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Pereskia aculeata Leaves as a Functional Food: Neurobehavioral and Redox Homeostasis Protection of Aged *Drosophila melanogaster* Against Paraquat Exposure

Jackeline De Miranda Schmidt[1] | Elize Musachio[1,2,3] | Débora Luísa Filipetto Pulcinelli[1] | Maria Eduarda Chelotti[1] | Nathália Cardoso de Afonso Bonotto[1] | Caroline Prado Rehbein[1] | Eliana Jardim Fernandes[4] | Gustavo Petri Guerra[4] | Maria Denise Schimith[2] | Ivana Beatrice Mânica Da Cruz[5] | Verônica Farina Azzolin[3,5] | Cindhy Suely da Silva Medeiros[1,3] | Caroline dos Santos Giuliani[6] | Aline Sobreira Bezerra[6] | Fernanda Barbisan[1,2,3]

[1]Biogenomics Laboratory, Federal University of Santa Maria, Av. Roraima 1000, Santa Maria 97105-900, Rio Grande do Sul, Brazil | [2]Postgraduate Program in Nursing, Federal University of Santa Maria, Av. Roraima 1000, Santa Maria 97105-900, Rio Grande do Sul, Brazil | [3]Postgraduate Program in Gerontology, Federal University of Santa Maria, Av. Roraima 1000, Santa Maria 97105-900, Rio Grande do Sul, Brazil | [4]Postgraduate Program in Biochemistry, Federal University of Pampa, Campus Uruguaiana, Road 472-Km 585, Uruguaiana 97501-970, Rio Grande do Sul, Brazil | [5]Open University of the Third Age Foundation, Av. Brasil 11430–Santo Antônio, Manaus 69029-040, Amazonas, Brazil | [6]Department of Food Technology and Science, Federal University of Santa Maria, Av. Roraima 1000, Santa Maria, Rio Grande do Sul 97105-900, Brazil

Correspondence: Fernanda Barbisan (fernanda-barbisan.1@ufsm.br)

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ABSTRACT

The aim of this study was to investigate the nutritional composition and functional properties of *Pereskia aculeata* Miller, known as Ora-pro-nóbis (OPN), and to evaluate whether dietary supplementation enhances resistance to paraquat-induced oxidative stress in aged *Drosophila melanogaster*. The nutritional and bioactive profile of OPN powder was characterized, and its functional impact was assessed using an experimental aging model. The flies were subjected to an aging protocol followed by dietary supplementation with OPN prior to an oxidative challenge. It was observed that prior supplementation with OPN preserved survival in flies exposed to PQ and protected locomotor function, an effect associated with modulation of acetylcholinesterase activity. OPN attenuated the oxidative damage and contributed to redox homeostasis, possibly by supporting endogenous antioxidant defenses. These results highlight the nutritional and functional potential of OPN, suggesting it as a promising dietary and therapeutic resource capable of strengthening the functional capacity of the antioxidant system and supporting functional resilience during aging.

1 | Introduction

The World Health Organization estimates that by 2050, 2 billion people, nearly one-fifth of the global population, will be elderly [1]. Human aging is recognized as a complex and inevitable physiological process, marked by cumulative and dynamic changes that gradually compromise homeostasis and increase vulnerability to disease [2]. Among the main factors involved, the imbalance between the production of reactive oxygen species (ROS) and the capacity of the endogenous antioxidant defense system stands out, leading to oxidative stress [3]. This imbalance not only accelerates the functional decline but also triggers a series of structural and molecular damages, including lipid peroxidation, protein oxidation, mitochondrial dysfunction,

Jackeline De Miranda Schmidt and Elize Musachio contributed equally to this work.

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DNA damage, activation of proinflammatory pathways, and enzymatic alterations [4, 5].

One of the most important targets of this process is the enzyme acetylcholinesterase (AChE), which is highly sensitive to oxidative imbalance, can be considered a marker of aging, and is associated with motor and cognitive deficit characteristics of neurodegenerative diseases [6]. These effects underscore the crucial role of endogenous antioxidant defenses, with reduced glutathione (GSH) being particularly relevant due to its detoxifying and regulatory functions. Elevated levels of GSH are associated with improved physical and cognitive performance in older adults, suggesting its importance in healthy longevity. However, GSH biosynthesis is significantly impaired during aging, favoring the accumulation of the oxidized form (GSSG) over the reduced form, resulting in decreased ability to neutralize ROS and conjugate xenobiotics [7, 8].

Therefore, dietary supplementation with natural antioxidants has widely been studied as a strategy to improve health and potentially increase longevity [9]. Bioactive compounds present in foods can increase endogenous antioxidant defenses and improve the organism's survival under stress, mainly by increasing the synthesis of endogenous antioxidant molecules, such as GSH, and modulating antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) [10].

Within this framework, *Pereskia aculeata* Miller, popularly known as Ora-pro-nóbis (OPN), stands out as an underutilized plant species native to Latin America, traditionally consumed as a nutritional resource and now attracting growing scientific interest [11]. Its leaves contain high levels of carotenoids, alkaloids, flavonoids, terpenes, and tocopherol (vitamin E), compounds associated with potential pharmacological properties, including antioxidant and neuroprotective effects [12]. Since natural deteriorating mechanisms occur during aging, such as the compromised antioxidant system, alternatives that help maintain redox balance and the body's ability to combat oxidative agents more efficiently are necessary. Therefore, based on the bioactive components found in OPN leaves, studying the effect of dietary supplementation focusing on the leaves of this plant emerges as a promising, yet still little-explored, perspective. Considering its bioactive composition and potential protective effects, the development of powdered OPN is a strategic approach, as it overcomes the limitations of consuming whole leaves and allows its incorporation into diets in a practical and accessible way. Despite preliminary evidence, most studies have focused on the fresh plant or extracts, and systematic investigations on the effects of OPN on aging and redox status in *in vivo* models are still limited.

To address this gap, it is necessary to employ a validated aging model organism capable of exhibiting both neurobehavioral and oxidative damage. In this sense, *Drosophila melanogaster* has widely been validated as an experimental model for aging studies, given its similarities to humans in cholinergic and antioxidant pathways [13]. The fly also presents attributes such as a short life cycle, ease of handling, low economic cost, exemption from ethics committees, and a positive response to antioxidant interventions, making it appropriate for investigating nutritional strategies in the context of aging [14, 15]. Santos et al. [10] used young flies to evaluate the effect of consuming OPN powder at different concentrations for 7 days on the basal redox and neurobehavioral systems. In contrast, this research offers the novelty of using a chronologically aged living organism to evaluate not only the effect of OPN supplementation on the basal redox system but also its effectiveness against a potent oxidative and neurotoxic agent.

