Toxicology Reports 13 (2024) 101766

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Acute toxicity of trypsin inhibitor from tamarind seeds in embryo and adult zebrafish (*Danio rerio*)

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ARTICLE INFO

Handling Editor: Prof. L.H. Lash

Keywords:
Tamarindus indica L.
Preclinical model
Embryotoxicity
Cardiotoxicity
Neurotoxicity
Genotoxicity

ABSTRACT

The trypsin inhibitor isolated from tamarind seeds (TTI) is being investigated for potential applications in the treatment of noncommunicable diseases (NCD), such as hypertension, obesity, and diabetes. This study aimed to assess TTI embryotoxicity and acute toxicity in adult zebrafish (*Danio rerio*). TTI was extracted and isolated from tamarind seeds. Embryonic and adult zebrafish were exposed for 96 hours to three concentrations of TTI (12.5, 25, and 50 mg/L). Zebrafish embryos (n=60 per group) were evaluated for survival, hatching, malformations, and potential developmental marker alterations, in addition to cardiotoxicity and neurotoxicity tests. For acute toxicity assessment in adults (n=20 per group), survival and locomotor and anxiety-like behaviors were assessed, along with genotoxicity (micronucleus) evaluation. Embryos exposed to TTI showed no significant adverse effects, presented normal heart rates and positive reflex response in the neurotoxicity tests. In adult fish, TTI did not cause mortality or significant behavioral changes, suggesting no neurotoxicity and no genotoxicity. Histopathological analyses of the whole body showed only changes in the liver and spinal cord, similar to those observed in the control group not exposed to TTI. These findings indicate TTI's biosafety and therapeutic potential in complex organisms. Further research is required to evaluate its long-term effects and efficacy in treating non-communicable diseases.

1. Introduction

The increasing prevalence of non-communicable chronic diseases (NCDs), such as hypertension, obesity, and diabetes, and consequently chronic metabolic inflammation and its complications, has driven the search for new treatment methods, leading to the development of new pharmaceuticals and nutraceuticals. Particularly, these advances have been made through the prospecting and screening of natural-origin molecules with underexplored therapeutic potential [1].

In this context, bioactive proteins, such as protease inhibitors, have demonstrated significant therapeutic potential due to their ability to regulate essential biological processes. These proteins are known to modulate proteolytic enzymes, which play crucial roles in biological events including wound healing, blood coagulation cascades, and digestion [2].

Protease inhibitors are recognized as key agents with diverse applications in biotechnology and medicine. They play a crucial role in enhancing our understanding of protein interactions and in the development of new compounds to manage pathological conditions and various diseases [3]. A significant portion of these bioactive compounds

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https://doi.org/10.1016/j.toxrep.2024.101766
Received 20 August 2024; Received in revised form 8 October 2024; Accepted 9 October 2024
Available online 10 October 2024

is produced by plants, which have historically been the source of many pharmaceutical drugs available today. A notable source of trypsin inhibitors is found in tamarind seeds (*Tamarindus indica* L.). The trypsin inhibitor isolated from tamarind seeds (TTI) has been studied in its isolated [4,5], purified [6–9], and nanoencapsulated forms [10–14], evaluated through *in vitro*, *in silico* [15,16], and preclinical studies, showing promise as a candidate for treating disease such as hypertension, obesity, and diabetes [17,18].

Previously, TTI has been attributed with satiety-inducing, anti-inflammatory, hypoglycemic, and hypolipidemic effects [4,5,19]. Despite previous studies on TTI [9,15,19], the biomedical application of these molecules still requires more specific tests and analyses to establish the safety of TTI in *in vivo* models, as recommended by the Food and Drug Administration (FDA), European Medicines Agency (EMA), and the Brazilian Health Regulatory Agency (ANVISA).

Thus, this study complements previous research by assessing the multilevel toxicity of TTI in the zebrafish *in vivo* model, as well as its toxicological potential during different ontogenetic stages of the organism. Zebrafish are increasingly used as a model organism for toxicology studies due to their small size, rapid development, and genetic similarity to humans [20–22]. Their embryos and larvae are particularly valuable for high-throughput screening of chemical toxicity, as they are transparent and develop quickly, facilitating the observation of developmental effects. Zebrafish have about 70–80 % genetic and physiological similarity to humans, making them relevant models for assessing the toxicity of new molecules [22,23].

Additionally, the small size of zebrafish embryos and larvae allows for the use of minimal quantities of test compounds, which is especially useful for screening new drug candidates. Their rapid development permits the assessment of toxicity during critical developmental stages. Toxicity endpoints in zebrafish include mortality, developmental abnormalities, behavioral changes, and molecular and cellular markers of toxicity, which can provide information on the potential therapeutic or harmful effects of the tested molecule [24–26].

In the literature, some studies have investigated the use of tamarind in zebrafish, focusing mainly on the fruit's pulp and peel. The study conducted by Lobitaña et al. [27] evaluated the embryotoxicity and teratogenicity of tamarind pulp extract on zebrafish eggs and larvae. Additionally, the hepatoprotective effects of tamarind peel extract [28] have also been assessed, as well as the chemical composition of this extract, investigating its in vitro and in vivo antioxidant capacity using zebrafish as an animal model [29]. However, to date, there are no records of studies evaluating, especially the impact of the trypsin inhibitor isolated from tamarind seeds (TTI) on zebrafish.

This study is the first to evaluate the toxicological safety of TTI at varying concentrations (12.5, 25, and 50 mg/L) across two life stages of the zebrafish (*Danio rerio*), specifically in embryos and adults. The toxicity of TTI was assessed using survival parameters, incidence of malformations, and assays for cardiotoxicity, neurotoxicity, and genotoxicity, leveraging TTI's known bioactive properties to conduct a comprehensive analysis throughout both embryonic development and adulthood.

2. Materials and methods

2.1. Ethical aspects

All experimental protocols and fish maintenance procedures were approved under protocol No. 301.035/2022 by the Ethics Committee on Animal Use of the Federal University of Rio Grande do Norte (CEUA-UFRN), following guidelines from the National Council for Animal Experimentation Control (CONCEA, Brazil) and in accordance with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments) [30]. Animal use adhered to the principles of sustainability's 3 R's: Replacement, Reduction, and Refinement, as well as OECD norms [30,31].

2.2. Obtaining and isolation of TTI

Tamarind (*Tamarindus indica* L.) was cultivated in a non-commercial family plantation in Cerro Corá, Rio Grande do Norte, Brazil. The Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), in Natal/RN (Brazil), botanically identified and registered the fruit in the National System for Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under number AF6CE9C.

The trypsin inhibitor isolated from tamarind seed (TTI) was obtained following the methodology of Carvalho et al. [4]. After peeling and pulping the fruit, cotyledons were ground to a 40-mesh flour at 6°C. Subsequently, Tris-HCl buffer 50 mM, pH 7.5, was added at a ratio of 1:10 (w/v) to extract the inhibitor. The solution was stirred for 3 hours at 20 ± 2°C, centrifuged (10,000 × rpm for 30 min at 4°C), and filtered to obtain the crude extract (CE).

Proteins were fractionated by sequential ammonium sulfate precipitation at saturation ranges of 0 %–30 % and 30 %–60 %, stirred at 20 ± 2°C, and centrifuged (10,000 × rpm for 30 min at 4°C). Precipitated fractions were suspended in Tris-HCl buffer 50 mM, pH 7.5, dialyzed, and designated as F1 (0 %–30 %) and F2 (30 %–60 %), stored at −20°C.

The protein fraction with the highest anti-trypsin activity underwent affinity chromatography on Trypsin-Sepharose CNBr 4B to isolate TTI. The column, calibrated with Tris-HCl buffer 50 mM, pH 7.5, eluted the non-retained protein peak with buffer and the retained proteins with HCl (5 mM) at 0.5 mL/minute, collected and stored at −4°C. The protein profile was evaluated by spectrophotometry at 280 nm. The retained proteins were dialyzed in Tris-HCl buffer 50 mM, pH 7.5, lyophilized, and tested for trypsin inhibition using 1.25 mM N-benzoyl-DL-arginine-p-nitroanilide (BApNA) as substrate [32]. The reaction was read on a spectrophotometer at 410 nm.

