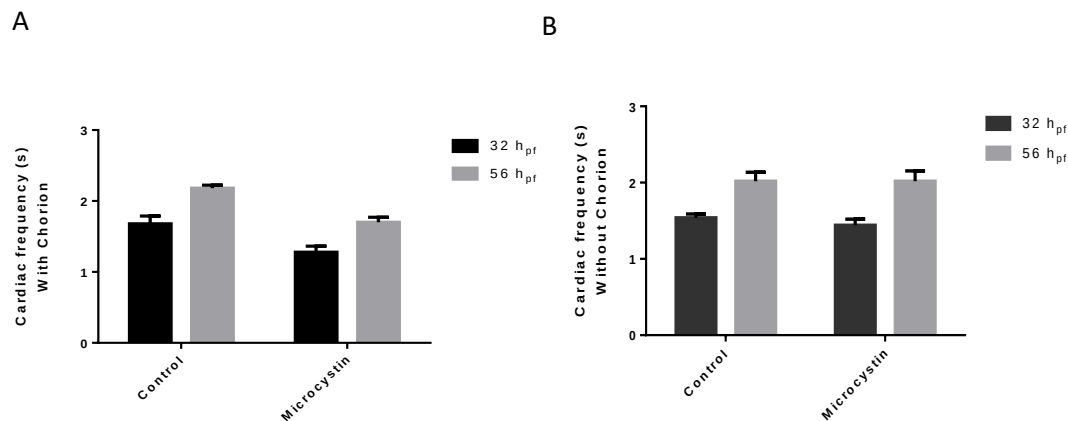


Figure 26: Effects of microcystin exposure on cardiac frequency (32 and 56 h_{pf}) of (A) chorionated and (B) dechorionated zebrafish embryos.

At 80 h_{pf} free swimming is analyzed, for this test only the no-corium test will be analyzed, because the chorion test was finalized at 56 h_{pf} and at this time the free-swimming is not evaluated, for the test without chorion statistic is not significant ($\chi^2 = 0.00$ p = 1.0), and thus both conditions present 100% of free swimming. In both tests, with or without chorion, microcystin showed no toxicity, as shown in **Figure 27** this result can be explained by the fact that exposure in our assay was only 24 h_{pf} and in the literature studies indicate that microcystin causes toxicity but their exposure was over 80 h_{pf} (Che et al, 2018;.. Li et al, 2011 and Wu, El, 2016.).

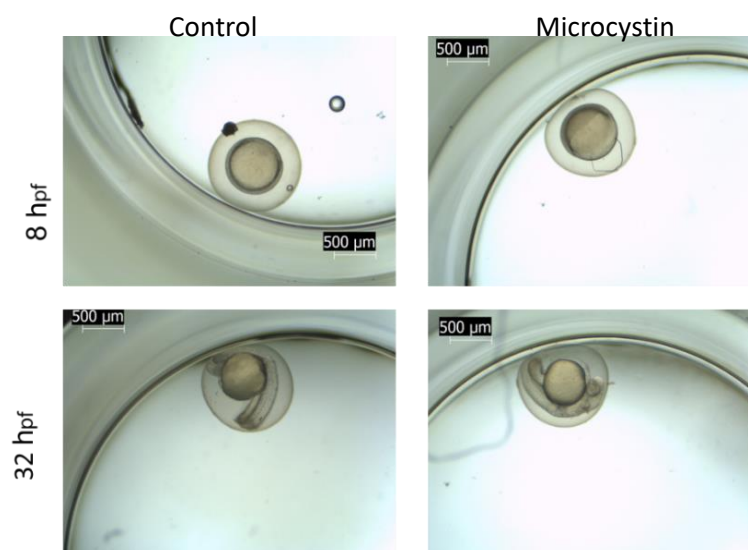


Figure 27: Effects of microcystin on development of zebrafish embryos, exposed to HEPES. E3 (Control) and 4mg/mL of microcystin at 8 and 32 h_{pf}

5.2.2 Kainic Acid

KA was selected because it is a natural neurotoxin, and the existing literature suggests it helps designing more effective treatments for epilepsy (Mussulini, et al., 2018 and Ojo, et al., 2019). The test condition were chosen according to the literature (Mussulini, et al., 2018 and Ojo, et al., 2019), and as reports only referred to adult zebrafish, the condition was adjusted to zebrafish embryos.

For the present assay the embryos were incubated from 2 to 80 h_{pf}. The first parameter evaluated is survival at 80 h_{pf}, because in this team is observed the accumulation of survival rate throughout the test and, therefore, can provide a total perspective of the whole survival rate, statistically no significance ($\chi^2=1, 2335$ and $P = 744959$), as observed in **Figure 28**

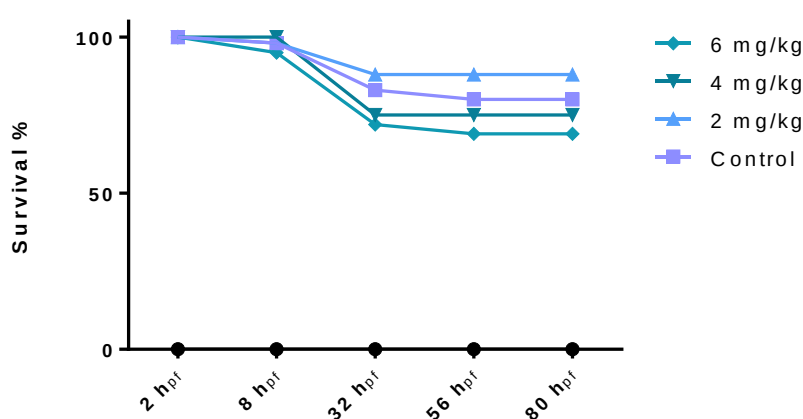


Figure 28: Effect of exposure to neurotoxin kainic acid on zebrafish embryos survival rate up to along 80 h_{pf}

The epibolic arch is analyzed at 8 h_{pf}, and statistically not significant ($P = 0,909031$) and, for this reason, embryonic development was similar across all conditions, as observed in **Figure 29A**. At 32 h_{pf}, the HTA is analyzed, but also in this one it has no significance ($P=0,591492$) as can be seen in **Figure 29B**.

B

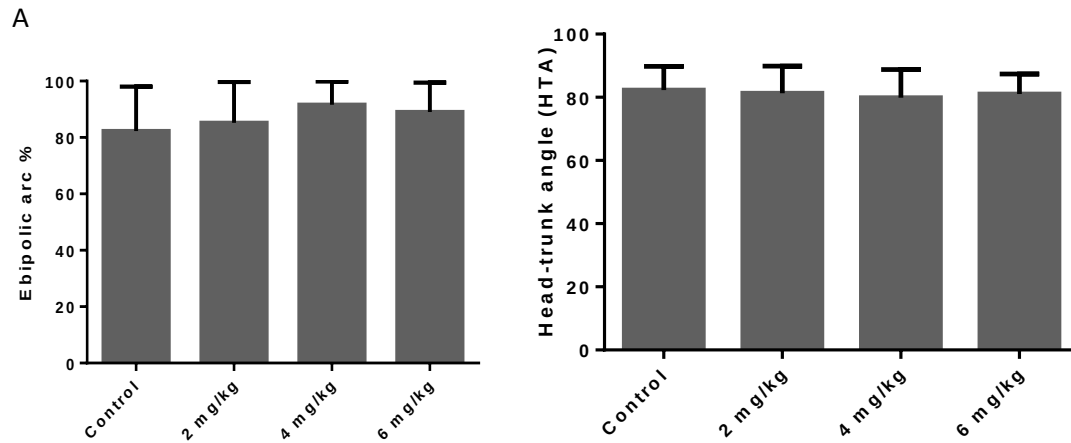


Figure 29: The effects of KA kainic acid exposure on arc epiboly (A) at 8 h_{pf}, and with on 32 h_{pf} the effect of KA to (B) HTA at 32 h_{pf}

At 32 h_{pf} spontaneous movements are analyzed, and statistically no significant ($\chi^2 = 1.504$, $P = 0.681310$), but KA conditions have a higher rate of spontaneous movements than control, as can be seen in **Figure 30A** these results were expected, as KA has an effect of mimicking the symptoms of epilepsy, so embryos incubated with KA have a higher spontaneous movement rate than controls. Although hatching rate is directly correlated with movement, embryos incubated with KA had a lower hatching rate than control, probably because of embryonic delays caused by KA toxicity, for the hatching stage statistically significant ($\chi^2 = 3.6610$, $P < 0.05$), as shown in **Figure 30B**. Cardiac frequency is evaluated at 32 and 56 h_{pf}, and statistically not significant ($P = 0.454050$), but when analyzing each condition and timepoints, it can be observed that there are differences between all conditions compared to the control, and for the condition of 6 mg/kg presents the lowest cardiac frequency, as observed in **Figure 30C**.

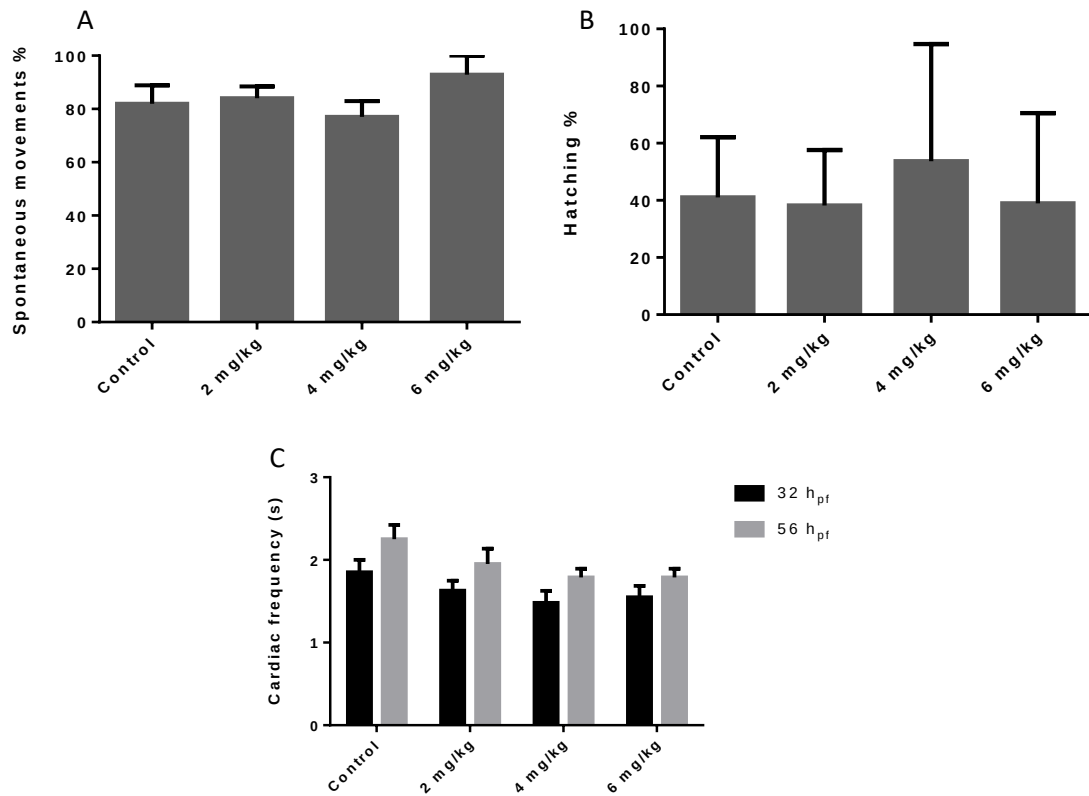


Figure 30: Effects of kainic acid exposure on zebrafish embryos. (A) spontaneous movements at 32 h_{pf}, (B) hatching rate at 56 h_{pf} and (C) cardiac frequency at 32 and 56 h_{pf}.

