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Research Article

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Acute Toxicity Study and Anti-Inflammatory Effect of Injectable Nanodispersion of *Bixa orellana* L. (Chronic In®) in *Danio rerio* (Zebrafish)

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Abstract

Medicinal plants hold great potential for developing drugs to treat inflammatory diseases. Bixa orellana L, popularly known as "annatto" or "urucum", stands out among various species. Due to its experimental at-tributes, the Zebrafish (Danio rerio) is a small tropical freshwater fish highlighted as an experimental model in pharmacological and toxicological tests of new drugs. This study aims to evaluate the acute toxicological effects and anti-inflammatory activity of the injectable nanodispersion of Bixa orellana (NBO, Chronic In®) in zebrafish. The stability of the NBO was assessed with an average particle size ranging from 53.15 ± 0.64 nm to 59.90 ± 3.63 nm. The acute toxicity study used intraperitoneal doses of NBO ranging from 250 to 1500 mg/kg. The results showed significant toxic effects at doses of 750, 1000, and 1500 mg/kg, with histopathological changes in the liver, kidneys, and intestine. The 50% lethal dose (LD50) calculation corresponded to 830.6 mg/kg. In silico tests, the detected toxicity mechanism may be related to inhibiting the cardiac potassium channel (hERG II) and/or the TNF tumor necrosis factor receptor-associated protein 1 (TRAP1). In the an-ti-inflammatory evaluation of NBO in a model of intraperitoneal edema induced by carrageenan in Zebrafish, a significant anti-inflammatory effect was observed at doses of 20 and 40 mg/kg (i.p), without significant histopathological alterations in the organs evaluated. In summary, NBO has an anti-inflammatory effect at low doses and demonstrated that doses of NBO ranging from 500 to 1500 mg/kg applied intraperitoneally were unsafe and showed toxic effects and lethality as the dose increased.

Keywords: *Bixa Orellana*, Anatto, Nanotechnology, Zebrafish

Abbreviations

The following abbreviations are used in this manuscript:

DFT: Density Functional Theory
GC-MS: Chromatograph coupled to mass spectrometry
HAI : Histopathological alterations index
LD50: 50% lethal dose
NBO : Injectable nanodispersion of Bixa orellana
OPBO : Standardized oil of Bixa orellana

pkCSM: Predicting small-molecule pharmacokinetic and toxicity properties
TNF: Tumor necrosis factor
TRAP1: TNF Receptor-Associated Protein 1

1. Introduction

Inflammation is an organism's defense mechanism that stimulates chemical mediators at the injury site. It is characterized by altered blood flow, vasodilatation, vascular permeability, and leukocyte recruitment, which result in clinical signs and symptoms such as pain, edema, hyperemia, and tissue dysfunction [1,2].

Medicinal plants hold significant potential for developing medicines to treat inflammatory diseases. Among the various species, *Bixa orellana* L., commonly known as "urucum" or "annatto", is a plant native to Brazil and certain regions of South and Central America [3,4]. Studies have proven its anti-inflammatory activity, and it has already been demonstrated that the presence of compounds such as bixin, tocotrienols, and geranylgeraniol contributes to its anti-inflammatory properties in the oil extracted from this species [5,6]. Tocotrienols are unsaturated forms of vitamin E and have potent anti-inflammatory and antioxidant activity [7].

The Zebrafish (*Danio rerio*) is a small tropical freshwater fish that, due to its experimental attributes, such as its small size, low cost, treatability, and genetics, has stood out as an experimental model in pharmacological and toxicological tests of new drugs [8,9]. This makes it suitable for validating new nano-formulated drugs from bioactive natural products with therapeutic potential [10].

Nanodispersions are pharmaceutical forms whose main characteristics are reduced particle size on nanometric scales, better bioavailability, decreased dose, and increased therapeutic effect. These characteristics favor their use as a treatment and reduce the possibility of causing toxicity [11].

Therefore, it is necessary to carry out studies that prove the pharmacological and toxicological activities of medicinal plants and their formulations so that it is possible to use their therapeutic properties. In this context, this study aimed to evaluate the acute toxicological and anti-inflammatory effects of the injectable nanodispersion of Bixa orellana oil (*Chronic In*®) in zebrafish (*Danio rerio*).

2. Materials and Methods

2.1 Bixa orellana Oil Obtainment and Chemical Analysis

B. orellana oil (Chronic®) was supplied by Ages Bioactive Compounds Co., batch 0012/2022. For the chemical analysis, δ-tocotrienol was dosed using a stock solution of δ-tocotrienol prepared in hexane (Sigma-Aldrich, MKCF5755, CG grade, Germany) at 10 mg/mL concentration. Other solutions were ready to build an analytical curve from this stock solution with concentrations of 2.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL, and 0.1 mg/mL. The analyses were performed using a gas chromatograph coupled to a mass spectrometer (GC-MS), following the methodology previously described by Rodrigues et al. [12].

2.2 Obtaining Polymeric Nanoparticles Containing *Bixa orellana* Oil

The nanoprecipitation method, adapted from Carvalho et al., synthesized a nanoparticulate polymeric system containing OPBO. For a final volume of 20 mL of nanodispersion (NBO), the following components were used: 1% purified *Bixa orellana oil*, 2% polyethylene glycol 4000 (PEG 4000), 2% Tween 80, 25% absolute ethanol, and 70% water for injections.

The organic phase was first prepared by solubilizing the OPBO and PEG 4000 in absolute ethanol. The mixture was stirred using a magnetic stirrer at 800 rpm for 20 minutes. The aqueous phase was prepared by solubilizing Tween 80 in water and stirring for 20 minutes. The organic phase was poured over the aqueous phase under agitation in a mechanical stirrer (Fisatom, SP, Brazil) for 20 minutes. After preparation, the nanodispersion obtained was filled into vials and subjected to characterization. The nanodispersion obtained was packaged into ampoules for injections and subjected to characterization.