Proteins were quantified using the Bradford method [33], with bovine serum albumin as a standard protein for obtaining a curve and linear equation, at the following concentrations: 0.05; 0.10; 0.15; 0.20; 0.25; 0.30; 0.35; 0.40 and 0.50 mg/mL. The readings were taken on a spectrophotometer at 595 nm (Biotek Epoch™ UV-Vis). To determine the isolation process and molecular mass estimation CE, F2, and TTI were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5 % bisacrylamide. Aliquots were applied to a gel prepared in plates measuring 10 ×14 cm and compared with the Amersham™ ECL™ Rainbow™ Marker - High range, which has molecular masses of 225, 76, 52, 38, 31, 24, 17, and 12 kDa. The run lasted approximately 1 hour under a constant current of 20 mA. Afterward, the gel was stained with Coomassie Blue [34].

2.3. Animals and housing

Wild-type adult zebrafish (*Danio rerio;* sex ratio 1:1 male:female) purchased from a fish farm, aged between 6 and 8 months (0.400 ± 0.300 g), were maintained at the FishLab of the Federal University of Rio Grande do Norte (UFRN). The fish were housed in an automated rack system (ZebTEC Active Blue Stand Alone - Tecniplast®) at constant temperature of 28°C, conductivity of 6 μOsm, and pH of 7.2. The laboratory photoperiod was set at 14 L:10D (Light:Dark), with lights on at 7:00 AM (ZT0). The animals were fed twice daily with commercial flaked food (Nutriflakes®, Brazil) with a nutritional composition of 35 % crude protein and approximately 30 % carbohydrates (calculated by the difference between 100 and the sum of the percentages of water, protein, fat, and ash according to the label information) (https://nutricon.ind.br/produtos/nutriflakes/) and Artemia sp. nauplii (Artemia salina do RN®, Brazil) 24 hours after hatching (live food).

2.4. Obtaining embryos

Adult zebrafish from the above stock were placed in breeding tanks (3 males: 2 females per tank), where males and females were physically separated (only visual and chemical contact) for 12 hours (overnight).

The separation was removed in the first hour of morning light, allowing the fish to spawn for 60 minutes. This procedure ensured precise timing of the fertilization window. Subsequently, eggs from different breeding matrices were collected from each breeding tank and transferred to Petri dishes, where they were observed under a magnifying glass to check for fertilization and blastula formation at 3 hours post-fertilization (hpf).

2.5. Embryotoxicity assessment

Embryos were transferred to 24-well plates (one per well) containing 2 mL of system water containing TTI at 12.5, 25, and 50 mg/L. Concentrations chosen were based in previous studies [5], considering the density of water, knowing that one kilogram of water occupies the volume of one liter. Twenty embryos were exposed to each concentration of TTI, and four embryos were used as internal controls on the plate. Additionally, system water was used as a negative control, and 3, 4-dichloroaniline (3,4 DCA) 4 mg/L was used as a positive control. The testing procedures followed the guideline for fish embryotoxicity test (FET) from OECD (protocol no. 236) [35]. The tests were performed with 20 eggs or larvae in triplicate. That way in total 60 eggs or larvae were used for each TTI concentration. Only the larvae neurotoxicity evaluation was carried out with 12 larvae in triplicate (in total 36 larvae).

Toxicity tests on zebrafish embryos lasted for 96 hours (from 3 to 96 days post-fertilization - hpf). Plates containing the eggs were maintained in incubators at 28°C and endpoints were assessed every 24 hours. Lethality, egg coagulation, absence of heartbeat, edemas, and changes in pigmentation were observed. Malformations in head, tail, heart, and spinal cord structures were monitored using a binocular stereoscopic microscope (80X) to observe development. Dead embryos were removed to prevent contamination, and spontaneous movements and hatching were recorded. The survival rates were the generated considering the total number of embryos exposed to different concentrations of TTI and the number of those that died after exposure and presented in percentages (%). Developmental assessment criteria were based on zebrafish embryogenesis descriptions by Kimmel et al. [36].

2.5.1. Larvae cardiotoxicity evaluation

Cardiotoxicity was assessed in 96 hpf larvae. The larvae were placed in 24-well plates and analyzed by observing heartbeats per minute (bpm) using a binocular stereoscopic microscope (LAB-2BZ - Zo, magnification 10x to 160x) connected to a computer for image projection and visual/manual counting of the beats. A Pasteur pipette was used to center the larvae. Twenty embryos from each group were recorded for one minute in triplicate to calculate the average bpm and evaluate cardiac function. Pericardial edema, absence of heartbeats, and any other malformations were documented.

2.5.2. Larvae neurotoxicity evaluation

At seven days post-fertilization (dpf), animals (n=12/group) were tested for optomotor response and avoidance response. The tests were conducted in triplicate.

In the Optomotor Response (OMR) test, 12 larvae were placed in a Petri dish on top of a computer screen displaying moving black and white stripes (1 cm/s upwards and downwards motion) for 60 seconds, followed by a free stimulus period (white screen) for 5 seconds, repeated 20 times. Behavior was recorded by a camera positioned above the Petri dish. At each interval, the number of larvae showing positive optomotor response (OMR+), following the stripe movement, and negative response (OMR-), moving opposite or in a different direction, was counted [37].

For the Avoidance Response test, we employed a moving black circle projection to assess larval behavior concerning their distance moved from the stimulus [37]. Aversive behavior was observed over 10 minutes, capturing images whenever the stimulus was positioned in the lower left side of the plate. These images were analyzed using ImageJ to determine larval distribution relative to the centroid. Larval coordinates (X and Y) were exported to Excel and divided into four quadrants: upper right, upper left, lower right, and lower left. The quadrant closest to the black circle stimulus was assigned a value of zero, and larvae in this quadrant also scored zero in the analysis. Quadrants were scored based on proximity to the stimulus: upper left, 1; lower right, 2; and upper right, 3. Each of the 12 larvae per group received a score indicating their position relative to the stimulus. An average close to 3 indicated more larvae were in the upper right quadrant, furthest from the aversive stimulus; an average close to 1 indicated more larvae in the upper left quadrant, closest to the black circle stimulus [38].

2.6. Adult acute toxicity assessment

Eighty adult zebrafish (6–8 months) from the stock population were randomly divided into four groups (n=20 per group) and exposed to TTI or water (control). Eight 2L-tanks (20×10 x10 cm) were previously prepared to receive 10 fish each. Every 2 tanks composed a treatment group. Three concentrations of TTI were used, 12.5, 25, and 50 mg/L. Control group was maintained in system water. The exposure time was 96-hour and fish were not fed during this period and the sample number followed the guideline for Fish Acute Toxicity Test from OECD (protocol no. 203) [39]. Observations were made every 24 hours and with the naked eye. It was recorded any visible anomalies in balance, appearance, and behavior. Mortality was also recorded.

2.7. Neurotoxicity assessment

By the end of the 96 h acute exposure time, animals were gently transferred and randomly assigned to behavioral test tanks. Behavioral tests were conducted between 2 PM and 5 PM and recorded using a Logitech® c920 HD Pro webcam. Neurotoxicity was evaluated through behavioral analyses, namely Novel Tank Test, Light-Dark Test, and Avoidance Response (n=20 for each group).

For the Novel Tank Test, typically used to measure anxiety-related parameters and locomotion, each fish was individually introduced into test tanks (22 ×15 x 22 cm) filled with system water. Behavior was recorded for 6 minutes by a camera positioned 40 cm from the tank. Selected behavioral parameters for analysis included average distance from the tank bottom, latency to the top, time spent at the top, average speed in motion, and total distance traveled.

The Light-Dark preference test, another test used for anxiety response evaluation, was conducted in a shuttle box tank half-covered with black plastic and half with white, with a central passage area (5 ×5 cm). Each animal was individually placed in the black side of the tank, and after 3 minutes of acclimation, the passage door was opened to allow the zebrafish to move between the black and white areas. Analyzed parameters included latency to enter the light area, time spent in the light area, number of transitions between the two areas, average speed, and distance traveled [40].

