

# Original Research Article

## Impact of Levodopa and Black Pepper Essential Oil in a Zebrafish Model of Rotenone-Induced Parkinson Disease

### ABSTRACT

Parkinson's disease, the second most common neurodegenerative disease, affects approximately 2 to 3% of individuals over the age of 65. It is characterized by the irreversible degeneration of cells in the central nervous system, compromising motor, physiological, and cognitive functions. The standard treatment is levodopa, a precursor of dopamine, whose prolonged use can cause motor complications, such as dyskinesia. Therefore, it is essential to develop alternative therapies that allow the dose of levodopa to be reduced without losing its efficacy, minimizing side effects. The present study evaluated the protective effect and toxicity of L-dopamine and *Piper nigrum* essential oil and their mixture in zebrafish embryos and larvae exposed to rotenone as an experimental model for Parkinson's-like disease. Parameters such as survival, hatching rate, teratogenicity, morphometry and behavioral tests (thigmotaxis, touch sensitivity, and optomotor response) were evaluated to investigate whether L-dopamine and *Piper nigrum* essential oil cause toxicity or can act as protective agents against the effects induced by rotenone, a highly toxic pesticide that causes behavioral alterations similar to symptoms of Parkinson's disease. Exposure to L-dopamine also resulted in defects in embryonic-larval development and behavioral alterations, showing a limited protective effect against toxicity induced by rotenone. On the other hand, *Piper nigrum* affected embryonic-larval development but showed no significant impact on zebrafish behavior. Furthermore, *Piper nigrum* showed no protective effect against defects induced by rotenone. It was concluded that rotenone is a chemical that induces phenotypes similar to Parkinson's disease in the zebrafish model. However, L-dopamine and *Piper nigrum* proved to be ineffective therapeutic alternatives to treat these symptoms in this model, presenting some toxic and synergistic effects. Thus, we emphasize the need to continue research in search of therapeutic approaches that can mitigate the effects of the disease induced by toxic agents such as rotenone in animal models.

**Keywords:** *Danio rerio*, Teratogenic effect, Mortality, Animal behavior.

### 1. INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, affecting between 2% and 3% of the population over 65 years of age (Balestrino and Schapira, 2020; Poewe et al. 2017). Furthermore, Mhanna et al. (2024) demonstrated that prodromal symptoms of PD, such as hyposmia, constipation, sleep disturbances and depression, can appear long before motor manifestations, which, because they are subtle and nonspecific, make diagnosis difficult in its early stages. PD is also responsible for significant morbidity, affecting 1 to 2 people in every 1,000 and totaling approximately 10 million individuals worldwide (Bang et al. 2021; Tysnes and Storstein, 2017). With the aging of the population, PD diagnoses have increased significantly, to the point of being considered a pandemic (Murakami et al. 2023). PD is an irreversible neurodegenerative

condition characterized by the progressive degeneration of dopaminergic neurons in a specific region of the substantia nigra (Kalia & Lang, 2015). Additionally, the frequent accumulation of Lewy bodies is observed, which is associated with the development of dementia (Gao, 2019). Currently available medications for treating PD aim to stimulate dopaminergic neurotransmission to alleviate motor symptoms; however, no existing drug is capable of completely curing the disease (Kalia & Lang, 2015).

To treat the symptoms of PD, levodopa (L-DOPA) is the medicine conventionally used in patients. When administered, this medication elevates dopamine levels in the brain, compensating for the deficiency caused by the degeneration of substantia nigra cells in PD disease (Liu et al. 2019). However, prolonged use of L-DOPA in high doses causes oxidative stress and is associated with unwanted side effects (Brinez-Gallego et al. 2023; Liu et al. 2019). Therefore, there is a need to evaluate new therapeutic alternatives that can reduce L-DOPA doses, as well as minimize possible toxic effects. Moreover, Mou et al. (2020) reported that oxidative stress, resulting from dopamine and its metabolism process, can induce the degeneration of dopaminergic neurons. Additionally, dopamine functions can increase intracellular calcium levels, further intensifying oxidative stress. Therefore, new therapeutic approaches are needed that allow the reduction of L-DOPA doses without compromising the effectiveness of PD treatment and minimizing motor and cognitive side effects (Naskar et al. 2015).

Natural bioproducts, such as essential oils, stand out as promising options due to their plant-based composition (Nascimento et al. 2020). Currently, around 64% of drugs are of natural origin, reflecting the interest in phytochemical molecules with therapeutic effects on neurodegenerative diseases, including PD (Valli et al. 2013; Shahpiri et al. 2016). In their study, Aebischer et al. (2021) reported that antioxidants from plants of the Lamiaceae family are significant in natural medicine and pharmacology. The evaluation of essential oils from these plants showed that thyme (*Thymus vulgaris*) and savory (*Saturejahortensis*) have the highest antioxidant capacity, due to the presence of thymol and carvacrol. Furthermore, marjoram, sage, and hyssop also demonstrated significant activity, possibly due to compounds such as terpinene and o-cymene, which may act synergistically to enhance the antioxidant effect.

In view of this, essential oils (EO) can gain importance, such as the EO of black pepper (*Piper nigrum* - PN), which has in its composition the phytochemical  $\beta$ -caryophyllene. Due to its anti-inflammatory properties and interaction with cannabinoid receptors,  $\beta$ -caryophyllene has been studied as a possible therapy for neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Horvath et al. 2012; Klauke et al. 2014). Studies in animal models have demonstrated that piperine may be effective in treating inflammation and neurodegenerative diseases such as PD (Chen et al. 2022; Sabina et al. 2011). Thus, it is essential to explore new therapeutic approaches, including different essential oils, using conventional animal models since traditional therapies may cause significant side effects.

The zebrafish has been used as an experimental model of PD both in its larval stage and in the adult stage for drug screening, thus gaining an important role in research involving not only PD but other chronic diseases (Andrade et al. 2023; Kalyn et al. 2019). Additionally, the combination of rapid neurological development and optical transparency during zebrafish embryogenesis makes it possible to investigate diseases such as PD (Kalyn et al. 2019), given that the dopaminergic projection in these animals is fully developed between 3- and 4-days post-fertilization (Du et al. 2016). In addition, the physiological and anatomical homology between zebrafish and humans is evidenced in most organs, including the nervous system (Vaz et al. 2018). Andrade et al. (2023) developed an embryo-larval model

of rotenone-induced PD. Thus, we used this model to evaluate the toxic and protective effect of drugs on parameters related to embryonic and neurobehavioral development.

Rotenone (RT) is a natural organic molecule and in excess, it degenerates dopaminergic neurons inducing their death and deforming Lewy bodies. This is due to the fact that RT causes the accumulation of  $\alpha$ -synuclein in rats treated with it (Doyle and Croll 2022). Yet, due to its lipophilic nature, RT can easily penetrate the blood-brain barrier and cell membranes, affecting various organelles, including mitochondria (Azimullah et al. 2023). Thus, generating clinical and pathological characteristics of PD, due to the inhibition of mitochondrial complex I (Rocha et al. 2022). This pesticide, although a natural lipophilic compound, is a prototype of an exogenous toxin that can induce Parkinson's-like disease phenotypes in animal models (Kalyn et al. 2019). The same authors emphasized that RT has been administered in rodents as an animal model for PD, as well as hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

Thus, although the literature regarding the use of  $\beta$ -caryophyllene as a protective agent in an embryo-larval model using RT is scarce and/or non-existent, it is worth noting that this sesquiterpene, whether alone or in combination, may have potential therapeutic effects. In this context, our study evaluated the protective effect and toxicity of L-DOPA and PN EO and their mixture in zebrafish embryos and larvae exposed to rotenone as an experimental model for Parkinson's-like disease. In our study, parameters such as survival, hatching rate, teratogenic effects, morphometry, and neurobehavioral tests were evaluated in order to know if L-DOPA and PN EO induce toxicity and can be used as protective agents in this animal model.