In experimental research, one of the most established methods for inducing oxidative stress in vivo is the use of paraquat (PQ), a highly toxic herbicide that undergoes reduction in the organism, generating a stable radical that reacts with oxygen to produce superoxide anion, a highly reactive species [16]. The accumulation of ROS and the subsequent depletion of reducing agents create an oxidative stress environment, causing damage to lipids, proteins, and DNA, as well as neurotoxicity, as demonstrated in several animal models. Therefore, this study aimed to characterize the proximate composition, chemical profile, and lipid profile of powdered OPN, and to evaluate whether dietary supplementation could confer resistance to PQ-induced oxidative stress in chronologically aged *Drosophila melanogaster*.

2 | Methods

2.1 | OPN Acquisition

The powdered OPN used in this study was commercially obtained from ApisNutri (https://www.apisnutri.com.br). According to the technical report provided, OPN leaves were collected, oven-dried, and ground into a dark green powder. All physicochemical and microbiological analyses were performed in accordance with the parameters established by Resolution RDC No. 623, March 9, 2022, of the Brazilian Health Regulatory Agency (ANVISA).

2.2 | Proximate Characterization of the Species

The proximate composition of the lyophilized sample was evaluated by determining the contents of moisture, ash, lipids, proteins, crude fiber, and carbohydrates, following the methods described by AOAC [17]. Total carbohydrate content was calculated by difference, subtracting from 100 the sum of the moisture, ash, lipid, crude fiber, and protein values.

2.3 | Fatty Acid Profile Analysis

Lipids were extracted according to the method of Bligh and Dyer [18], followed by methylation of fatty acids as described by Hartman and Lago [19]. Fatty acid composition was analyzed by gas chromatography using a flame ionization detector (GC-FID) (Agilent 6890, Agilent Technologies, USA) equipped with a DB-23 capillary column. Chromatographic conditions were adjusted according to standard protocols for fatty acid methyl ester separation. Individual fatty acids were identified by comparison of retention times with a certified FAME Mix 37 standard (Supelco, USA). The results were expressed as relative percentages of total identified fatty acids, following the analytical recommendations described by Visentainer [20].

2.4 | Chemical Characterization by Spectrophotometry

The chemical characterization of OPN powder was performed using established spectrophotometric methods, as described below. All analyses were conducted in triplicate, and the results

were calculated based on analytical calibration curves. Reagents were obtained from Sigma-Aldrich Co. (St. Louis, USA).

The total phenolic content was determined using the Folin–Ciocalteu method, following Singleton and Rossi [21]. The results were expressed as gallic acid equivalents per gram of OPN (mg GAE/g).

The total flavonoid content was quantified using the aluminum chloride colorimetric assay, according to Marinova et al. [22], and expressed as catechin equivalents per gram of OPN (mg CATEQ/g).

Condensed tannins were determined using the vanillin–HCl assay, as described by Morrison [23], with minor adaptations to accommodate the OPN matrix. The results were expressed as catechin equivalents per gram of sample (mg catechin/g).

The total alkaloid content was assessed using the colorimetric method described by Sreevidya and Mehrotra [24], based on the formation of a colored complex with Dragendorff® reagent, and the results were expressed as bismuth nitrate equivalents per gram of OPN (mg BN/g).

2.5 | Antioxidant Capacity Assay

The antioxidant capacity of OPN powder was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, following a slightly modified version of the method described by Choi et al. [25]. Different concentrations of OPN extracts were incubated with the DPPH solution, and the absorbance was measured spectrophotometrically after a fixed reaction period. Rutin was used as a positive control. Antioxidant activity was expressed as percentage of DPPH radical inhibition, and the IC_{50} value (concentration required to inhibit 50% of the radical) was calculated by regression analysis.

2.6 | *Drosophila melanogaster* Stock Acquisition and Maintenance

Wild-type *Drosophila melanogaster* (Harwich strain) was obtained from the Biogenomics Laboratory at the Federal University of Santa Maria. Flies were maintained under standard laboratory conditions, as previously described by Meichtry et al. [26], and fed a conventional cornmeal-based diet throughout the experimental period.

Dietary supplementation and experimental exposures were conducted exclusively during the aging and oxidative challenge phases, as described in the subsequent sections.

2.7 | Experimental Design

Male and female flies were randomly allocated into four groups: control (CTL), PQ, OPN, and OPN + PQ. The experimental design comprised three sequential stages: (i) aging of flies, (ii) supplementation with OPN, and (iii) acute exposure to PQ as illustrated in Figure 1. After these interventions, behavioral and biochemical marker analyses were performed by examiners who were unaware of the experimental groups.

2.7.1 | Aging Protocol

Flies are considered elderly after 40 days of age [5]. Therefore, we developed an aging protocol with a total of 200 flies for each independent treatment. *Drosophila melanogaster* flies of both sexes were removed from the stock up to 1 day of age and transferred to vials containing a standard diet until they were 38 days old.

2.7.2 | OPN Supplementation Protocol

At 38 days of age, flies were divided into two experimental groups ($n = 100$ per group). The supplemented group received a diet containing 10% OPN powder (1 g of OPN per 10 g of standard diet) *ad libitum* (concentration chosen according to Santos et al. [10]. Supplementation was maintained for 7 days, tempo standard in *Drosophila melanogaster* dietary protocols and sufficient to detect relevant metabolic and physiological changes [27, 28].

2.7.3 | Protocol for Exposure to PQ

At the end of 45 days of life (after 7 days of exposure), *both* the supplemented and nonsupplemented groups were subdivided: half of the individuals ($n = 50$) were exposed to PQ and the other half ($n = 50$) remained in the control solution. Thus, 50 supplemented and 50 nonsupplemented flies were transferred to vials containing filter paper soaked in a 10-mM PQ solution diluted in 5% sucrose, as described by Ueda et al. [29]. The exposure was performed acutely, for 24 h. The remaining groups (50 supplemented and 50 nonsupplemented) were transferred to vials containing only 5% sucrose. Thus, four experimental groups were established: (I) CTL – nonsupplemented, exposed only to sucrose; (II) PQ – nonsupplemented, exposed to PQ; (III) OPN – supplemented, exposed only to sucrose; and (IV) OPN + PQ – supplemented, exposed to PQ. After the three experimental protocols (aging, supplementation, and exposure to PQ), the flies, then 46 days old, were subjected to in vivo and ex vivo tests, as described in the sections below.

2.8 | In Vivo Tests

2.8.1 | Mortality Rate Assessment

The mortality rate was assessed by counting the number of flies that died after 24 h of exposure to PQ. Each group consisted of approximately 50 flies. For this analysis, three independent experiments were performed ($n = 3$). The result was expressed as a percentage of flies.