At 56 h_{pf} the yolk extension, which is statistically significant ($P < 0.05$), is analyzed between all KA conditions and the control and there are differences, and at 4 mg/kg it has a yolk extension lower than the control. Already at 6 mg/kg yolk extension is greater than control, this shows that there is a negative effect on lipid metabolism, as observed in **Figure 31A**. At 80 h_{pf} free swimming is analyzed, and statistically there is significance ($P < 0.05$), indicating this (**Figure 31B**).

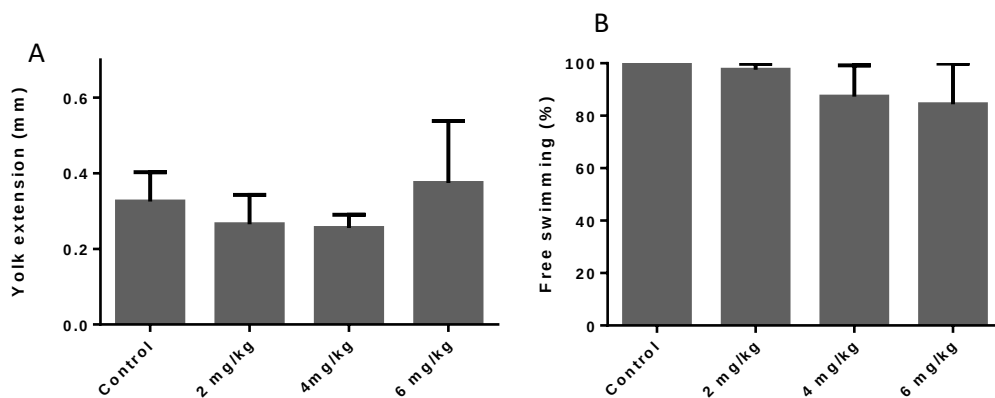


Figure 31: Effects of kainic acid exposure (A) on yolk extension at 32 h_{pf}, and (B)- on free swimming at 80 h_{pf}.

The results obtained indicate that KA can induce spontaneous movements in embryos, it can also cause damage to embryonic development, but without causing significant delays, as seen in **Figure 32**, so KA can be used in more complex tests to find new therapies against neurological diseases such as KA may indicate whether or not a compound is neuroprotective. The results of this assay were used to choose a condition to test the neuroprotective effect of CUR.

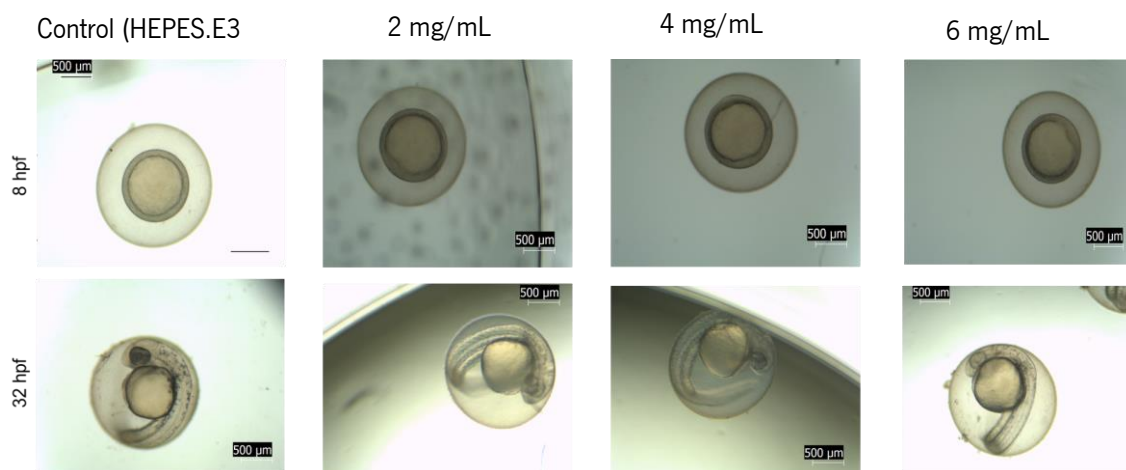


Figure 32: Effects of kainic acid on development of zebrafish embryos exposed to HEPES.E3 (control), and 2, 4 and 6 mL/kg of kainic acid at 8 and 32 h_{pf}.

5.3 Toxicity and bioactivity assessment of products of natural origin to biomedical uses

5.3.1 Curcumin and curcumin-loaded liposomes

Curcumin has a neuroprotective and anticancer effect (Marslin, et al., 2017), but in its free form it has low bioavailability and is rapidly metabolized by the organism (Marlin, et al., 2017), therefore, it is crucial to use curcumin-loaded nanoparticles to improve curcumin's bioavailability, so that it can better evaluate its bioactives. Exosomes are nanovesicles which perform intercellular communication, so are an excellent tool to encapsulate and deliver various types of molecules (Lu, et al., 2019 and Stremersch, et al., 2016). Liposomes also mimic the constitution of biological barriers which renders them very biocompatible. Also, the possibility to functionalize these with molecules that target the BBB is also an advantage when compared to natural exosomes (Lu, et al., 2019 and Stremersch, et al., 2016). The use of DODAP (1,2 Dioleoyl-3-dimethylammonium propane) serves to increase the encapsulation efficiency of the molecule (Semple, et al., 2001).

Embryos were exposed to the following conditions: CUR free 8 or 20 μM , 8 μM and 20 μM curcumin loaded liposomes with or without DODAP and 40 or 200 μM empty liposomes with or without DODAP, incubation started at 2 h_{pf} and ended at 80 h_{pf}. The first parameter analyzed was survival at 80 h_{pf}, statistically not significant ($\chi^2=12,35965$ and $P=0.261713$), as shown in **Figure 33**, but observing each condition the best survival rates are: Control (90%), Exo-lip 8 μM with DODAP (93%) and Exo-lip 20 μM without DODAP (90%).

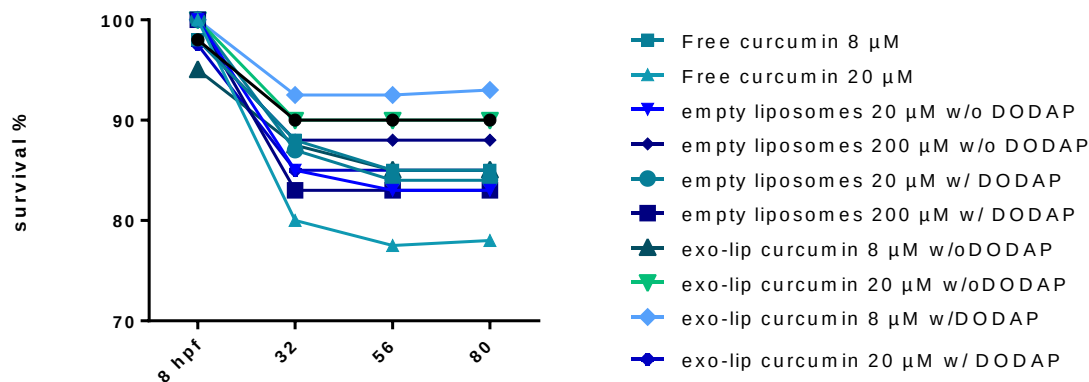


Figure 33: Effects of curcumin and curcumin-loaded liposomes exposure on cumulative survival rate of zebrafish along embryonic development.

At 8 h_{pf} epibolic arc revealed no relevant statistical differences among groups ($P=0,537540$), as it can be seen at **Figure 34A**. Also for spontaneous movements no significant differences were found among groups ($\chi^2=12,330$, $P=0.263$), although a trend (not statistically relevant) can be highlighted when test conditions are compared to control, namely: Exo-lip 8 μM without DODAP (93.75%), CUR free 20 μM (92.22%) and empty liposomes 8 μM without DODAP (87%) showed higher spontaneous movements rate than control (78.89%) (see **Figure 34B**).

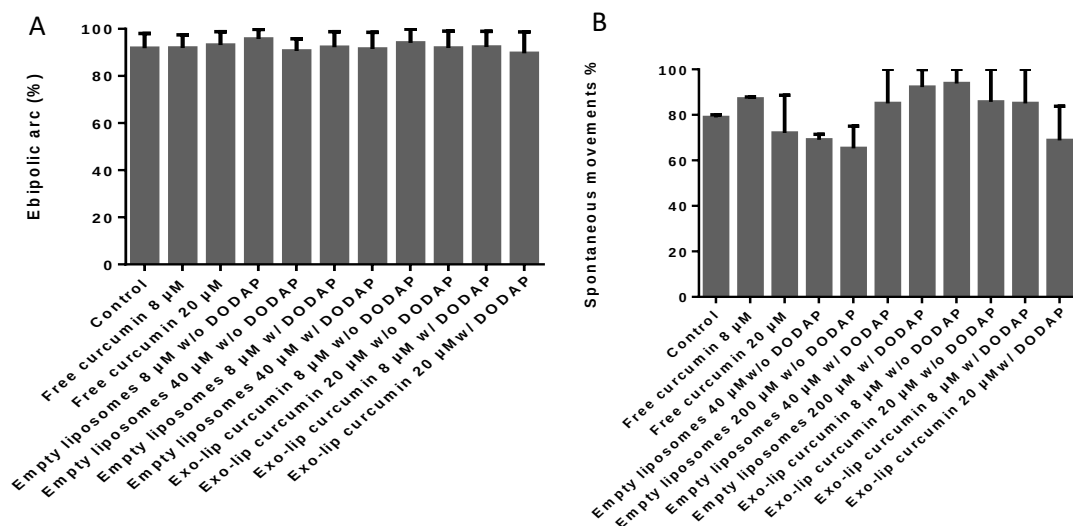


Figure 34: Effects of curcumin and curcumin-loaded liposomes exposure on zebrafish embryos (A) - ebipolic arc at 8 h_{pf}, and (B) on spontaneous movements at 32 h_{pf}.

