

Article

Effects of Quercetin in Free Form and Nanoemulsion in an In Vivo Model of Parkinson's Disease

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Abstract

Background/Objectives: Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by motor and cognitive impairments due to dopaminergic neuron loss. The neurotoxin MPTP is commonly used to model PD, as it selectively targets these neurons. Quercetin (QU), a flavonoid with antioxidant properties, has shown neuroprotective potential, but its poor solubility limits clinical application. Nanoemulsions (NEQU) have emerged as a strategy to enhance its bioavailability and efficacy. **Methods:** To evaluate the neuroprotective and antioxidant effects of QU and NEQU, zebrafish larvae were exposed to MPTP (50 μ M) and assessed for survival, locomotion (total distance traveled), morphological parameters, reactive oxygen species (ROS), lipid peroxidation (via MDA), and reduced glutathione (GSH) levels. **Results:** Only NEQU pre-treatment reversed MPTP-induced locomotor deficits. Both QU and NEQU (2.5 μ M) significantly reduced ROS production and lipid peroxidation, with no effect on GSH levels. Notably, MPTP exposure led to a significant reduction in head size, an unprecedented finding in zebrafish PD models, indicating neurotoxicity. Morphometric analysis showed no change in total body length. However, MPTP significantly decreased swim bladder size and increased yolk sac size. Treatment with QU and NEQU attenuated these swim bladder alterations; no significant differences were observed in other parameters. **Conclusions:** These findings suggest that quercetin, particularly when nanoencapsulated, is a promising candidate for further development as a therapeutic agent to mitigate PD-related neurodegeneration.

Keywords: neurodegeneration; nanomedicine; neuroprotection



Academic Editor: Milan T. Makale

Received: 1 August 2025

Revised: 15 October 2025

Accepted: 31 October 2025

Published: 20 November 2025

Citation: Vian, C.d.O.; De Aguiar, R.F.; Marinho, M.A.G.; Mackmillan, V.P.; Alves, C.M.; Rodrigues, J.L.; de Miranda, F.B.; Dora, C.L.; Horn, A.P.; Hort, M.A. Effects of Quercetin in Free Form and Nanoemulsion in an In Vivo Model of Parkinson's Disease. *Future Pharmacol.* **2025**, *5*, 68.

<https://doi.org/10.3390/futurepharmacol5040068>

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1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide, second only to Alzheimer's disease. PD affects millions of individuals, with an

estimated 10 million diagnosed cases globally. The primary symptoms of PD, resulting from neurological dysfunction, include tremors, muscular rigidity, postural instability, mental confusion, insomnia, speech impairments, and smooth muscle spasms. Beyond motor dysfunction, these symptoms profoundly compromise patients' well-being and independence, as they are frequently accompanied by varying degrees of cognitive decline [1,2]. In addition to motor manifestations, PD patients may also present with non-motor symptoms such as sleep disturbances, depression, and cognitive impairments, further complicating disease management [3].

Most cases of PD are of unknown origin, or idiopathic, although approximately 10% are associated with genetic factors. Despite this, many cases of PD remain undetected, and its precise triggers are still not fully understood [4]. Physiologically, the symptoms arise from the degeneration of dopaminergic neurons in the substantia nigra (SN) pars compacta, which project to the striatum. This degeneration leads to a marked reduction in dopamine (DA) levels and tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis within the striatum, ultimately impairing neural communication. The neuropathological hallmarks of PD include striatal dopamine deficiency and the presence of intracellular Lewy bodies, filamentous aggregates formed by the accumulation of α -synuclein (α -syn) protein [5]. Nevertheless, therapeutic interventions for PD remain restricted to symptomatic management, with no available cure or strategies to halt disease progression. The most common current treatment is levodopa (L-Dopa), which alleviates motor symptoms by temporarily compensating for dopamine loss; however, it is associated with significant and undesirable side effects [6].

Therefore, a promising strategy for CNS diseases is the use of antioxidant phytochemicals such as quercetin (QU) (3,3',4',5,7-pentahydroxyflavone), a polyphenolic secondary metabolite found in various vegetables and fruits, including onions, broccoli, apples, and grapes [7]. QU is well known for its antioxidant properties, enabling it to neutralize free radicals that cause cellular damage. In addition to its anti-inflammatory, antiviral, and neuroprotective effects, QU has demonstrated pharmacological activity in PD by modulating multiple molecular pathways. For instance, QU can inhibit the mTOR pathway, which regulates cell growth and metabolism, thereby promoting autophagy, a crucial process for the clearance of aggregated proteins and damaged organelles characteristic of PD [8,9]. Although the role of phytochemicals in mitigating oxidative stress is well established in the pathogenesis of PD, the direct relationship between antioxidants and the inhibition of α -synuclein (α -syn) aggregation remains under active investigation. Nevertheless, growing evidence indicates that antioxidant compounds such as polyphenols may influence Lewy body formation, as oxidative stress is a critical factor in triggering and accelerating this process. Moreover, α -syn aggregation involves the structural conversion of monomers into amyloid fibrils, and polyphenols have been shown to modulate protein misfolding and enhance autophagy. Thus, QU may act not only by counteracting oxidative stress but also by inhibiting α -syn aggregation and Lewy body formation, both central hallmarks of PD progression [10]. Additionally, QU activates the transcription factor Nrf2, which regulates the expression of antioxidant genes through the antioxidant response element (ARE), thereby protecting cells against oxidative stress [11–13]. QU also inhibits the activation of the nuclear factor kappa B (NF- κ B) pathway, a master regulator of inflammation, contributing to the reduction in neuroinflammation. These combined effects, along with the regulation of additional pathways such as MAPK and PI3K/Akt, position QU as a potent modulator of essential cellular processes that support neuronal survival in PD [14,15]. Consequently, QU has gained increasing attention in studies on flavonoids as therapeutic agents for neurodegenerative diseases [15–18]. However, the poor oral bioavailability of QU remains a major limitation, particularly after single-dose administration, due to absorption barriers

related to dietary macronutrients. Pharmacokinetic studies in humans have shown that QU exhibits an oral bioavailability of only ~2%, underscoring the urgent need for strategies that improve its absorption and therapeutic efficacy [19]. The relatively large molecular size and polarity of QU hinder its passive diffusion across biological membranes, while its high lipophilicity results in poor aqueous solubility, further limiting absorption [19,20].

The blood–brain barrier (BBB) is a significant obstacle in the treatment of CNS diseases, including PD, as it serves as a stringent defense against potentially toxic substances. This barrier is composed of specialized endothelial cells, astrocytes, pericytes, and neurons, responsible for maintaining homeostasis and protecting the brain from unwanted compounds. However, this selectivity also severely limits the effectiveness of treatments that need to cross the BBB to reach the brain tissue [21]. In the case of therapies involving QU, crossing the BBB is particularly challenging due to its chemical structure and physical properties, necessitating the development of drug delivery strategies, such as the use of nanocarriers or chemical modifications, aimed at enhancing its permeability and ensuring that the compound effectively reaches the CNS [17,22,23].

In this context, nanostructured carrier systems have emerged as a promising strategy for pharmaceutical formulations, with particular emphasis on lipid nanoemulsions (NEs) [22]. These heterogeneous systems consist of one fluid dispersed in another immiscible liquid, stabilized by one or more emulsifying agents. NEs have demonstrated several advantages, including increased therapeutic efficacy and improved drug bioavailability. In some cases, they also enable controlled release of the encapsulated compounds [24]. Furthermore, the biocompatibility of the materials used in the production of NEs makes them suitable for administration through multiple routes. Lipid NEs can also provide a slower and more sustained release of the encapsulated agents, maintaining their concentrations and activity at the target site. This property facilitates the transport of drugs across semi-permeable membranes, thereby broadening their therapeutic applications [25]. Moreover, the nanometric size range (10–200 nm) enhances permeability across biological membranes, thereby potentiating the therapeutic effect. Nevertheless, this increased permeation capacity may also lead to physiological alterations, not only due to the chemical composition of the encapsulated active compound but also as a consequence of the formulation constituents, such as lipids and surfactants. Therefore, although nanoencapsulation offers significant benefits, potential adverse effects related to excipient toxicity and the behavior of nanoparticles within complex biological systems should be carefully considered [26–28].

Currently, zebrafish (*Danio rerio*) models are being employed in the evaluation of neurodegenerative disorders, since it effectively mimics human neurophysiological and neuropharmacological actions [1]. It is a widely accepted animal model for studies of different neurological disorders, including memory dysfunctions. The basic structure of the CNS in zebrafish contains all the major structures found in the mammalian brain, and the same neurotransmitters, such as GABA, glutamate, dopamine, norepinephrine, serotonin, histamine, and acetylcholine, which are present in the interneuronal systems. In particular, it is postulated that a portion of the dopaminergic system in zebrafish (*Danio rerio*) (mainly in the ventral diencephalic region) is functionally homologous to the nigrostriatal pathway in mammals [1]. Thus, this study aims to evaluate the possible neuroprotective effects of a NEs containing QU in an experimental model of PD, induced by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in zebrafish larvae.