2.8.2 | Behavioral Tests to Assess Locomotor Capacity

The open field test described by Connolly [30], with adaptations by Musachio et al. [13], assessed the locomotor and exploratory activity of flies. The flies (five per group) were individually evaluated in a shallow arena (3 cm high to limit flight movement and encourage walking), with a lid marked with 1 cm^2 quadrants on the surface. Activity was quantified by counting the number of quadrants traversed in 1 min. The test was performed in duplicate for each fly, and the average of these values was calculated.

The negative geotaxis test was performed following the method of Ref. [31] to analyze the climbing ability of flies. Five flies from each group were used and individually placed in vertically positioned tubes, and the time required to reach a height of 8 cm was recorded, with a maximum duration of 2 min. Both behavioral tests were performed in five independent experiments ($n = 5$).

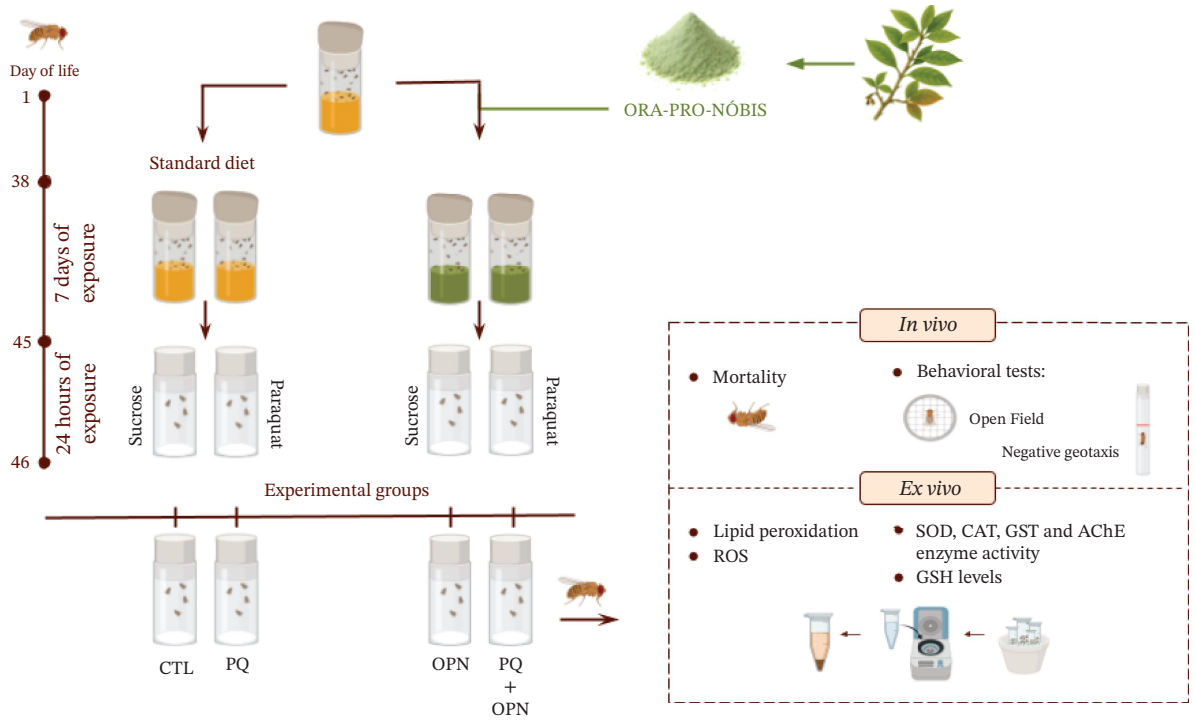


FIGURE 1 | Experimental protocol design.

2.9 | Ex Vivo Analyses

2.9.1 | Supernatant Preparation

The prepared supernatant consists of homogenized tissue from the whole body of a set of 10 flies from each experimental group. For fly homogenization, 20 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer (pH 7.0) dissolved in water was used for TBARS, ROS, AChE, SOD, CAT, and GST analyses. In the preparation of the homogenate used for GSH determination, the flies were homogenized in 0.5 M tris(hydroxymethyl)aminomethane (Tris–HCl) buffer (pH 8.0), because in addition to buffering, it does not chemically interfere with the sulfhydryl groups [32, 33]. Centrifugation times varied according to the protocol for each assay: SOD, CAT, GST, and AChE, 1.000 rpm for 10 min; TBARS and GSH, 10.000 rpm for 5 min; and ROS, 3.570 rpm for 5 min. After centrifugation, the precipitate was discarded and the supernatant preserved for analysis, as described below.

2.9.2 | Assessment of Lipid Peroxidation

Lipid peroxidation was assessed by quantifying thiobarbituric acid reactive substances (TBARS), specifically detecting malondialdehyde, as described by Ohkawa et al. [34], adapted by Musachio et al. [13]. For each group, 100 μL of the supernatant was added to a reaction mixture containing thiobarbituric acid (8% pH 3.2), acetic acid (pH 3.4), sodium sulfate (1.2% SDS), and water. Samples were incubated at 95°C for 2 h, after which absorbance was measured at 532 nm. Values were normalized by protein concentration and expressed as nmol MDA/mg of tissue.

2.9.3 | Quantification of ROS

The quantification of ROS was performed following the protocol of Pérez-Severiano et al. [35], based on the oxidation of 2′,7′-dichlorofluorescein diacetate (DCFDA). For this, supernatant was mixed with HEPES buffer and DCFDA (1 mM), incubated for 1 h at room temperature, protected from light. Fluorescence was quantified at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. The values were expressed as a percentage relative to the control group.

2.9.4 | Quantification of Reduced GSH Levels

To quantify GSH, the method of Jollow et al. [36], adapted by Shakya and Siddique [37], was used, where the free sulfhydryl group (-SH) reduces 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). The supernatant was incubated with Tris–HCl buffer (0.5 M, pH 8) and DTNB (5 mM) for 20 min in the dark, and the absorbance was read at 412 nm. The results were expressed as a percentage relative to the control group.

2.9.5 | AChE Enzyme Activity

AChE activity was evaluated based on Ellman et al. [38], with adaptations from Lucas et al. [39], which consists of the reaction of thiocholine with DTNB. The supernatant was added to the reaction mixture containing KPi buffer (0.1 M, pH 8.0), DTNB (10 mM), and ACh (7.25 mM)), and the absorbance was rapidly monitored at 412 nm for 2 min. Enzyme activity was expressed in μmol ACh/h/mg of protein.

2.9.6 | Determination of SOD Activity

SOD activity was evaluated according to Kostyuk and Potapovich [40], based on the enzyme's inhibitory effect on quercetin oxidation. The supernatant were incubated in HEPES buffer and KPi/EDTA (0.025 M/0.1 mM, pH 10), TEMED, and quercetin. Absorbance was monitored at 406 nm for 2 min. Enzymatic activity was expressed in U/mg of protein, corresponding to 50% inhibition of quercetin oxidation.