At 32 h_{pf}, the yolk volume was analyzed and statistically not significant ($P=0,168265$), but when analyzing each condition, a difference was observed between them, the condition empty liposome 8 μ M without DODAP has similar values with control, the other condition of curcumin and curcumin-loaded liposomes, see Figure 35, the yolk volume value is lower than control and this result indicates a putative effect on lipid metabolism.

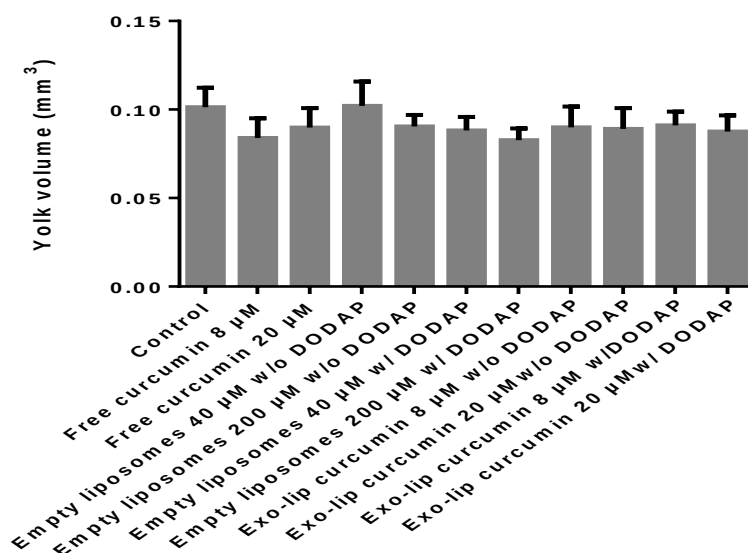


Figure 35: Effects of curcumin and curcumin-loaded liposomes exposure on zebrafish embryos yolk volume at 56 h_{pf}.

At 56 h^{pf} hatching was analyzed, and was shown to be statistically significant ($\chi^2=92.98$ and $P<0.05$), but between the conditions there are differences in hatching rate, highlighting that Empty liposomes 20 μM without DODAP (60.3%), Exo-lip 8 μM or 20 μM without DODAP (85% and 75% respectively) and Exo-lip 8 μM with DODAP (85%), have a higher hatching rate than the control (44, 8%), Empty liposomes without DODAP 8 μM (19.5%) have a lower hatching rate, as shown in **Figure 36A**. At 32 and 56 h^{pf}, cardiac frequency was analyzed, statistically not significant ($P= 0,341243$), but there are differences between conditions, at 32 h^{pf}, CUR free 8 μM , Exo-lip 20 μM without DODAP and Exo-lip 8 μM with DODAP have a higher cardiac frequency than control, whereas at 56 h^{pf} only the condition CUR free 8 μM has a higher than normal cardiac frequency (**Figure 36B**), these results indicate that these conditions alter the cardiac frequency, indicating possible toxicity.

Finally, at 80 h^{pf}, free swimming is analyzed and statistically significant ($P<0.05$), as shown in **Figure 36C**. The results indicate CUR 20 free μM causes developmental delays and is therefore toxic, but empty 8 or 20 μM with DODAP liposomes and 8 or 20 μM with DODAP curcumin-loaded liposomes do not cause embryonic developmental delays and have good survival rate (**Figure 37**).

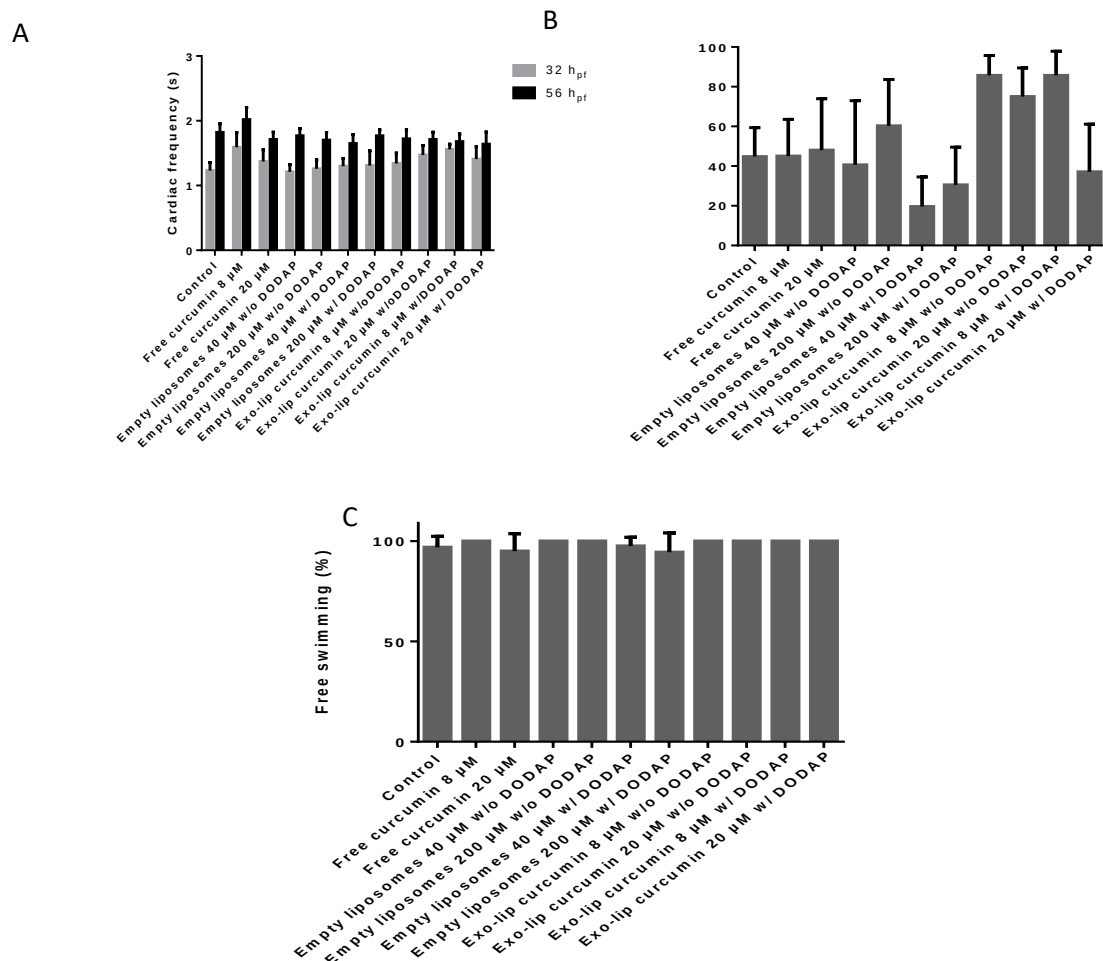


Figure 36: Effects of curcumin and curcumin-loaded liposomes exposure on zebrafish embryos (A) cardiac frequency at 32 and 56 h_{pf}, (B)- hatching rate at 56 h_{pf}, and (C) free swimming at 80 h_{pf}.

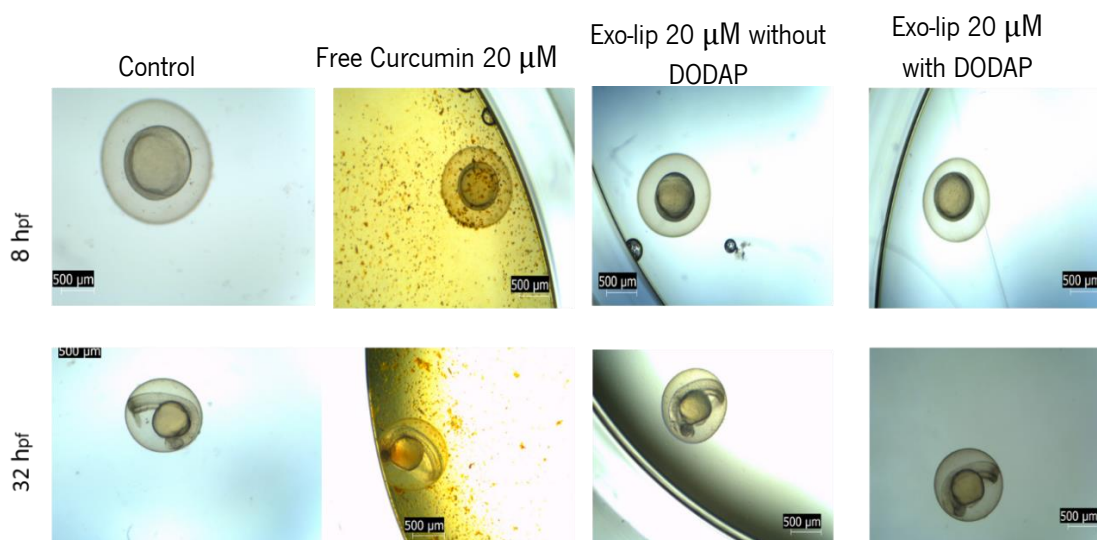


Figure 37: Effects of curcumin and curcumin-loaded liposomes on development of zebrafish embryos exposed to control, free curcumin 20 µM, Exo-lip 20 µM with or without DODAP, at 8 and 32 h_{pf}.

5.3.1.1 Curcumin and curcumin-loaded liposomes to counteract microcystin-induced toxicity

Exposure to neuroprotective agents during embryogenesis can prevent the emergence of neurodegenerative diseases (Kumar, et al., 2012), or the use of neuroprotective compounds can protect the nervous system against toxins, so it is important to identify potential neuroprotectors against neurotoxins, to know and understand their effect on the embryonic neurodevelopment. CUR is a polyphenol of natural origin generally nontoxic, and it has been recognized as competent neuroprotective agent to eliminate ROS (Meo, et al., 2019), since it acts as antioxidant bioactive. Literature indicates that studies with CUR prove its benefits to neurological health (Marslin, et al., 2017 and Meo, et al., 2019).

First, survival at 80 h_{pf} will be analyzed for the non-statistically significant chorion assay ($\chi^2=6.1904$ and $P=0.185371$), see **Figure 38A**, but when analyzing each condition the survival rate is lower at CUR free 20 µM (70%) than in the control (90%), for the non-chorus test there is also no significance ($\chi^2 = 7.6595$ and $P = 0.104878$), and the CUR free 20 µM condition has the lowest survival rate (70%). than control (100%) These results show that CUR free 20 µM has a toxic effect, causing mortality of embryo (see **Figure 38B**).