2. Materials and Methods

2.1. Chemicals and Solutions

Quercetin (QU) (purity $\geq 95\%$), tricaine methanesulfonate (MS-222, 98% purity), 2',7'-dichlorofluorescein diacetate (DCF), butylated hydroxytoluene (BHT), sodium dodecyl

sulfate (98.5%), thiobarbituric acid, bovine serum albumin, methylcellulose, Folin–Ciocalteu and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) reagent were purchased from Sigma Aldrich (St. Louis, MO, USA). The stock solution of free quercetin was prepared in dimethyl sulfoxide (DMSO) and stored at $-20\text{ }^{\circ}\text{C}$. Test solutions of both free QU and nanoemulsified QU (0.1, 0.5, 1 μM) were prepared by dissolving the required volume of the stock solution in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl_2 , 0.33 mM MgSO_4 , pH 7.2, and dissolved oxygen (8 mg/L). The tricaine methanesulfonate solution was prepared in E3 medium, and the pH was adjusted to 7.2 ± 0.2 .

2.2. Zebrafish Maintenance

Adult *Danio rerio* specimens were obtained from Aquário Mania Peixes Ornamentais Ltda. (Pelotas, RS, Brazil; CNPJ: 91.169.532/0001-02; CNPJ: 91.169.532/0001-02), located at R. General Neto, 639—Centro, Pelotas, RS, Brazil (96015-280). The adult zebrafish, originally sourced from wild populations, were acquired from this local commercial supplier and maintained in a recirculating aquatic system (Altamar, São Paulo, SP, Brazil) using 10-L tanks at a density of one fish per liter. Standard maintenance conditions included pH of 7.2–7.4, temperature of $28 \pm 1\text{ }^{\circ}\text{C}$, conductivity of 400–600 $\mu\text{S}/\text{cm}$, water hardness ranging from 75 to 200 mg/L CaCO_3 , and a 12-h light/dark cycle. The fish were fed two to three times daily with brine shrimp (*Artemia salina*) and commercial dry flakes, a supplementary food (Tetra[®], Melle, Germany). For breeding, males and females were kept in a 1:1 ratio overnight. To prevent the fish from consuming their eggs, tanks were equipped with a mesh cover for egg collection, allowing only eggs to pass through a mesh placed at the bottom of the tank. The following morning, fertilized eggs were collected approximately 45 min after the lights were turned on. The collected eggs were placed in cell culture plates with E3 embryo medium (NaCl 5 mM, KCl 0.7 mM, CaCl_2 0.33 mM, and MgSO_4 0.33 mM), pH 7.2, dissolved oxygen $>6.3\text{ mg/L}$, and total hardness 65 mg/L (as CaCO_3). Viable embryos (non-coagulated) were selected and maintained in a biochemical oxygen demand (BOD) incubator at $28\text{ }^{\circ}\text{C}$ with a 12-h light/dark cycle until the end of the experiment. The Institutional Animal Care and Use Committee of the Federal University of Rio Grande (FURG) approved the experimental procedures (Institutional Animal Ethics Committee CEUA-FURG, protocol no. Pq019/2021, approved on 26 May 2022).

2.3. Development of Quercetin-Containing Nanoemulsions

NEQU was prepared using a hot solvent diffusion method combined with the phase inversion temperature (PIT) technique [22–24]. Castor oil (150 mg) and lecithin (20 mg) were dissolved in acetone:ethanol (60:40, *v/v*; 5 mL) at $60\text{ }^{\circ}\text{C}$ and added to 50 mL of an aqueous phase containing PEG-660 stearate (1.5% *w/v*), preheated to $80\text{ }^{\circ}\text{C}$, under stirring (700 rpm). The suspension was cooled to room temperature, and solvents were removed under reduced pressure (239 mbar, 5 min; 58 mbar, 3 min; 23 mbar, 9 min). The final volume was adjusted to 20 mL and filtered through an 8 μm cellulose nitrate membrane. For NEQU, 10 mg of QU was incorporated into the organic phase; free QU was solubilized in castor oil at the same proportion.

2.4. Particle Size and Zeta Potential Measurements

Particle size and zeta potential were measured using a Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK). Samples were diluted in distilled water and analyzed at $25\text{ }^{\circ}\text{C}$ by dynamic light scattering (DLS) with a detection angle of 90° . The hydrodynamic radius was calculated using the Stokes–Einstein equation. For zeta potential determination, samples were diluted in ultrapure Milli-Q[®] water and analyzed by laser Doppler electrophoresis under an applied voltage of $\pm 150\text{ mV}$. Measurements were performed in triplicate ($n = 3$) [29].

2.5. Experimental Design

Embryos were collected 1 h post-fertilization (hpf), and the viable ones were selected and maintained at 28 °C in an incubator. At 4 days post-fertilization (dpf), the larvae were randomly allocated into 96-well plates, with one animal per well, in a total volume of 200 µL. Initially, a survival curve was performed to determine the concentrations of free quercetin (QU at 1, 2.5, 5, 7.5, 10, and 25 µM) as well as the percentages of its vehicle, DMSO (0.3% and 0.8%, corresponding to concentrations of QU at 10 and 25 µM, respectively). The nanoemulsion contains quercetin (NEQU at 1, 2.5, 5, and 7.5 µM), along with the free nanoemulsion (NE at 2.5, 5, and 7.5 µM) and MPTP (50, 75, 100, 125, and 150 µM). Each compound was analyzed separately. This procedure was essential for determining future associations of these compounds. The concentrations used in this study were based on the literature [30–32].

Subsequently, the following concentrations were determined: (1) Control, which received only E3 medium; (2) MPTP at 50 µM; (3) DMSO at 0.08%, used as the dilution vehicle for the highest concentration of QU; (4) DMSO at 0.08% + MPTP; (5) QU at 1 µM + MPTP; (6) QU at 2.5 µM + MPTP; (7) NE at 2.5 µM, used as the dilution vehicle for the higher concentration of nanoemulsified quercetin; (8) NE at 1 µM + MPTP; (9) NE at 2.5 µM + MPTP; (10) NEQU at 1 µM + MPTP; (11) NEQU at 2.5 µM + MPTP. After 24 h of pre-treatment (at 5 dpf), the medium was renewed, and MPTP was added at a concentration of 50 µM. At 7 dpf (48 h of MPTP exposure), the animals were subjected to behavioral, morphometric, and oxidative analyses. Larvae showing visible malformations, lack of motor response, or signs of distress before or during the experiment were excluded. Randomization was used to allocate experimental units to control and treatment groups using a simple random method without specific software. In addition, all analyses were conducted by blinded observers who were unaware of the experimental group identities in order to minimize potential interpretation bias.

Quercetin-containing nanoemulsions (NEQU) were developed by the Laboratory of Nanotechnology Applied to Health at the Federal University of Rio Grande (FURG). Mean particle size of approximately 20 nm. The compounds of the nanoemulsion (NE) include QU, castor oil, soybean lecithin as co-surfactant, PEG-660 stearate surfactant, and the organic solvents acetone and ethanol. The lipid nanocarriers have already been characterized and were prepared using the hot solvent diffusion technique combined with the phase inversion method. The stock formulations were prepared at a concentration of 1.5 mg/mL. The number of animals varied according to the type of analysis performed and was determined following the methodological recommendations and literature references specific to each assay.

2.6. Determination of Survival Rate

The survival rate of zebrafish larvae was assessed daily. Larvae were exposed to the treatments from 5 dpf, and survival was recorded by counting the number of live larvae at 6 dpf and 7 dpf. Mortality was defined by the absence of heartbeat. The percentage of survival was calculated for each group based on the number of surviving larvae relative to the total larvae at the beginning of the experiment. The analysis was performed using GraphPad Prism software 9.0.

2.7. Total Distance Traveled

Seven dpf larvae with no morphological alterations, placed in 96-well plates containing one larva per well with 220 µL of treatment, were subjected to locomotion analysis under darkness (infrared light) at 25 frames per second using the DanioVision system (Noldus Information Technology®, Wageningen, The Netherlands). Larvae were acclimated for 20 min in darkness at 28 °C ($n = 40$ –60 animals/group). The behavioral test for total

distance traveled was quantified for 50 min. The total distance traveled (mm) was used to assess locomotor and behavioral responses) [33–35].

2.8. Morphological Analysis Procedure

The morphological analysis was conducted at 7 dpf. The larvae were anesthetized with MS-222 (0.02%) and immobilized in a 3% methylcellulose solution, then positioned laterally on microscopy slides for image capture using a G1200 digital microscope (Mustool[®], Shenzhen, China). The morphometric measurements included total body length, defined as the distance from the mouth of the larva to the pigmented tip of the tail; the height of the yolk sac, measured along a perpendicular axis to the larva's longitudinal axis, from the point of maximum vertical extension to the start of the swim bladder; the height of the swim bladder, measured along its maximum vertical axis; and cranial measurement, taken from the line of the eye to the upper lip of the larva, following the contour of the head. Measurements were performed using ImageJ software (version 1.53t, National Institutes of Health, Bethesda, MD, USA for Windows), based on the obtained images [36,37].