2.9.7 | Determination of CAT Activity

CAT activity was determined according to Aebi [41], with adaptations from Paula et al. [42]. The assay is based on the enzyme's ability to degrade hydrogen peroxide (H_2O_2). The supernatant was added to the reaction mixture containing KPi/EDTA buffer (0.25 M/2.5 mM, pH 7.0), H_2O_2 (30%) and Triton X-100, and the absorbance was quickly measured in a spectrophotometer at 240 nm. The results were expressed as CAT units per mg of protein (U/mg).

2.9.8 | Determination of GSH-S-Transferase (GST) Activity

GST activity was determined according to Habig et al. [43], with adaptations from Musachio et al. [13], where enzyme-catalyzed conjugation occurs with the substrate 1-chloro-2,4-dinitrobenzene (CDNB), thus forming a quantifiable product. The supernatant was incubated with KPi/EDTA buffer (0.25 M/2.5 mM, pH 7.0), GSH (100 mM), and 1-chloro-2,4-dinitrobenzene (CDNB) (50 mM). Enzymatic activity was normalized by the protein content and expressed in mU/mg of protein.

2.9.9 | Total Protein Quantification

The protein content of the sample was analyzed according to the Bradford [44] colorimetric assay. The method is based on the interaction between Coomassie Brilliant Blue G-250 and basic and aromatic amino acid residues, which can be quantified spectrophotometrically at 595 nm.

2.10 | Statistical Analysis

All chemical characterization analyses were performed in triplicate, with results expressed as mean± standard deviation (SD). The antioxidant capacity assay was evaluated by IC_{50}.

The statistical analysis of the results from the in vivo and ex vivo assays was performed using GraphPad Prism software, version 6 (San Diego, CA, USA), using the following statistical model: $Y_{ij} = \mu + O_i + P_j + O \times Y_{ij} + e_{ij}$, where Y_{ij} represents the observed value; μ is the overall mean; O_i is the fixed effect of OPN; P_j is the fixed effect of PQ; $O \times Y_{ij}$ is the fixed interaction effect between OPN and PQ; and e_{ij} is the random experimental error. Therefore, a two-way ANOVA was used for all data, with Tukey's test employed for multiple comparisons. Probability values less than 0.05 ($p \leq 0.05$) were considered statistically significant.

3 | Results

3.1 | Chemical Characterization and Fatty Acid Profile of the Sample

The proximal composition and concentration of total phenolic compounds, flavonoids, tannins, and alkaloids of the species are described in Table 1. The profile and quantification of fatty acids present in the sample are described in Table 2.

When evaluating the proximate composition of OPN, a low moisture content (7.46 g%), low lipid fraction (2.24 g%), high ash (16.10 g%), protein (19.76 g%), and fiber (33.33 g%) contents were identified. Regarding bioactive compounds, OPN presented a high concentration of total phenolics (320.32 mg GAE/g), significant levels of tannins (130.92 mg CATEQ/g) and flavonoids (16.53 mg CATEQ/g), and traces of alkaloids (12.16 mg NB/g).

TABLE 1 | Proximal composition, total phenolic, flavonoid, tannin, and alkaloid content of OPN.

Component	Mean ± standard deviation
Moisture (g%)	7.46 ± 0.12
Ash (g%)	16.10 ± 0.14
Protein (g%)	19.76 ± 0.15
Lipid (g%)	2.24 ± 0.03
Crude fiber (g%)	33.33 ± 2.77
Carbohydrate (g%)	21.12 ± 2.86
Caloric value (kcal)	183.66 ± 12.28
TPC (mg GAE/g)	320.32 ± 0.01
Flavonoids (mg CATEQ/g)	16.53 ± 0.002
Tannins (mg CATEQ/g)	130.92 ± 0.02
Alkaloids (mg NB/g)	12.16 ± 0.003

Note: CATEQ = catechin equivalent; NB = bismuth nitrate equivalent; TPC = phenolic content; the total value was determined from three replicate measurements performed for each compound analysis.
Abbreviation: GAE = gallic acid equivalent.

The fatty acid profile results showed a predominance of SFA (41.19%), with lauric (C12:0), tridecanoic (C13:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), arachidic (C20:0), behenic (C22:0), and lignoceric (C24:0) acids being the most prominent. MUFA accounted for 8.73%, with oleic acid (C18:1n9) being the most abundant. Among PUFA, α-linolenic acid (C18:3n3)

TABLE 2 | Fatty acid profile of OPN (*Pereskia aculeata)*.

[1]Fatty acid	OPN (*Pereskia aculeata*)
C12:0	0.28
C13:0	1.25
C14:0	0.79
C16:0	29.46
C16:1n7	0.19
C18:0	6.48
C18:1n9	8.54
C18:2n6	8.72
C18:3n3	32.11
Long-chain SFA (≥ C20:0)	2.38
SFA	41.19
MUFA	8.73
PUFA	40.83
PUFA/SFA	0.99
$\sum n-6$	8.72
$\sum n-3$	32.11
$\sum n-6/n-3$	0.28

[1]values expressed as a percentage of the total area of the chromatograms; SFA (saturated fatty acids = C12:0; C13:0; C14:0 C16:0; C18:0 and long-chain SFA ≥ C20:0); MUFA (monounsaturated fatty acids = C16:1n7; C18:1n9); and PUFA (polyunsaturated fatty acids = C18:2n6c; C18:3n3).