2.3 Characterization of *B. orellana* Nanodispersion (NBO)

For stability purposes, nanodispersion was characterized on the day of preparation and after 7 and 30 days. The visual aspect, phase separation, and sedimentation were evaluated macroscopically. The particle size, polydispersity index, and Zeta potential were assessed in triplicate following the methodology described by Borges et al. using Zeta-Sizer equipment (Malvern Pan-Analytics, England) [13].

2.4 pkCSM: Predicting Small-Molecule Pharmacokinetic and Toxicity Properties

The pkCSM platform was utilized to evaluate the pharmacokinetic and physicochemical characteristics of the therapeutic compound δ-tocotrienol. The SMILES notation for δ-tocotrienol was obtained from the PubChem database and input into the pkCSM tool. The compound's molecular structure allowed for the prediction of various properties, including absorption, solubility, distribution, metabolism, excretion, and toxicity [14].

2.5 Molecular Docking

Protein receptors were obtained from the Protein Data Bank. Using Molegro software, the crystal structures of the target receptors were pre-processed by removing ions, water molecules, and existing ligands. The receptor molecules were then modified by adding hydrogen atoms through Autodock Vina and saved in pdbqt format. ChemDraw was utilized to minimize each compound and convert them into mol2 format, which was subse-quently converted into pdb format using Molegro. The compounds were then converted into pdbqt format using AutoDock tools. Ligand-centered maps were generated with AutoGrid, using a grid size of 90 Å × 90 Å × 90 Å. All other parameters were set to their default values. AutoGrid and AutoDock Vina were employed to construct the grid maps, and Discovery Studio 4.5 software was utilized to analyze the 2D interactions be-tween the compounds and target receptors [15,16]. This study evaluated the interaction of zebrafish TNF tumor necrosis factor receptor-associated protein 1 (TRAP1)

(PDB ID: 6D14 at 2.50 Å resolution) with the ligand δ-tocotrienol.

2.6 Density Functional Theory (DFT) Computational Analysis

Density Functional Theory (DFT) calculations were performed using the B3LYP functional with the 6-311G(d,p) basis set. This approach was applied to study the properties of δ-tocotrienol. The B3LYP method is widely recognized for its cost-effectiveness and reliability in predicting the geometries of both natural and synthetic molecules in quantum chemistry. All computations were done using the GAUSSIAN 09W software package, and the optimized molecular structures were visualized with GaussView 6.0 [17].

2.7 Non-Covalent Interaction Analysis (NCI)

The natural bond orbital (NBO) calculations were performed at the same level of theory using the NBO pro-gram 3.1 embedded in Gaussian. A visual study of the non-covalent interaction (NCI) was achieved using a combination of Multiwfn, visual molecular dynamics (VMD), and Gnuplot programs [18].

2.8 Animals and Ethical Aspects

This study was submitted to the Animal Use Ethics Committee of the Federal University of Amapá (CEUA-UNIFAP) and was approved under Protocol No. 001/2023. The experiment used zebrafish, *Danio rerio*, of the Wild strain, six months old, measuring between 3.5-4.0 cm in length, weighing 400-600 mg, purchased from Aqua New Aquários e Peixes Co. ME (PE, Brazil), kept in the Zebrafish Platform of the Drug Research Laboratory at the Federal University of Amapá - UNIFAP, Amapá, Brazil. The animals were kept in water under controlled temperature, feeding, and light/dark cycle conditions.

2.9 Acute Toxicity Study and Determination of LD50

To perform the acute toxicity tests, the animals were randomly divided into five experimental groups of eight animals and fasted for 24 hours before the treatments commenced. Subsequently, intraperitoneal doses of 250 mg/kg, 500 mg/kg, 750 mg/kg, 1000 mg/kg, and 1500 mg/kg of *Bixa orellana* Nanodispersion (Chronic In®) were administered. After administration, the animals were kept in isolated aquariums to observe behavioral parameters. To determine the 50% lethal dose (LD50), the number of deaths in each group was counted ac-cording to the dose administered, following the methodology described by Santos et al. [19].

2.10 Behavioral and Mortality Analysis

The behavior and mortality of the animals were evaluated according to the parameters described by Souza et al. (2016) at 1 hour, 3 hours, 9 hours, 24 hours, and 48 hours after treatment. Behavioral reactions were characterized in three stages: Stage I: 1) increase in swimming activity and 2) tail axis tremors; Stage II: 1) Circular swimming and 2) Posture loss; Stage III: 1) Motility loss; 2) Deposition at the bottom of the aquarium and 3) Death. Mortality was assessed continuously, and the fish were considered dead when the movement of the operculum and the response to mechanical stimulation could no longer be detected. After toxicity testing, all surviving fish were euthanized.

2.11 Edema Induction by Carrageenan and Experimental Design

Edema was induced in adult zebrafish following the method described by Borges et al. [13]. The animals were individually anesthetized with cooled water (8-10 °C) for approximately 3 minutes, then 20 µL of 1% kappa carrageenan solution (Sigma Co., Lot 16HO616) in PBS was applied intraperitoneally. All animals were weighed individually on an analytical scale before edema induction and 5 hours after carrageenan application to assess their final weight. Following euthanasia, a histopathological study was conducted.

The animals were randomly divided into six experimental groups (n = 12/group), treated intraperitoneally, and edema was induced 1 hour after treatment.

The following groups were chosen: **CON Group** - PBS solution (20 µl) and distilled water (20 µl); **DCF Group** - Injectable diclofenac 25 mg/kg; **VEH Group** - Nanodispersion vehicle 20 µl (2% polyethylene glycol 4000 (PEG 4000), 2% Tween 80, 25% absolute ethanol and 70% water for injections) (20 µl); **NBO 10 Group** - *B. orellana* nanodispersion 10 mg/kg; **NBO 20 Group** - *B. orellana* nanodispersion 20 mg/kg; **NBO 40 Group** - *B. orellana* nanodispersion 40 mg/kg.