2.9. Sample Preparation

For the analysis of oxidative parameters, the zebrafish larvae were euthanized at 7 dpf using tricaine (MS-222) at a concentration of 300 mg/L, in accordance with Brazilian guidelines and laws [38]. Zebrafish larvae (1:10 larvae/ μ L) were homogenized in buffer containing Tris-HCl (100 mM), EDTA (2 mM), and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (5 mM), pH 7.75. The samples were homogenized for 40 s, 40 s at speed 6 in an IKA Ultra-Turrax T10 Basic with an ice bath, centrifuged at $10,000 \times g$ for 20 min at 4 °C and the supernatant was used to quantify lipid peroxidation and GSH levels. For the quantification of reactive oxygen species, the larvae (1:4) were homogenized in buffer containing sucrose (0.5 M), Tris base (20 mM), EDTA (1 mM), dithiothreitol (1 mM) and KCl (0.15 M), pH 7.60 and were analyzed immediately after homogenization. The results of the oxidative parameters were normalized by the total protein content contained in the samples, which was quantified by the Lowry method [39].

2.10. Reduced Glutathione (GSH)

Reduced glutathione (GSH) levels were measured using 5,5-dithiobis-2-nitrobenzoic acid (DTNB, Sigma-Aldrich, St. Louis, MO, USA). For this, 240 μ L of each sample were precipitated with 50% trichloroacetic acid and centrifuged at $20,000 \times g$ for 10 min at 4 °C. Then, the reaction was assembled in a transparent microplate, using 100 μ L of sample supernatant, 200 μ L of Tris base buffer (0.4 M; pH 8.9) and 10 μ L of DTNB. Absorbance was measured using a microplate reader (Elx 800, BioTek, Winooski, VT, USA) at a wavelength of 405 nm. Data were expressed as μ mol of GSH/mg protein [40].

2.11. Measurement of Lipid Peroxidation Product

Lipid peroxidation was quantified by analyzing thiobarbituric acid reactive substances (TBARS). The samples were incubated with butylated hydroxytoluene (BHT), thiobarbituric acid (TBA, 0.8%), acetic acid (20%, pH 3.4), and sodium dodecyl sulfate (SDS, 8.1%) for 30 min in a hot water bath at 95 °C. After incubation, 500 μ L of n-butanol was added, and the samples were centrifuged at $3000 \times g$ for 10 min at 15 °C. TBARS were quantified using a fluorimeter (Victor 2, Perkin Elmer) with excitation/emission wavelengths of 535/595 nm. The data were expressed as nmol MDA/mg protein, with tetramethoxypropane (TMP) used as a standard [41].

2.12. Determination of Reactive Oxygen Species (ROS)

At 7 days post-fertilization (dpf), ROS content was measured using samples processed as described in Section 2.8. ROS levels were analyzed using the fluorescent compound 2,7-dichlorofluorescein-diacetate (H2DCF-DA; Sigma-Aldrich, St. Louis, MO, USA). The samples were homogenized, and the supernatant was immediately incubated with H2DCF-DA (16 μ M) and analyzed for 60 min, with readings every 5 min, in a fluorimeter (Victor 2, Perkin Elmer) with a wavelength of 485/520 nm (excitation/emission). Data were expressed as fluorescence units per minute (FU.min) [42].

For representative in vivo images of the groups, larvae were washed with E3 medium and transferred to 6-well plates (1 larva per well) containing 2 mL of E3 medium. Subsequently, the larvae were exposed to H2DCF-DA at a final concentration of 0.4 mM at 28 °C for 1 h. After the treatment exposure, the larvae were washed three times with E3 medium and then immersed in 0.076 mM tricaine anesthetic for 1 min in E3 medium. The larvae were subsequently immobilized with 1.5% methylcellulose in E3 medium for imaging through the lateral surface of their bodies. Images were acquired using an inverted fluorescence microscope (Olympus IX81, Melville, NY, USA) with excitation/emission wavelengths of 485/535 nm and an exposure time of 77.76 ms [43].

2.13. Statistical Analysis

Statistical analyses were conducted using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA. Activation ID: 7c4c55fb-e591-42b6-9167-848618250650). Survival rates were assessed using the Mantel–Cox test and expressed as a percentage of survival. Outliers were identified using the ROUT (Relative Outlier Test). Data normality was evaluated with the Shapiro–Wilk and Kolmogorov–Smirnov tests. When normality was met, one-way ANOVA was performed, followed by post hoc comparisons using Tukey’s test. In the cases where the requisites were not fulfilled, Kruskal–Wallis non-parametric test was applied and followed by Dunn’s post hoc. Parametric data are presented as mean $\pm$ SEM whereas median $\pm$ interquartile range values for non-parametric data. The level of significance was set at $p \leq 0.05$. All experiments were conducted in triplicate, except for the reactive oxygen species (ROS) analysis, which was performed in duplicate.

3. Results

3.1. Nanoemulsion Characterization

The quercetin-loaded nanoemulsion (NEQU) was obtained by applying the hot solvent diffusion approach in association with the PIT method, as detailed in the Materials and Methods. The formulation exhibited a mean particle diameter close to 20 nm and a PDI of 0.21. A similar physicochemical profile was observed for the blank nanoemulsion, as presented in Table 1.

Table 1. Physicochemical characteristics of the quercetin-loaded nanoemulsion. PDI = polydispersity index; NE = nanoemulsion; NEQU = quercetin-loaded nanoemulsion. Values are presented as mean $\pm$ SD.

Formulation	Size (nm)	PDI	ζ -Potential (mV)
NE	19.83 $\pm$ 1.02	0.16 $\pm$ 0.010	−5.08 $\pm$ 0.10
NEQU	19.82 $\pm$ 0.72	0.21 $\pm$ 0.010	−5.34 $\pm$ 0.05

3.2. Survival Rate

For the QU curve and its vehicle DMSO, the data obtained demonstrated that the survival rate of zebrafish at 4 to 5 days post-fertilization (dpf) was not significantly affected by the different concentrations of QU. Survival rates remained above 90% across all ex-

perimental conditions. Specifically, both the Control group and the group treated with DMSO at 0.3% μM exhibited 100% survival. QU at 2.5, 5, and 7.5 μM re-sulted in survival rates of 97%. In contrast, higher concentrations of QU at 25 μM and DMSO at 0.8% were associated with a slight decrease in survival, with rates of 94% (Figure 1A). These results suggest that, under the experimental conditions employed, free quercetin did not have a significant effect on the survival of the zebrafish model until the concentration of 25 μM .

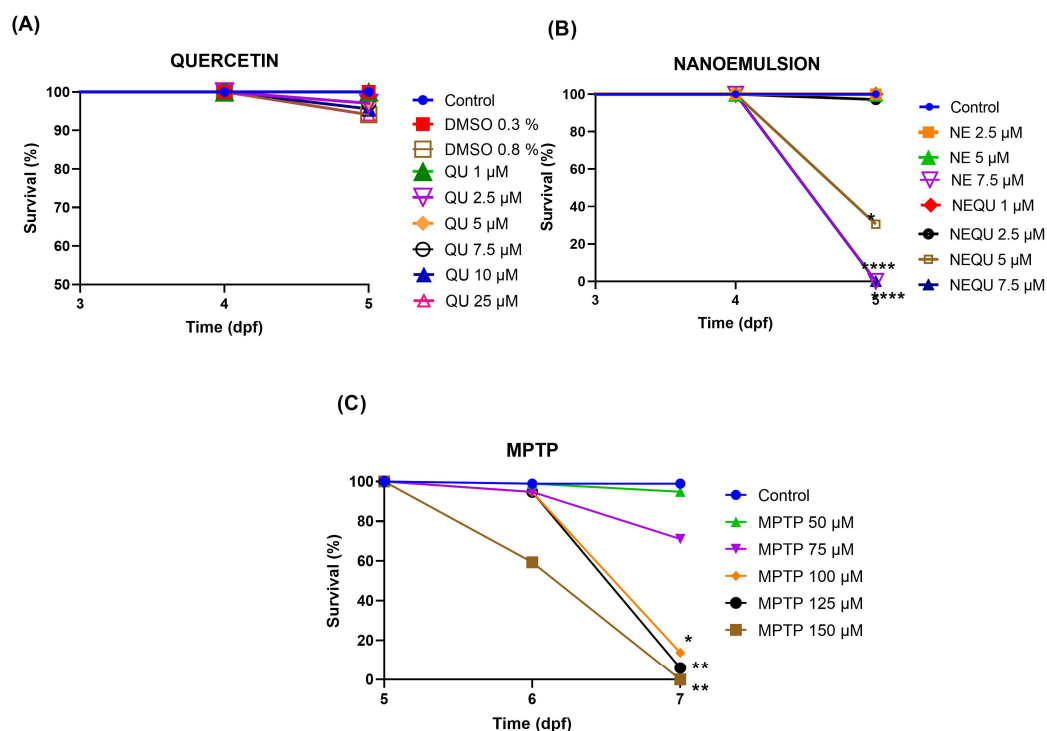


Figure 1. Survival rate of animals exposed to free quercetin (QU) (A) nanoemulsified quercetin (NEQU) (B) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (C) Concentration -response curves were analyzed with the Log-rank test; group differences were assessed by one-way ANOVA for survival followed by Dunnett's post hoc test. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; vs. control ($n = 30$ animals per group).