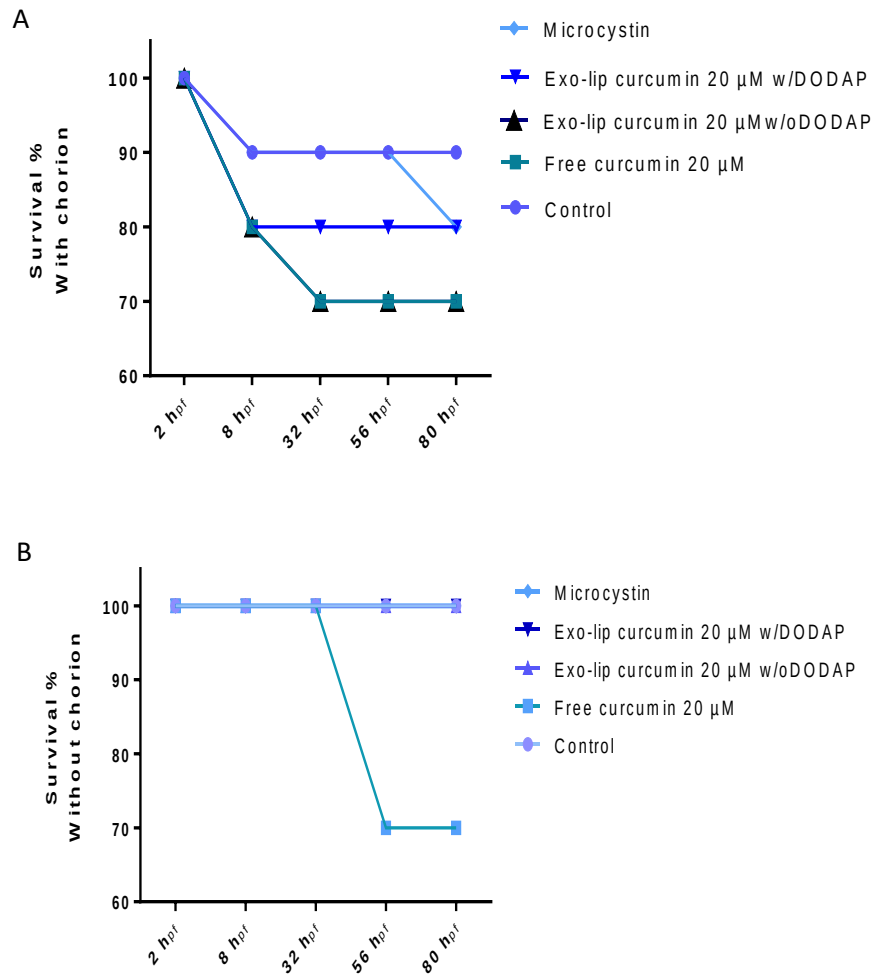


Figure 38: Effects of free and encapsulated curcumin (Exo-lip) on survival of zebrafish exposed to microcystin. (A) assay with chorion (B) assay without chorion

At 32 h_{pf} spontaneous movements were analyzed, in the test with chorion is statistically significant ($\chi^2=34.8484$, $P<0.05$), as shown in **Figure 39A**, conditions with the lowest spontaneous movement rate are CUR free 20 μM and microcystin, both with 50%, but for the without chorion assay there is no significant ($\chi^2= 4.2105$, $P=0. 378265$), but if each condition is analyzed, the one with the lowest movement rate is CUR free 20 μM (60%), while all other conditions, including control, have 70%, as shown in **Figure 39B**. Cardiac frequency was analyzed at 32 and 56 h_{pf}, for the test with chorion, there is statistically significant ($P <0.05$), but the CUR free 20 μM condition has the lowest cardiac frequency compared to the other conditions, as observed in **Figure 39C**. For the test without chorion, there is no significance ($P= 0,339874$), but has cardiac frequency differences at 56 h_{pf}, at this time all conditions have a lower cardiac frequency than control. (**Figure 39D**).

B

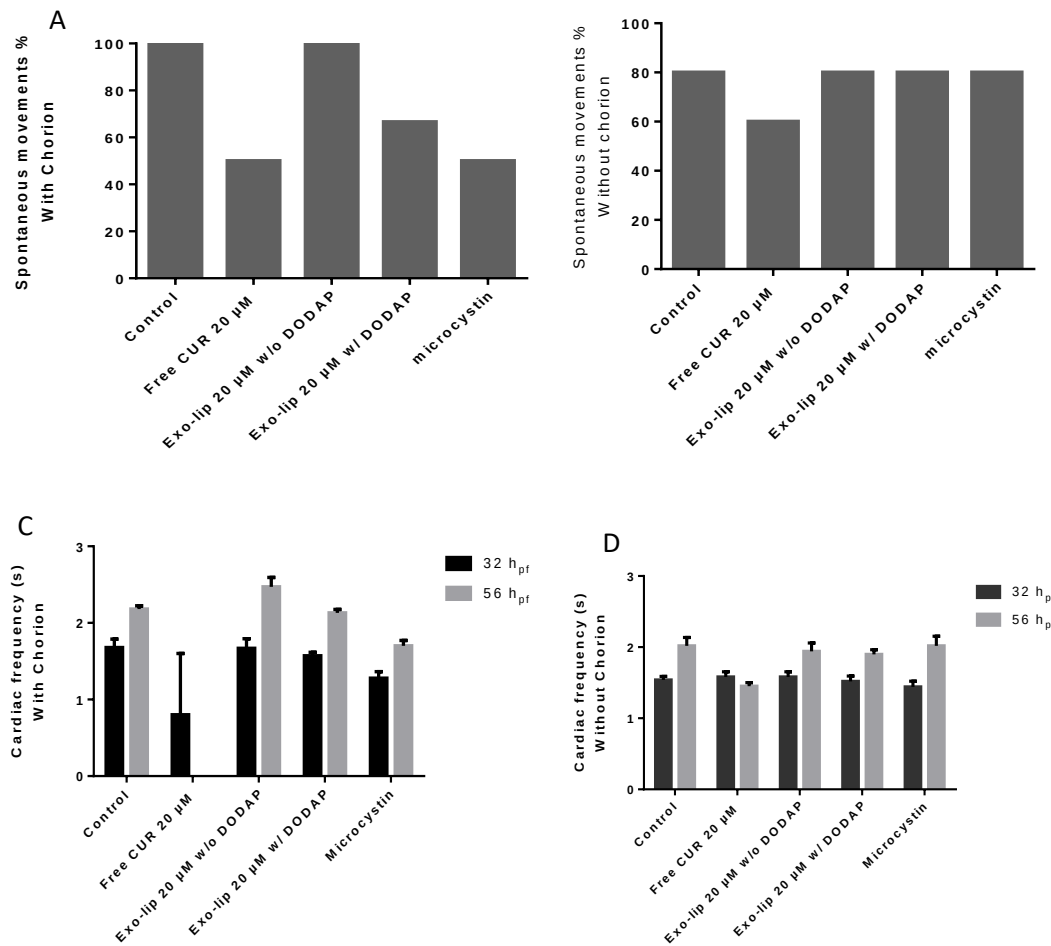


Figure 39: Effects of free and encapsulated curcumin (Exo-lip) (A-B) on spontaneous movements (32 h_{pf}) and (C-D) cardiac frequency (32 and 56 h_{pf}) of zebrafish exposed to microcystin.

At 56 h_{pf} hatching was also analyzed, assay with chorion is statistically significant ($P < 0.05$), but the 20 μ M CUR free and microcystin conditions have a hatching rate of 0%, indicating that there was a significant developmental delay embryonic (**Figure 40A**). The test without chorion also presents statistical significance ($P < 0.05$), but the conditions of CUR free 20 μ M and Exo-lip 20 μ M without DODAP have the lowest hatching rates (0 and 60%, respectively), as observed in **Figure 40B**. At 80 h_{pf} free-swimming was analyzed, but only for the test without chorion, because the test with chorion ended at 56 h_{pf}, and thus there is no data on this parameter, for the test without chorion statistically significant ($P < 0.05$). However, when analyzing each condition, the CUR free 20 μ M has a lower free swimming rate (50%), while the other conditions, including the control, have a 100% free swimming rate. The curcumin compound has fluorescence, and we used this to evaluate whether the compound was incorporated into either its free form or the liposomes in the embryos BBB, because the BBB is formed and functional from 72 h_{pf}, so in this team they were made fluorescence images (**Figure 41C**) to prove curcumin incorporation.

The results indicate that CUR free 20 μM causes significant delays in embryonic development, and thus is toxic to embryos, the same is not observed if the same concentration of curcumin in liposomes is used, whereas microcystin was not observed significant effects of developmental delays, This can be explained by the fact that in our study embryos were exposed only for 24 h_{pf} , whereas in other studies found in the literature the exposure of embryos to microcystin is over 80 h_{pf} (Che, et al., 2018; Li, et al., 2011 and Wu, in el., 2016).

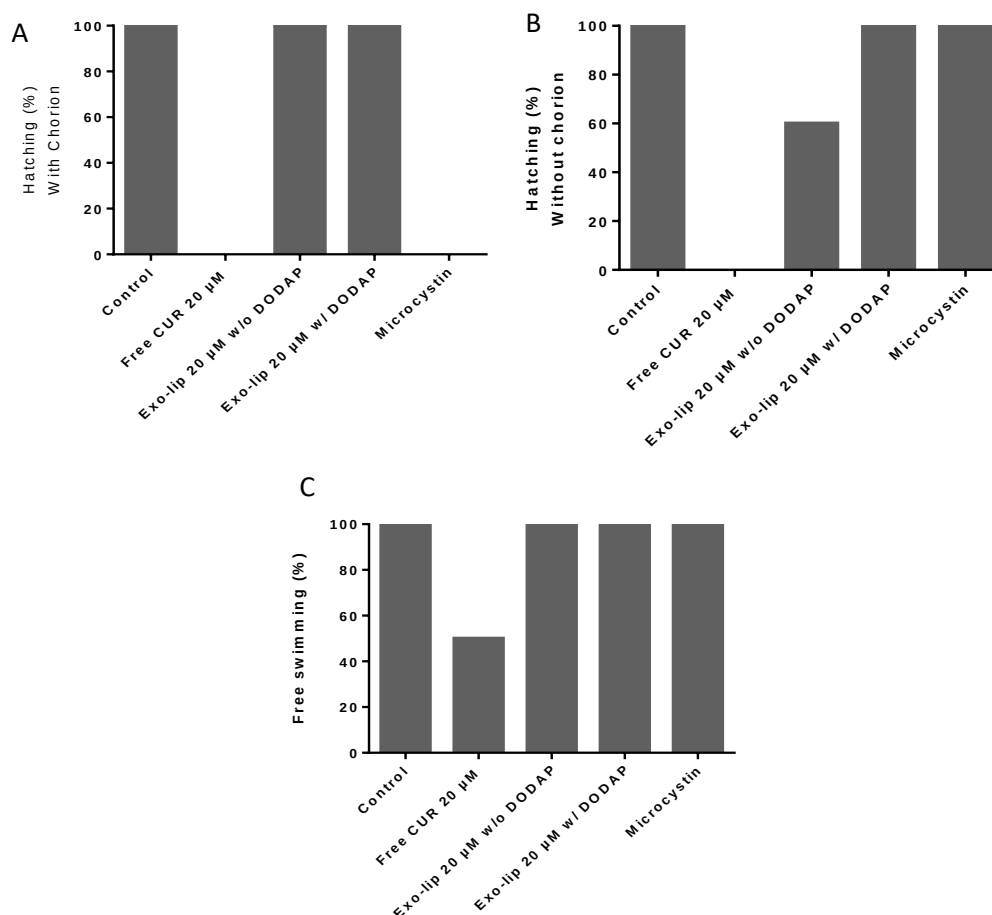


Figure 40: Effects of free and encapsulated curcumin (Exo-lip) (A) on hatching rate (56 h_{pf}) and (B) free swimming of zebrafish without chorion (80 h_{pf}) exposed to microcystin.