2.12 Organ Preparation for Histopathological Analysis

After the tests, histopathological analysis was conducted on the animals' organs for toxicity (liver, intestine, and kidneys) and edema (liver and kidneys). The animals were stored in labeled cassettes and immediately fixed in Bouin's solution. Decalcification was then performed in a 7% EDTA solution for 48 hours. The material was dehydrated in increasing concentrations of ethyl alcohol, diaphanized in xylene, and embedded in paraffin (Inlab). Histological sections were then obtained at a thickness of 5 µm using a microtome (SLEE Medical) and stained with Hematoxylin and Eosin. The analysis was conducted under an optical microscope (Olympus BX41-Micronal Microscope) [20].

2.12.1 Evaluation of Histopathological Changes in the Toxicity Test

The histopathological alterations index (HAI) was determined by the extent of tissue alterations observed in the liver, intestine, and kidneys. The alterations can be classified into stages I, II, and III. The HAI value indicates whether the organ is healthy (0 to 10), has mild to moderate alterations (11 to 20), moderate to severe alterations (21 and 50), or contains irreversible alterations (> 100) [21,22]. Thus, the indices were calculated according to the following equation:

$$I = \frac{\sum_{i-1}^{na} ai \; + 10 \; \sum_{i-1}^{nb} bi \; + 10^2 \sum_{i-1}^{nc} ci}{N}$$

Where: **a**: first stage alterations; **b**: second stage alterations; **c**: third stage alterations; **na**: number of alterations considered as the first stage; **nb**: number of alterations considered as the second

stage; nc: number of alterations considered as the third stage; N: number of fishes analyzed per treatment.

2.8.2 Statistical Analysis

Data were expressed as the mean ± standard error of the mean for each experimental group. GraphPad Prism (version 7.0) was used for the statistical analysis of the results obtained. ANOVA (one-way) was applied, followed by the Tukey-Kramer test to compare the means between the treated animals and the control groups, with significant values set at $p < 0.05$.

3. Results

3.1 Characterization of the Nanoformulation Obtained from *B. orellana* Oil (NBO)

As analyzed by gas chromatography, the chromatogram (Figure 1) enabled the integration of the peak corresponding to δ-tocotrienol ($C_{27}H_{40}O_2$), with a retention time of 28.6 minutes. The average concentration of δ-tocotrienol was 0.725 ± 0.062 mg/mL (72.0 ± 1.0%), calculated from the straight-line equation obtained for δ-tocotrienol at various concentrations. The linearity coefficient (R^2) result was 0.9973, and the resulting straight-line equation was $y = 8.30 \times 106\ x - 2.818 \times 106$.

The nanoprecipitation method, adapted from Carvalho et al., prepared a nanoparticulate formulation containing OPBO. In this method, no visual changes related to loss of stability were observed over the evaluation period. The formulation showed no phase separation or precipitation of components, demonstrating a visibly stable appearance, a slightly yellowish color, and a pH of 5.8.

In this study, the average size of the nanoparticles obtained was stable, 53.15 ± 0.64 nm, with a PDI of 0.574 ± 0.032 on the first day. On the 30th day, 59.90 ± 3.63 nm was observed with a PDI of 0.574 ± 0.032. Additionally, the nanoformulation exhibited a zeta potential of 19.86 ± 0.60 mV on day zero and 19.66 ± 1.45 mV on the 30th day of analysis (Table 1).

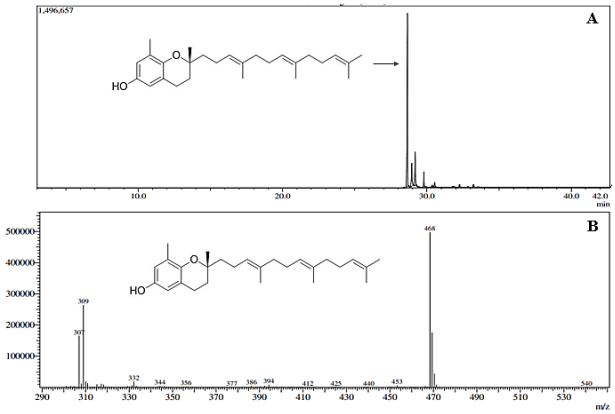

Figure 1: (A) Chromatographic Profile of OPBO with the Main Peak Corresponding to δ-Tocotrienol ($C_{27}H_{40}O_2$) at a Retention time of 28.6 min. (B) Fragmentation Spectrum Corresponding to δ-Tocotrienol with a Molecular ion of 309 m/z

	Zeta Potential (mV)	Average size (nm)	PDI
Day 0	19.86±0.60	53.15±0.64	0.533±0.008
Day 7	15.26±0.32	58.06±0.76	0.594±0.007
Day 30	19.66±1.45	59.90±3.63	0.594±0.014

Table 1: Analysis of the Zeta Potential (mV), Average Size (nm), and Polydispersity Index (PDI) of the Nanodispersion (NBO)

Analyzed at 0, 7, and 30 days after obtaining the NBO, showing the average and standard deviation (n = 3).

3.2 In Silico Analysis pkCSM, Molecular Docking and Functional Density Theory

Table 2 provides the ADMET and toxicity profile of δ-tocotrienol, highlighting its safety and pharmacokinetic attributes (Figure 2). The predicted lethal dose (LD50) for δ-tocotrienol is 500 mg/kg, classifying it under toxicity class 4, which indicates low acute toxicity. It does not exhibit AMES toxicity, implying it is non-mutagenic. The maximum tolerated dose for humans is predicted to be 0.538 mg/kg, demonstrating its relative safety. While it does not inhibit hERG I channel, it is a known inhibitor of hERG II channels, which could have implications for cardiac safety.