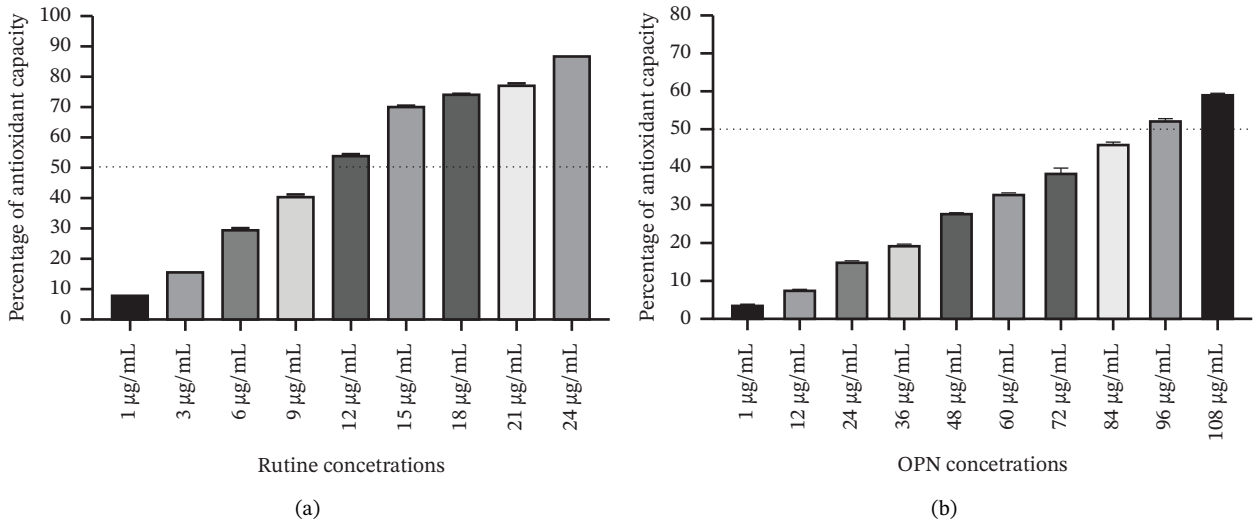


FIGURE 2 | DPPH radical scavenging activity. (a) Rutin. (b) OPN. All were expressed as a percentage of antioxidant capacity (%AC) at different concentrations. The dashed line represents the IC_{50} value.

showed the highest concentration (32.11%), followed by linoleic acid (C18:2n6) at 8.72%. The n-6/n-3 ratio was 0.28, and the polyunsaturated/saturated (PUFA/SFA) ratio was 0.99.

3.2 | Antioxidant Capacity Assay

The DPPH radical scavenging activity (Figure 2) of OPN powder was determined at concentrations of 1, 12, 24, 36, 48, 60, 72, 84, 96, and 108 μg/mL, obtaining the straight-line equation $y = 0.6237x + 38{,}501$ ($R^2 = 0.9976$). For a positive control, rutin was used at concentrations of 1, 3, 6, 9, 12, 15, 18, 21, and 24 μg/mL, obtaining the straight-line equation $y = 10{,}269x - 0.4936$ ($R^2 = 0.9756$). The 50% concentration to inhibit DPPH radicals (IC_{50}) was obtained with about 96 μg/mL (52. 57%) for OPN and approximately 12 μg/mL (54.66%) for the rutin standard.

3.3 | Mortality Rate

When evaluating the mortality rate (Figure 3) of aged flies after 24 h of exposure to PQ, it was observed that the group supplemented with OPN and exposed to PQ showed a decreased mortality rate compared to the nonsupplemented group ($p = 0.0005$).

3.4 | Locomotion Tests and AChE Activity

The AChE activity (Figure 4(a)) of flies supplemented with OPN and exposed to PQ did not differ significantly from the control group, suggesting the preservation of cholinergic activity. On the other hand, flies not supplemented with OPN and exposed to PQ had a significant reduction in AChE activity compared to the control ($p < 0.0001$).

In the open field test (Figure 4(b)), OPN supplementation increased the locomotor activity of flies exposed to PQ, which covered a greater number of quadrants in 1 minute compared to flies exposed without supplementation ($p = 0.0009$). The flies supplemented and not exposed to PQ did not differ significantly from the control group. In the negative geotaxis test, the climbing time of flies (Figure 4(c)) was significantly shorter in the group supplemented with OPN + PQ compared to the group exposed to PQ alone ($p < 0.0001$), indicating preservation of locomotor capacity.

3.5 | Oxidative Stress Parameters and Cellular Metabolic Activity

3.5.1 | ROS Levels and Lipid Peroxidation

When quantifying ROS levels (Figure 5(a)), aged flies supplemented with OPN and exposed to PQ showed a significant reduction in ROS production compared to the group exposed to PQ without supplementation. However, ROS levels remained higher than the control group. This indicates that OPN provided partial protection against oxidative stress. Lipid peroxidation, assessed by MDA levels (Figure 5(b)), was significantly lower in flies supplemented with OPN and exposed to PQ compared to the nonsupplemented and exposed groups ($p = 0.0001$).

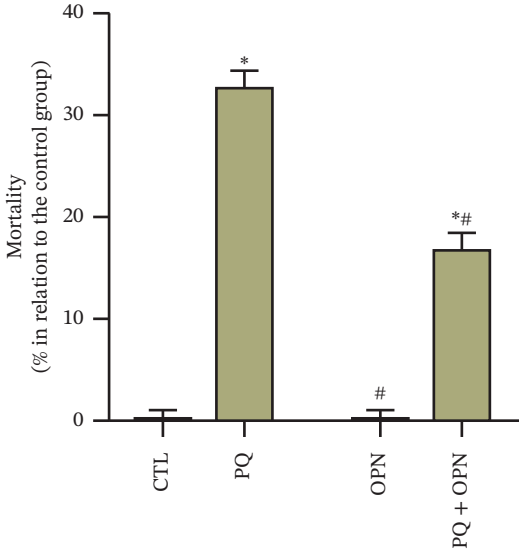


FIGURE 3 | Evaluation of the mortality rate of aged *Drosophila melanogaster* supplemented and nonsupplemented flies with Ora-pro-nóbis (OPN), and those exposed/not exposed to paraquat (PQ) for 24 h. The asterisk indicates a statistically significant difference ($p \leq 0.05$) compared to the control group. The hash indicates a statistically significant difference compared to the PQ group.