For the NEQU and its vehicle NE, the survival assessment results indicated significant differences between the experimental groups. The Control group, as well as the groups treated with NE at the concentrations of 2.5 μM and 5 μM , exhibited 100% survival. However, the concentration of 7.5 μM of NE resulted in 100% mortality at 5 dpf. In the NEQU-treated group, the concentration of 1 μM also maintained 100% survival, while the concentration of 2.5 μM resulted in a slight decrease, with a survival rate of 97.2%. In contrast, the concentration of 5 μM of NEQU was associated with a marked decline, with a survival rate of only 30.5%. The highest concentration of NEQU (7.5 μM) led to a 100% mortality (Figure 1B). At the end of 5 dpf, the estimated CL_{50} values were 4.651 μM for NEQU (95% CI = 4.172 to 5.130 μM) and 6.250 μM for NE (95% CI = 5.731 to 6.769 μM). These results indicate that, although lower concentrations of both compounds are not harmful to zebrafish survival, exposure to higher doses leads to marked lethality.

The survival results when animals were exposed to different concentrations of MPTP exhibited significant variation over the dpf. In the Control group, the survival rate was 100% at 5 dpf and 99% at 6 and 7 dpf. The group treated with MPTP at 50 μM showed a survival rate of 99% at 6 dpf and 95% at 7 dpf. The concentration of MPTP at 75 μM resulted in a survival rate of 95% at 6 dpf and 71% at 7 dpf. In the group treated with MPTP at 100 μM , survival was 95% at 6 dpf but dropped drastically to 14% at 7 dpf. The concentration of 125 μM of MPTP showed a survival rate of 95% at 6 dpf and a marked decrease to

6% at 7 dpf. Finally, the group exposed to MPTP at 150 μM had a survival rate of 60% at 6 dpf and no survivors (0%) at 7 dpf (Figure 1C). At the end of 7 dpf, the estimated CL_{50} for MPTP was 83.06 μM (95% CI = 76.81 to 89.31 μM). These findings indicate that lower concentrations do not significantly affect zebrafish survival, whereas higher doses of MPTP lead to pronounced lethality.

Based on the tested concentrations, it can be concluded that 50 μM MPTP is not toxic, as higher concentrations resulted in a sharp decline in the survival of zebrafish larvae. So, based on our results, we have chosen QU at concentrations of 1 and 2.5 μM ; MPTP at the concentration of 50 μM and NEQU at concentrations of 1 and 2.5 μM to continue our investigations (Figure 1A,B).

The results demonstrated that exposure of zebrafish to MPTP (50 μM) combined with either QU or NEQU did not significantly affect survival rates throughout the dpf. Across all experimental groups, including the control, vehicle groups (DMSO or NE), and treatments with QU or NEQU at concentrations of 1 and 2.5 μM , survival remained close to 100% up to 7 dpf. These findings indicate that both QU and NEQU, at the tested concentrations, exhibited no toxicity when co-administered with MPTP, thereby maintaining zebrafish viability (Figure 2A,B).

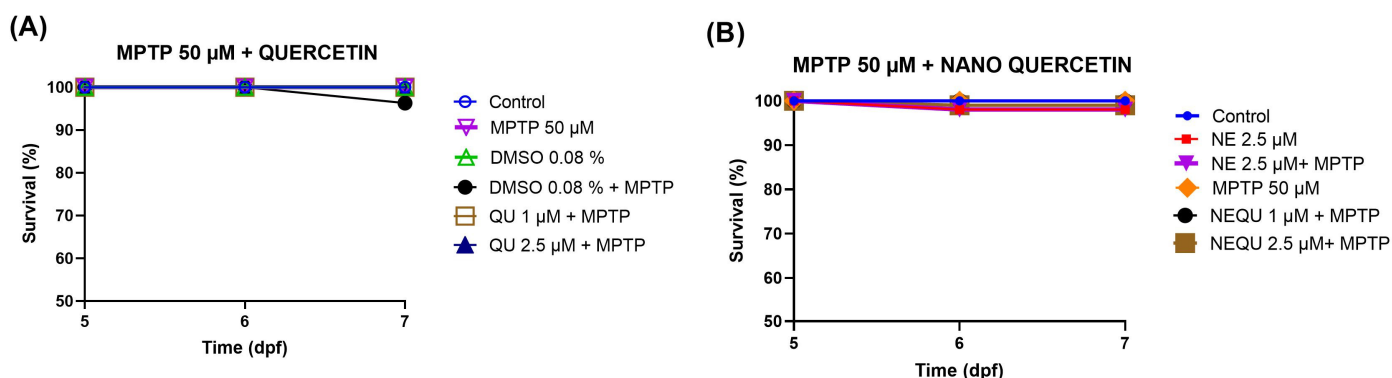


Figure 2. Survival rate of animals exposed to MPTP combined with different concentrations of QU (A) and NEQU (B). Concentration-response curves were analyzed with the Log-rank test; group differences were assessed by one-way ANOVA for survival followed by Dunnett's post hoc test. vs. control ($n = 30$ animals per group).

3.3. Behavioral Test

The total distance traveled by *Danio rerio* larvae was monitored over a 50-min period to assess the effects of different treatments on locomotor activity. The control group exhibited a significantly greater distance traveled compared to the group exposed to 50 μM MPTP, confirming the motor impairment induced by the neurotoxin. Larvae treated with DMSO (0.03% and 0.08%) showed no statistically significant differences relative to the control, indicating that the vehicle, at the tested concentrations, did not interfere with locomotion. Among the treated groups, larvae exposed to NEQU 2.5 μM + MPTP demonstrated a significant recovery of locomotor activity compared to the QU 2.5 μM + MPTP group (Figure 3). These results suggest that the nanoemulsion containing quercetin is more effective in mitigating MPTP-induced motor deficits than free quercetin. Additionally, the DMSO 0.08% + MPTP group showed partial improvement in the distance traveled, suggesting a potential cytoprotective effect of the solvent. In contrast, treatment with free quercetin (QU 2.5 μM + MPTP) did not produce significant recovery of locomotor activity, reinforcing the superiority of nanoformulated quercetin (NEQU) as a delivery system and enhancer of neuroprotective efficacy.