Properties	δ-tocotrienol
Predicted LD50 (mg/kg)	500
Predicted Toxicity Class	4
AMES toxicity	No

Max. tolerated dose (human)	0.538
hERG I inhibitor	No
hERG II inhibitor	Yes
Oral Rat Acute Toxicity (LD50) (mol/kg)	2.047
Oral Rat Chronic Toxicity (LOAEL) (log mg/kg, bw/day)	2.810
Hepatotoxicity	No
Skin Sensitisation	No
T. Pyriformis toxicity (log ug/L)	1.931
Minnow toxicity (log mM)	-2.229

Table 2: ADMET and Toxicity Profile δ-Tocotrienol

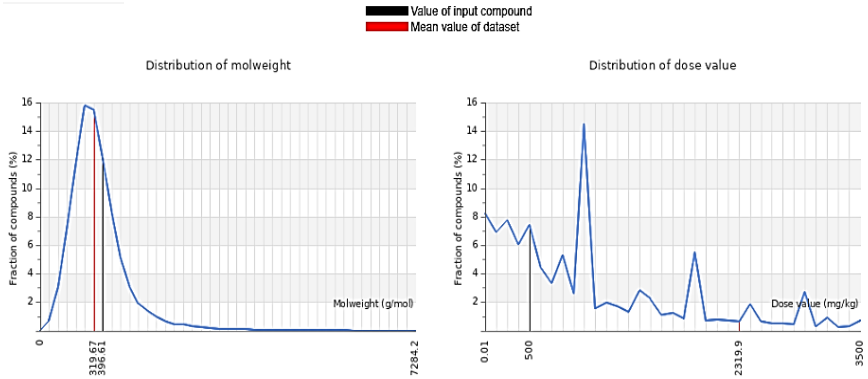

Figure 2: Comparison of Molecular Weight and Dose-Dependent Profiles of δ-Tocotrienol

δ-tocotrienol	
Binding Energy (kcal/mol)	-6.0
Ligand efficiency	-0.210
Estimated Inhibition constant {(Ki) (μM)}	40.5

Table 3: Results of Binding Interactions of the Compounds with Target TRAP1

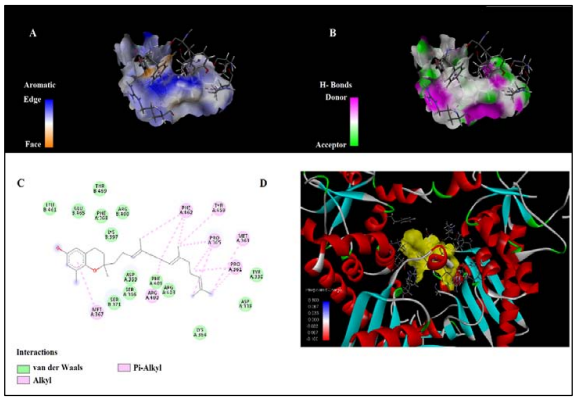

Figure 3: Interactions of δ-Tocotrienol with Target TRAP1: A) Aromatic Surface Representation B) H-Bond Distribution, C) 2D Ligand Interaction, D) 3D Representation of Docked Poses

Table 4 and Figure 3 present the predicted molecular interactions between δ-tocotrienol and the target protein structure TRAP1. The interactions predominantly involved hydrophobic contacts, such as alkyl and π-alkyl interactions, which are crucial for stabilizing the binding of δ-tocotrienol within the protein's active site. Alkyl interactions were observed with residues PRO361, PRO365, and ARG400, ranging from 3.14 Å to 4.60 Å. Specific contacts included δ-tocotrienol's C21 interacting with PRO365 at a distance of 3.14 Å and C27 interacting with MET363 at a distance of 4.33 Å. These interactions highlight key hydrophobic engagements contributing to the compound's binding affinity.

Additionally, π-alkyl interactions were identified with aromatic residues TYR459, PHE462, and MET367 at distances ranging from 4.25 Å to 4.95 Å. Multiple π-alkyl contacts were observed between PHE462 and various regions of δ-tocotrienol, underlining the aromatic ring's role in stabilizing the binding. These findings underline δ-tocotrienol's potential for binding to TRAP1, driven by a combination of alkyl and π-alkyl in-teractions. This makes it a promising candidate for further therapeutic exploration targeting this protein.

The Density Functional Theory (DFT) analysis of δ-tocotrienol provides insight into its electronic properties, as summarized in Table 5. The energy of the highest occupied molecular orbital (EHOMO) is -0.1923 eV, indicating its ability to donate electrons. The lowest unoccupied molecular orbital energy (ELUMO) is 0.0051 eV, reflecting its potential to accept electrons. The energy gap (ΔE) between these orbitals is 0.1974 eV, indicating a balance between the molecule's stability and reactivity (Figure 4).