Avoidance Response involved assessing zebrafish exploring behavior before and after the exposure to a predator model, simulating a fear and avoidance scenario. The predator model used was a bird predator made with plaster, simulating the natural predator of zebrafish [41]. The bird model was attached to a mobile iron system that could be manually moved to approach just above the water surface [42]. Each zebrafish was individually placed in a circular tank (24 cm diameter x 5 cm water column) for 5 minutes habituation. Subsequently, the bird model was moved close to one quadrant of the tank and then removed. Behavior was recorded by camera for 6 minutes, 3 minutes before and 3 minutes after the bird approach. Parameters such as locomotion, freezing time, average speed, distance traveled, time spent in the predator quadrant, and visiting frequency were analyzed before and after exposure to the predator.

Behavioral data collected from recordings were analyzed using ANY-Maze™ software (Stoelting, CO, USA). The program allows for detailed

assessment of behavioral patterns across different experimental contexts, providing a thorough understanding of the observed responses.

2.8. Micronucleus assay

Following the behavioral tests, the animals were anesthetized by immersion in ice and addition of clove oil (40 mL/L). The ice reduces metabolism and minimizes stress, while the clove oil, which contains the natural anesthetic eugenol, acts quickly and safely during the experiments. Subsequently, biological material was collected for the micronucleus assay, following described protocols [26,43].

Blood samples were collected from each fish through an incision in the pectoral fin using a 0.5 M EDTA pipette. Slide smears were prepared (5 animals/group; 2 slides/animal), air-dried, fixed with methanol for 10 minutes, and stained with 5 % Giemsa in PBS for 5 minutes. A total of 1000 mature erythrocytes per slide (2000 erythrocytes per group) were analyzed using an optical microscope (Olympus Microscope CX22, USA) at 1,000X magnification to detect the presence of micronucleus (MN) formation in erythrocytes. The data were analyzed using one-way ANOVA.

2.9. Qualitative histopathological evaluation

After anesthesia and euthanasia with ice and clove oil, five animals from each concentration and the control group (H_2O) were collected for histopathological analyses. The zebrafish were individually placed in cassettes and fixed in 10 % formalin for 24 hours, dehydrated in a graded alcohol series (70 %, 80 %, 90 %, 100 %), cleared in xylene, and embedded in paraffin. Sections of 4 μm thickness were obtained using a microtome (Leica RM2235, Buffalo Grove, IL, USA). Whole-body histopathological analyses were performed using equidistant transverse sections, stained with hematoxylin and eosin, and covered with a coverslip.

A pathologist from UFRN blindly analyzed a representative sample of five slides from each group using a light microscope and a Leica Microsystems DFC 450 C camera. The images were captured with a 40x objective lens using an image analyzer and Proview Optika software.

2.10. Statistical analysis

The data were analyzed using GraphPad Prism 10.1.0. Mean ± standard deviation was used. Normality and homogeneity were checked using Shapiro-Wilk and ANOVA tests, respectively. Embryotoxicity up to 96hpf was evaluated using a Kaplan-Meier curve, with comparisons made using the log-rank test (Mantel-Cox). Malformations were compared using one-way ANOVA followed by Dunnett's post hoc test. Larval behavior and cardiotoxicity were analyzed using one-way ANOVA followed by Dunnett's post hoc test. Adult behavior was assessed using one-way ANOVA followed by Dunnett's post hoc test. Avoidance behavior was measured with a behavior change index. Genotoxicity was evaluated using one-way ANOVA followed by Dunnett's post hoc test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. TTI characterization

For the isolated TTI, the second protein fraction, named F2, was saturated with 30–60 % ammonium sulfate and subjected to affinity chromatography on a Trypsin-Sepharose CNBr 4B column. The protein profile was assessed by spectrophotometry at 280 nm (Fig. 1 A). Additionally, the non-reducing and denaturing SDS-PAGE gel stained with Coomassie Blue confirmed the isolation of TTI, indicated by the presence of protein bands with an estimated molecular mass of around 20 kDa (Fig. 1B).

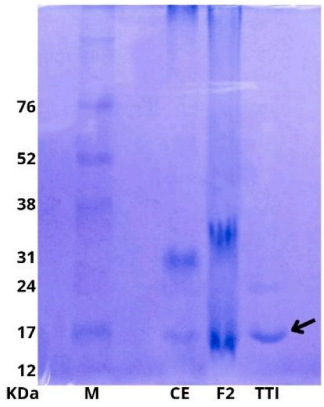

Fig. 1. 12.5 % denaturing polyacrylamide gel electrophoresis (SDS-PAGE) of protein fraction 2 (F2) obtained from tamarind (*Tamarindus indica* L.) seed flour crude extract subjected to affinity chromatography on Trypsin-Sepharose 4B CNBr. stained with Coomassie Blue. M: Molecular Weight Marker (Amersham™ ECL™ Rainbow™ Marker – High Range); CE: Crude Extract; F2: Protein fraction 2 (30–60 % ammonium sulfate saturation). TTI: Tamarind trypsin inhibitor indicated by the black arrow.

3.2. Embryotoxicity assessment

After conducting toxicity tests on zebrafish embryos during 96 h, exposing them to different concentrations of TTI, survival rate and hatching rate were analyzed at each larval developmental stage (Fig. 2). The control group presented survival rate of 86.7 %. In contrast, the zebrafish embryos exposed to the toxic compound 3,4 DCA (positive control) showed a significantly lower survival rate (5 %) compared to the control and treated groups. The survival rates of embryos exposed to TTI at concentrations of 12.5, 25, and 50 mg/L were 85 %, 91.7 %, and 86.7 %, respectively (Fig. 2A). The log-rank test demonstrated statistical significance for the positive control group compared to the other groups ($df = 4$, $p < 0.001$). No statistical significance in survival rate was observed in the remaining groups compared to the negative control ($p > 0.05$). The larval hatching rate (Fig. 2B) did not vary significantly between the groups exposed to TTI ($df = 3$, $p = 0.7922$). Generally, most larvae had successfully hatched by 72 hpf.

Regarding malformations, zebrafish embryos exposed to the three concentrations of TTI did not exhibit significant morphological changes when compared to the control group of animals. However, the embryo exposed to 3,4 DCA presented an elevated number of malformations ($p<0.0001$) (Fig. 3). However, the group of zebrafish exposed to 12.5 mg/L of TTI presented some malformations, but these were not statistically significant ($p<0.05$) when compared to the fish exposed to 3,4 DCA, the positive control, or to the other groups (Fig. 3 A). All types of abnormalities were found in the group of zebrafish exposed to 3,4 DCA. The groups exposed to TTI 12.5 mg/L showed a higher number of pericardial edema (n=7 out of 60 animals) and yolk sac edema (n=7 out of 60 animals), which were statistically different from the control group ($p=0.0118$), however, these malformations accounted for less than 10 % of the sample (Fig. 3B).

3.3. Larvae cardiotoxicity

For the evaluation of cardiotoxicity, heartbeats were counted at 96hpf (Fig. 4). The average heart rate obtained was 152 bpm for the control, 153 bpm for the TTI 12.5 mg/L group, 157 bpm for the TTI 25 mg/L group, and 171 bpm for the TTI 50 mg/L group. While the control group was statistically different ($p<0.0001$) from the TTI 25 mg/L and 50 mg/L groups (ANOVA, $F = 122.4$, $p<0.0001$), all groups presented values within the normal range for zebrafish larvae (120–180 bpm).

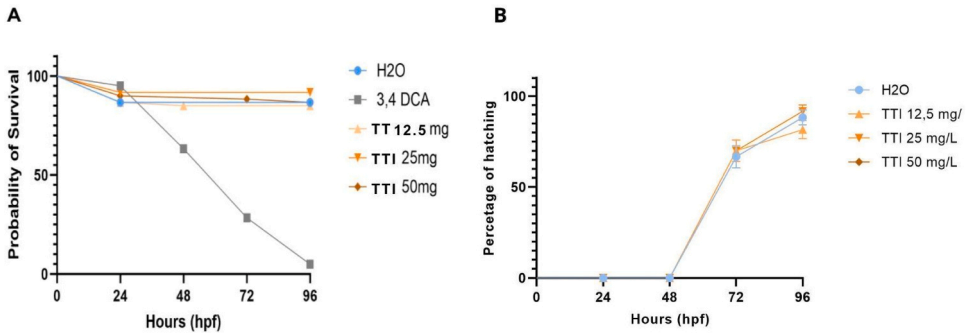

Fig. 2. Zebrafish embryos (A) survival and (B) hatching during 96 h exposed to trypsin inhibitor isolated from tamarind seeds (TTI). Control group (water), 3,4 DCA (positive control), TTI 12.5 mg/L, TTI 25 mg/L, and TTI 50 mg/L (N=60 per group). (A) The Kaplan-Meier survival curve followed by the log-rank test revealed statistical significance for the positive control compared to the other groups ($p<0.0001$). (B) Hatching rate of zebrafish embryos recorded every 24 hpf. Log-rank test did not indicate statistical significance ($p>0.05$).