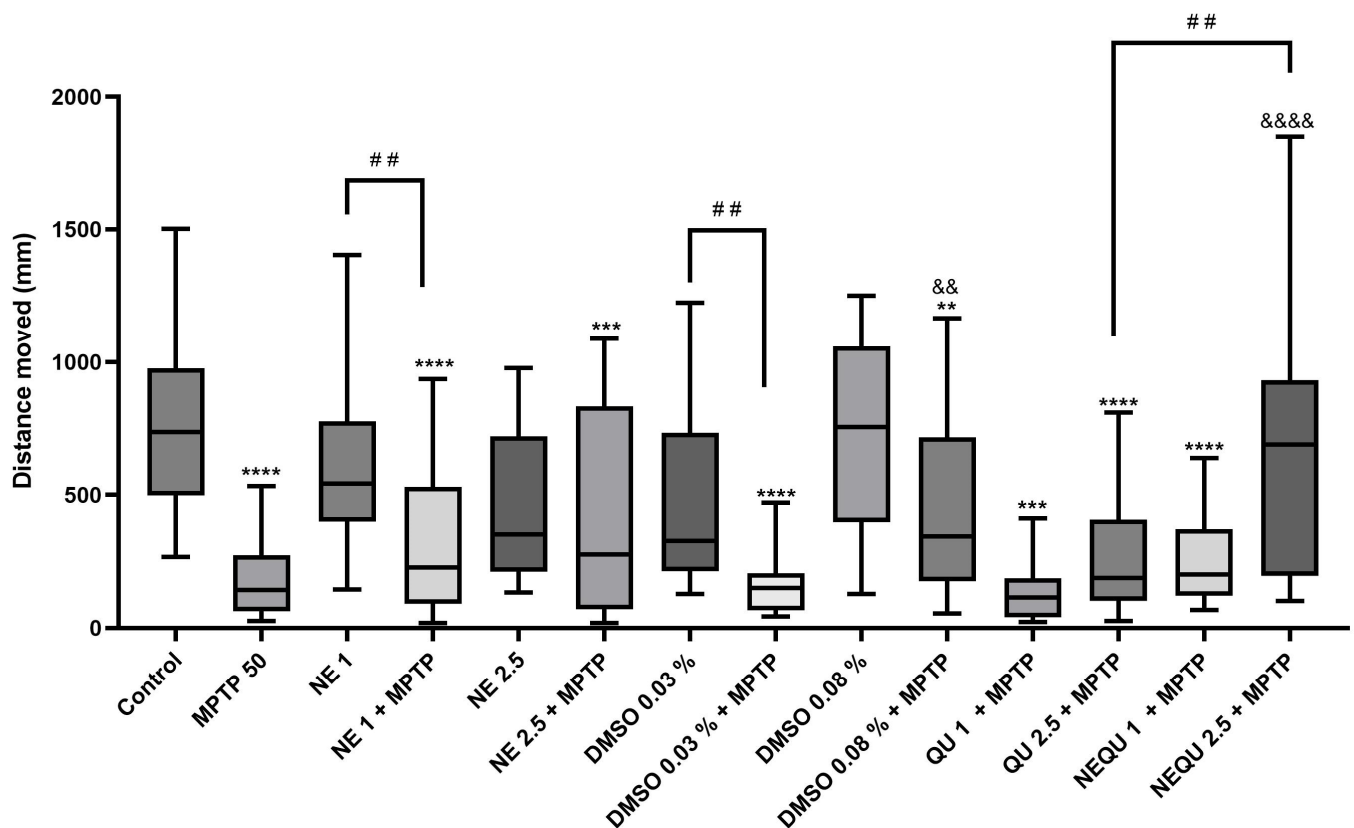


Figure 3. Effects of MPTP, QU and NEQU on spontaneous swimming behavior, measured by the total distance moved. Swimming behavior was analyzed over a 50-min period ($n = 40\text{--}45$ animals/group). Data are expressed as median $\pm$ interquartile range and were analyzed using the Kruskal–Wallis test followed by Dunn’s post hoc test. * represents significant difference in relation to control group. & in relation to MPTP-treated group. # indicates comparisons between selected treatment groups. (**, && and ## $p < 0.01$; *** $p < 0.001$; **** and &&&& $p < 0.0001$).

3.4. Morphological Analysis Results

The results of the morphometric analysis of body size (Figure 4B) showed no statistically significant differences among the groups. Head size analysis (Figure 4C) revealed that larvae treated with 50 μM MPTP exhibited a significant reduction of approximately 13% compared with the control group, with a mean head length of $\sim 441\ \mu\text{m}$. A similar reduction was also observed in the QU 2.5 μM + MPTP and NEQU 2.5 μM + MPTP groups, both of which showed significantly smaller head sizes relative to the control. In contrast, treatments with DMSO 0.08%, DMSO 0.08% + MPTP, NE 2.5 μM , or NE 2.5 μM + MPTP did not produce significant differences when compared to the control. These results indicate that head size is particularly sensitive to MPTP-induced toxicity and that the reduction persisted even in the presence of free or nanoemulsified quercetin.

When swim bladder size was assessed (Figure 4D), larvae treated with 50 μM MPTP showed a significant 35% reduction compared to the control group. Treatment with QU 2.5 μM and NEQU 2.5 μM partially attenuated this reduction. Analysis of yolk sac size (Figure 4E) revealed that larvae exposed to MPTP exhibited a significant increase of approximately 17% relative to the control, indicating morphological alterations induced by the neurotoxin. A similar enlargement was also observed in the QU 2.5 μM + MPTP group. Interestingly, treatment with NE 2.5 μM + MPTP attenuated this effect, resulting in yolk sac size values comparable to those of the control group. In contrast, NEQU 2.5 μM + MPTP, as well as DMSO or NE administered alone or in combination with MPTP, did not induce significant differences relative to the control.

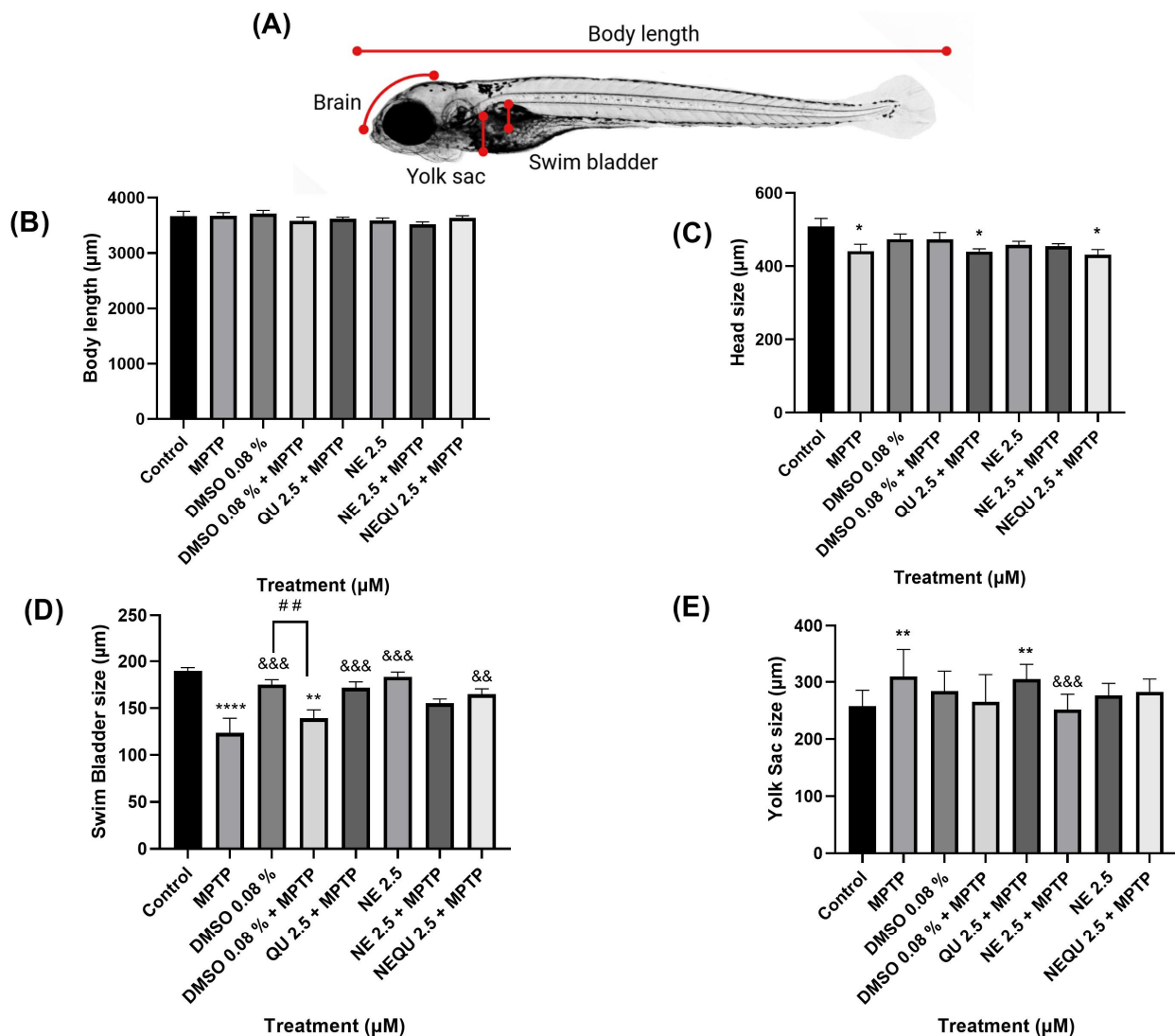


Figure 4. Morphometric analysis. (A) Lateral view of a representative larva illustrating the measured parameters, scale bar: 200 μm, (B) body length, (C) Head size, (D) swim bladder size and (E) yolk sac size ($n = 20$ animals per group). Data are presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test * represents significant difference in relation to control group. & in relation to MPTP-treated group. # indicates comparisons between selected treatment groups. (* $p < 0.05$; **, && and ## $p < 0.01$; &&& and **** $p < 0.0001$).

3.5. Reduced Glutathione (GSH) and Measurement of Lipid Peroxidation Product

The levels of GSH in zebrafish larvae are presented in Figure 5A. The control group showed the highest values, whereas treatment with MPTP alone caused a significant 42% reduction. The DMSO 0.08% + MPTP group exhibited a 36% decrease compared with the DMSO 0.08% group, indicating that MPTP compromised the antioxidant defense under these conditions. Similarly, the NE 2.5 μM + MPTP and NEQU 2.5 μM + MPTP groups showed reductions of 34% and 29%, respectively, compared with the NE 2.5 μM group, confirming that MPTP-induced oxidative stress was not prevented by either quercetin formulation.

Lipid peroxidation, assessed through TBARS levels (Figure 5B), revealed that treatment with 50 μM MPTP caused a significant 246% increase compared with the control group, indicating marked oxidative damage induced by the neurotoxin. In contrast, QU 2.5 μM + MPTP and NEQU 2.5 μM + MPTP significantly reduced TBARS levels by 62% and

55%, respectively, demonstrating that both free and nanoemulsified quercetin conferred strong protection against MPTP-induced lipid damage.