Amino acid	Interacting	Distance (Å)
A:PRO361 - :[001	Alkyl	4.02
A:PRO365 - :[001	Alkyl	3.86
A:ARG400 - :[001	Alkyl	4.60
:[001:C21 - A:PRO365	Alkyl	3.14
:[001:C26 - A:PRO365	Alkyl	4.11
:[001:C27 - A:PRO361	Alkyl	4.19
:[001:C27 - A:MET363	Alkyl	4.33
A:TYR459 - :[001:C21	Pi-Alkyl	4.74
A:PHE462 - :[001:C16	Pi-Alkyl	4.61
A:PHE462 - :[001	Pi-Alkyl	4.95
A:PHE462 - :[001:C21	Pi-Alkyl	4.25
:[001 - A:MET367	Pi-Alkyl	4.78

Table 4: Predicted Interactions of Docked Conformations of δ-Tocotrienol Against Structure of TRAP1

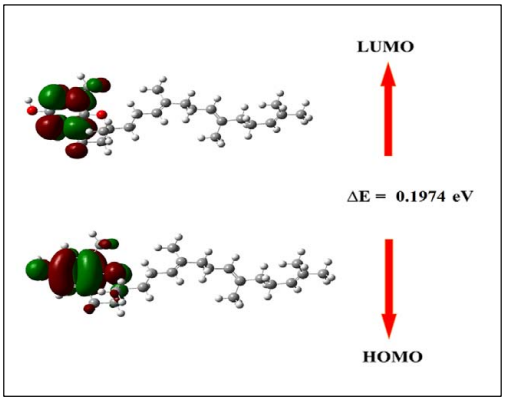

Figure 4: HOMO-LUMO Images of δ-Tocotrienol

Parameters(eV)	δ-tocotrienol
EHOMO (eV)	-0.1923
ELUMO (eV)	0.0051
ΔE(LUMO-HOMO) (eV)	0.1974
Ionization Energy I = -E(HOMO)(eV)	0.1923
Electron Affinity A = -E(LUMO) (eV)	-0.0051
Electronegativity index χ = (I + A)/2 (eV)	0.0936
Chemical potential μ = - χ	-0.0936
Chemical Hardness η = (I - A)/2 (eV)	0.0987
Softness 1/η eV−1	10.13
Electrophilicity index ω = μ2/2 η	0.0444

Table 5: Key Parameters in Density Functional Theory Analysis and Their Values

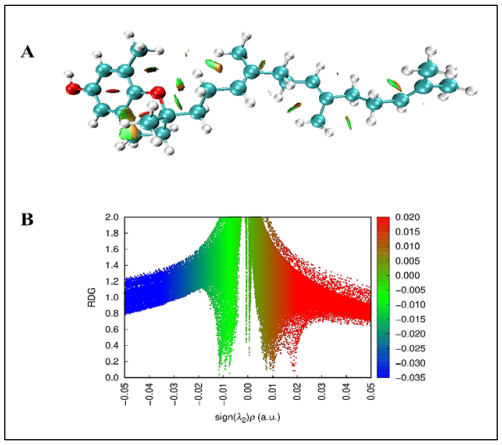

Figure 5: A) Reduced Density Gradient Colored Map b) RDG Plot of δ-Tocotrienol Molecule

3.3 Evaluation of Acute Toxicological Effects and LD50 in Zebrafish

In the acute toxicity test, the animals were administered different doses of the injectable nanodispersion of *Bixa orellana* oil **(Chronic in®)** via intraperitoneal injection. They showed behavioral alterations classified as Stages I, II, and III. The animals were then subjected to observational screening, resulting in alterations that began with an increase in the animal's excitability, loss of posture, and deposition of the animal at the bottom of the aquarium, ultimately leading to death.

The toxicity assessment (Table 5) observed that the 250 mg/kg NBO dose did not elicit any behavioral changes indicative of toxicity. The 500 mg/kg dose resulted in 2 deaths within 48 hours of treatment. In contrast, the animals treated with the 750 mg/kg dose exhibited stage II and III behaviors and mortality within less than 24 hours of administration. For the high dosages of 1000 and 1500 mg/kg of NBO, the evolution of all stages was noticeable in a short period and resulted in significant mortality within 3 hours of evaluation.

				GROUPS (N=8)		
Time	**Stages**	**NBO 250 mg**	**NBO 500 mg**	**NBO 750mg**	**NBO 1000 mg**	**NBO1500 mg**
	I	1 (8)	1 (8)	1 and 2 (8)	1 (8)	1 (8)
1h	II	1 (8)	1 (8)	1 and 2 (8)	1 and 2 (8)	1 and 2 (8)
	III				1 and 2 (8)	1, and e 3 (8)
	I	1 (7)	1 (8)			
3h	II	1 (5)	1 (5)	1 and 2 (8)	1 and 2 (8)	
	III		1	1 and 2 (8)	1, 2 and 3 (6)	
	I	1 (5)	1 (6)			
9h	II	1 (3)	1 (5)	1 and 2 (8)		
	III			1 and 2 (8)		
	I					
24h	II		1 (6)	2 (8)		
	III			1, 2 and 3 (3)		
	I	1 (3)	1 (8)			
48h	II		1 and 2 (3)			
	III		3 (2)			

Table 6: Behavioral Alterations were Evaluated in the Toxicity test of Different Concentrations of NBO - *Bixa Orellana* Nanodispersion oil (Chronic in®) in Zebrafish

Stage I: 1) increased swimming activity and 2) tremors in the tail axis; Stage II: 1) Circular swimming and 2) Posture loss; Stage III: 1) Motility loss; 2) Deposition of the animal at the bottom of the aquarium and 3) Death. (n) Number of animals with alterations.

The liver, kidneys, and intestines were analyzed in the histopathological evaluation of the animals submitted to the toxicity test (Figure 6). In the assessment of the liver, it was observed that with increasing doses, there was an increase in alterations, especially in the groups treated with doses of 750, 1000, and 1500 mg/kg, which showed significant nuclear

degeneration, cytoplasmic degeneration, nuclear atrophy, and tissue necrosis. In the other organs, alterations were evident at doses above 750 mg/kg, such as in the photomicrographs of the kidneys, where leukocyte infiltration, tubular degeneration, and tissue necrosis were observed. Photomicrographs of the zebrafish intestine revealed alterations, including goblet cell hypertrophy, displacement of the lamina propria, and villous degeneration.