Figure 41: Internalization of curcumin on embryos of zebrafish. (A) free curcumin 20 μ M, (B) Exo-lip 20 μ M with DODAP, and (C) Exo-lip 20 μ M without DODAP.

5.3.1.2 Curcumin and curcumin-loaded liposomes to counteract kainic acid-induced toxicity

KA was selected because it is a natural neurotoxin, and the existing literature suggests it helps designing more effective treatments for epilepsy (Mussulini, et al., 2018 and Oyo, et al., 2019). The test condition were chosen according to the literature (Mussulini, et al., 2018 and Oyo, et al., 2019), and as reports only referred to adult zebrafish, the condition was adjusted to zebrafish embryos.

Zebrafish embryos were exposed to test neuroprotective conditions (curcumin and curcumin-loaded liposomes) from 2 to 32 h_{pf}, after which these were exposed to the neurotoxin kainic acid from 32 to 80 h_{pf}. In **Figure 42**, zebrafish embryonic cumulative survival rate can be observed along development. Free CUR 20 μ M induced the highest embryonic lethality rate (47%), indicating that at this concentration it has not an efficient neuroprotective effect. In contrast, free CUR 8 μ M and liposomes curcumin 20 μ M with DODAP showed the highest survival rates (78% and 75%, respectively), thus demonstrating competence to act as neuroprotective agent.

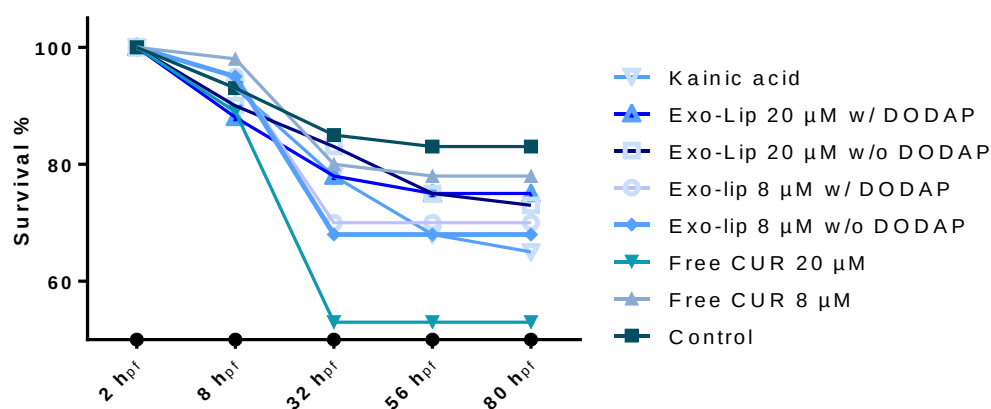


Figure 42: Effects on survival rate of zebrafish embryos pre-exposed (2-32 h_{pf}) to putative neuroprotective agents free and liposome-encapsulated curcumin followed by exposure (32-80 h_{pf}) to the neurotoxin kainic acid.

At 32 h_{pf}, the following parameters are evaluated: spontaneous movements, cardiac frequency and HTA. In spontaneous movements, statistically significant ($\chi^2=44.015$ and $P<0.05$), as can be seen in **figure 43**, the condition with more protective effects was incubation with liposomes curcumin 8 μ M without DODAP (83%), a result higher than that of control (52.7%), whereas the condition liposomes curcumin 20 μ M without DODAP showed no protective effects as spontaneous movements were 21%. As expected, KA also had a high spontaneous movements index (55%), because this neurotoxin induces epilepsies-like symptoms.

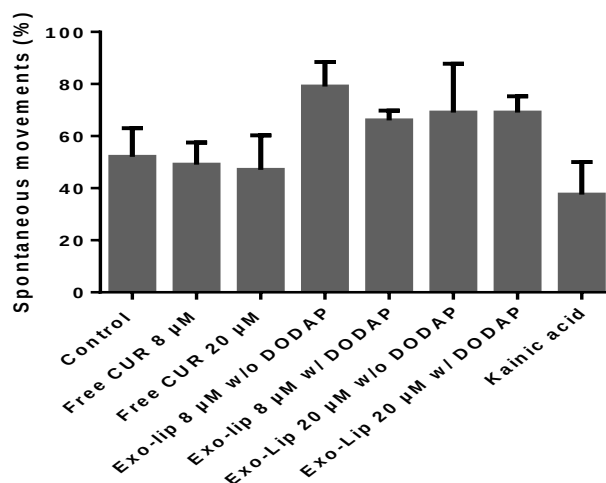


Figure 43: Effects of curcumin and curcumin-loaded liposomes on zebrafish embryos spontaneous movements at 32 h_{pf}.

Cardiac frequency was assessed at two distinct endpoints (32 and 56 h_{pf}), and statistically significant ($P < 0.05$), the results show that when embryos first exposed with neuroprotectors at 32 h_{pf} only the condition liposome CUR 20 μ M has a cardiac frequency similar to the control, and after

exposure to KA, the cardiac frequency of embryos decreased compared to control, whereas compared to embryos exposed to KA only, the cardiac frequency is slightly lower than control, indicating that curcumin and Exo-lip had a positive effect on KA-treated embryos. Nevertheless, liposomes CURM 20 μ M with DODAP demonstrated the most normal pattern of effect as the treated embryos exhibited cardiac frequencies close to that of control group, as noted in **figure 44**.

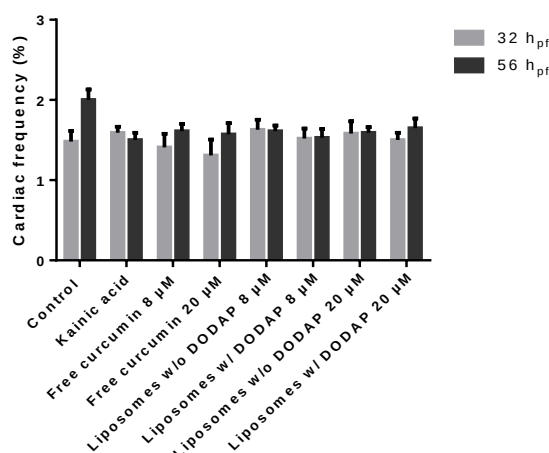


Figure 44: Effects of pre-exposure of curcumin and curcumin-loaded liposomes followed by exposure to KA on zebrafish embryonic cardiac frequency at 32 and 56 h_{pf}.

Next parameter analyzed was the hatching rate at 56 h_{pf}, statistically significant ($P < 0.05$), after zebrafish embryos were pre-incubated with the neuroprotective agents (2-32 h_{pf}), these were exposed to the neurotoxin KA (32 h_{pf} onwards), Exo-lip 8 μ M with DODAP and Exo-lip 20 μ M without DODAP showed similar effect to that of control, whereas free CUR 8 μ M, free CUR 20 μ M and KA lead to hatching rates below 60%, as observed in **Figure 45A**. For free swimming, statistically, the data demonstrated significant differences among groups ($P < 0.05$), and the lowest percentage of zebrafish embryos exhibiting this feature was induced by free CUR 20 μ M, whereas free CUR 8 μ M and Exo-lip 20 μ M with DODAP did not affected them., thus indicating competence to act as protective agents against KA-induced effects (**Figure 45B**).

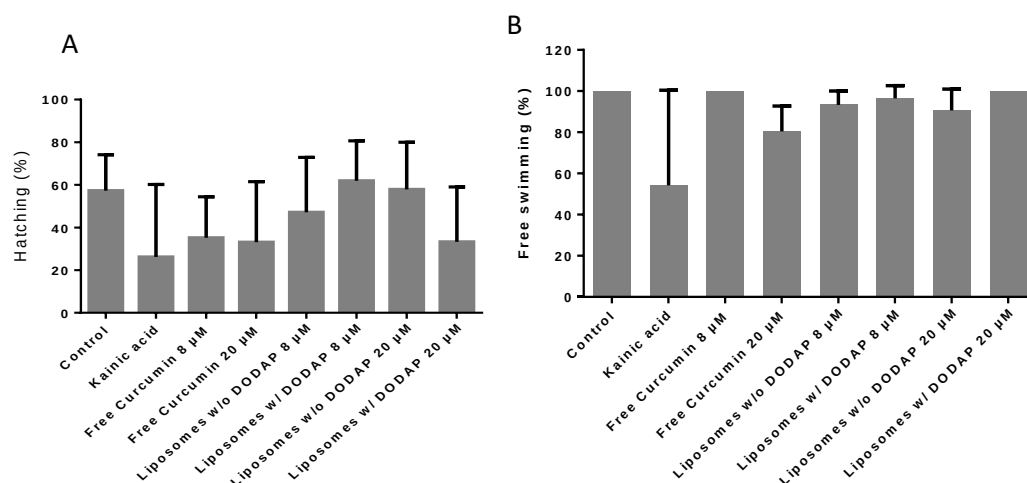


Figure 45: Effects of pre-exposure of curcumin and curcumin-loaded liposomes followed by exposure to KA on zebrafish embryonic (A) hatching rate at 56 h_{pf} and (B) free swimming at 80 h_{pf}.

5.4 *Cystoseira baccata* and *Cystoseira tamariscifolia*

Algae of genus *Cystoseira* produce bioactive compounds such as antioxidant, anti-inflammatory, antiviral, antifungal and antibacterial (Chan, et al, 2002; N. Gonzalez-Ballesteros, et al, 2016 and Souza, et al., 2017), which can be used for new therapies to various human diseases such as cancer and neurodegenerative disorders. *Cystoseira* has a diversity of secondary metabolites, e.g: steroids, phenolic compounds and terpenoids, are vitamins, fatty acids, carbohydrates and triglycerides (Souza, et al., 2017).

ZET assays with algae first analyzed the effects of *Cystoseira baccata* (CB) extract exposure on zebrafish embryos survival rate along development. No statistically significant ($\chi^2=5.1884$, $P=0.3933$), differences were found between test concentrations. Among these, 0.0625 and 0.03125 g/mL of CB extract led to the lowest survival rate (77.5% and 70.0%, respectively), as shown at **Figure 46A**. At 8 h_{pf}, epibolic arc was analyzed and significant statistical differences were found among groups ($F(5,11)=9.197$, $P<0.05$), with only 0.0325 g/mL of CB extract exerting a different effect as compared to all the other test concentrations. Further replicates are required to confirm this result, as it can be due to a cumulative error on metric analysis, rather than an actual significant effect (**Figure 46B**).