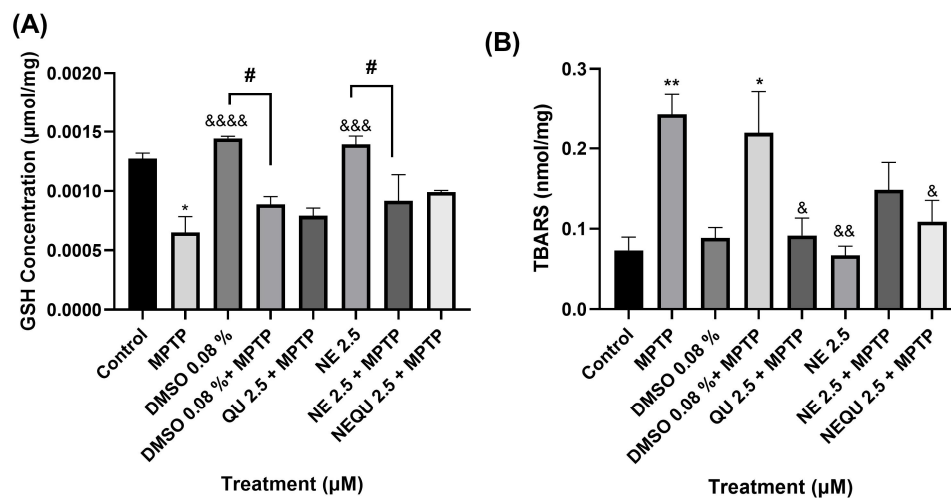


Figure 5. Effects of QU, NE e NEQU on the levels of GSH (A) and TBARS (B) in zebrafish larvae exposed to MPTP. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. Data are presented as mean $\pm$ SEM. * represents significant difference in relation to control group. & in relation to MPTP-treated group. (pool of 50 animals per group). # indicates comparisons between selected treatment groups. (*, # and & $p < 0.05$; ** and && $p < 0.01$; &&& $p < 0.001$; &&&& $p < 0.0001$).

3.6. Reactive Oxygen Species (ROS) Levels

The results of ROS quantification shown in Figure 6A indicate that the treatment with 50 μ M MPTP induced a significant increase of approximately 10% in ROS production compared to the control. However, the combination of both vehicles with MPTP (DMSO and NE 2.5 + MPTP) resulted in approximately 43% and 39% increases in ROS production when compared to the MPTP alone group.

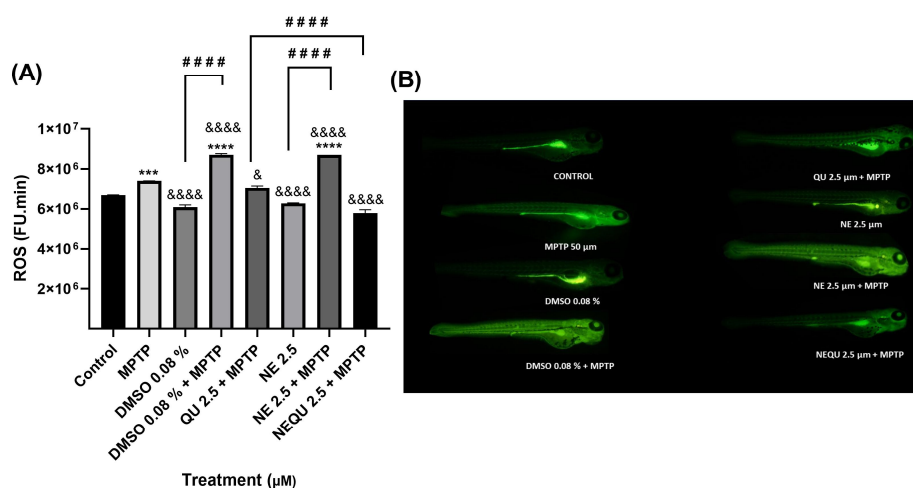


Figure 6. Effect of QU, NE, and NEQU compounds on reactive oxygen species (ROS) production in zebrafish model treated with MPTP. Quantification of ROS (in fluorescence units, FU/min) across different experimental groups (A). Representative images of ROS fluorescence in zebrafish (B). One-way ANOVA followed by Tukey's post hoc test ($n = 32$ animals per group). Data are presented as mean $\pm$ SEM. * represents significant difference in relation to control group. & in relation to MPTP-treated group. # indicates comparisons between selected treatment groups. (& $p < 0.05$; *** $p < 0.001$; &&&&, ##### and **** $p < 0.0001$).

Moreover, both QU and NEQU were able to reverse the oxidative effects caused by the DMSO and NE 2.5 vehicles associated with MPTP, resulting in significant reductions of 19% and 33% in ROS levels, respectively, compared to their respective vehicles. Figure 6B presents representative images of ROS fluorescence *in vivo* in the experimental groups, demonstrating that both QU and NEQU successfully mitigated the oxidative effects of DMSO + MPTP and NEBR + MPTP.

4. Discussion

Since current treatments for PD can only alleviate clinical symptoms without slowing disease progression, there is still no cure for this condition, resulting in a significant demand for effective agents or strategies to prevent or attenuate its course [6]. Although QU has demonstrated neuroprotective properties both *in vitro* and *in vivo*, the effects of the free molecule remain controversial due to its low bioavailability [44]. Consequently, the free form of QU shows reduced efficacy, limiting its potential clinical application in the treatment of CNS disorders. Therefore, the development of a drug delivery system containing QU may enhance its therapeutic applicability, reinforcing the need for new pharmacological alternatives that minimize these limitations.

The results of this study clearly demonstrate that MPTP successfully induced PD-like features in zebrafish larvae. Compared with the control group, larvae exposed to MPTP exhibited significant alterations across all analyzed parameters, including motor deficits, morphometric changes such as reduced brain size, and oxidative imbalance. These findings confirm that the PD model was well established, providing a solid foundation for evaluating the therapeutic strategies under investigation. In this context, quercetin emerges as a potential neuroprotective agent capable of mitigating oxidative damage and improving MPTP-induced motor deficits. Free quercetin at high concentrations (25 μ M) did not exhibit lethal effects, supporting its non-toxic therapeutic potential (Figure 1A). In contrast, NEQU displayed significant toxicity at concentrations above 5 μ M, leading to high mortality rates, as shown in the survival curve (Figure 1B). Our findings highlight the dual role of nanoencapsulation: on the one hand, enhancing drug delivery and bioavailability, but on the other hand, potentially amplifying dose-dependent cytotoxicity. This phenomenon has also been reported in the literature, where certain nanoemulsion formulations improved pharmacological effects at lower doses but induced significant cytotoxic responses at higher concentrations due to excipient composition and nanoparticle-specific interactions with biological systems [45]. Therefore, while nanoemulsions represent a promising delivery system for poorly soluble compounds such as quercetin, a careful balance between efficacy and safety must be established. In this context, dose optimization, together with additional *in vivo* pharmacokinetic and toxicological studies, is essential [46]. This indicates the need to investigate the specific components of the NEQU formulation that may contribute to the observed toxicity. The potential toxicity of the vehicle limited the evaluation of higher concentrations.

The high mortality associated with NEQU, as shown in Figure 1B, and with its vehicle NE starting at concentrations of 5 μ M may be attributed to factors related to nanoemulsion formulation, including the presence of surfactants and the concentration of their components [47]. Smaller organisms, such as zebrafish larvae, are particularly susceptible to these formulations, as their reduced size increases the likelihood of particle interactions with cells and cellular components, thereby exacerbating toxicity. The use of surfactants such as Poloxamer 188 and Solutol HS15, although essential for nanoemulsion stabilization, can also contribute to toxic effects [33]. At higher concentrations, these compounds may exert cytotoxic activity. Furthermore, droplet size also influences toxicity: the smaller the droplets, the greater the penetration into tissues and the potential for toxicity, since

smaller organisms have a reduced capacity to metabolize or eliminate these particles efficiently. Together, these factors explain the increased toxicity of NEQU and NE compared with free quercetin, underscoring the importance of optimizing formulations to minimize adverse effects [48–50].

The results demonstrating that the nanoemulsion vehicle exerted a positive effect against MPTP toxicity may indicate that components of the formulation themselves contributed to neuroprotection. In particular, the use of castor oil as the oil phase is noteworthy, since vegetable oils are known to contain bioactive compounds such as polyphenols, which possess intrinsic antioxidant properties [39]. Castor oil, which comprises more than 90% of unsaturated hydroxyl fatty acids in the formulation, has been extensively reported for its antioxidant and anti-inflammatory activities. These characteristics not only reduce oxidative stress but may also provide neuroprotective benefits, supporting its potential as a therapeutic excipient in the context of PD [51,52]. Thus, we acknowledge that it is not possible to conclusively attribute all the observed effects exclusively to the nanoencapsulation of quercetin, since both DMSO and the components of the NE, particularly castor oil, exhibit intrinsic biological effects.

Analyzing the motor behavior results (Figure 3), a comparison between the DMSO 0.08% group and the DMSO 0.08% + MPTP group revealed that DMSO, a solvent commonly used as a vehicle for hydrophobic compounds in in vivo experiments, provided notable protection against the effects of MPTP. This protective effect can be attributed to the cytoprotective properties of DMSO, as previously described in the literature. Studies indicate that DMSO may mitigate CNS injury by scavenging free radicals that trigger inflammatory processes [53–57]. It is important to note, however, that depending on the concentration, DMSO can exert distinct effects, acting either as a protective agent or as a cytotoxic compound. Regarding motor behavior, it was also evident that QU did not exert protective effects against MPTP at the tested concentrations. In contrast, the NEQU 2.5 μ M + MPTP group showed significant improvement compared with QU at the same concentration, suggesting that the nanoformulation may act as a delivery system for QU in the CNS, thereby mitigating the motor impairments associated with PD [1].