## 2. MATERIAL AND METHODS

### 2.1 Maintenance and breeding of animals

The experiments were carried out at the Laboratory of Ecophysiology and Animal Behavior (LECA) of the *Universidade Federal Rural de Pernambuco* (UFRPE). The methods were previously approved by the Ethics Committee on the Use of Animals (CEUA) (License No. 3581030221) of the same institution. Adult animals of the species zebrafish (*Danio rerio*, wild type strain, nine months old) were raised and housed in the vivarium. These animals were housed in 80-liter aerated aquariums, fed three times a day, twice with extruded commercial feed (30% crude protein), and once with *Artemia salina* (*Artemia* spp.). The photoperiod was controlled at 10/14 h (light/dark), the temperature at  $26 \pm 1$  °C, and the pH at  $7.0 \pm 0.5$ . In addition, other abiotic parameters such as dissolved oxygen, ammonia, nitrite, and nitrates were maintained at acceptable levels for the species (OECD 2013; Westerfield 2000).

To obtain the eggs, adult animals were separated according to sex in breeding tanks for zebrafish (Zebclean, Alesco), using males and females in an 8:4 ratio (Westerfield 2000). After spawning, viable eggs were collected and evaluated by a light microscope (with LED lamp) at 1-hour post-fertilization (hpf) (Cadena et al. 2020a). Only eggs whose spawning was more than 90% viable were used (OECD 2013). The eggs were randomly placed in sterile polystyrene chambers with a capacity of 80 mL (16 experimental groups containing fifteen eggs per chamber), kept in an incubator with the same physicochemical parameters described above, and considered optimal for the species.

### 2.2 Chemicals, solvents and solutions

The reagents used in this study, rotenone (RT) (CAS# 83-79-4, purity  $\geq 95\%$ ) and dimethyl sulfoxide (DMSO) (CAS# 67-68-5, purity  $> 99\%$ ), were purchased from Sigma (St. Louis,

MO, USA) and *Dinâmica* (Indaiatuba, SP, Brazil), respectively. Initially, a stock solution containing 0.02 mg/L of rotenone was prepared, which was subsequently diluted in dechlorinated water to reach a nominal concentration of 11.25 µg/L of RT (Andrade et al., 2022), used to induce parkinsonian effects in zebrafish.

For the treatment of these effects, the drugs levodopa (L-DOPA, CAS# 59-92-7) and benserazide hydrochloride (CB, CAS# 10035-04-8), purchased from commercial establishments, were used. *Piper nigrum* essential oil (EO) (CAS# 8006-82-4, purity > 99%) was obtained from Lazlo (Belo Horizonte, MG, Brazil). To prepare the solutions, 100 mg L-DOPA and 25 mg CB tablets were macerated to reach nominal concentrations of 0.20 mg/L L-DOPA (1000 mM) and 0.05 mg/L CB (170 mM), respectively. Then, 0.05 g of *P. nigrum* EO was dissolved in DMSO (0.1% V/V), resulting in a nominal concentration of 5.00 mg/L. The nominal concentration of DMSO used was less than 0.01%, i.e., 50 times lower than the concentration known to cause behavioral changes (Hedge et al., 2023). This solution was diluted with dechlorinated water to reach a concentration of 1.00 mg/L of EO. *Piper nigrum* essential oil was previously characterized by the manufacturer, by means of gas chromatography, which identified the following main components: β-caryophyllene (25.5%), limonene (19.8%), β-pinene (13.8%), eugenol (7.2%), terpinen-4-ol (5.8%) and α-pinene (4.1%). All other reagents used in this study were of analytical or chromatographic grade. The final nominal concentrations of each experimental group are described in Table 1.

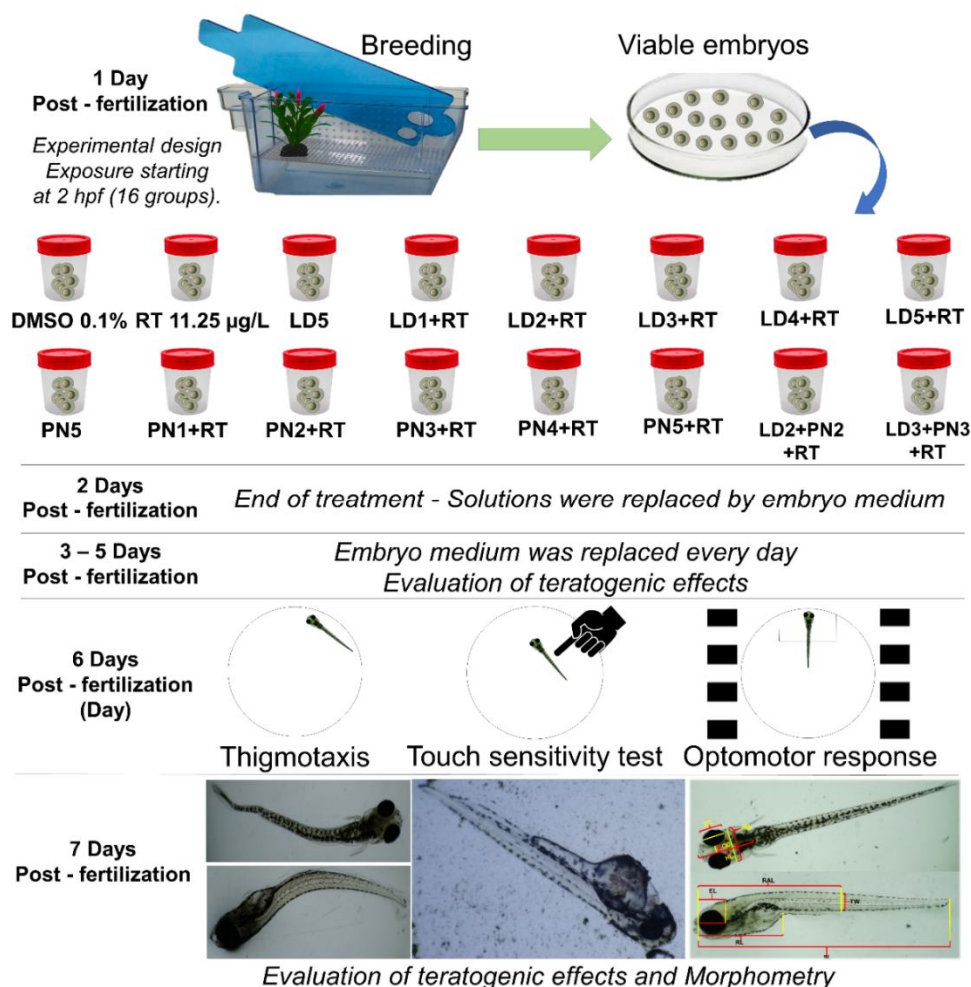
### 2.3 Assessment of the toxicity of chemical compounds and their mixtures

The toxicity tests of the chemical compounds and their mixtures were carried out according to the methodology described below. After packaging and exposing the eggs (2 hpf) to the different concentrations mentioned above (Table 1), 16 experimental groups were evaluated with 5 replicates for each group with 15 animals per polystyrene chamber (≈ 1200 animals) for 2 to 144 hpf. The *P. nigrum* EO stock solution has been renewed daily. The stock solution of L-DOPA was renewed every two days due to its stability in solution (Pappert et al. 1996). Figure 1 presents a schematic of the endpoints evaluated during the toxicity tests.