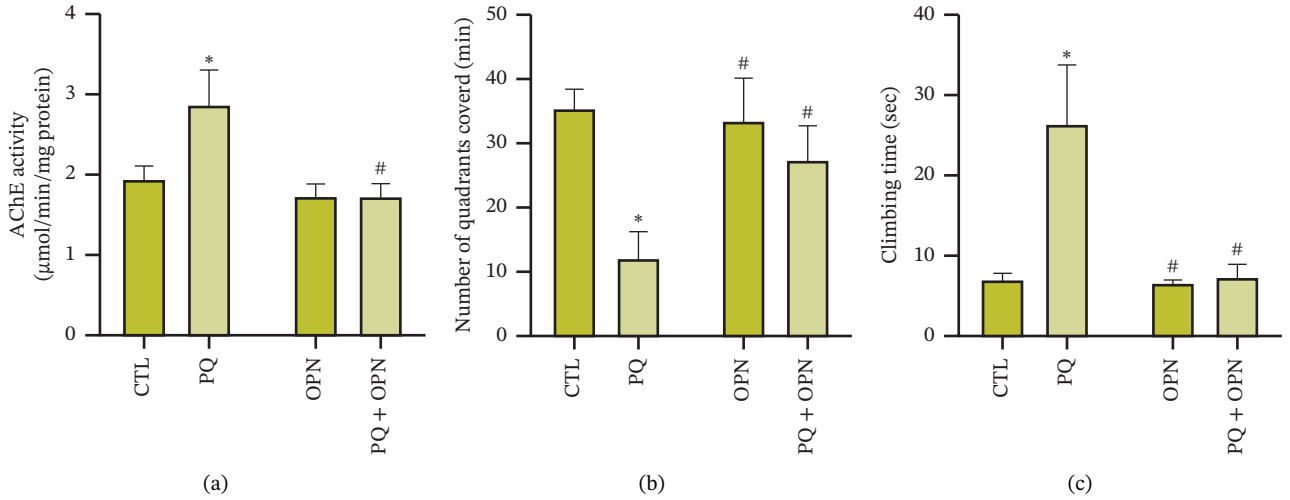


FIGURE 4 | Assessment of acetylcholinesterase (AChE) enzyme activity (a) and exploratory capacity by the open field test (b) and negative geotaxis (c) in aged *Drosophila melanogaster* supplemented/nonsupplemented with Ora-pro-nóbis (OPN) and exposed/unexposed to paraquat (PQ) for 24 h. The asterisk indicates a statistically significant difference ($p \leq 0.05$) compared to the control group. The hash indicates a statistically significant difference compared to the PQ group.

Furthermore, MDA levels in the supplemented flies were statistically similar to those in the control group, suggesting that OPN supplementation provided complete protection against PQ-induced lipid damage.

3.5.2 | Enzymatic Activity of SOD, CAT, GST, and GSH Levels

As an enzymatic antioxidant defense, the activity of SOD (Figure 6(a)) and CAT (Figure 6(b)) was evaluated. Supplementation with OPN in aged flies exposed to PQ preserved the activity of both antioxidant enzymes at the control level. Those belonging to the OPN + PQ group showed greater functioning of both SOD and CAT compared to those not supplemented and exposed to PQ ($p < 0.0001$).

In the assessment of nonenzymatic antioxidant defense, measured by GSH levels (Figure 6(c)), dietary supplementation with OPN significantly increased basal GSH levels in unexposed flies compared to those not supplemented and not exposed ($p < 0.0001$). In flies exposed to PQ, supplementation did not significantly increase GSH levels, but they remained at levels similar to the control group.

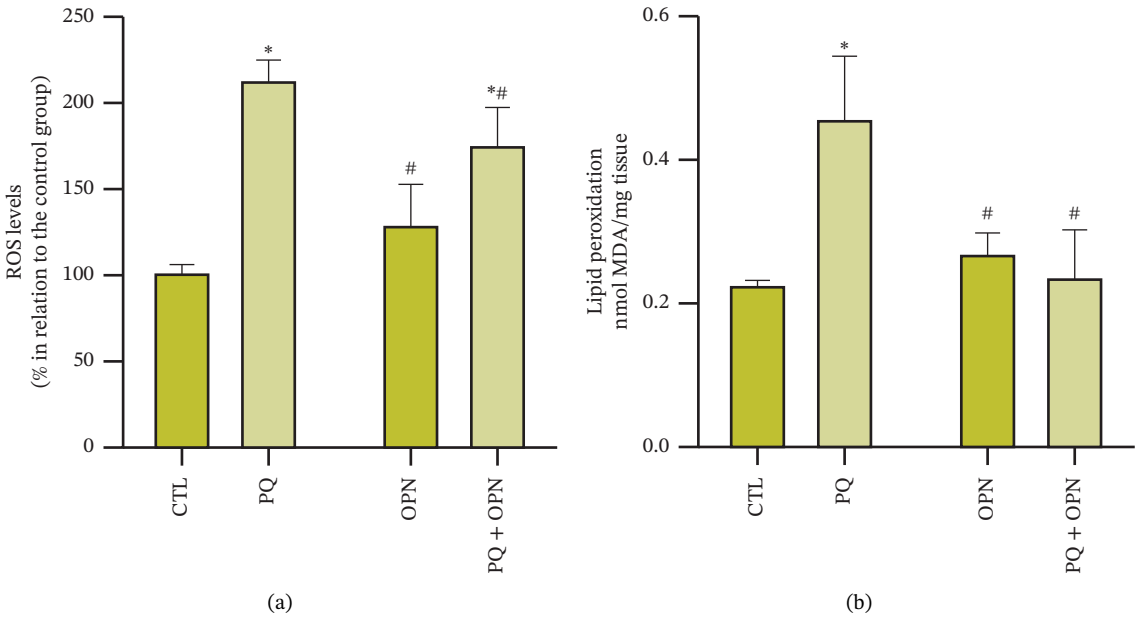


FIGURE 5 | Assessment of reactive oxygen species (ROS) levels (a) and lipid peroxidation by malondialdehyde (MDA) production (b) in aged *Drosophila melanogaster* supplemented/not supplemented with Ora-pro-nóbis (OPN) and exposed/not exposed to paraquat (PQ) for 24 h. The asterisk indicates a statistically significant difference ($p \leq 0.05$) compared to the control group. The hash indicates a statistically significant difference compared to the PQ group.