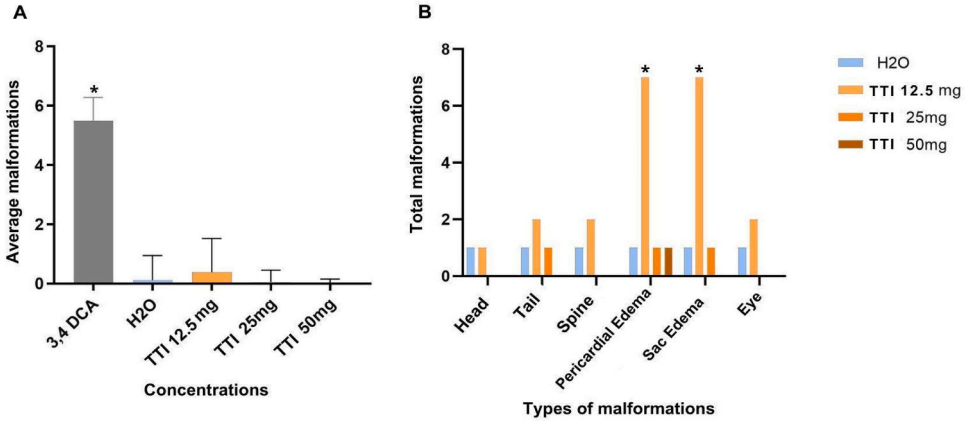

Fig. 3. (A) Average and (B) total of phenotypic malformations observed during 96 hours of development in zebrafish exposed to trypsin inhibitor isolated from tamarind seeds (TTI) at different concentrations. Zebrafish embryos (N=60) were exposed to TTI treatments (12.5, 25 and 50 mg/L) for 96 hours, and two control groups were used for comparison. Negative control group was exposed to system water and Positive control was exposed to 3,4 DCA (N=60). (A) One-way ANOVA showed a difference in the average malformations between the 3,4 DCA group and the other groups ($p<0.0001$). (B) Two-way ANOVA followed by Dunnett's multiple comparisons test showed a statistically significant difference in the TTI 12.5 mg/L group compared to the control group for two malformation types ($p=0.0118$). Asterisk (*) above the bars indicates statistical significance.

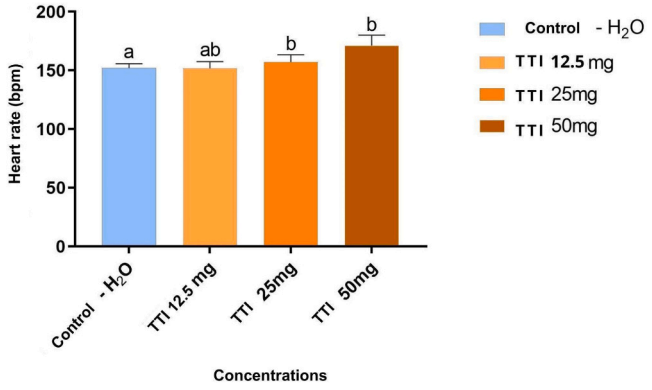

Fig. 4. Heart rates (beats per minute - bpm) of zebrafish larvae at 96 hours post-fertilization (hpf). Embryos were exposed to trypsin inhibitor isolated from tamarind seeds (TTI) at three concentrations (12.5, 25 and 50 mg/L) or water (control group) (n=60). Bars represent average values ± SD. Different letters on bars indicate statistical significance between groups (One-way ANOVA, $p<0.05$).

3.4. Larvae neurotoxicity

The optomotor response of 7dpf larvae showed higher positive optomotor responses (OMR+) than negative responses (OMR-). The accuracy of the response was 78.7 % for the control group, 94.0 % for the TTI 12.5 mg/L, 85.6 % for the TTI 25 mg/L, and 94.4 % for the TTI 50 mg/L. The groups' response was statistically significant (ANOVA, $F = 17.54$, $p<0.001$). Post hoc test indicated that the control group presented lower OMR+ than the other groups ($p<0.05$), and also indicated differences between TTI 12.5 mg/L and TTI 25 mg/L ($p=0.0069$), and TTI 25 mg/L and TTI 50 mg/L ($p=0.0038$). Despite being statistically different from the control group, TTI treated groups showed higher accuracy in OMR (Fig. 5 A).

For the avoidance response, the results were like the OMR, and all groups showed higher positive responses than negative ones. There were no differences between treated and control groups (ANOVA, $F = 0.2770$, $p=0.8419$) (Fig. 5B).

3.5. Adult acute toxicity

After 96-hour exposure to TTI, all groups of adult zebrafish presented 100 % survival rate and no observable abnormalities. Individuals were then tested for specific behavioral responses.

In the novel tank test, which evaluates anxiety-like and exploratory behaviors, no alterations were observed in the parameters analyzed

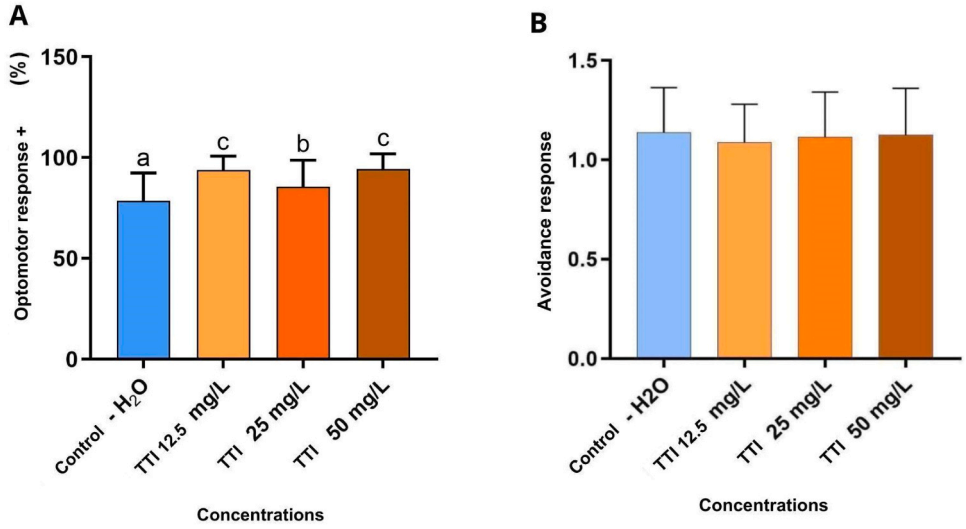

Fig. 5. (A) Optomotor response and (B) Avoidance response evaluated in 7dpf zebrafish larvae. Embryos were exposed to trypsin inhibitor isolated from tamarind seeds (TTI) at three concentrations (12.5, 25 and 50 mg/L) or water (control group) from 3 to 96 hpf. At 7dpf, 12 individuals from each group were tested for behavioral response, in triplicate. (A) Optomotor response was statistically different between groups, indicated by different letters above bars (ANOVA, $p<0.05$). (B) No statistical significance was observed for the avoidance behavior.

(Fig. 6). Swimming profiles were analyzed based on the total distance traveled (ANOVA, F = 0.02486, p=0.9946; Fig. 6A) and average speed (ANOVA, F = 0.07454, p=0.9734; Fig. 6B), with no statistical significance between groups. For the distance from the bottom (ANOVA, F = 0.6485, p=0.5872; Fig. 6C) and latency to enter in the top area (ANOVA, F = 0.5483, p=0.6516; Fig. 6D), no differences were observed between groups treated with TTI and control.