**Table 1. Concentrations of the individual chemical compounds and their mixtures were used for toxicity assessment and protection against the toxic effects of rotenone exposure using the zebrafish model.**

Chemical compound	Name of the experimental groups	Concentration	Rotenone
Control (DMSO 0.1%)	DMSO	-	0
Rotenone (RT)	RT	-	11.25 µg/L
Levodopa (LD)	LD	1000 mM	0
Levodopa (LD)	LD1+ROT LD2+ROT LD3+ROT LD4+ROT LD5+ROT	0.125 mM 0.250 mM 0.500 mM 0.750 mM 1,000 mM	11.25 µg/L
<i>Piper nigrum</i> (PN)	PN	10.00 µg/mL	0
<i>Piper nigrum</i> (PN)	PN1+ROT PN2+ROT PN3+ROT	1.25 µg/mL 2.50 µg/mL 5.00 µg/mL	11.25 µg/L

	PN4+ROT PN5+ROT	7.50 µg/mL 10.00 µg/mL	
Levodopa (LD) + <i>Piper nigrum</i> (PN)	LD2+PN2+RT  LD3+PN3+RT	0.250 M + 2.50 µg/mL  0.500 M + 5.00 µg/mL	11.25 µg/L

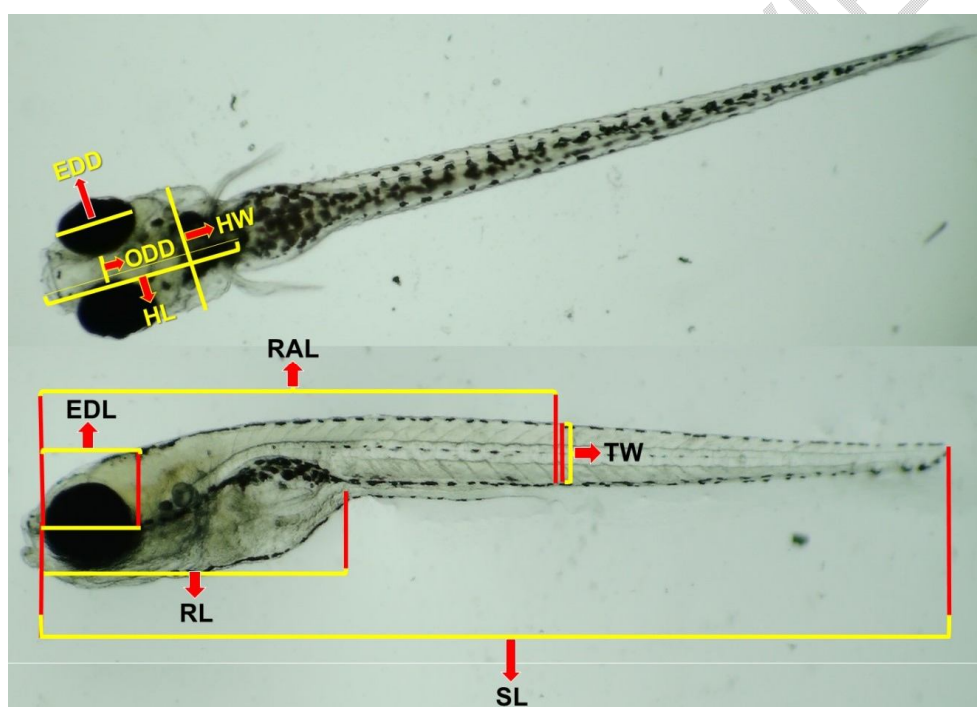


**Figure 1. A diagram of experimental endpoints evaluated during the toxicity tests.**

Mortality and embryonic and larval development (24 -144 hpf) of zebrafish were used as a response variable to evaluate the toxicity of the chemical compounds and their mixtures, and the animals were exposed to different concentrations (Table 1). With the aid of an optical microscope (400x, 1000x), zebrafish embryos and larvae were observed using the attached camera and photographed (Hayear Mod. HY-2307 and S-EYE software 1.42.474). Mortality was observed daily, being presented as a percentage (%) according to the OECD (2013). The hatching of the embryos was observed daily to obtain the percentage of the hatching rate, which is an indicator parameter of the teratogenic effects. Embryonic development was evaluated daily based on the observation of the following teratogenic effects: developmental delay, reduction in pigmentation, absence of somites, pericardial edema, yolk sac edema, coagulation area, spine deformation, tail deformation, eye malformation, absence of swim bladder inflation (Cadena et al. 2020a; Cadena et al. 2020b; OECD 2013; Silva et al. 2019).

Mortality and teratogenic effects were observed qualitatively by dichotomous response (present or absent). These results were presented in the form of a percentage (%) of affected animals, where an affected animal was considered when it showed at least one teratogenic effect or mortality induced by the chemical compound.

The morphometry analysis was performed in 144 hpf, after the larvae were euthanized in immersion with concentrated eugenol solution, according to the Brazilian Guidelines for the Practice of Euthanasia (Brasil 2018). With the aid of an optical microscope (400x, 1000x) and camera attached to it (Hayear Mod. HY-2307 and S-EYE 1.42.474 software), the animals were photographed for morphometric measurements of the dorsal (Cadena et al. 2020a) and lateral positions (Sales Cadena et al. 2021) (Figure 2). In the dorsal position, head length (HL), ocular distance in dorsal position (ODD), eye diameter (ED), and head width (HW) were evaluated (Cadena et al. 2020a). In the lateral position, eye diameter in lateral position (EDL), rump length (RL), rump anus length (RAL), larvae standard length (SL), and tail width (TW) were also evaluated (Sales Cadena et al. 2021).



**Figure 2.** Morphometric measurements were performed on zebrafish larvae with 144 hpf. Dorsal position: Head length (HL), ocular distance in dorsal position (ODD), eye diameter (ED), and head width (HW). Lateral position: Eye diameter in lateral position (EDL), rump length (RL), rump anus length (RAL), larvae standard length (SL), and tail width (TW).

#### **2.4. Evaluation of the toxicity and protective effects of chemical compounds in a Parkinson's-like model**

Larvae with no visible morphological alterations exposed by 144 hpf to the chemical compounds and their mixtures (Table 1) were used for behavioral tests. These tests were conducted according to modified methodologies from Cadena et al. (2020a). Briefly, the larvae were removed from the polystyrene chambers and placed in 48-well plates, each well containing one larva. The plates were placed under ambient light, on a white base, in the

morning ( $\approx 27^{\circ}\text{C}$ ). The thigmotaxis test (TH) is efficient for assessing anxiety-like behavior (Basnet et al. 2019; Cadena et al. 2020a). The TH test was performed after 10 minutes of acclimatization of the larvae and the response of the tendency to be close to or far from the wall of the plates was recorded. TH was considered positive when the larva was positioned close to the wall and negative when the larva was away from the wall. Next, the touch sensitivity (TS) test was performed to evaluate the larval response to mechanical stimuli. These stimuli were applied to the tail or head region of the larvae and the response was recorded. The larva was considered to have responded to this stimulus (positive TS) when it showed escape behavior and did not respond to the stimulus (negative TS) when it remained stationary. The scan sampling method (Silva et al. 2019) was used to collect behavioral data, considering the evaluation of the dichotomous response, being positive when the larva responded to the stimulus and negative when it did not.