The development and morphology of the swim bladder are widely used as indicators of the overall health of zebrafish larvae. Previous studies have shown that a functional swim bladder typically develops around 4 dpf. Since MPTP was administered at 5 dpf, it is possible that the neurotoxin interfered with the final stages of swim bladder development. This organ plays a crucial role in balance and locomotion, regulating buoyancy and enabling fish such as zebrafish to maintain their position in the water column with minimal energy expenditure. The development of the swim bladder in zebrafish (*Danio rerio*) can be compromised by multiple factors. Genetic mutations, particularly in genes regulating swim bladder formation, can result in structural abnormalities, while environmental conditions such as temperature, oxygen levels, nutritional deficits, and exposure to toxic substances may also impair its development, ultimately compromising balance and motor performance [58–61]. MPTP, a neurotoxin that primarily targets the central nervous system, can also indirectly impair peripheral organ development and function, including that of the swim bladder, by disrupting neural homeostasis and inducing motor dysfunction [62]. This interpretation supports our findings (Figure 4D), which indicate that MPTP-induced CNS impairment may extend to peripheral organs [63]. Interestingly, our results also show that QU and NEQU attenuated the reduction in swim bladder size, suggesting an overall protective effect on organismal health.

Another important aspect concerns the structure of the yolk sac, which plays a critical role in the nutrition and early development of zebrafish and other vertebrates. Although not directly related to motor deficits or PD, the yolk sac is frequently examined in studies

of embryonic development and toxicology, as its size can reflect the impact of neurotoxic or mutagenic substances on development [64]. In zebrafish models, exposure to neurotoxins such as MPTP can interfere with normal yolk sac absorption, leading to developmental delays or abnormalities. Such alterations may indicate systemic dysfunctions that can include motor impairments resulting from central nervous system dysregulation, similar to those observed in PD models. While no direct association between yolk sac size and PD has been established, yolk sac morphology can serve as an important marker of systemic effects in experiments evaluating the developmental toxicity of neurotoxic agents. In addition to biochemical and behavioral outcomes, our findings suggest a relationship between morphological alterations and motor performance in the zebrafish PD model. Specifically, preservation of swim bladder size was associated with improved locomotor activity, supporting a positive relationship between these parameters. Conversely, an increase in yolk sac size coincided with reduced distance moved, suggesting a negative relationship with motor performance. Although a formal correlation analysis was not performed, the observed patterns across treatment groups (Figure 3) support the interpretation that MPTP-induced morphological changes contribute to motor impairments and that treatments capable of preserving these structures may indirectly promote functional recovery. To our knowledge, this is the first study to demonstrate such an effect in a PD model.

The reduction in head size observed in larvae treated with MPTP can be attributed to several interconnected mechanisms affecting head development. MPTP is known to induce neuronal death, particularly in regions of the CNS, leading to a decrease in neuronal populations and, consequently, reduced development of head structures. These alterations suggest that decreased thickness and volume in prefrontal, medial, and lateral temporal regions may foreshadow cognitive decline in PD [65]. In addition, MPTP triggers oxidative stress, resulting in the production of ROS that cause cellular damage and contribute to reduced head volume. Inflammatory responses induced by MPTP, including glial cell activation, further promote neuronal death and disrupt neurogenesis, ultimately impairing head growth and morphology [14].

Moreover, MPTP toxicity can compromise the energy metabolism of neurons, limiting their ability to counteract PD-related mechanisms. This aligns with previous findings reporting accelerated volume reduction in the occipital and temporal lobes, the inferior parietal lobule, as well as in the insula, putamen, and the basal nucleus of Meynert. In these studies, white matter was less affected than gray matter, with the most impacted regions including cortical areas, the amygdala, and nuclei of the basal forebrain, but not the basal ganglia [65]. These observations underscore the need for further investigation into the mechanisms underlying such effects, as understanding these processes is essential for developing treatments for neurodegenerative diseases.

The reduction in head size observed in larvae treated with MPTP may be interpreted as an adaptive response to neuronal injury caused by neurotoxicity, reflecting structural adjustments to compensate for damage. For instance, Mak et al. (2017) demonstrated that ventricular atrophy is associated with cognitive decline in PD patients, suggesting that changes in head structure, such as reduced head size, may have significant implications for cognition and overall well-being [66–68]. Furthermore, inflammation induced by MPTP may contribute to head volume reduction through glial cell activation, which, while part of the neural repair process, can also disrupt normal development [69]. Importantly, our results demonstrated that neither QU nor NEQU were able to prevent the recurring damage associated with MPTP-induced reduction in head size. A limitation of the present study is the absence of a direct assessment of dopaminergic (DA) neuron loss, a core pathological hallmark of Parkinson's disease. Future studies including immunohistochemical and

molecular analyses of DA neurons will be essential to complement and strengthen the current findings.

The potential interaction of the quercetin nanoemulsion with the blood–brain barrier (BBB) is an important consideration when evaluating its neuroprotective effects. Previous studies have shown that nanoparticle size is a critical determinant of BBB permeability, with smaller particles demonstrating a higher capacity to cross and reach the brain parenchyma [70]. In our study, the nanoemulsion presented an average size of approximately 20 nm, which falls within the range reported to favor BBB permeation. Moreover, recent findings indicate that quercetin-loaded nanoparticles can enhance neuroprotection while preserving BBB integrity and reducing neuronal damage under oxidative stress conditions [71]. Although the present work did not directly investigate BBB penetration, these data support the plausibility that the nanoemulsified formulation could reach the brain and contribute to the observed protective effects. Future studies specifically designed to evaluate BBB transport and safety will be essential to confirm this hypothesis.

Several studies have highlighted the potential of QU to modulate GSH levels by inducing its synthesis [72,73]. This effect is dose-dependent, as high concentrations of QU can alter GSH homeostasis through the action of glutathione peroxidase (GSH-Px). In this pathway, hydrogen peroxide (H_2O_2) is converted into water (H_2O), while GSH is oxidized to GSSG (oxidized glutathione or glutathione disulfide). Glutathione reductase (GSH-Rd) then catalyzes the reduction of GSSG back to GSH, mainly in the liver and red blood cells, thereby maintaining the balance between reduced and oxidized glutathione. This equilibrium is essential for the antioxidant defense system but may be highly sensitive to the administered dose of quercetin. At low concentrations, however, quercetin has paradoxically been reported to inhibit GSH levels, with some studies observing this effect at concentrations around 0.5% [72–75].

In our study (Figure 5A), the results related to GSH levels indicate that although QU possesses antioxidant potential through modulation of GSH pathways, it was unable to increase GSH levels when tested in the PD model. Importantly, our findings confirm the robustness of the zebrafish PD model, in which GSH levels were reduced by 49% compared with the control group. These results are consistent with previous reports; for instance [33], demonstrated that zebrafish larvae exposed to MPTP alone exhibited a significant 23.85% reduction in GSH levels. Together, these data confirm that a compromised antioxidant system is a hallmark of the zebrafish PD model [33].

The decrease in GSH levels, one of the primary endogenous antioxidants, is directly associated with exacerbated oxidative stress, which contributes to neurodegeneration. Although some studies suggest that QU may enhance antioxidant defenses in certain contexts, the lack of effect on GSH levels observed here may be related to factors such as the concentration administered, the duration of exposure, or the specific conditions of the experimental model. These findings underscore the complexity of the interaction between QU and the antioxidant system, particularly under stress conditions. They also suggest that the dose of QU may influence physiological GSH levels, potentially stimulating its synthesis at higher concentrations while reducing its effectiveness at lower doses due to its impact on the GSH redox cycle. It is important to note that the reduction in lipid peroxidation observed in our study was not accompanied by a complete normalization of GSH levels. This apparent discrepancy is consistent with previous reports indicating that, while antioxidant treatments can attenuate ROS accumulation and lipid peroxidation, the restoration of endogenous antioxidant systems such as glutathione is often incomplete. This reflects the complex and multifactorial nature of redox imbalance in Parkinson's disease models. The observation that both free quercetin and nanoencapsulated quercetin reduced lipid peroxidation but did not restore GSH levels suggests that the neuroprotective effect

of quercetin is not exclusively dependent on the glutathione pathway. In the context of MPTP-induced ROS generation, quercetin may act directly by scavenging these species before they inflict significant damage on cellular membranes, thereby preventing lipid peroxidation. Beyond this direct antioxidant activity, another plausible mechanism involves the activation of the transcription factor Nrf2, which regulates cellular antioxidant and anti-inflammatory responses [76]. Once translocated into the nucleus, Nrf2 induces the expression of several antioxidant enzymes, including GST, HO-1, SOD, and CAT, which mitigate oxidative stress through pathways parallel to the glutathione system [15]. In this context, the improvement in lipid peroxidation observed in our study may have been mediated by preferential activation of these enzymes rather than by GSH synthesis, particularly if intracellular GSH pools were severely depleted by MPTP and required more time or stronger stimulation to achieve significant recovery.