For the light dark preference test, a protocol that evaluates anxiety-like and risk-taking behaviors, the group treated with the lower TTI concentration presented reduced distance traveled (ANOVA, F = 5.186, p=0.0031; Fig. 7A) and average speed (ANOVA, F = 3.857, p=0.0142; Fig. 7B), although neither the TTI groups differed from the control group ($p>0.05$). For latency to enter the white area (ANOVA, F = 3.826, p=0.0147; Fig. 7C), the animals exposed to TTI 25 mg/L showed reduced time to reach the white side than TTI 12.5 mg/L (p=0.0119), but they were not statistically different from the control group ($p>0.05$). However, time spent in the white side did not differ between groups (ANOVA, F = 0.7985, p=0.4999; Fig. 7D). Regarding the number of transitions between areas (Fig. 7E), all animals exposed to TTI exhibited fewer transitions compared to the control group (ANOVA, F = 7.082, p=0.0004).

In the avoidance test (Fig. 8), no differences were observed between the zebrafish groups in terms of average speed (ANOVA, F = 0.417, p=0.742; Fig. 8B), distance traveled (ANOVA, F = 2.896, p=0.052; Fig. 8A), and time spent in the predator area (ANOVA, F = 1.139, p=0.349; Fig. 8C). However, the freezing behavior, that is a total absence of apparent movements except for those responsible for breathing (ANOVA, F = 4.961, p=0.007; Fig. 8D), and the frequency of entry into the predator's target area (ANOVA, F = 3.80, p=0.020; Fig. 8E) differed between groups. Fish exposed to TTI 25 mg/L showed a significant increase in freezing compared to the untreated control group (p=0.007). For the frequency of visits to the predator's target area, the TTI 12.5 mg/L group showed a significant increase (p=0.02) in entries compared to the TTI 50 mg/L group, which reduced visits to the predation area, but the groups did not differ from the control.

different concentrations. Adult zebrafish were treated with 12.5, 25 and 50 mg/L TTI (n=20), or were maintained in water (control group, N=20). Then, each individual was tested in a circular arena, where a bird model reached the water surface and was then removed. Fish behavior was recorded for 3 min before and 3 min after the bird exposure. Behavior was analyzed for distance traveled (A), speed (B), time spent in the area where the bird arrived (C), freezing (D), and visit frequency to the area where the bird arrived (E). Different letters above bars indicate a statistical significance between groups (One-way ANOVA, $p<0.05$).

3.6. Micronucleus assay

In the genotoxicity evaluation, micronucleus percentage was 0.00 % for both the control (untreated) group and the TTI 12.5 mg/L groups, while TTI 25 mg/L showed 0.05 % (1: 2000) of micronucleus and TTI 50 mg/L 0.10 % (2: 2000). These results were not significant (ANOVA, F= 1.833 p=0.2192; Fig. 9).

3.7. Histopathological evaluation

After whole-body qualitative histopathological analyses, no signs of damage were observed in the tissue structures of the zebrafish compared to the animals in the unexposed group. However, through qualitative analyses, the presence of both microvesicular and macrovesicular steatosis was recorded in the liver of all animals from the evaluated groups, both with and without TTI exposure (Fig. 10A). Additionally, for all groups of zebrafish, mononuclear inflammatory infiltrate was observed in the spinal cord, located around the medullary canal and distributed in the gray matter, including in the group without TTI exposure (Fig. 10B).

4. Discussion

In this study, the trypsin inhibitor isolated from tamarind seeds (TTI) was analyzed for acute embryo and adult toxicity in zebrafish. It was observed that TTI did not present toxicity during the embryonic stage (from 3hpf to 96hpf) or in the adult stage when considering the evaluated parameters and concentrations (12.5, 25 and 50 mg/L). This study supports the biosafety of TTI and enables the use of this protein in research testing its efficacy. This research contributes to the development of treatments and therapies where TTI can be used to reduce comorbidities associated with metabolic diseases [5,16,19].

Here, TTI was isolated and visualized using SDS-PAGE, showing a predominant protein band with an estimated molecular mass of approximately 20 kDa (Fig. 1), corroborating previous studies by Ribeiro et al. [19], Carvalho et al. [4], and Costa et al. [5]. Therefore, it can be stated that the new batch of TTI exhibited the same biochemical

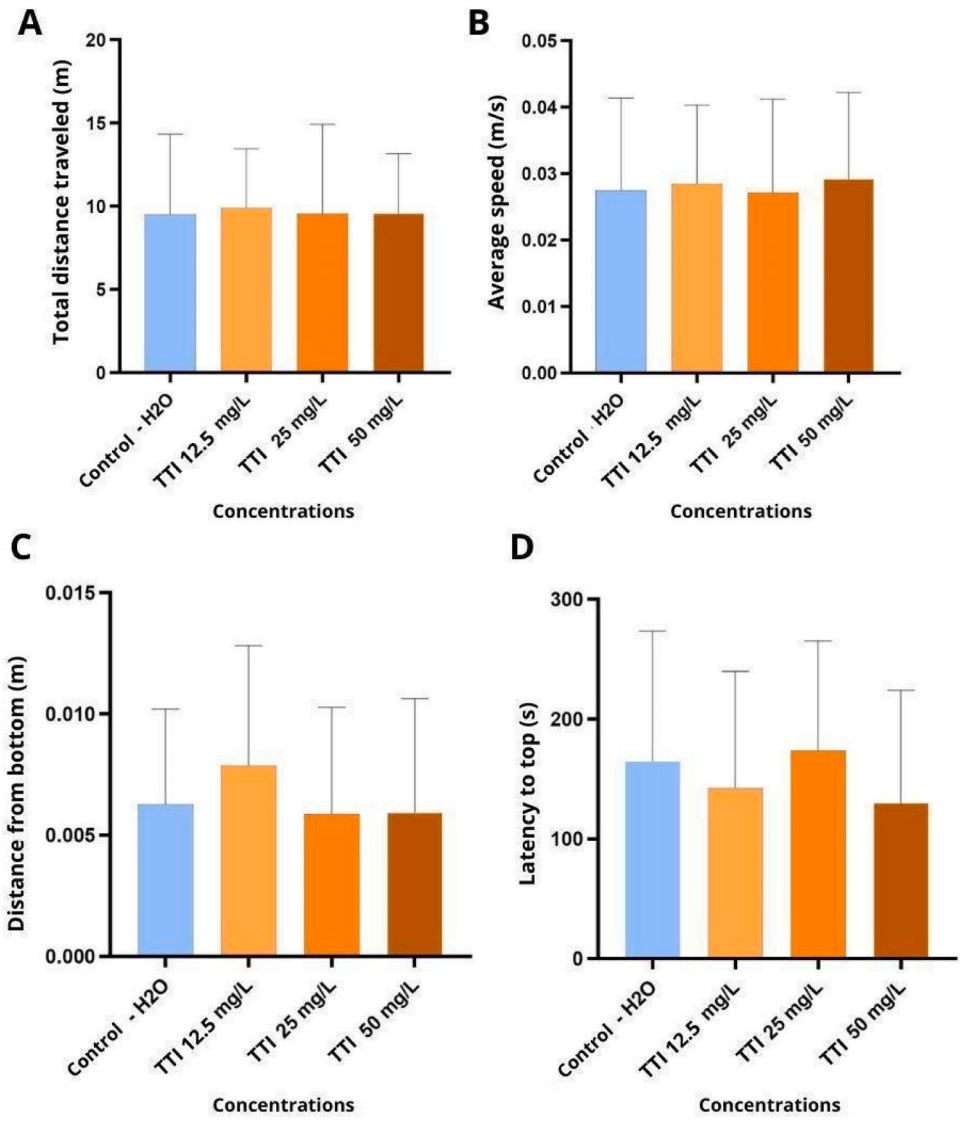

Fig. 6. Novel Tank Test conducted with adult zebrafish after 96 hours of exposure to TTI at different concentrations. Adult zebrafish were treated with 12.5, 25 and 50 mg/L TTI (n=20), or were maintained in water (control group, n=20). Individuals were tested in the novel tank for 6 min. Behavior was analyzed for distance traveled (A), speed (B), distance from the bottom (C), and latency to enter the top area (D). One-way ANOVA showed no statistical significance for the parameters ($p>0.05$).

properties identified in previous studies, attesting to the consistency in obtaining this molecule.