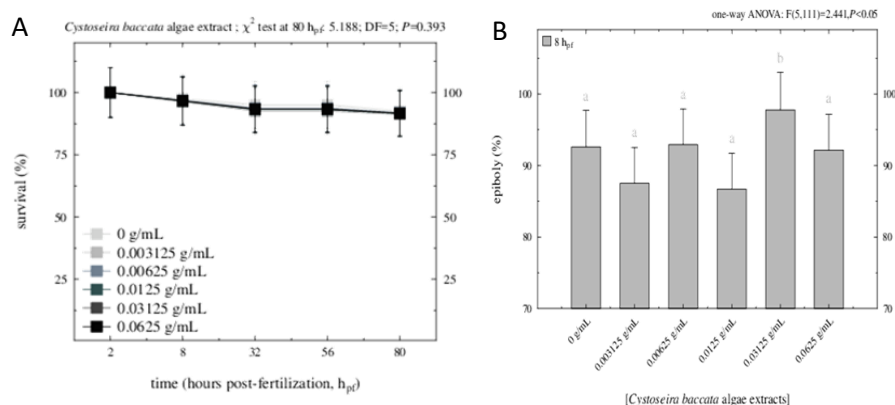
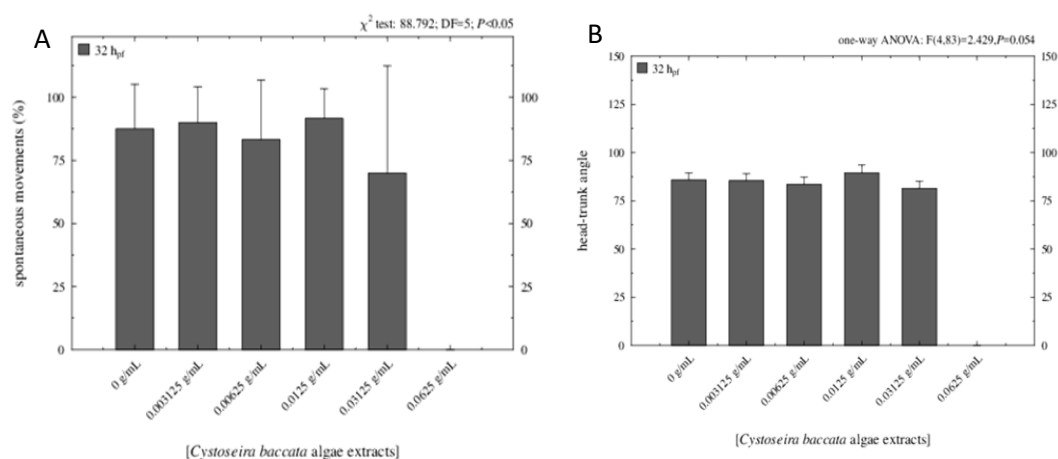


Figure 46: Effects (A) on cumulative survival rate along development and (B) epiboly arc at 8 h_{pf} of zebrafish embryos exposed to different test concentrations of CB extract.

At 32 h_{pf}, the analysis of spontaneous movements, statistically significant ($\chi^2=88.79$, $P<0.05$), shows that the condition 0.03125 g/mL extract induces low rate of spontaneous movements, which may reflect an effect at the level of the primitive spinal network, thus preventing embryo movements. When incubated with 0.625 g/mL extract, the embryos had a zero spontaneous movement rate, because these embryos were with a development level similar to that of 10 h_{pf}, as observed in **figure 47A**. Another parameter analyzed at 32 h_{pf} is HTA, statistically not significant ($P=0.688$), although at condition 0.0625g/mL extract the embryos had angle zero because embryonic development was delayed (**Figure 47B**). At 32 and 56 h_{pf}, the cardiac frequency was evaluated and showed no significant ($P=0.722$); at 32 and 56 h_{pf} there are differences between conditions and control, and for extract concentrations 0.0125; 0.03125 and 0.0625 g/mL, the cardiac frequency drops dramatically and at the highest extract concentration it records zero because the embryos are delayed in development, while in the other two conditions the cardiac frequency has decreased because The extract had a negative effect on the formation of embryo secondary neuromotor circuits, as observed in **figure 47C**.



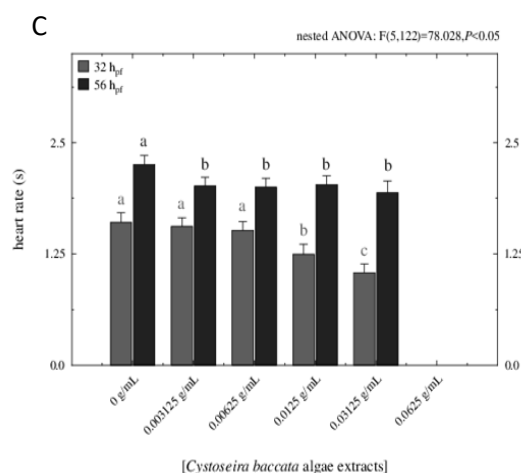


Figure 47: Effects of CB extract exposure (A) on spontaneous movements at 32 h_{pf}, (B) on HTA at 56 h_{pf} and (C) on heart rate at 32 and 56 h_{pf} of zebrafish embryos

At 56 h_{pf}, hatching rate was analyzed and differences among test concentrations were showed to be statistically relevant ($\chi^2=92.6297$, $P<0.05$). For 0.0625 g/mL of CB extract no records are shown because embryos at this test concentration were delayed in their development (**Figure 48A**). Regarding free-swimming, it can be observed that the lowest rate of free swimming recorded was for 0.03125 g/mL extract of CB, while for the highest concentration tested (0.0625 g/mL of CB extract) no records are shown because embryos were delayed in their embryonic development (**Figure 48B**). In general, it can be concluded that 0.0625g/mL of CB extract causes either lethality and severe sub-lethal toxicity leading to delays in zebrafish embryonic development, while the lowest test concentrations (0.003125 and 0.00625 g/mL of CB extract) were nontoxic (**Figure 49**).

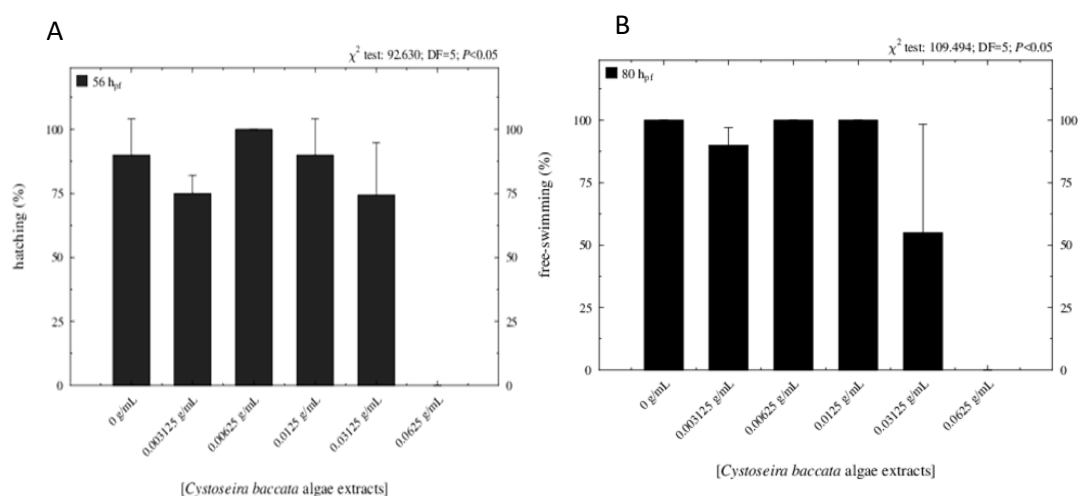


Figure 48: Effects of CB extract exposure on zebrafish embryos (A) hatching rate (56 h_{pf}) and (B) free swimming (80 h_{pf}).

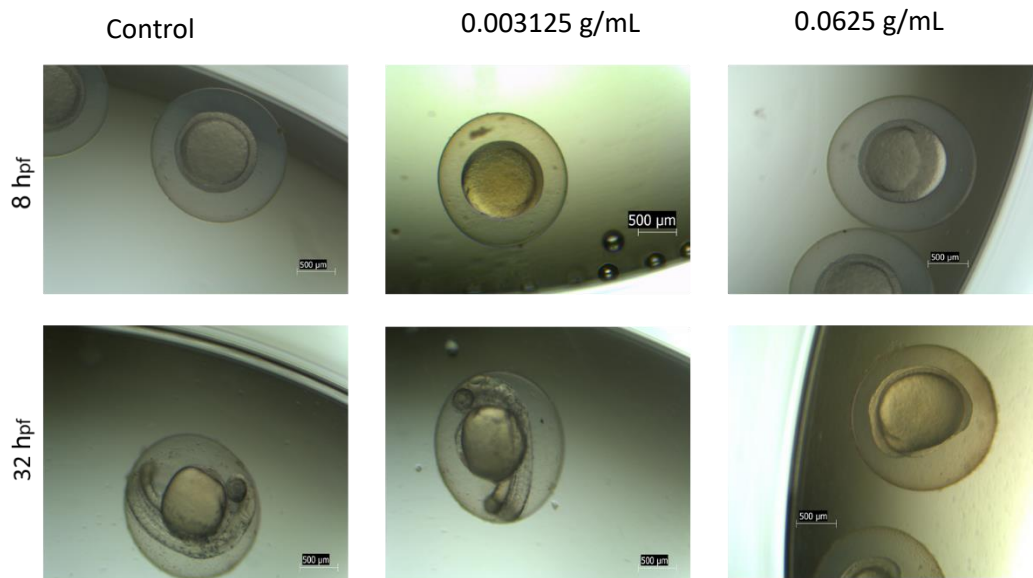


Figure 49: Effects of CB extract exposure on development of zebrafish embryos at 8 and 32 h_{pf}

The CT assay was performed, after characterization of the toxicological profile of CB extract, with the following conditions: 0.00625; 0.003125 and 0.03125 g/mL, as in the previous CB assays the lowest conditions showed no toxicity and their bioactives had positive effects on embryo development, whereas condition 0.03125 g/mL did cause some damage to embryo development.

For the survival rate, at 80 h_{pf}, statistically is significant ($P < 0.05$), but the extract concentration 0.03125 g/mL causes the highest mortality rate, while the other conditions had similar results to the control, as observed in **figure 50A**. At 8 h_{pf}, epiboly arc data showed statistic is significant ($P < 0.05$), but only 0.00625 g/mL extract led to differences compared to embryos in control condition (**Figure 50B**).