ROS play a crucial role in the pathology of PD, contributing to oxidative stress and neurodegeneration [76]. In this study, both QU and NEQU were effective in reducing ROS levels in zebrafish larvae exposed to MPTP, which is known to elevate ROS production. In contrast, the vehicles DMSO and NE, when combined with MPTP, increased oxidative stress. The reduction in ROS observed with QU and NEQU indicates a significant protective effect against MPTP-induced oxidative damage, supporting the restoration of cellular redox balance. These results emphasize not only the capacity of these formulations to decrease oxidative load but also the importance of antioxidant strategies in treating neurodegenerative conditions. The increase in ROS levels observed in the Vehicle + MPTP groups compared with MPTP alone can be explained by the pharmacological properties of the carriers. MPTP requires metabolic conversion by monoamine oxidase-B (MAO-B) into MPP⁺, the active metabolite that inhibits mitochondrial Complex I and triggers excessive ROS production [15]. DMSO is known to rapidly penetrate cellular membranes and the blood–brain barrier, thereby enhancing intracellular compound release. This likely facilitated greater uptake of MPTP by target neurons, leading to elevated intracellular levels of MPP⁺ and exacerbated oxidative stress [77]. Similarly, nanoemulsions improve solubility, stability, and transport of molecules across biological barriers, which may have increased the bioavailability and neuronal delivery of MPTP [78,79]. These findings support the interpretation that the apparent pro-oxidant effect of the carriers does not result from intrinsic toxicity but rather from enhanced MPTP delivery to dopaminergic neurons, leading to greater MPP⁺ formation and amplified oxidative stress.

Studies on lipid peroxidation in MPTP models are essential for understanding how oxidative stress contributes to neurodegeneration, particularly in PD. MPTP is a neurotoxin that induces PD-like symptoms by promoting excessive ROS production. This ROS overproduction leads to lipid peroxidation, a process that damages the membranes of dopaminergic neurons, primarily located in the substantia nigra, a brain region critical for movement control. Impaired mitochondrial function accelerates the death of these neurons [80,81].

Research has demonstrated that lipid peroxidation is directly associated with the loss of dopaminergic cells in MPTP models, making it an important marker of neurodegeneration in PD. Strategies targeting oxidative stress, such as the use of antioxidants like quercetin, have shown potential in reducing ROS levels and protecting neurons. Quercetin, in particular, is recognized for its strong antioxidant properties and is under investigation for its protective effects in PD models [15,82–84].

QU protects against lipid peroxidation by inhibiting the formation of lipid degradation products, thereby preserving cellular integrity. This antioxidant action is particularly important under conditions of oxidative stress, where lipid peroxidation contributes to cellular damage and neurodegeneration. Our findings are consistent with this evidence

(Figure 5B), showing that both QU and NEQU reduced lipid peroxidation, as indicated by lower MDA levels. This reduction suggests that both free and nanoencapsulated quercetin exert significant protective effects against oxidative damage, reinforcing their therapeutic potential in PD [7,18,84].

The positive effects observed in this study for QU, both in its free form and as a nanoencapsulated compound, can be attributed to its nature as a flavonol that exerts antioxidant activity by neutralizing a variety of free radicals, including hydroxyl, peroxy, and superoxide species. One of its primary mechanisms of action is the induction of antioxidant gene expression through activation of the Nrf2 pathway [85]. In addition, QU contributes to the inactivation of nitric oxide and the prevention of lipid oxidation, both of which are processes associated with MPTP exposure [86]. A limitation of this study is that the carriers employed (DMSO and NE containing castor oil) possess intrinsic biological activity, which may have influenced the observed outcomes. Future studies using inert carriers, such as nanoemulsions formulated with pharmacologically neutral oils, will be required to better isolate the specific contribution of quercetin nanoencapsulation.

The BBB is a highly specialized system that separates the brain from the systemic circulation, preserving the neural microenvironment and protecting nervous tissue from potentially toxic agents. This barrier is formed by endothelial cells tightly connected through occluding junctions, supported by pericytes and enveloped by astrocytic end-feet. Such an organization confers high selectivity to molecular transport, restricting passive diffusion and allowing only the controlled passage of nutrients, ions, and signaling molecules essential for cerebral homeostasis [87]. Among the factors that influence the ability of a compound or nanocarrier to cross the BBB, particle size plays a decisive role. Nanosystems with diameters below 100 nm, particularly those ranging between 10 and 50 nm, tend to exhibit a higher probability of crossing the barrier, possibly by exploiting receptor-mediated or adsorptive transcytosis pathways on the endothelial surface [88–90]. The nanoemulsion developed in this study presented an average droplet diameter of approximately 20 nm, which falls within the range generally considered favorable for BBB interaction. Lipid-based systems of this type not only enhance the solubility and systemic stability of poorly soluble drugs but also facilitate their diffusion through biological membranes [89,91]. Another relevant component of the formulation is quercetin, known for its strong antioxidant potential and neuroprotective activity. When incorporated into nanostructured systems, its bioavailability and stability increase substantially, overcoming the limitations imposed by its polarity and low solubility in the free form [91]. Thus, the combination of the nanoemulsion lipid matrix with quercetin results in a promising system for applications targeting the CNS.

Nevertheless, it is essential to acknowledge the limitations of the experimental model used. The zebrafish (*Danio rerio*) larvae employed in this study are at an early developmental stage, during which the BBB is not yet fully formed. Structures such as tight junctions begin to differentiate around three days post-fertilization (dpf), but the barrier achieves full functional maturity only near the tenth dpf [92,93]. Therefore, at 4–5 dpf, the developmental stage used for exposure in this study, the BBB is only partially developed. This condition allows the observation of preliminary neuroactive or neuroprotective effects but does not reflect the full selectivity observed in adult mammals. Consequently, the results presented here should be interpreted as indicative of potential neuroprotective mechanisms rather than definitive evidence of effective BBB penetration. Further investigations using endothelial BBB models or mammalian systems will be required to confirm whether this nanoemulsion can indeed cross the barrier and exert direct therapeutic effects within neural tissue.

5. Conclusions

This study demonstrated that quercetin, both in its free and nanoemulsified forms, may help mitigate some of the damage caused by PD, alleviating the toxic effects of MPTP in zebrafish larvae. Our results showed that quercetin (QU) and nanoemulsified quercetin (NEQU) exert protective effects on the central nervous system. In the zebrafish model, this protection was reflected not only in biochemical and behavioral outcomes but also in the preservation of morphological structures such as the swim bladder. Although this alteration does not directly correspond to the motor symptoms observed in clinical PD, in zebrafish larvae swim bladder functionality is essential for proper locomotion and balance, both of which are negatively affected by MPTP exposure. Thus, preservation of this structure should be interpreted as an indirect marker of improved motor performance within this experimental model. In addition, both formulations demonstrated antioxidant properties that contributed to reducing damage caused by lipid peroxidation and reactive oxygen species. Notably, only NEQU was able to reverse the motor impairments induced by MPTP, as evidenced by the analysis of total distance traveled, suggesting that the neuroprotective effect may be partly attributed to the properties of the nanoformulation. This work also reinforces the value of zebrafish as a model for research in neuroscience and toxicology. Finally, our findings open new avenues for future studies that may contribute to the development of novel strategies for the treatment of neurodegenerative diseases. Further investigations exploring higher concentrations of free quercetin, compared with the nanoemulsion formulation, may provide complementary insights into its efficacy, an aspect to be addressed in subsequent studies.

Author Contributions: Conceptualization, C.d.O.V., A.P.H. and M.A.H.; methodology, C.d.O.V., M.A.G.M., V.P.M., C.M.A., J.L.R., F.B.d.M., C.L.D., A.P.H., R.F.D.A. and M.A.H.; software, C.d.O.V., F.B.d.M. and R.F.D.A.; validation, C.d.O.V., A.P.H. and M.A.H.; formal analysis, C.d.O.V., A.P.H. and M.A.H.; investigation, C.d.O.V., M.A.G.M., V.P.M., C.M.A., J.L.R. and F.B.d.M.; data curation, C.d.O.V. and M.A.G.M.; writing—original draft preparation, F.B.d.M.; writing—review and editing, C.d.O.V., A.P.H. and M.A.H.; visualization, C.d.O.V., A.P.H. and M.A.H.; supervision, A.P.H. and M.A.H.; project administration, C.d.O.V., A.P.H. and M.A.H.; funding acquisition, M.A.H. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES) [001].

Institutional Review Board Statement: The animal study protocol was approved by the Animal Use Ethics Committee of the Federal University of Rio Grande (protocol no. Pq019/2021, approved on 26 May 2022).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are available from the corresponding author, M.A.H., upon reasonable request.

Conflicts of Interest: The authors declare no potential conflicts of interest.

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