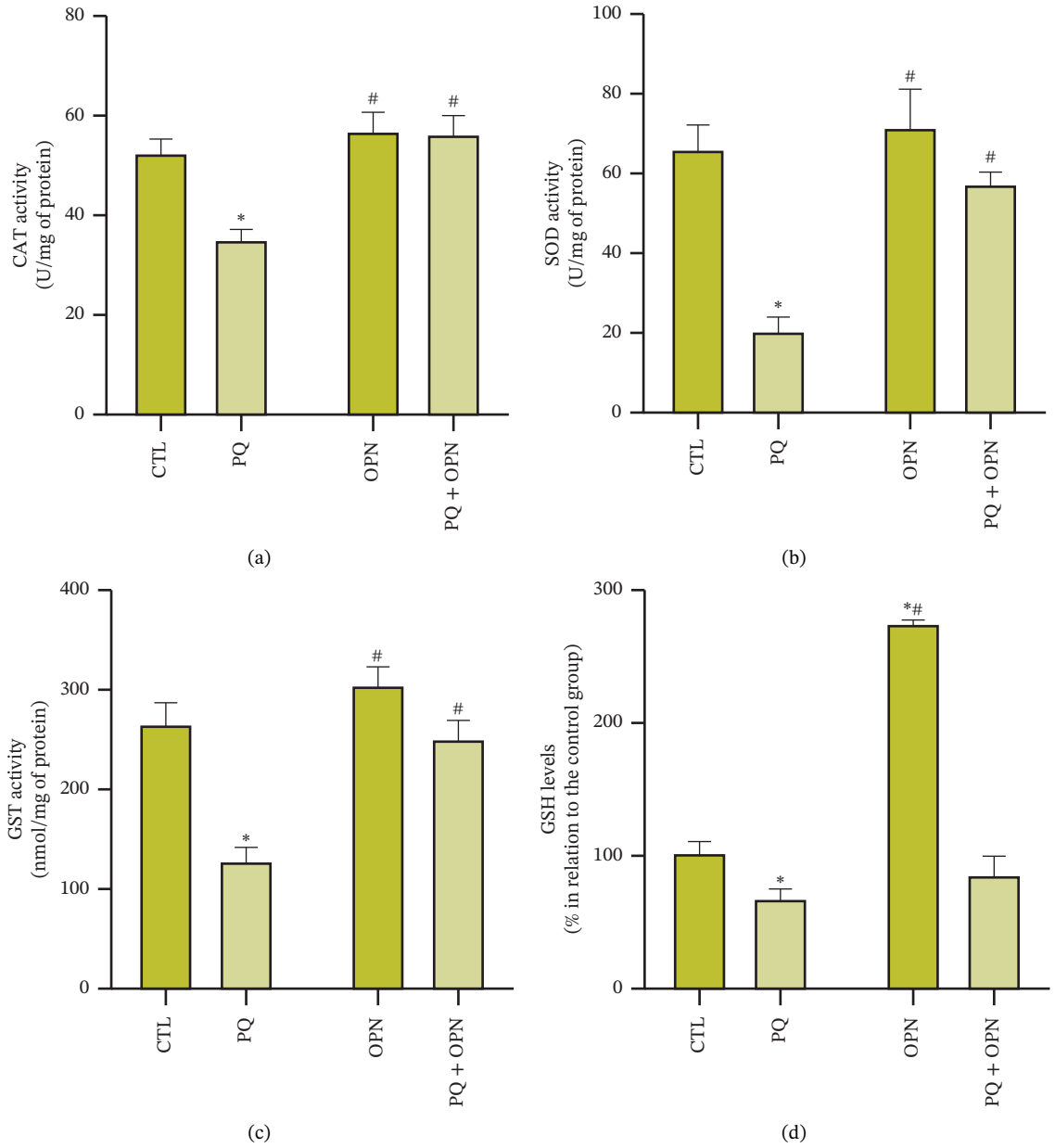


FIGURE 6 | Assessment of the activity of enzymes superoxide dismutase (SOD) (a), catalase (CAT) (b), glutathione S-transferase (GST) (c), and reduced glutathione (GSH) (d) in aged Drosophila melanogaster supplemented/not supplemented and exposed/not exposed to PQ for 24 h. The asterisk indicates a statistically significant difference ($p \leq 0.05$) compared to the control group. The hash indicates a statistically significant difference compared to the PQ group. CTL: Control, PQ: Paraquat, OPN: Ora-pro-nóbis, PQ + OPN: Paraquat + Ora-pro-nóbis.

Regarding detoxification enzyme activity, assessed by GST levels (Figure 6(d)), OPN supplementation significantly increased enzyme activity compared to flies exposed to PQ without supplementation ($p < 0.0001$). Furthermore, in the groups exposed to PQ, supplementation maintained GSH levels like those of the control group.

4 | Discussion

Advancing age is intrinsically associated with increased vulnerability to oxidative stress, either due to physiological changes inherent to aging or continuous exposure to environmental and metabolic agents that generate ROS [3]. This reality underscores the need for studies that identify viable, readily accessible alternatives effective in protecting against oxidative damage, thereby contributing to healthy aging in the population. In this sense, the ease of consuming natural foods with antioxidant properties may be a promising alternative to help older adults maintain redox homeostasis and physiological function. Ideally, dietary supplementation should also protect against toxic contaminants to which the aging body is exposed daily, thereby reducing its susceptibility to oxidative damage and chronic diseases. Alternatives to make OPN consumption more convenient and extend its shelf life involve obtaining its powder form, which can be diluted directly in water or juices, used in the preparation of recipes, or in processed foods for consumption [45–47].

Regarding the proximate composition of OPN, the high levels of protein, ash, and fiber are notable, enhancing the plant's

nutritional value and making its use in diets advantageous and promising. Furthermore, it is important to highlight the low lipid fraction, which demonstrates a reduced caloric density from fats, and the low moisture content, which confers greater stability and shelf life to commercial OPN. Proteins represent more than 20% of the mass of its leaves and are sources of essential and non-essential amino acids related to human needs. Thus, OPN constitutes an alternative to animal protein for people with dietary restrictions. Some authors suggest that the peptides and amino acids present, in addition to contributing to the nutritional value of the plant, may be associated with its antioxidant effect [48]. The high amount of ash indicates a rich mineral profile which, according to Oliveira et al. [49], includes iron, magnesium, manganese, zinc, and calcium, essential nutrients for the human body. Studies involving the use of OPN in flour form have demonstrated that its consumption can increase satiety and improve intestinal motility due to its high fiber content [46]. This nutritional profile reinforces the potential of OPN as a sustainable plant-based ingredient in the development of functional foods and diets aimed at disease prevention.

The α-linolenic and linoleic acids found in our OPN powder are essential in the diet, as the human body does not synthesize them and they are metabolic precursors of PUFAs, such as omega-3 (n3) and omega-6 (n6), which have opposing effects on metabolism. While n-3 fatty acids have protective effects against chronic, metabolic, and degenerative diseases, n-6 fatty acids are associated with inflammatory processes and the development of pathophysiological conditions, especially those of cardiovascular origin [51]. Therefore, the predominance of SFA and the high content of α-linolenic acid observed in our study indicate that OPN presents a lipid profile with potential beneficial effects on health. The low n-6/n-3 ratio obtained is considered nutritionally favorable by international organizations, which recommend a maximum limit of 10:1 to maintain an adequate balance between inflammatory and anti-inflammatory processes and thus reduce the risk of disease [52]. Furthermore, the PUFA/SFA ratio observed in OPN is also within the recommended values for cardiovascular health, reinforcing the potential of the plant's lipid composition.

Furthermore, OPN is classified as a nonconventional edible plant (NCEP), a characteristic that further enhances its nutritional and functional value. Because it is easy to grow, resistant to adverse conditions, and inexpensive, OPN represents an accessible alternative for both rural and urban populations. This reinforces not only its scientific relevance but also its socioeconomic and environmental potential, aligning with current demands for sustainable functional ingredients and food security strategies [52].

Antioxidants protect the body from intracellular oxidative processes caused by ROS, such as lipid peroxidation. Flavonoids and phenolic compounds of plant origin belong to the group of non-enzymatic antioxidants and can act by eliminating alkylperoxyl radicals that attack the lipids of cell membranes, thus contributing to reduce lipid peroxidation [53]. The antioxidant effect of flavonoids is linked to their basic chemical structure of aromatic rings with one or more attached hydroxyl groups, as these groups are responsible for donating hydrogen atoms to free radicals, forming stable complexes and reducing the ROS concentration [54].