The doses of TTI used in this study (12.5, 25, and 50 mg/L) were selected based on preliminary preclinical experiments [12], which evaluated their safety and efficacy in cell cultures and mammals. It is essential to consider the differences in absorption and metabolism dynamics between species to ensure the relevance of the results. Additionally, this approach underscores the need for futher studies to enhance the understanding of TTI's effects in broader contexts.

Previous toxicity tests with TTI conducted in cell cultures showed no interference of 0.3 and 0.6 mg TTI/mL on CHO-K1 cells [15] and the absence of cytotoxic effects of nanoencapsuled TTI in concentrations of 0.5; 2.5, and 5.0 mg/mL on Caco-2 and CCD-18Co cells [12]. *In vitro* toxicity encompasses a range of nonspecific changes that impact cellular functions, leading to cell death. These tests detect various toxic effects and help determine the initial concentrations for acute toxicity tests in pre-clinical studies [44,45]. As an initial approach for evaluating the safety, *in vitro* tests offer a forefront approach, however subsequent evaluations in animal models are crucial to demonstrate how the bioactive compounds behave in a complex organism [46,47].

As far as we know, only the study by Costa et al. [12] has tested the subacute blood toxicity of nanoencapsuled TTI in an animal model. However, these authors used only a single concentration (12.5 mg/kg) administered orally to Wistar rats and observed no effects on hemoglobin, hematocrit, and total leukocyte count. Thus, the present results provide novel data by addressing the acute toxicity of TTI in a comprehensive study using zebrafish.

In this study, embryotoxicity and acute toxicity evaluations using TTI were conducted with three concentrations, 12.5, 25, and 50 mg/L. Regarding embryotoxicity, no effects of TTI at any concentrations were observed on survival and hatching rates (Fig. 2). Although a few malformations were observed for embryos treated with 12.5 mg TTI/L (Fig. 3), it was not statistically relevant, encompassing less than 10 % of the sample. Similarly, Jegadheeshwari et al. [48] evaluated the embryotoxicity of another trypsin inhibitor, extracted from *Dioscorea bulbifera* L. (DbGTi), in zebrafish and observed normal development and hatching. In the embryotoxicity tests, exposure begins at a critical developmental stage called gastrulation and continued for 96 h until the mobile larval stage, in which both survival and hatching rates were considered important endpoints for the animal viability [49,50]. Any

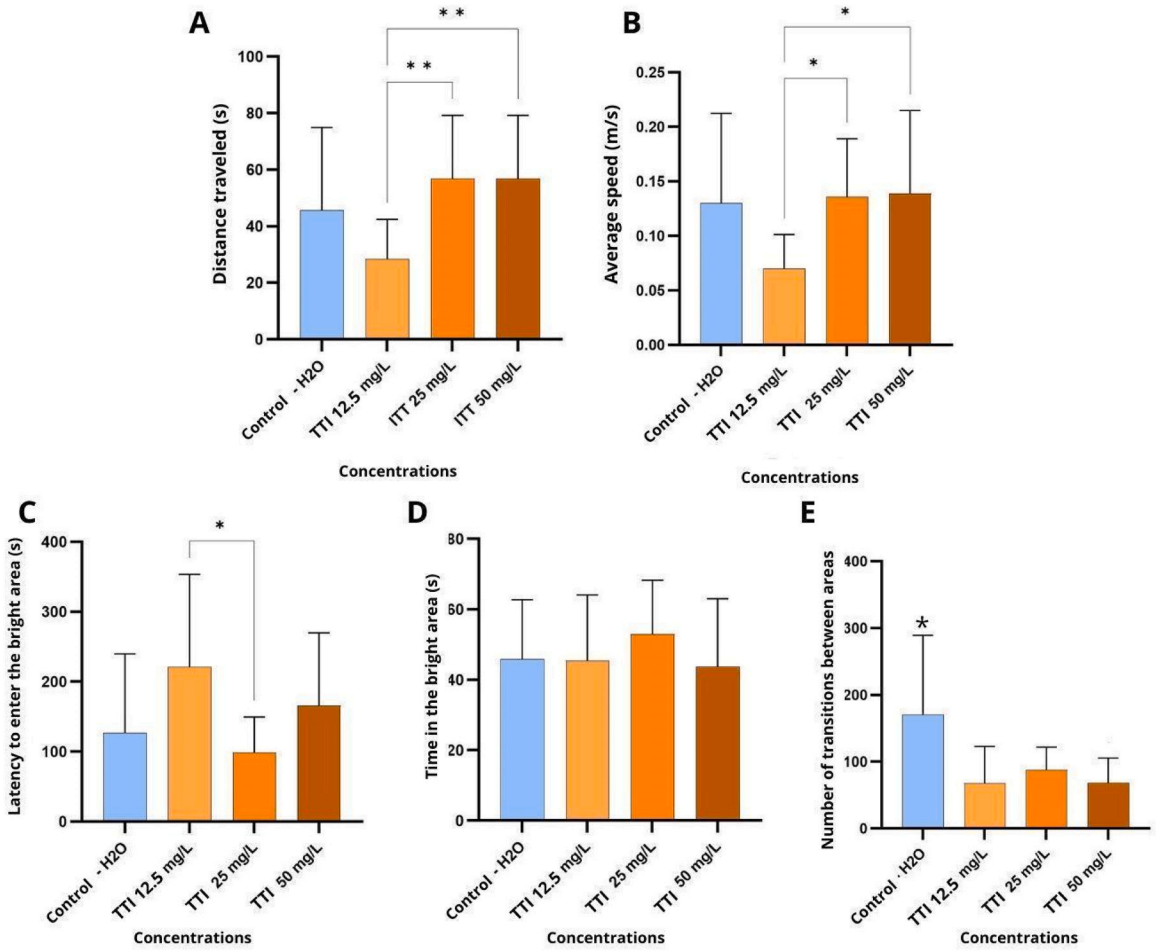

Fig. 7. Light-Dark Test conducted with adult zebrafish after 96 hours of exposure to Trypsin Inhibitor isolated from tamarind seeds (TTI). Adult zebrafish were exposed to 12.5, 25 and 50 mg/L TTI and water for the control group (n=20). After that, individual fish were tested in the Light-Dark tank for 6 min. Behaviors assessed were distance traveled (A), speed (B), latency to enter the white area (C), time spent in the white area (D), and number of transitions between areas (E). Asterisk above bars indica statistical significance (One-way ANOVA: * $p<0.05$; ** $p<0.005$).

alterations in these parameters may indicate toxicity [50,51]. Thus, the results obtained here support the safety of TTI for early stages of development.

In addition to morphological development, cardiotoxicity was evaluated by assessing heart rate, an important parameter of cardiac function [52]. Although all evaluated groups showed heart rate values within the normal range for zebrafish larvae [53], an increase in this value was observed in animals exposed to TTI (Fig. 4). This data indicates increased cardiac workload due to TTI exposure, but since these values are uncommon in the literature, they cannot be used as indicators of toxicity. Therefore, more studies addressing cardiovascular function in greater depth should be developed to clarify any potential cardiological effects. Moreover, long term exposure regimes should be carried on evaluating possible chronic effects.

In terms of neurotoxicity-related responses, evaluated here using the optomotor and avoidance tests (Fig. 5), no indicators of potential neurotoxicity were detected. Nervous functions related to reflex (spinal cord integrity) and decision-making (brain integrity) [54] were preserved in the groups of animals exposed to TTI, indicating neural safety of the protein.

Other studies evaluating the toxicity of known neurotoxic substances, such as pesticides, indicate the tests used here as reliable indicators of neural function [38]. Conversely, substances with low neurotoxic potential can be suggested using optomotor and avoidance response tests, such as hydroxyapatite-based biomaterials [37] and cantaloupe melon extracts [55].

Although data indicating the embryonic toxicological safety of TTI were evidenced, studies with adults were also conducted to evaluate whether TTI could also have toxic effects and cause neural function alterations. In the study conducted by Patriota et al. [56], the acute *in vivo* toxicity evaluation of the trypsin inhibitor from Moringa oleifera flower (MoFTI) in adult female Swiss mice revealed that a single intraperitoneal concentration of MoFTI (300 mg/kg) did not result in mortality or changes in body weight, and food and water consumption. Similarly, no mortality was observed in animals exposed to TTI at any concentration tested here.