The optomotor response (OMR) was used to assess the visual acuity of the animals (Basnet et al. 2019; Cadena et al. 2020a). The plates with the larvae were placed on a Full HD monitor (Dell E2211Hc) in a horizontal position and submitted to a video with a total duration of 75 seconds, with black and white lines moving to the right. The video simulates a stream of water to count the number of larvae that followed the direction of the stimulus (Brastrom et al. 2019). The video, captured by a cell phone camera, consisted of 5 s of white (acclimatization) for the initial time (TI) and 30 s of alternating lines in the right direction. OMR was evaluated in two different areas, representing low alignment (70% of the well) and high alignment (30% of the well) (Cadena et al. 2020a).

## **2.5. Statistical analysis**

Data normality was verified by the Shapiro-Wilk test with  $p < 0.05$ . Parametric data such as hatching rate, teratogenic effects, thigmotaxis tests, and touch sensitivity were analyzed by one-way ANOVA. When the difference was significant, the means were compared using Tukey's test, with  $p < 0.05$ . For the non-parametric data, the optomotor response, the MCNemar test was used. For statistical analyses, the Origin Pro Academic 2015 software (Origin Lab. Northampton, MA, USA) was used.

## **3. RESULTS AND DISCUSSION**

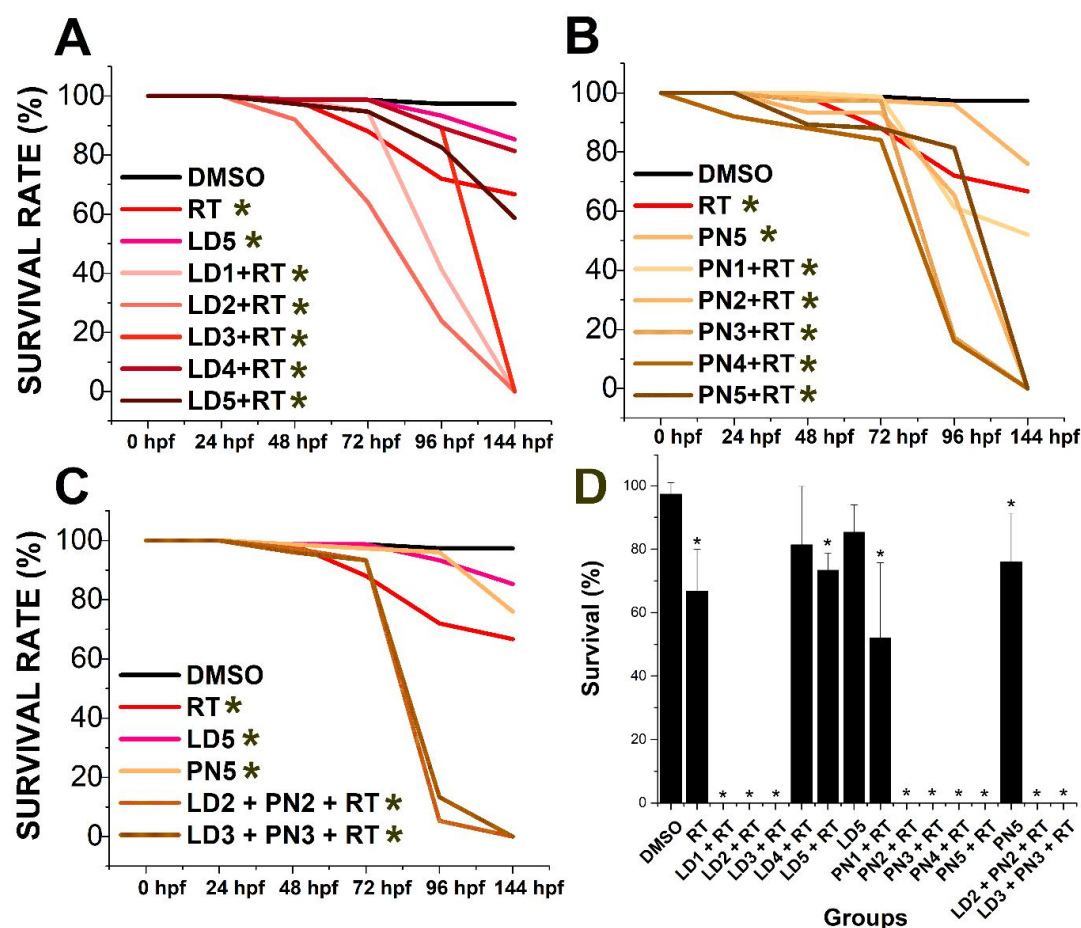
### **3.1 Results**

#### **3.1.1 Evaluation of the toxicity of chemical compounds in the embryo-larval development of zebrafish**

We evaluated the toxicity of chemical compounds and their mixtures in embryo-larval development by 96 hpf. The results are presented in Figures 3, 4, and 5. Figure 3 shows the effect that the chemical compounds had on the survival of the animals. The group treated with DMSO had an observed mortality rate of no more than 10%, which is in accordance with the standard recommended by the OECD (2013), validating the data obtained. Figure 3A shows a decrease in the survival of animals when exposed to RT, especially in the larval period. LD also affected the survival of the animals with toxicity. Also, in Figure 3A, the groups with the lowest concentrations of LD+RT (LD1+RT, LD2+RT, and LD3+RT) from 72 hpf, showed survival lower than 10%, showing that LD in low concentrations did not protect the animals from the toxic effect of RT. While LD4+RT and LD5+RT showed higher survival rates, between 60 and 80%, protecting the animals from the toxic effect of RT. In Figure 3B, the animals exposed to PN had a survival rate between 75 and 100%. However, PN did not protect against the toxic effect of RT and presented possible synergy, as it reduced the survival of the animals as the concentration of PN increased. Figure 3C shows the



interaction between PN and LD, showing that, when mixed, they did not have a protective effect, but a greater toxic effect. In addition, Figure 3D shows the survival of the animals at 144 hpf. It was possible to observe that only the LD4+RT group had a protective effect against the toxic effect of RT. PN and LD mixtures had no protective effect. Finally, regardless of the chemical compound used, the toxic effects are more observed from 72 hpf, when the zebrafish is already in the larval stage and does not have the chorion as protection.