In Cruz et al. [48], both the inhibition of the DPPH radical, observed in an antioxidant assay, and the inhibition of lipid peroxidation and ROS generation, observed in cell lines, were correlated with the total phenolic and flavonoid content of OPN. Thus, the presence of flavonoids and phenolic compounds identified in the OPN powder used in our study may also have contributed to the intracellular antioxidant effects observed in vivo assays, resulting in lower lipid peroxidation and reduced ROS in aged flies supplemented with OPN and exposed to PQ. This finding reinforces the plant's potential as a sustainable source of nutraceuticals and promising food ingredients.

Flies previously supplemented with OPN and subsequently exposed to PQ showed reduced AChE catalytic capacity compared to those exposed only to PQ, demonstrating an anticholinesterase action exerted by OPN, since, as established in the literature, PQ is capable of increasing AChE activity [16]. In the study, Torres et al. [55] attributed the anticholinesterase effect of OPN to the terpenes, alkaloids, flavonoids, and phenolic compounds present in the plant. According to Tamfu et al. [56], phenolic compounds bind to the enzyme's active site, and flavonoids altered the conformation of this site, leading to the inhibition of AChE binding to its substrate and consequently reducing cholinesterase activity. The lower the AChE activity, the lower the degradation of the neurotransmitter ACh, and consequently, the greater the availability of this molecule in the synaptic cleft, which is directly related to the execution of movements [39]. Therefore, the reduction in enzyme activity observed in our study can also be attributed to the phytoconstituents found and may have directly contributed to the improvement in the locomotor capacity of the flies, also observed in our study by the open field and negative geotaxis tests, in the group previously supplemented with OPN and subsequently exposed to PQ. Thus, based on the observed neurofunctional preservation, dietary supplementation with OPN emerges as a potential adjunct in the treatment or prevention of neurodegenerative diseases, such as Alzheimer's, whose prevalence increases with aging.

Regarding the antioxidant assay, it was observed that the OPN used in our study as a dietary supplement for flies, despite containing a mixture of various phytoconstituents, with those responsible for the antioxidant action in low concentrations, was highly effective in scavenging the DPPH radical, albeit less effective than quercetin, a pure reference compound. This finding corroborates the results of Maciel et al. [52], who demonstrated antioxidant activity in the aqueous extract of OPN using the same assay, correlating it with the presence of phenolic compounds.

In addition to exogenous antioxidants such as bioactive molecules present in OPN, it is important to highlight the role of endogenous antioxidant substances present in the body, such as GSH, which acts directly in the detoxification of xenobiotics and functions as an essential cofactor for several enzymes [57]. The present study demonstrated that prior supplementation with OPN maintained GSH levels at similar levels to those of the control group after exposure to PQ.

A significant increase in GSH levels was also observed in flies supplemented with OPN, when compared to the control group. The increase in intracellular GSH levels may be due to the presence of its precursors in the OPN incorporated into the diet. Evidence highlights that the presence of isorhamnetin was able to stimulate intracellular GSH levels in cell lines [48], which correlates with the increase in basal GSH levels observed in our study. Furthermore, the previous identification of high sulfur levels in OPN leaves suggests the presence of the amino acid cysteine [58], an amino

acid rich in sulfhydryl groups (-SH) necessary for GSH synthesis [59]. Therefore, the combination of SH groups and the phenolic compound isorhamnetin could explain the increased GSH levels associated with OPN supplementation.

In addition, OPN supplementation preserved GST activity at levels similar to the control after exposure to PQ. GST is a detoxifying enzyme that conjugates GSH with xenobiotic compounds to antagonize their oxidative action [59]. Because PQ can bind to the active site of the GST enzyme, saturating it and reducing available GSH levels, its activity tends to be compromised. However, in the present study, the preservation of GST activity may be related to the increase in the GSH substrate, promoted by OPN, favoring the maintenance of enzyme–substrate homeostasis, even under conditions of oxidative stress. This finding related to GSH and GST is relevant, considering that this is a chronologically aged organism that received prior supplementation before being exposed to a highly oxidizing agent, PQ.

PQ is a herbicide whose toxicity mechanism involves the generation of superoxide anion, a highly toxic reactive species [16]. Following the classical neutralization mechanism, SOD dismutes the superoxide radical, converting it into hydrogen peroxide (H_2O_2), a less reactive species that CAT subsequently eliminates in the form of water and oxygen [60]. Regarding the activity of the antioxidant enzymes CAT and SOD, prior supplementation with OPN in aged flies maintained their activity at basal levels, even after exposure to PQ. This result can be attributed to the dietary antioxidant effect exerted by OPN itself, which mitigates the oxidative stress caused by PQ without requiring a compensatory response from endogenous enzymes, converting oxidative products into products that are less toxic to the organism.

5 | Conclusion

Taken together, the results showed that OPN presents a remarkable nutritional and functional profile, combining high levels of protein, fiber, and minerals with significant concentrations of bioactive compounds. Its lipid composition, with a predominance of α-linolenic acid and an exceptionally low n-6/n-3 ratio, demonstrates cardioprotective and metabolic potential. These findings reinforce the use of OPN as a sustainable plant-based ingredient for the development of functional foods and diets aimed at promoting health.

In summary, the results of this study demonstrate that dietary supplementation with OPN exerts potential neuroprotective and antioxidant effects in a model of chronological aging using *Drosophila melanogaster*. OPN's ability to modulate biomarkers of oxidative stress, preserve locomotor function, and reduce mortality when exposed to an oxidizing agent known as PQ reinforces its potential as a promising nutritional strategy. Although further studies are needed to fully elucidate its mechanisms of action and validate its efficacy in superior models and clinical trials, this work contributes to consolidating OPN as a potential candidate in functional food formulations and supplements aimed at healthy longevity.

Author Contributions

Jackeline De Miranda Schmidt and Elize Musachio: conceptualization, methodology, investigation, data curation, formal analysis, and writing–original draft. Débora Luísa Filipetto Pulcinelli, Maria Eduarda Chelotti, Nathália Cardoso de Afonso Bonotto, Cindhy Suely da Silva Medeiros, and Caroline Prado Rehbein: investigation, methodology, and data curation. Eliana Jardim Fernandes and Caroline dos Santos Giuliani: investigation and data curation. Gustavo Petri Guerra and Aline Sobreira Bezerra: formal analysis and methodology. Maria Denise Schimith, Verônica Farina Azzolin, and Ivana Beatrice Mânica Da Cruz: conceptualization and funding acquisition. Fernanda Barbisan: conceptualization, methodology, formal analysis, writing–original draft, and supervision. All authors: writing–review and editing. Jackeline De Miranda Schmidt and Elize Musachio contributed equally to this work.

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Ethics Statement

Ethical review and approval for this study were waived because it is an invertebrate model organism.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

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