In the behavioral tests, the novel tank test (Fig. 6), commonly applied to access locomotion and anxiety-like response [57,58], did not indicate any behavioral alterations due to TTI exposure. In contrast, the study by Da Silva Júnior et al. [26], which evaluated the response after exposure to retene (a hydrocarbon derived from biomass burning), indicated an increase in swimming speed along with a decrease in total distance traveled, indicating signs of hyperactivity in the animal. Although no effects were observed in this type of test, risk-taking response (light-dark test) and anti-predatory response (avoidance test) were also evaluated to complement the neurotoxicological assessment.

In the risk-taking test (Fig. 7), conducted through the light-dark test, where the animal is placed in a dark environment and exposed when entering the light area [59,60], it was observed that animals exposed to the lowest concentration of TTI (12.5 mg/L) reduced swimming speed and increased latency to enter the light area compared to other TTI groups, but not to the control. Scototaxis, the main parameter indicative

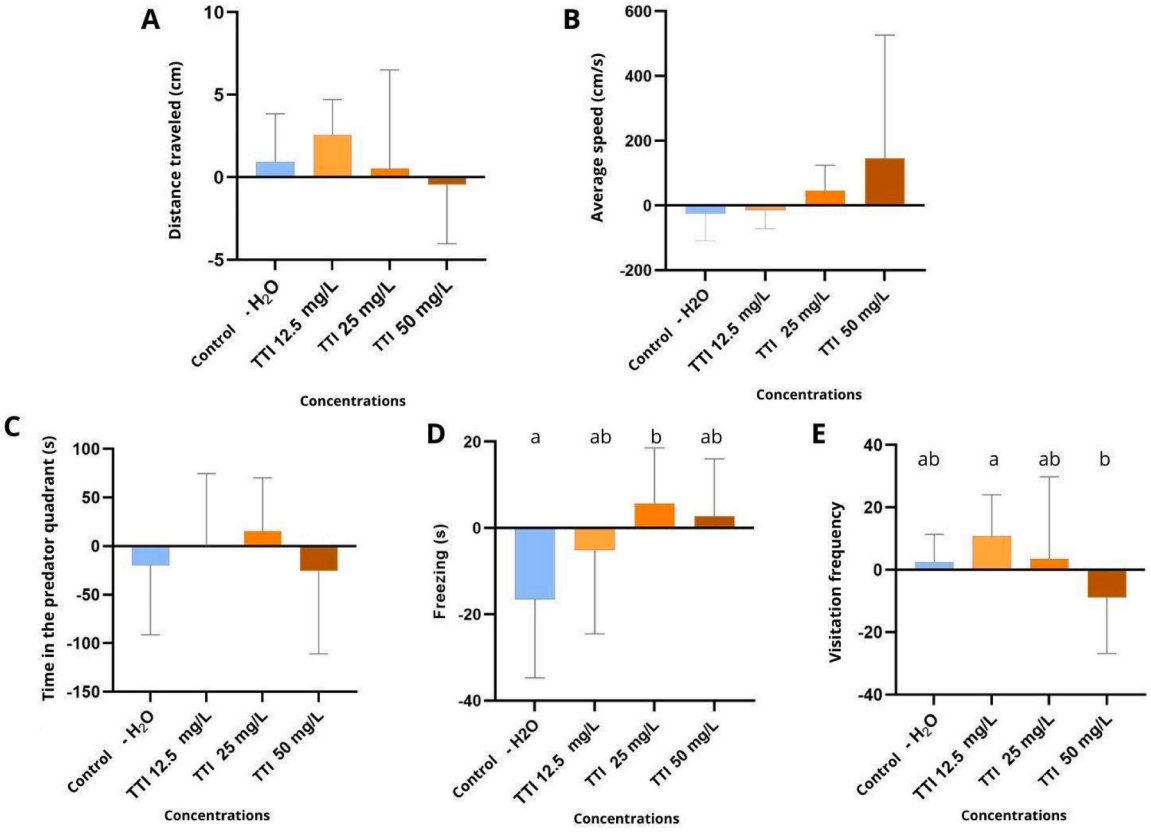

Fig. 8. Avoidance response assessed in adult zebrafish after 96 hours of exposure to TTI at.

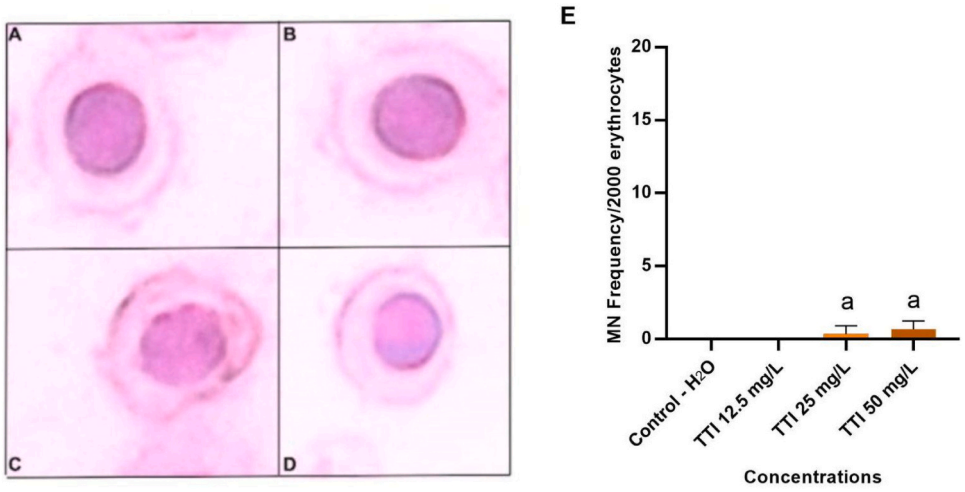

Fig. 9. Erythrocytes from adult zebrafish observed after 96 hours of TTI exposure (1,000x magnification). (A) Control group, (B) TTI 12.5 mg/L, (C) TTI 25 mg/L, and (D) TTI 50 mg/L. (E) Micronucleus frequencies in zebrafish blood. The results are expressed as the number of micronuclei per 2000 randomly counted erythrocytes. Equal letters on bars indicate that there was no statistical significance between the groups. (ANOVA, p>0,05).

of increased anxiety in this test [61,62], was not observed in the other groups. This result, along with the new tank test, suggests a low anxiogenic effect of TTI. The occasional increase in anxiety response observed in the zebrafish group exposed to 12.5 mg/L of TTI may be attributed to an unknown environmental disturbance, as other anxiety indicators were not observed in this group during the analysis.

Finally, regarding the avoidance test, which measures the animal's anti-predatory response, it was observed that the group of animals exposed to 12.5 mg/L of TTI showed an increase in the number of entries into the predator area, indicating reduced anxiety. This result contradicts the indication of increased anxiety-like response in the light-dark test, reinforcing the hypothesis that some disturbing event occurred during the light-dark test recording, inducing this behavior. Other studies reinforce that complementary tests evaluating anxiety-like behavior and other behavioral parameters should be conducted in a block to avoid biases in the analyses [42].