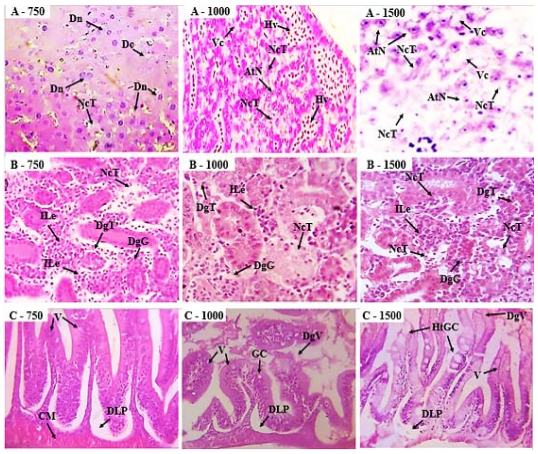

Figure 6: A - Photomicrographs of zebrafish liver in longitudinal sections of the groups (750, 1000, 1500 mg/kg). Dn (nuclear degeneration); Dc (cytoplasmic degeneration); Vc (cytoplasmic vacuolization); Hv (hypervascularization); AtN (nuclear atrophy); NcT (tissue necrosis). B - Photomicrographs of zebrafish kidneys in longitudinal sections of the groups (750, 1000, 1500 mg/kg). ILe (leukocyte infiltrate); DgT (tubular degeneration); NcT (tissue necrosis). C - Photomicrographs of zebrafish intestines in longitudinal sections from the 750, 1000, and 1500 mg/kg groups. The intestinal tissue shows villi (V), goblet cells (GC), muscle layer (CM), goblet cell hypertrophy (HtGC), and displacement of the lamina propria (DLP). DgV (villous degeneration)

In the determination of the histological alteration index (Figure 7) of the liver, kidney, and intestine organs of zebrafish exposed to treatments with 250, 500, 750, 1000, and 1500 mg/kg of NBO (Chronic in®), a significant increase in histological damage ($p < 0.01$) was observed in the organs evaluated from the 750 mg/kg dose with an index of moderate alterations (11-20) in both the liver, kidneys and intestine. For the 1000 and 1500 mg/kg doses, mild to severe alterations were observed (21-50), with highly significant values ($p < 0.001$) concerning the groups with lower doses (250 and 500 mg/kg), indicating a correlation between the higher the dose and the greater the histopathological damage.

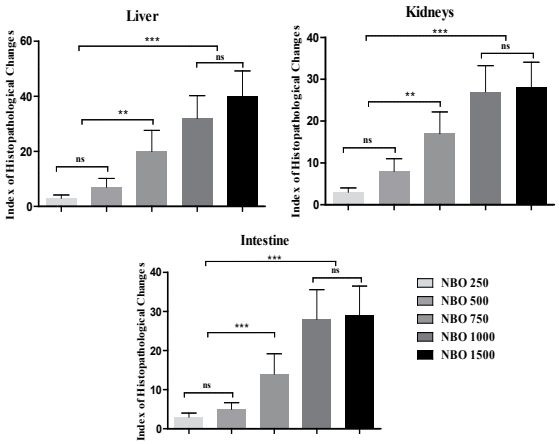

Figure 7: Index of histological changes in zebrafish liver, kidneys, and intestines exposed to treatments with 250, 500, 750, 1000, and 1500 mg/kg NBO (Chronic in®). The values represent the mean and standard devia-tion. ** ($p < 0.01$) and *** ($p < 0.001$) represent statistically significant results

When determining the 50% lethal dose (LD50), the percentage of dead animals was compared to the increasing NBO doses (Figure 8). It was observed that deaths occurred as the administered dose increased. Thus, 100% lethality of the animals was observed in the group receiving a 1500 mg/kg dose within a maximum of 1 hour. 1000, 750, and 500 mg/kg doses showed deaths between 3 and 48 hours, with a lethality rate of 75%, 37.5%, and 25%, respectively. Based on the data analysis using linear regression (Figure 8) and obtaining the straight-line equation $y = 0.08176x—17.91$, the LD50, which has a value of 830.6 mg/kg, was determined, representing the dose that kills 50% of the animal population.

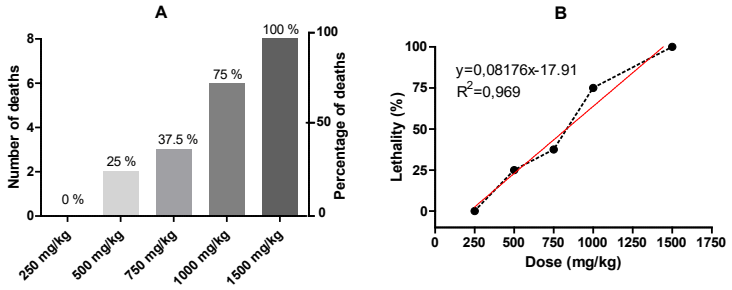

Figure 8: Absolute number and percentage of deaths in the groups treated with different doses of *B. orellana* nanodispersion. B. Lethality curve according to the administered dose and straight-line equation y = 0.08176x-17.91. A lethal dose of 50% (LD50) of 830.6 mg/kg was calculated.

3.4 Anti-inflammatory Activity of *Bixa orellana* Nanodispersion Oil

The inflammation study evaluated the evolution of edema (Figures 9 and 10). In the negative control group treated with the NBO vehicle (VEH), there was intense edema during the evaluation period. On the other hand, in the groups treated with doses of NBO, a significant reduction ($p < 0.001$) in edema was observed in the groups treated with 20 and 40 mg/kg, as well as in the group treated with diclofenac 25 mg/kg (DCF). In the control group treated with PBS solution, it was impossible to observe edema formation, as no carrageenan was administered, and the animals remained stable throughout treatment. Regarding the percentage of edema inhibition, it was observed that the DCF group showed the highest rate of inhibition, with 68.42 ± 6%. The groups treated with NBO at 20 and 40 mg/kg showed 59.0% and 58.5% inhibition percentages, respectively. However, in the group treated with NBO at 10 mg/kg, no inhibition more significant than 10% was observed.