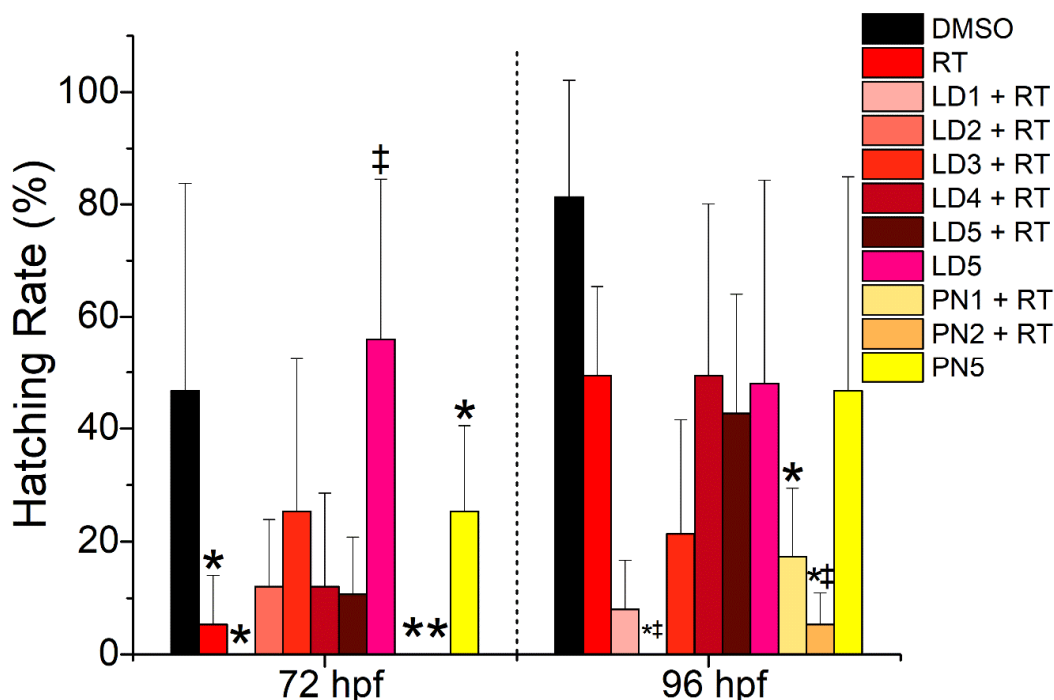


**Figure 3.** Effect of time and group on the survival rate (%) of zebrafish exposed to rotenone, levodopa, and essential oil of *Piper nigrum* up to 144 hpf (A, B, and C). Survival (%) of zebrafish at 144 hpf (D). The effect of time and group (A, B, and C) was studied by two-way ANOVA ( $F(19, 398) = 3.7$ ,  $p < 0.001$ ) followed by Tukey's test ( $p < 0.05$ ). For survival rate, each experimental group was compared to the 0.1% DMSO group by one-way ANOVA ( $F(15.79) = 86.79$ ,  $p < 0.001$ ) followed by Tukey's Test ( $p < 0.05$ ) at 144 hpf (D). Legend: DMSO 0.1% - Dimethylsulfoxide; RT – Rotenone; LD – Levodopa; PN – *Piper nigrum*. \*Significant difference ( $p < 0.05$ ) between the DMSO group compared to the other groups.

In Figure 4, the results revealed that zebrafish eggs hatched between 72 and 96 hpf. RT slowed the hatching rate only by 72 hpf, because at 96 hpf, this effect was not observed. LD5 did not exert toxicity at the times studied. On the other hand, LD1+RT showed time-independent toxicity. Regarding the LD2+RT, LD3+RT, LD4+RT, and LD5+RT groups, no toxicity was observed regardless of time. Thus, we can conclude from these results that LD showed protective effects against the toxic effects of RT in relation to the hatching rate. PN5



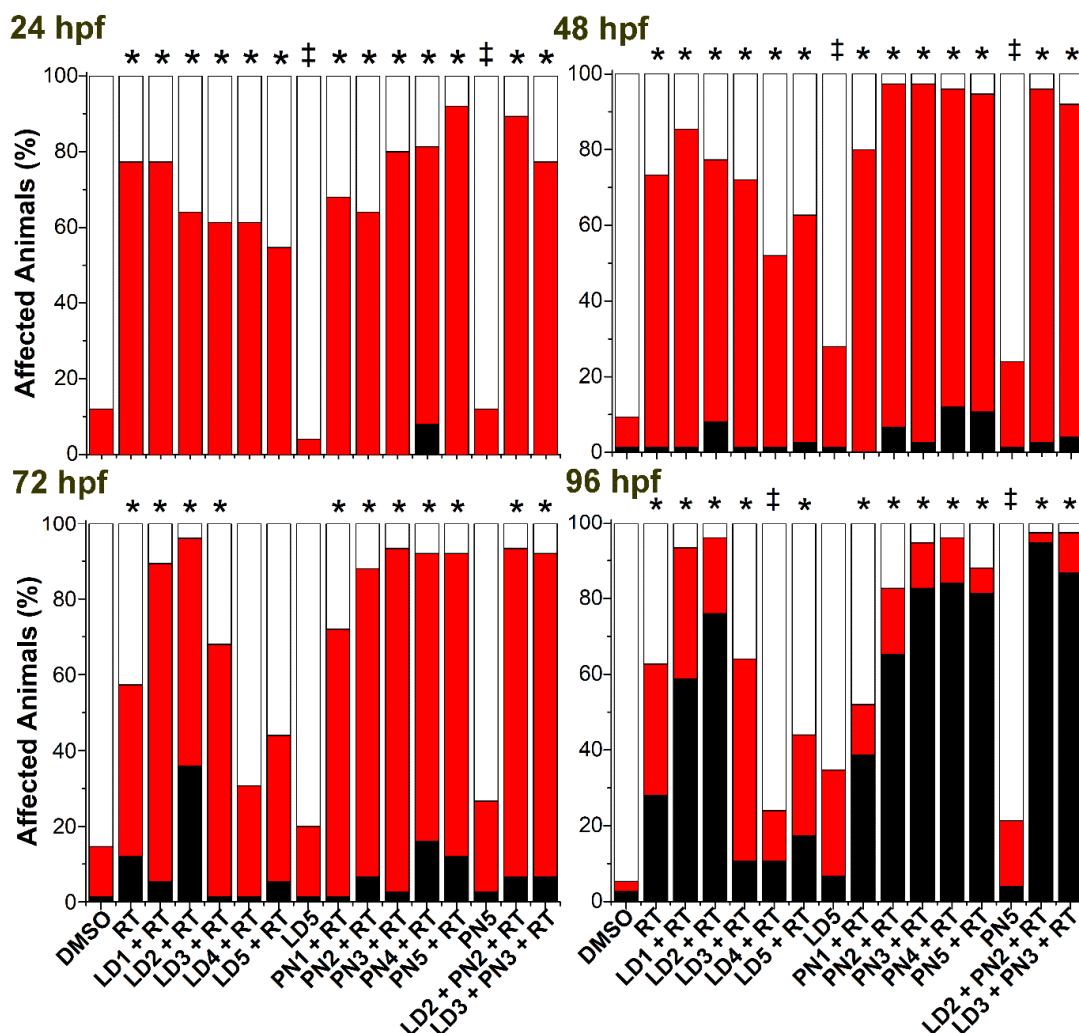
affected the hatching of the animals only at 96 hpf. On the other hand, PN1+RT, and PN2+RT had toxicity in the two times studied, revealing that PN associated with RT presented possible synergy, increasing toxicity. Also in Figure 4, the PN3+RT, PN4+RT, and PN5+RT groups, as well as the mixtures, did not have their hatching rates evaluated due to the earlier death of the animals.



**Figure 4.** Hatch rate (%) of zebrafish exposed to rotenone, levodopa, and *Pipernigrum* essential oil at 72 and 96 hpf. Each experimental group was compared with the 0.1% DMSO group and RT (11.25 µg/L) by one-way ANOVA followed by Tukey's test ( $p < 0.05$ ) at 72 hpf ( $F(15, 74) = 5.9$ ,  $p < 0.001$ ) and 96 hpf ( $F(15, 75) = 8.7$ ,  $p < 0.001$ ). \*Significant difference ( $p < 0.05$ ) between the DMSO group compared to the other groups; †Significant difference ( $p < 0.05$ ) between the RT group (11.25 µg/L) compared to the other groups. Legend: DMSO 0.1% - Dimethylsulfoxide; RT - Rotenone; LD - Levodopa; PN - *Piper nigrum*.

The results presented in Figure 5 show the percentage of affected animals in the period from 24 to 96 hpf. RT affected the development of the animals during the entire evaluation period where it was possible to observe all the teratogenic effects studied, this being the most toxic compound. However, LD5 only affected animals in the period between 48 and 72 hpf, where several teratogenic effects were observed, such as yolk sac edema, absence of pigmentation, absence of swim bladder, absence of somites, and developmental delay. This showed that, early in development, the chorion protected against toxic effects. LD did not protect against the toxic effects of RT with the exception of the LD4+RT group at 72-96 hpf and LD5+RT at 72 hpf. This indicated a protective effect of LD at higher concentrations on the toxic action of RT, corroborating the findings found in the survival of the animals (Figure 3). PN5 did not show toxicity at the time studied and also did not protect against the toxic effects of RT. In addition, there was probably a synergistic effect between PN and RT, increasing their toxicity, since there was a high mortality rate after 96 hpf, corroborating the survival findings (Figure 3). On the other hand, the mixtures, during the entire time studied,

showed a high percentage of affected animals, showing the fact that these associated compounds presented synergy-increasing toxicity.