A B

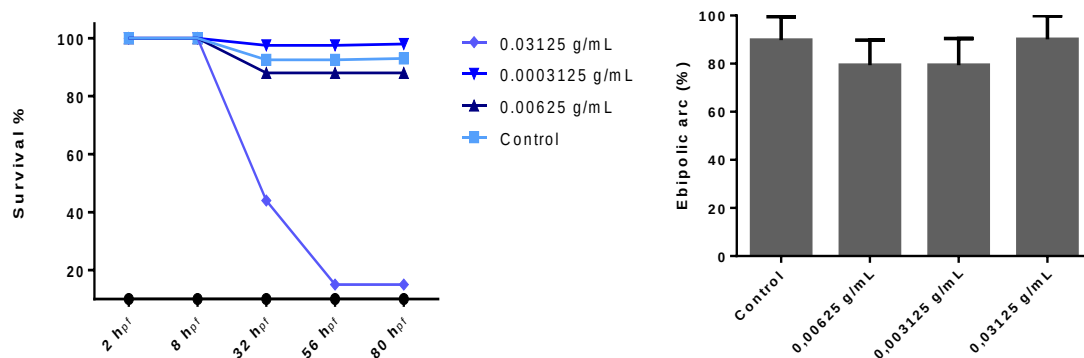


Figure 50: Effects of CT extract exposure on zebrafish embryos (A) cumulative survival rate along development and (B) epibolic arc at 8 h_{pf}

At 32 h_{pf}, HTA was evaluated and for this test there was is statistic significant ($P < 0.05$), although 0.03125 g/mL extract did led to results that differ to all other conditions, including the control (**figure 51A**). At 32 h_{pf}, the yolk volume and egg volume were also analyzed; from yolk there is no significant ($P = 0,347823$), but there is significant in egg volume ($P < 0.05$), only condition 0.03125 g/mL extract had statistical differences compared to other conditions, including the control. At 56 h_{pf} yolk extension was evaluated, statistically not significant ($P = 0,227252$), but there are differences between all conditions, but that of 0.03125 g/mL extract, as embryos are with delayed embryonic development, It was not possible to evaluate this parameter (**figure 51B**). The pupil surface was analyzed, statistically there is no overall difference ($P > 0.05$), but there are statistical differences between the condition 0.03125 g/mL extract in comparison with the other conditions, because the embryos have developmental delays and do not have the eye and pupil formed (**table 2**).

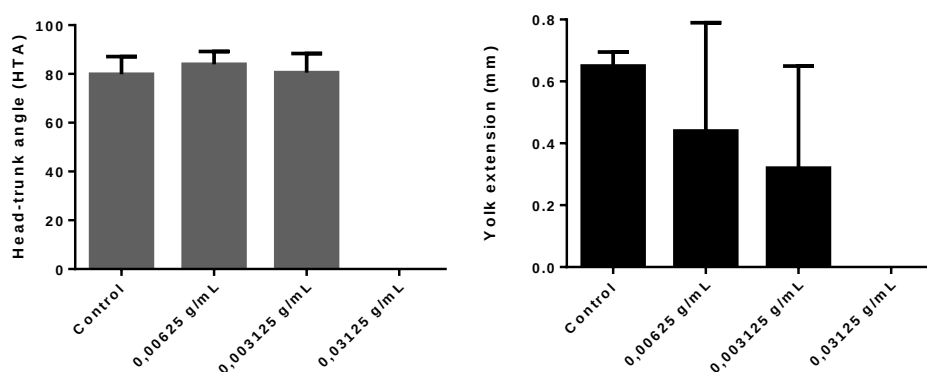


Figure 51: Effects of CT extract exposure on zebrafish embryos (A) HTA (32 h_{pf}) and (B) yolk extension (56 h_{pf}).

Table 2 Effects of CT extract exposure on zebrafish embryos yolk volume and pupil surface, at 32 h_{pf}.

Test concentration (g/mL)	Yolk volume (mm ³)	Test concentration (g/mL)	Pupil surface (mm ²)
0	0,09	0	0,01
0,003125	0,09	0,003125	0,07
0,00625	0,09	0,00625	0,01
0,03125	0,15	0,03125	0,00

At 32 h_{pf} spontaneous movements results had statistically significant ($\chi^2=62.737$ and $P < 0.05$), but at condition 0.03125 g/mL extract no movement was detected because the embryos of this condition are behind the time. indicating that the primary spinal network has not developed (**Figure 52A**). The cardiac frequency analyzed at both 32 and 56 h_{pf} had statistically significant ($P > 0.05$) and statistically there is a difference between the control and all the conditions tested. With incubation with 0.03125 g/mL extract there is no cardiac frequency due to severe developmental delays (**Figure 52B**). Finally, free swimming results had statistically significant ($P < 0.05$) but there are statistical differences between control and condition 0.03125 g/mL extract, as embryos development is delayed and no hatching occurred, so no larvae exhibit free-swimming behavior, indicating that the higher concentration tested is much more toxic (**Figure 52C**).

With these results, there is an indication that the higher concentration (0.03125 g/mL) has a negative effect on embryonic development and apparently does not allow the embryo to develop normally, stopping the developmental progression of 9 or more. embryos. At 10 h_{pf}, as shown in **Figure 53**, the embryos with the lowest condition (0.00625 g/mL) show similar embryonic development to the control, indicating that this condition has no toxicity, as can be seen in **Figure 53**. However, When analyzing the effect of the two algae (CB and CT), we observed that the extract of the CT has a more toxic effect than CB, because in the CB test the condition 0.03125 g/mL causes a slight damage to embryonic development, since in CT the same condition causes severe developmental damage.

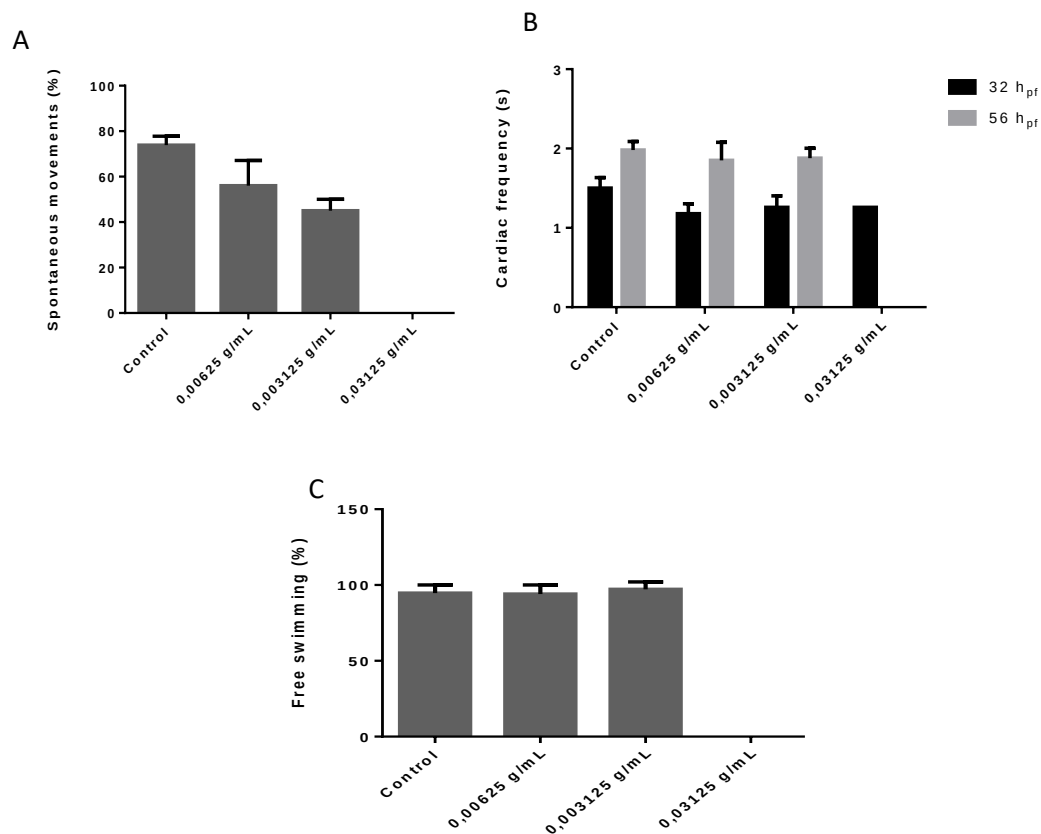


Figure 52: Effects of CT extract exposure on zebrafish embryos (A) spontaneous movements at 32 h_{pf}, (B) cardiac frequency at 32 and 56 h_{pf}, and (C) free-swimming at 80 h_{pf}

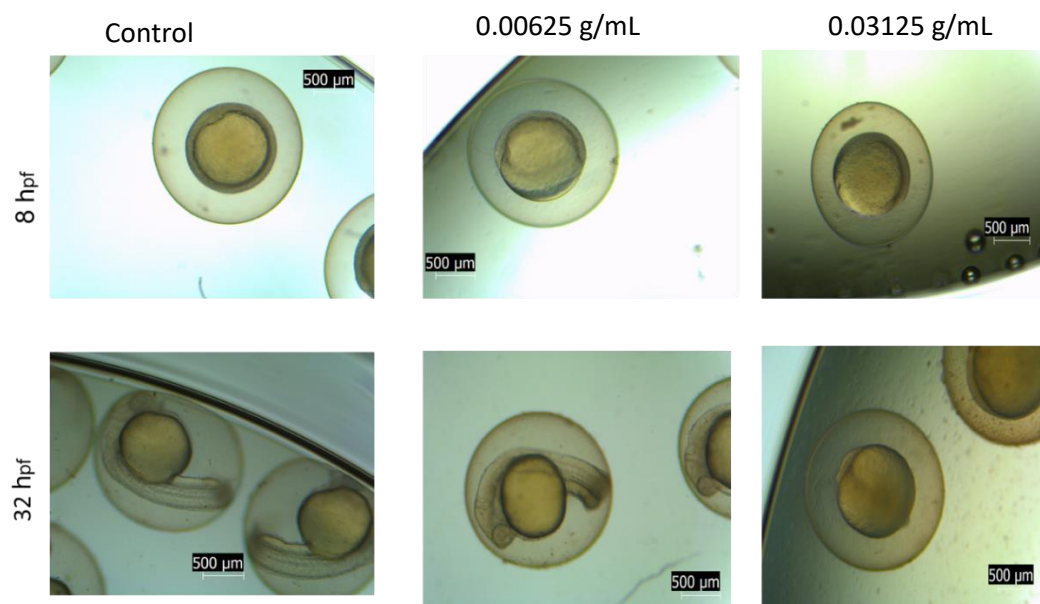


Figure 53: Effects of CT extracts on the development of zebrafish embryos exposed to control, 0.00625 and 0.03125 g/mL at 8 and 32 h_{pf}.