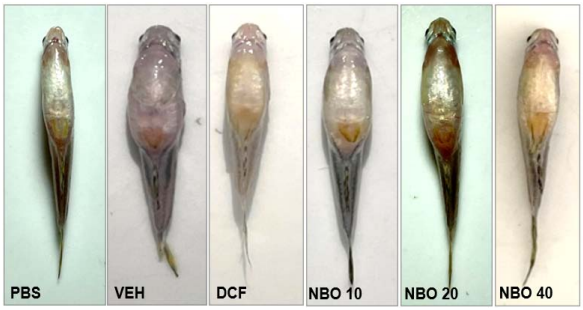

Figure 9: Treatment effects on edema formation in zebrafish after 5h of treatment. (PBS), DCF (Diclofenac 25 mg/kg), VEH (Nanodispersion vehicle), NBO10 (NBO 10 mg/kg), NBO 20 (NBO 20 mg/kg) and NBO 40 (40 mg/kg)

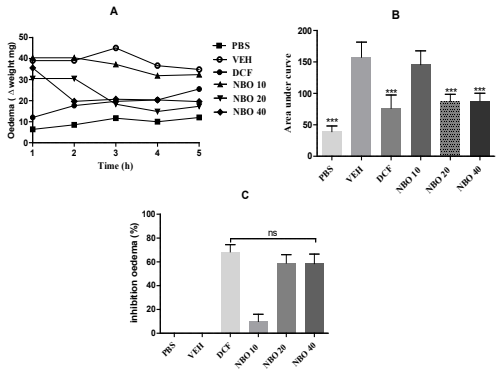

Figure 10: Treatment effect on intraperitoneal edema induced by carrageenan. (A) Evolution of edema over time. (B) The area under the curve of the results. (C) Percentage of edema inhibition. Groups (PBS), DCF (Diclofenac 25 mg/kg), and NBO doses of 10, 20, and 40 mg/kg. *** ($p < 0.001$) represents statistically sig-nificant results compared to the NBO vehicle group

3.5 Histological Evaluation of the Organs of Animals Submitted to the Inflammation Test

In the histopathology evaluation of the animals submitted to the inflammation test, the liver and kidneys were analyzed to assess the possibility of tissue alterations. In the analysis of the liver (Figure 11), all the groups analyzed showed structural aspects within the normal range; no tissue conditions indicated toxicity or damage. Normal hepatocytes were observed, some with cytoplasmic vacuolization and increased hyperemia (blood vessels).

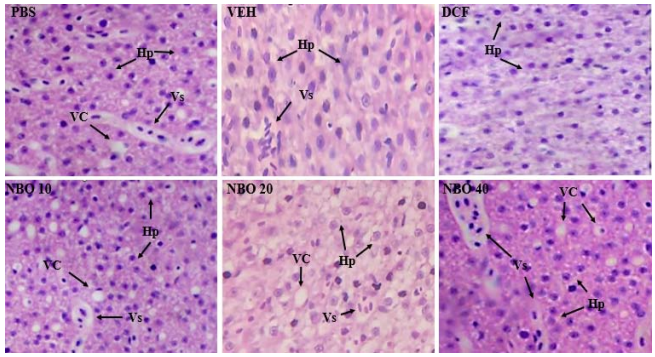

Figure 11: Photomicrographs of zebrafish liver in longitudinal sections from the groups (PBS), DCF (Diclofenac 25 mg/kg), VEH (Nanodispersion Negative Control), NBO 10 (NBO 10 mg/kg), NBO 20 (NBO 20 mg/kg) and NBO 40 (NBO 40 mg/kg). Hp (normal hepatocytes; VC (cytoplasmic vacuolization); Vs (blood vessel).

In evaluating the renal histopathology of the Zebrafish (Figure 12), the kidneys showed few alterations, with normal renal tubules, except for the VEH group, which exhibited intense leukocyte infiltration, tissue damage, and tubular degeneration. In the groups treated with NBO at 10 and 20 mg/kg, a slight leukocyte infiltrate was observed, with no tissue damage, except in the NBO 40 mg/kg group, which showed slight tubular damage and a leukocyte infiltrate.

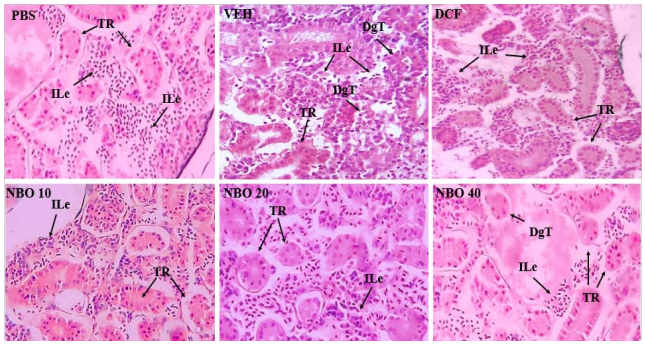

Figure 12: Photomicrographs of zebrafish kidneys in longitudinal sections from the groups (PBS), DCF (Diclofenac 25 mg/kg), VEH (Nanodispersion Negative Control), NBO 10 (NBO 10 mg/kg), NBO 20 (NBO 20 mg/kg) and NBO 40 (NBO 40 mg/kg). TR (renal tubules) ILE (leukocyte infiltrate); DgT (mild tubular degeneration)

4. Discussion

The present study demonstrates that the injectable nanodispersion of *Bixa orellana* oil (NBO) exhibits physicochemical characteristics consistent with pharmaceutical stability, notably a particle size below 60 nm, low polydispersity index, and stable zeta potential. These features align with findings by de Oliveira Carvalho [11] who reported that nanosystems with reduced particle sizes (50–200 nm) exhibit enhanced stability, bioavailability, and reduced aggregation. The observed zeta potential values above +15 mV further support the colloidal stability of the nanodispersion, as previously indicated by Zielińska et al. [23-29].