**Figure 5.** Percentage of animals affected during 24, 48, 72, and 96 hpf exposed to rotenone, levodopa, and *Piper nigrum* essential oil. Each experimental group was compared with the 0.1% DMSO and ROT (11.25  $\mu\text{g/L}$ ) group by one-way ANOVA followed by Tukey's test ( $p < 0.05$ ) at 24 hpf ( $F(15, 79) = 12.92$ ,  $p < 0.05$ ), 48 hpf ( $F(15, 79) = 15.23$ ,  $p < 0.05$ ), 72 hpf ( $F(15, 79) = 14.50$ ,  $p < 0.05$ ), 96 hpf ( $F(15, 79) = 20.42$ ,  $p < 0.05$ ). Legend: DMSO 0.1% - Dimethylsulfoxide; RT – Rotenone; LD – Levodopa; PN – *Piper nigrum*. \*Significant difference ( $p < 0.05$ ) between the DMSO group 0.1% and the other groups; ‡Significant difference ( $p < 0.05$ ) between the RT group and the other groups.

The morphometric analyses are presented in Table 2. Regarding head morphometry (Table 2A), the RT group showed a reduction in head length (HL) and ocular distance in dorsal position (ODD), showing that this chemical compound exerted toxicity in relation to head morphometric parameters. On the other hand, LD5 also showed toxicity, as it affected HL, ODD, and head width (HW). LD4+RT and LD5+RT groups also showed toxicity in the same morphometric parameters described above (Table 2A). Reductions in the same

morphometric parameters mentioned above were also observed in the PN5 and PN1+RT groups. This shows that PN did not protect the animals from the toxic action of RT. In addition, the lateral morphometry shown in Table 2B showed that the RT group showed toxicity in relation to eye diameter in lateral position (EDL) and larvae standard length (SL). LD5 affected all morphometric parameters analyzed and the LD4+RT and LD5+RT mixtures also affected these parameters, showing that there was no protective effect. The PN5 group showed differences only in EDL, while the PN1+RT group showed reductions in EDL, rump anus length (RAL), and SL, indicating that PN had no protective effect against RT in relation to morphometry.

**Table 2. Morphometry of zebrafish larvae in 144 hpf exposed to rotenone, levodopa, and *Piper nigrum* essential oil. Data were expressed as Mean  $\pm$  Standard Deviation and the results were compared by one-way ANOVA followed by Tukey's test ( $p < 0.05$ ). \*Significant difference ( $p < 0.05$ ) between the DMSO group: 0.1% in relation to the other groups; ‡Significant difference ( $p < 0.05$ ) between the RT group (11.25  $\mu$ g/L) and the other groups. Legend: DMSO 0.1% - Dimethylsulfoxide; RT - Rotenone; LD - Levodopa; PN - *Piper nigrum*. Morphometric analyses of zebrafish larvae with 144 hpf exposed to rotenone, levodopa, and *Piper nigrum* essential oil. Legends - Dorsal position: Head length (HL), ocular distance in dorsal position (ODD), eye diameter (ED), and head width (HW); Lateral position, eye diameter in lateral position (EDL), rump length (RL), rump anus length (RAL), larvae standard length (SL), and tail width (TW).**

TABLE 2 (A) Dorsal position morphometry					
Groups	HL (mm)	ED (mm)	ODD (mm)	HW (mm)	
DMSO	.738.000 ± 0.085	0.172 ± 0.165	0.346 ± 0.045	0.536 ± 0.060	
RT	.681.000 ± .080*	0.152 ± 0.110	0.316 ± 0.054*	0.511 ± 0.062	
LD4+RT	.579.000 ± .056*‡	0.140 ± 0.084	0.289 ± 0.037*‡	0.453 ± 0.049*‡	
LD5+RT	.573.000 ± .039*‡	0.138 ± 0.031	0.277 ± 0.025*‡	0.457 ± 0.0441*‡	
LD5	.597.000 ± .440*	0.124 ± 0.034	0.298 ± 0.019*	0.469 ± 0.032*	
PN1+RT	.652.000 ± .053*‡	0.139 ± 0.032	0.305 ± 0.037*	0.496 ± 0.045*	
PN5	.658.000 ± .058*	0.110 ± 0.032	0.312 ± 0.023*	0.489 ± 0.039*	
TABLE 2 (B) Lateral position morphometry					
Groups	EDL (mm)	RL (mm)	RAL (mm)	SL (mm)	TW (mm)
DMSO	0.368 ± 0.040	1.213 ± 0.111	2.032 ± 0.124	3.526 ± 0.289	0.259 ± 0.024
RT	0.342 ± 0.046*	1.204 ± 0.097	1.975 ± 0.182	3.377 ± 0.280*	0.251 ± 0.030
LD4+RT	0.302 ± 0.039*‡	1.119 ± 0.077*	1.833 ± 0.112*‡	3.151 ± 0.225*‡	0.232 ± 0.025*‡
LD5+RT	0.284 ± 0.040*‡	1.066 ± 0.093*‡	1.817 ± 0.091*‡	3.099 ± 0.174*‡	0.231 ± 0.025*‡
LD5	0.308 ±	1.117 ±	1.842 ±	3.208 ±	0.242 ±

	0.044*	0.073*	0.079*	0,130*	0.021*
PN1+RT	0.329 ±	1.172 ±	1.913 ±	3.311 ±	0.259 ±
	0.051*	0.084	0.149*	0,185*	0.030
PN5	0.338 ±	1,164 ±	1.956 ±	3.508 ±	0.266 ±
	0.034*	0,072	0.109	0,175	0.020

### 3.1.2 Evaluation of the toxicity and protective effects of chemical compounds in a Parkinson's-like model

At 144 hpf, tests were performed in a Parkinson's-like model only with animals that had no visible teratogenic effects. First, the thigmotaxis test was performed, the results of which are shown in Figure 6A. There were no detectable behavioral changes in the animals exposed during embryo-larval exposure to the chemical compounds since the ability of the larvae to move close to the plaque wall was not affected by RT, LD, PN, and their mixtures. The touch sensitivity test was used to evaluate how the animals responded to mechanical stimuli when exposed to chemical compounds and their mixtures. According to the results in Figure 6B, this parameter was also not affected.