Regarding genotoxicity *in vivo* models, the micronucleus (MN) assay is a fundamental component in these tests, evaluating the safety of different substances. Micronuclei are fragments or complete chromosomes expelled from the nucleus, either spontaneously or as result of

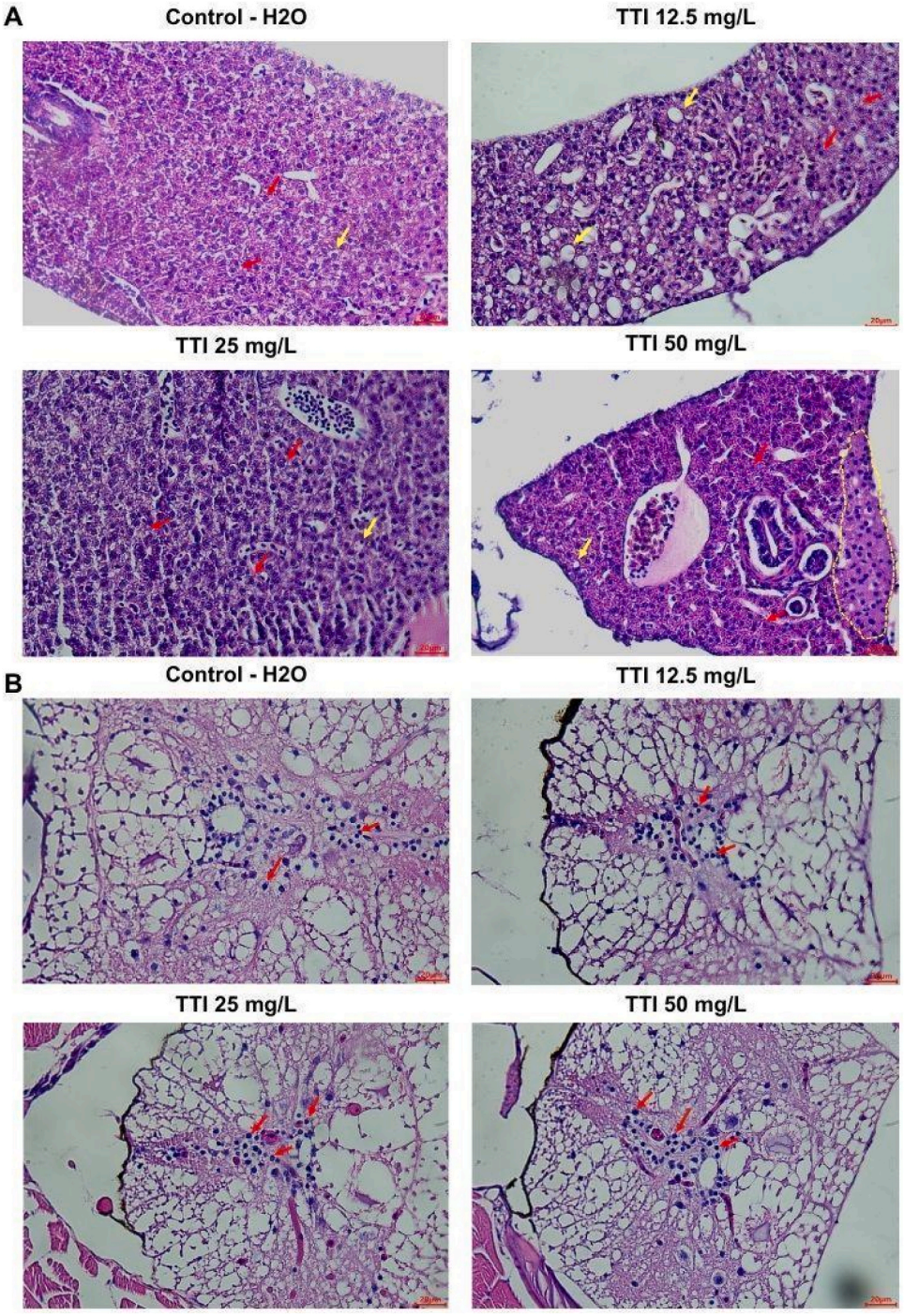

Fig. 10. Photomicrographs of representative liver and spinal cord slides from adult zebrafish after 96 hours of exposure to TTI at different concentrations. Adult zebrafish exposed to various treatments for 96 hours, at 40x objective. (A) Photomicrographs of the liver: Microvesicular steatosis (red arrow); Macrovesicular steatosis (yellow arrow); Area of necrosis (dotted outline). (B) Photomicrographs of the spinal cord: Mononuclear inflammatory infiltrate in the gray matter (red arrow). Control - H2O: no exposure; TTI 12.5: exposed to 12.5 mg/L of trypsin inhibitor isolated from tamarind seeds; TTI 25: exposed to 25 mg/L of trypsin inhibitor isolated from tamarind seeds; TTI 50: exposed to 50 mg/L of trypsin inhibitor isolated from tamarind seeds. Groups (n=5 animals/group).

DNA damage, leading to genetic damage [63]. In this study, the blood erythrocytes of zebrafish exposed to the three TTI concentrations were analyzed (Fig. 9). Similarly, the results indicated that this molecule did not exhibit genotoxic properties, as no representative numbers of micronuclei were observed in the erythrocytes evaluated at the tested concentrations. This result reinforces other indicators of low TTI toxicity presented so far. In another study, Valadez-Vega et al. [64] analyzed a protein extract rich in enzyme inhibitors from *Amaranthus hypochondriacus* seeds, where they observed that the highest concentration (250 mg/kg) exerted genotoxic activity in male CD-1 mice, inducing MN production. Unlikely, TTI concentrations tested here did not cause genotoxic effects in adult zebrafish.

In addition to the analyses mentioned, this study also assessed the histopathological effects of TTI. The importance of histological analysis in toxicological screening is well established [65]. Therefore, corroborating the results obtained, histopathological analyses conducted on adult zebrafish exposed to TTI showed no histopathological changes distinct from the unexposed group. However, certain findings were

observed in specific organs, such as mild steatosis in the liver and non-specific mononuclear inflammatory infiltrate in the spinal cord, similar to those found in the zebrafish group not exposed to TTI. Thus, these lesions are not directly related to TTI exposure. These data are consistent with previous studies conducted on Wistar rats exposed to TTI (25 mg/kg and 50 mg/kg), where the absence of harmful effects on the morphology of the evaluated organs (liver, stomach, kidneys, and pancreas) was observed [19].

It is important to highlight that the absence of cross-contamination between groups was ensured by the methodology adopted. Thus, a hypothesis to explain these histopathological findings, even in the animals not exposed to TTI, could be that the damage may have been caused by the diet provided to the animals during their growth, development, and maintenance. Studies have highlighted that the carbohydrate content in commercial feeds requires attention, as zebrafish do not have a specific requirement for this macronutrient, although they possess enzymes for its digestion and metabolism [66]. Excess of carbohydrates can lead to hepatic steatosis [67], and combination of elevated glucose levels and insulin resistance can result in medullary damage [68–70].

In the review study conducted by Chang et al. [67], different dietary models were analyzed. The results indicate that high-fructose diets, as well as periods of fasting following fertilization, can induce liver damage similar to hepatic steatosis and NASH (non-alcoholic steatohepatitis) in zebrafish. Coincidentally, the animals used in this study were subjected to these same conditions throughout their growth period, including a 96-hour fasting phase during the experiment. Additionally, the commercial feed in the zebrafish diet contained approximately 30 % carbohydrates, calculated by subtracting the combined percentages of water, protein, fat, and ash from 100 % according to the label information (https://nutricon.ind.br/produtos/nutriflakes/).

Furthermore, after 19 days of glucose exposure to induce type 2 diabetes (DM2), adult zebrafish exhibited increased markers of peripheral inflammation, neuroinflammation, and neuronal apoptosis, as well as anxiety-like behavior [67]. These results support the hypothesis that the considerable carbohydrate content in the diet provided in this study may have been a determining factor for both the increased fat production in the liver and the inflammation in the spinal cord.

Although this study presents a series of indicators of the toxicological safety of TTI, it is important to note that TTI was used in an aqueous solution, encountering the organism via the cutaneous route in embryos and adults. Since this molecule has potential gastrointestinal activity, its effects should be evaluated through the oral route. This aspect should be addressed in future studies using this experimental model, as well as in those already conducted with Wistar rats [9,19]. Additionally, conducting more in-depth studies on cardiovascular function is important to verify the results obtained here. Future studies should also investigate biomarkers of oxidative stress and inflammation, which are usually associated with the toxicity of substances.

5. Conclusions

The trypsin inhibitor isolated from tamarind seeds (TTI) was successfully extracted and isolated, presenting toxicological safety in embryotoxicity and acute adult toxicity assessments in zebrafish. The results indicate the biosafety of TTI across various developmental stages and in different tests. TTI did not cause mortality or significant behavioral changes in larvae and adult animals. Even at high concentrations, there were no indicators of acute toxicity, confirming the safety of the compound. In the genetic test, it did not show genotoxicity or histopathological changes in the tissues compared to animals not exposed to TTI. This study provides a multilevel and comprehensive evaluation of the toxicological safety of TTI, covering ontogenetic stages and various tests. These results support the safety of the molecule.

Funding

This research was also supported by the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES, Finance Code: 001). AHAM and ACL are supported by National Council for Scientific and Technological Development (CNPq), process 303094/2022–2 and 308245/2023–7, respectively.

CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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