The silico pharmacokinetic and toxicity analyses of δ-tocotrienol, the major compound in NBO, revealed low acute and chronic toxicity, absence of hepatotoxicity and skin sensitization, and no AMES mutagenicity. However, the predicted inhibition of the hERG II channel raises a safety concern at high concentrations, consistent with literature that attributes potential cardiotoxicity to drugs interacting with the lipophilic hERG pore [30,31]. These results align with in vivo data demonstrating safety at lower doses and toxicity at higher concentrations.

In the acute toxicity assay, zebrafish administered 750 mg/kg or higher doses of NBO exhibited severe behavioral alterations and histopathological lesions in the liver, kidneys, and intestines, culminating in a calculated LD50 of 830.6 mg/kg. These findings reflect organ-specific sensitivity to toxic agents, as described by Carvalho [21], reinforcing the value of zebrafish as a reliable model for toxicological screening [32-39]. The behavioral patterns observed initial excitability followed by loss of posture and eventual immobility are consistent with prior studies on zebrafish toxicity induced by phytotherapeutic compounds [19,20].

Histopathological evaluations confirmed a dose-dependent increase in tissue damage, particularly at 750 mg/kg or higher doses. Hepatic lesions included nuclear and cytoplasmic degeneration, vascular congestion, and necrosis. Renal alterations, such as tubular degeneration and leukocyte infiltration, corroborated findings from Omar et al and Souza et al who highlighted these changes as markers of nephrotoxicity. Notably, intestinal damage, including goblet cell hypertrophy and villous degeneration, was dose-dependent [40,41].

Interestingly, the predicted interaction between δ-tocotrienol and TRAP1, as evidenced by a moderate binding energy of -6.0 kcal/mol and an inhibition constant (Ki = 40.5 μM), suggests a possible mechanism of mito-chondrial-mediated toxicity. TRAP1 is a mitochondrial heat shock protein (HSP) known for its anti-apoptotic and cytoprotective roles [35,36]. The inhibition of TRAP1, as demonstrated in studies on cancer models, can impair cellular stress responses and promote apoptosis, thereby supporting its involvement in the observed toxicity profile [37,38].

In contrast, the anti-inflammatory activity of NBO was significant at doses of 20 and 40 mg/kg, comparable to the reference drug diclofenac (25 mg/kg). This effect is consistent with earlier reports of tocotrienol-mediated downregulation of inflammatory pathways, including the inhibition of COX-2 and PLA2 [7,44-48]. Carrageenan-induced edema is mediated by pro-inflammatory mediators such as prostaglandins and histamine; the inhibition of these pathways by NBO supports its therapeutic potential.

After anti-inflammatory assays, histological analysis of liver and kidney tissues confirmed the absence of significant tissue alterations at therapeutic doses. Only minor findings, such as discrete vacuolization and leukocyte infiltration, were observed, notably in the vehicle group. This supports the safety of NBO at effective anti-inflammatory concentrations.

Finally, prior studies using *Bixa orellana* extracts in rodents at doses of up to 4000 mg/kg have shown no acute toxicity or mortality [32–34], indicating a good safety margin for oral administration. However, the injectable route and nanoformulation may alter pharmacodynamics and bioavailability, which could explain the higher toxicity observed at elevated doses in the zebrafish model.

Altogether, the data supports the therapeutic potential of NBO as a low-dose anti-inflammatory agent while emphasizing the importance of dose optimization to avoid toxic effects. The zebrafish model, supported by in silico analyses, proved valuable for assessing both the efficacy and safety of this novel nanoformulation.

5. Conclusions

The results of this study demonstrate that the injectable nanodispersion of *Bixa orellana* (NBO) exhibits a significant anti-inflammatory effect in zebrafish, particularly at doses of 20 and 40 mg/kg, without causing relevant histopathological changes in the evaluated organs. On the other hand, doses above 500 mg/kg induced progressive acute toxicity, with behavioral alterations, histological damage in the kidneys, liver, and intestines, and a median lethal dose (LD_{50}) estimated at 830.6 mg/kg.

In silico analyses, it suggests that the potential mechanism underlying NBO toxicity may involve the inhibition of the cardiac hERG II channel and TRAP1 protein, indicating a possible risk of adverse effects at higher doses. These findings reinforce the safety and efficacy of NBO at low doses as an anti-inflammatory agent while highlighting the pharmacological safety limits associated with its use in injectable formulations.

Therefore, this study contributes to the advancement of nanoformulated herbal medicines by providing relevant experimental evidence on the anti-inflammatory activity and toxicological profile of NBO, establishing a foundation for future preclinical and clinical studies aimed at its therapeutic application.

Patents

The work is part of patent BR 10 2023 006029 3 A2, "Composition, the process for obtaining polymeric nanoparticles containing *Bixa orellana* oil standardized in total tocotrienols, the process of the polar microparticulate system or natural microcluster, and its uses," deposited at the National Institute of Industrial Property in Brazil.

Author Contributions: Investigation, L.P.G., N.N.D.C., H.O.C., B.V.M.F., V.M.V., G.C.F., and D.E.S.G.; formal analysis, T.M.L., and I.D.R.P.; data curation, H.R.S.; software, E.C.A.; conceptualization, supervision, project administration, and funding acquisition, J.C.T.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The authors followed the legislation guidelines for animal experimentation established by the National Council for Animal Experimentation of Brazil (CONCEA). The Ethics Committee on the Use of Animals (CEUA) of the Federal University of Amapá-UNIFAP approved the project under Protocol No. 001/2023.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

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