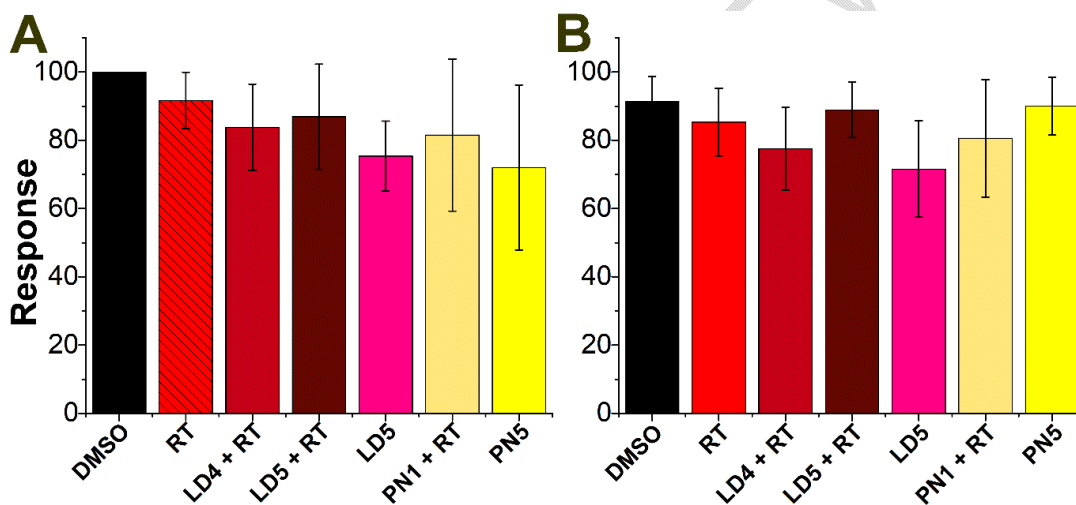


Figure 6. Percentage of behavioral response of zebrafish exposed to rotenone, levodopa, and *Piper nigrum* essential oil at 144 hpf when evaluated by the Thigmotaxis Test (A) and Touch Sensitivity Test (B). Each experimental group was compared with the 0.1% DMSO group and RT (11.25 µg/L) by one-way ANOVA followed by Tukey's Test ( $p < 0.05$ ) (Thigmotaxis ( $F(6, 34) = 2.03$ ,  $p < 0.05$ ), Touch Sensitivity ( $F(6, 34) = 1.92$ ,  $p < 0.05$ )). Legend: DMSO 0.1% - Dimethylsulfoxide; RT - Rotenone; LD - Levodopa; PN - *Piper nigrum*. \*Significant difference ( $p < 0.05$ ) between the DMSO group: 0.1% in relation to the other groups; ‡Significant difference ( $p < 0.05$ ) between the RT group and other groups.

The results of the OMR analysis are shown in Figure 7. The animals exposed to RT did not respond to the stimulus and were considered affected. The LD5, LD4+RT, and LD5+RT groups did not respond to the stimuli, thus showing that LD alone and in its highest concentrations interfered with the optomotor response of the animals and did not protect against the toxic effects of RT. Also in Figure 7, PN5 affected the optomotor response of the animals, but at a lower concentration (PN1+RT) it protected against the toxic effects of RT.

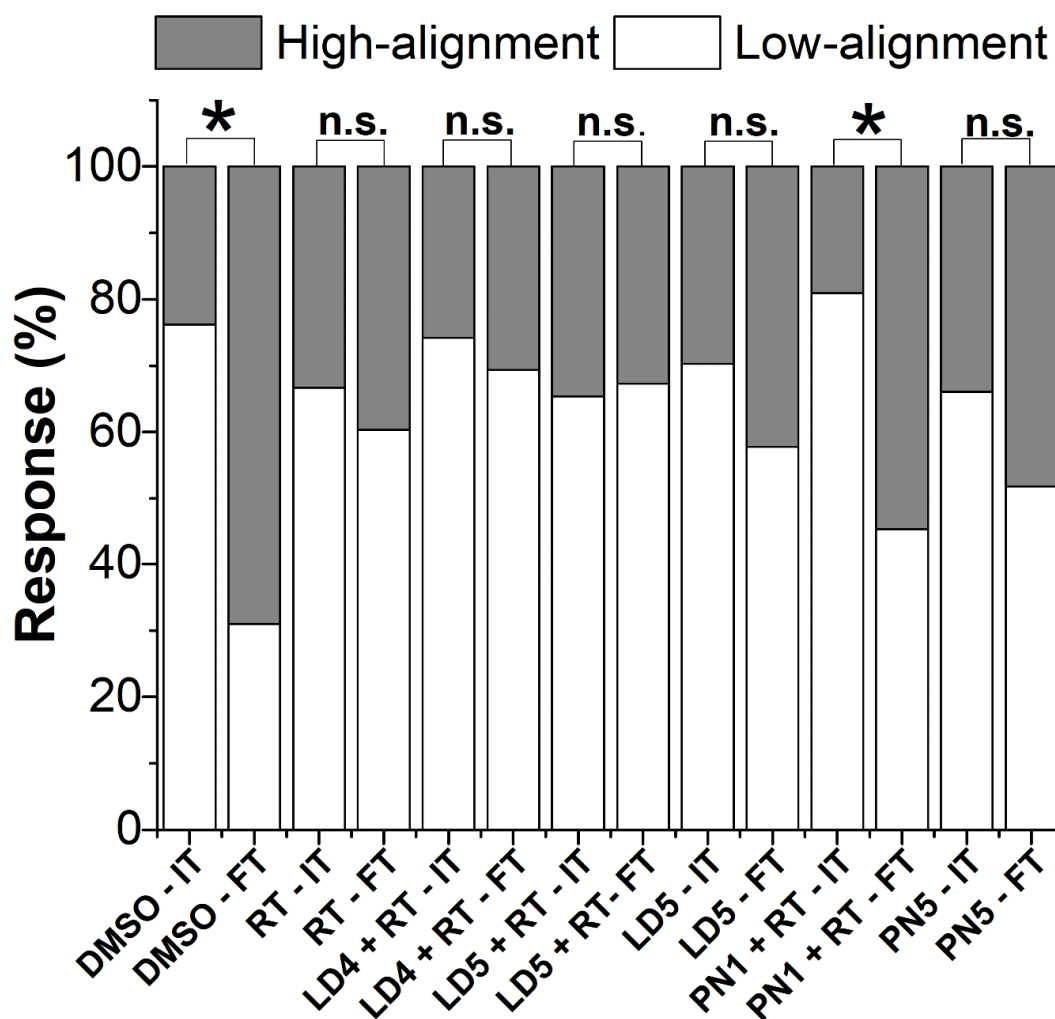


Figure 7. Percentage of optomotor response (OMR) of zebrafish larvae in 144 hpf exposed to rotenone, levodopa, and essential oil of *Piper nigrum*. comparing the Initial Time (IT) with the Final Time (FT) of each experimental group. Results were compared using McNemar's symmetry test. \*Significant difference ( $p < 0.05$ ) between the different times; n.s.: No significant difference ( $p > 0.05$ ). Legend: DMSO 0.1% - Dimethylsulfoxide; RT – Rotenone; LD – Levodopa; PN – *Piper nigrum*.

Finally, we can conclude with the results obtained that RT was a chemical compound that induced several types of defects in the embryo-larval development of zebrafish and affected the optomotor response of the animals. LD also presented defects in embryo-larval development and affected behavior, with its effect as a protector against RT-induced defects being low in the animal model studied. Finally, PN also affected embryo-larval development but did not affect zebrafish behavior and did not have a protective effect.

### 3.2 Discussion

The zebrafish has been used in the field of neuropharmacology as a model for drug screening, thus gaining an important role in the study of the treatment of neurodegenerative

diseases due to its brain regions homologous to those of humans (Barros et al. 2020; Khan et al. 2017). Also, the evaluation of the toxicity of natural products such as the PN EO used in our study can be performed in animal models since the toxicity studied in these models is correlated with human toxicity (Jayasinghe and Jayawardena 2019). In our study, we evaluated the toxic effects of RT on zebrafish embryos and larvae as an experimental model for Parkinson's-like disease. In addition, we investigated the toxic and protective effects of L-DOPA and PN and their association as a possible therapeutic alternative to treat these embryos with PD-like symptoms obtaining interesting results, discussed below.