Conclusions

Zebrafish is a model organism for the discovery of new therapies for human diseases e.g: neurodegenerative, cardiac, behavioral and muscular diseases (Santoriello, et al., 2012). With the need for non-animal testing, zebrafish embryo is a viable alternative similar to *in vitro* cell models (OECD, 2006). Besides, genome of zebrafish is fully sequenced and has characteristics homologous to human genome, with 76% of genetic homology (Cassar, et al., 2019 and Sieber, et al., 2019). Other factors can be highlighted that make embryonic and larvae stages more advantageous than adults (a period of the life cycle of zebrafish protected by current regulation to animal experimentation, OECD, 2006), namely: small, transparent, rapidly developing, *ex utero* and low maintenance cost.

In the present study, tests were performed to establish zebrafish embryos as a model for neurotoxicity studies using KA and MC compounds. KA results showed that conditions of 4 and 6 mg/kg caused significant developmental delays at different embryonic stages; Dechorionated embryos exposed to MC have their embryonic development altered, as MC is highly toxic and causes damage to embryonic development. in embryonic development. But by testing curcumin and curcumin-loaded liposomes, being KA-induced toxicity, it has been shown that curcumin is an effective neuroprotective agent against KA toxicity and aided in the development of the CNS of embryos. For this reason it is important to have tests to evaluate the toxicity and bioactivity of natural products for in vivo biomedicine applications.

Finally, these studies corroborated zebrafish embryogenesis as a reliable tool for assessing biological effects of neurotoxic and neuroprotective agents earlier on the life cycle of a vertebrate model, and the protocol used can be discussed for future modeling of drugs bioactivity towards diseases and disorders associated with human brain.

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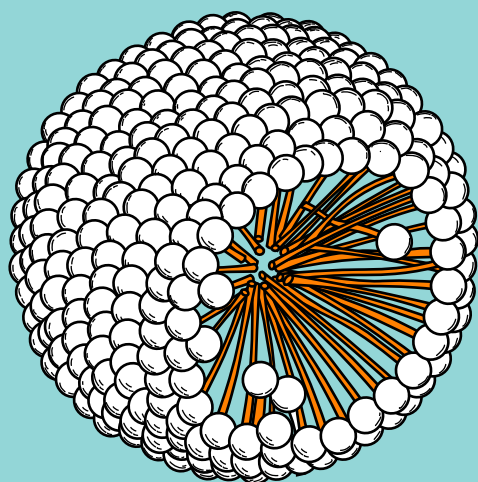
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Annex

Attached are summaries of posters that were presented during the development of this work.

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P26

Developmental neurotoxic effect of nanoparticles: towards real-time assessment using zebrafish embryos as vertebrate model

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Exposure to neurotoxic agents during embryonic development can cause irreparable short- and long-term damage to vertebrates, including for humans, as it may affect the central nervous system (CNS) and the blood brain barrier (BBB)[1]. The reported CNS susceptibility during embryogenesis suggests that there may be a critical window of exposure during brain development. Nowadays, therapies using nanoparticles are an important alternative for most of the diseases, since they allow, for example, more effective and localized delivery of drugs and higher success in treatments [2]. However, the exposure to them at an embryonic stage can cause neurotoxic damages.

In this study, the putative toxicity of two different nanoparticle formulations was evaluated, namely gold nanoparticles, which were produced by Green synthesis, and liposomal nanoparticles. Both have known and interesting bioactivities, such as anti-inflammatory, antioxidant, antifungal and antibacterial[3][4].

Thus, the capacity of the neuroprotective effect of the nanoparticles in contact with toxins was tested, the evaluation was done through the ZET assay, with the endpoints shown in the figure and following parameters, mortality, epiboly, malformation, heart beat, spotaneus movements, hedd-trunk angle, hatching and free-swimming.

2D cell models are easy to reproduce, however they fail to mimic real conditions. Animal models are better than 2D models, however many ethical problems are involved. Zebrafish embryos compose a low-cost and highly informative non- animal alternative to evaluate early developmental responses associated with neurotoxicity[5][6]. As model has multiple advantages for its use in toxicity tests including *ex-utero* fertilization, transparency of the embryos which allow observations at real time, rapid development and high fecundity[5][6]. Zebrafish embryos possess neural development characteristics similar to the vertebrate ones, which makes them ideal to assess embryo neurotoxicity *in vivo*.

Obtained results show that zebrafish is a profitable and reliable model organism for neurotoxicity tests. Nevertheless, there is a need to continue these neurotoxic tests in more complex models, so that these nanoparticles can become an alternative therapy.

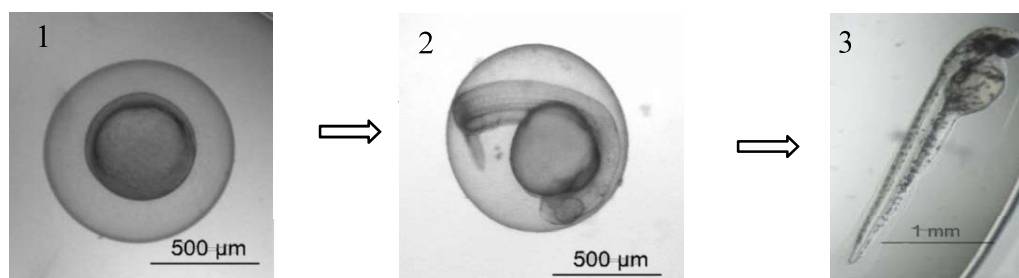


Figure 1: Different endpoints of zebrafish development at 1-8 h_{pf}, 2- 32 h_{pf} and 3- 56 h_{pf} *h_{pf}= hours post fertilization.

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P19

Novel liposomes for Alzheimer's disease treatment

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Alzheimer's disease is one of the most debilitating neurological diseases, afflicting an ever-increasing number of a majorly elderly population worldwide^[1]. Curcumin is a phytochemical which has been described to aid in the neuroprotection of the brain against oxidative stress insults and to reduce amyloid- β accumulation in the brain afflicted by Alzheimer's disease^[2]. However, its solubility in human blood and its pharmacokinetics is very unfavorable, leading to rapid elimination from the blood stream. Moreover, it is poorly absorbed by the intestines, limiting oral administration.

The use of liposomes to encapsulate and carry curcumin to the brain is already being proposed for therapy. We developed a novel type of liposomes, with a unique mixture of phospholipids mimicking cellular components, that can encapsulate curcumin in a highly efficient manner. These liposomes have an adequate size to cross the human blood brain barrier and are not cytotoxic to fibroblast and neural cell lines. Moreover, curcumin is incorporated in these cells and has a neuroprotective effect^[3], reducing ROS production and increasing cell viability upon incubation with an oxidative stress inducer. Furthermore, these liposomes do not show any *in vivo* toxicity in a zebrafish embryotoxicity model, with curcumin being incorporated without side effects. The functionality of zebrafish blood brain barrier (BBB), which is similar in structure to the human one, is fully obtained 3 days after fertilization, and provides valuable information^[4]. With this system we can diminish the production of ROS, and presumably slow the progression and alleviate the symptoms of Alzheimer's disease.

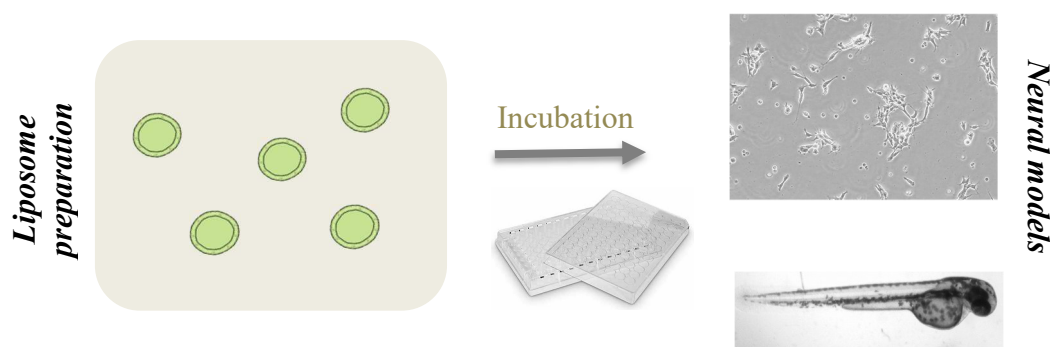


Figure 1: Schematic overview of the production and validation of the novel mimetic liposomes in models of neural oxidative insult.

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P22

Green synthesis of gold nanoparticles led by algae *Cystoseira tamariscifolia* and evaluation of their biological activity

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In recent years, the development of efficient green chemistry methods for synthesis of metal nanoparticles has emerged as an eco-friendly alternative for the production of well-characterized nanoparticles ¹. Gold nanoparticles are presently under intensive study due to their attractive physicochemical and biological properties as well as their potential applications in the development of new technologies for nanomedicine, both in therapy and diagnosis ^{2,3}. Seaweed phytochemicals, including hydroxyl, carboxyl, and amino functional groups, can serve both as effective metal-reducing agents and as capping agents to provide a robust coating on metal nanoparticles in a single step ⁴.

In this study, we tested if *Cystoseira tamariscifolia* (CT) is a potential agent involved in the reducing and stabilizing processes for the green synthesis of gold nanoparticles. An aqueous extract of this macroalga was prepared and used to produce spherical, stable, polycrystalline nanoparticles with a mean diameter of 7.6 ± 2.2 nm for Au@CT (gold nanoparticles produced in CT extracts), as demonstrated by UV-vis spectroscopy, TEM, HRTEM, STEM and zeta potential measurements. Moreover, the biomolecules present in the extract and nanoparticles were characterized by Fourier Transformed Infrared Spectroscopy (FTIR). *In vitro* antioxidant activity assays also showed that CT extract has a high reducing power, phenolic content and DPPH scavenging activity.

Effects of Au@CT and CT extract alone on cellular metabolism were evaluated by tetrazolium-based colorimetric cellular assay (MTT), and on cell membrane integrity by lactate dehydrogenase activity (LDH) assay. Wound-healing assay revealed impacts on cell proliferation and migration capacity. These assays were performed to investigate whether Au@CT and CT extract alone affect cell viability in mouse (L929 cell line) and human (BJ5-ta cell line) fibroblast cells as *in vitro* models. Lower concentrations of the alga extract and derived nanoparticles did not cause any cytotoxicity. This, together with the wound-healing assay, indicates a potential positive role in cell regeneration.

The zebrafish embryotoxicity (ZET) assay, recommended by OECD to evaluate acute toxicity, was performed to obtain a correlation between *in vitro* and *in vivo* toxicity. These embryos are translucent, allowing direct, real time observation and the evaluation of whole organism responses, from mortality to more specific parameters such as neurotoxicity. The results show that toxicity is evident only at very high concentrations

These results reveal that green synthesis in CT extracts of non-toxic, bioactive nanoparticles have very desirable features with potential applications in biomedicine.

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