Regarding toxicity, RT affected the survival and initially the hatching rate of animals exposed to 11.25 µg/L, occurring between 72 and 96 hpf. It caused teratogenic effects and altered the behavior of animals, which are potential Parkinson's-like effects. The same was observed in the study conducted by Andrade et al. (2023) who tested different RT concentrations (5 – 20 µg/L) and observed a high percentage (between 50 and 90%) of animals with teratogenic effects when exposed to high RT concentrations, as well as a delay in hatching. One reason for these effects to intensify after 72 hpf is the fact that zebrafish is in its larval stage and the absence of chorion facilitates the action of toxic chemical compounds (Santos et al. 2023). Probably, chorion may have reduced the toxic effects of RT in the early stages as observed in our work. This could be explained because the chorion has low permeability and low selectivity, since the yolk membrane, composed of three different and contiguous layers in the epiboly stage, exhibits electrical properties, as does the yolk nucleus and produces a relatively low selectivity to the entry of chemical compounds (Bonsignorio et al. 1996; Sanchis-Otero et al. 2023). In addition, RT may have its entry into the chorion reduced, thus reducing toxicity in the early stages of development.

Based on our results, RT is a highly toxic pesticide, which corroborates the findings of Melo et al. (2015). In our study, we used an RT concentration of 11.25 µg/L, within the range studied by Melo et al. (2015). In addition, the same authors reported that RT caused embryotoxicity, and effects such as lack of tail detachment, late formation of somites, pigmentation deficiency, cardiac edema, and caudal deformities, among others. In our study, the exposure of zebrafish embryos to RT caused the absence of pigmentation, spinal deformation, and formation of edemas. These effects were also observed by Melo et al. (2015) who justified the mutagenic, cytotoxic, and embryotoxic action of RT by the effect it has on oxidative stress since concentrations >1 µg/L of RT affected the oxidative stress enzymes CAT and GST and at concentrations >10 µg/L affected AChE, respectively.

In thigmotaxis and touch sensitivity tests, we evaluated in a simple way the anxiety and response to mechanical stimuli of the animals, and these parameters were not affected when the animals were exposed to RT. However, we emphasize that these neurobehavioral tests are performed with animals that did not show visible teratogenic effects (Cadena et al. 2020a), which could indicate that they are more resistant animals. However, in a more complex test such as OMR, we visualized the neurotoxicity of RT, as the animals needed to visualize the line and follow the stimulus generated by the video, thus resulting in a more complex test, especially involving the visual acuity of the animals (Brastrom et al. 2019). Therefore, if the animals did not respond, it is suggested that they were affected from a neurobehavioral point of view. This effect on OMR may be related to RT neurotoxicity, which is a possible symptom of Parkinson's-like disease. This was likely because RT crosses the blood-brain barrier, destroying dopaminergic neurons and deforming Lewy corpuscles (Azimullah et al. 2023), affecting animal movement. This may have explained the neurotoxic effects of RT on zebrafish found in our work, but it was only possible to detect using more complex neurobehavioral tests such as OMR.

L-DOPA is widely used for the treatment of PD; however, it also induces toxic effects as observed in our study. In our findings, we visualized several L-DOPA-induced toxic effects on embryo-larval development and a partial protective effect against RT. Usually, the literature presents published studies on the protective effects of L-DOPA against Parkinson-like symptom-inducing chemical compounds by evaluating neurobehavioral endpoints (Brinez-Gallego et al. 2023; Ilie et al. 2022). L-DOPA in humans has been reported to induce stress by increasing the activity of the Hypothalamic-Pituitary-Adrenal axis by elevating cortisol levels (Idalencio et al. 2021). In addition, Brinez-Gallego et al. (2023) reported that L-DOPA induces oxidative stress in zebrafish. Considering that zebrafish have high genetic similarity with mammals and that high cortisol levels in zebrafish embryos produce cardiotoxicity such as edema (Nesan and Vijayan 2012), this could explain the teratogenic effects described in our work in the early stages of zebrafish development. In view of this, we raise the following question: Could L-DOPA and RT, both of which induce oxidative stress, produce toxic effects combined? Ilie et al. (2022) observed that L-DOPA did not protect against the effects of oxidative stress induced by RT and, probably, the toxic effects observed in our study using L-DOPA against RT are more related to the toxic effects of RT. In our study, LD4 showed a possible protective effect. This leads us to reflect that the therapeutic range of L-DOPA use in our animal model was very narrow or even non-existent. In our results, L-DOPA affected OMR and did not protect against the toxic effects of RT when we analyzed behavioral tests. We observed that L-DOPA reduced the response to OMR. Brinez-Gallego et al. (2023) reported a decrease in swimming speed induced by L-DOPA compared to zebrafish larvae from the control group. In addition, Ilie et al. (2022) reported differences in the swimming pattern of adult zebrafish when exposed to L-DOPA and L-DOPA+RT. Finally, Brinez-Gallego et al. (2023) reported that L-DOPA is underactive and suggests that L-DOPA could cause damage to neurons linked to locomotor activity, which could explain our findings.

Regarding the PN essential oil (EO), which has the components  $\beta$ -caryophyllene, limonene,  $\alpha$ - and  $\beta$ -pinene, eugenol, among others, we expected low toxicity and protective effects against RT, but these were not the findings of our study. We observed high mortality, reduced hatching rate, affected morphometry, and toxicity in behavioral parameters when animals were exposed to NP and PN+RT. Although PN oil has the major component  $\beta$ -caryophyllene, the other components present in PN EO are known in the literature to induce toxic effects on zebrafish such as pinene (Şişman et al., 2023), eugenol (Tao et al. 2023) and limonene (Szaszkiwicz et al. 2021).  $\alpha$ -Pinene at high concentrations (320 and 640 mg/L) may induce teratogenic effects such as the absence of somites, lordosis, pericardial and yolk sac edema, and tail deformation (Şişman and Ceylan 2023) whose mechanism of toxicity is unknown. Eugenol, on the other hand, can induce growth and hatching retardation and the absence of swim bladder inflation due to inhibition of the Wnt/ $\beta$ -catenin signaling pathway (Tao et al. 2023). Finally, limonene may reduce locomotor response in zebrafish whose mechanism is also unknown (Szaszkiwicz et al. 2021). Even though the PN EO had these compounds in low concentrations, we could not rule out the possible toxic effects that they could induce. Based on our findings, they were more evident than the possible protective effects of  $\beta$ -caryophyllene. Silva et al. (2023) also observed toxic effects on zebrafish embryos and larvae when exposed to Lemongrass (*Cymbopogon flexuosus*), Thyme (*Thymus vulgaris*) and Oregano (*Origanum vulgare*) EOs. These findings showed the adverse effects of these oils on zebrafish embryos and larvae and revealed essential oil toxicity (Silva et al. 2023). Therefore, we conclude that the LE of NP did not have a protective effect against RT in our animal model.



## 4. CONCLUSION

RT was a highly toxic pesticide, which induced various types of defects in the embryonic-larval development of zebrafish and affected the animals' behavior, which are potential effects similar to those of PD. LD also presented defects in embryonic-larval development and affected behavior, having a low protective effect against RT in the animal model studied. Finally, PN also affected embryonic-larval development but did not affect the behavior of the zebrafish and had no protective effect. LD and PN proved to be ineffective therapeutic alternatives to treat these symptoms in this model, presenting some toxic and synergistic effects. Our research emphasizes the importance of exploring new treatment methods to alleviate the impact of Parkinson's-like disease induced by toxic substances such as RT in animal models.

## DISCLAIMER (ARTIFICIAL INTELLIGENCE)

During the preparation of this work the author(s) used Microsoft Copilot in order to correct the language of the manuscript.

## ETHICAL APPROVAL

All tests involving animals were previously approved by the Ethics Committee for Animal Use of the Universidade Federal Rural de Pernambuco, under License No. 3581030221.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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