ORIGINAL ARTICLE

NEUROPROTECTIVE EFFECT OF AQUEOUS YERBA MATE (*ILEX PARAGUARIENSIS*) EXTRACT IN A PARKINSON'S DISEASE IN VITRO MODEL

Elize Musachio[1], Tábada Samantha Marques Rosa[2], Verônica Farina Azzolin[3], Moisés Henrique Mastella[4], Izabella Paz Danezi Felin[5], Marta Medeiros Frescura Duarte[6], Maria Denise Schimith[7] Aron Ferreira da Silveira[8], Ivana Beatrice Mânica da Cruz[9] Fernanda Barbisan[10]

Highlights: (1) Yerba mate reduced apoptosis and cytotoxicity in the PD *in vitro* model. **(2)** Extract modulated antioxidant enzymes and oxidative stress pathways. (3) Yerba mate protected against oxidative DNA damage in SH-SY5Y cells.

PRE-PROOF

(as accepted)

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[1] Federal University of Santa Maria - UFSM. Santa Maria/RS, Brazil. https://orcid.org/0000-0002-2577-2379

[2] Federal University of Santa Maria - UFSM. Santa Maria/RS, Brazil. http://lattes.cnpq.br/0737340290641833

[3] Open University of the Third Age Foundation – FUnATI. Manaus/AM, Brazil. https://orcid.org/0000-0002-8191-5450

[4] Federal University of Santa Maria - UFSM. Santa Maria/RS, Brazil. https://orcid.org/0000-0001-6990-6079

[5] Federal University of Santa Maria - UFSM. Santa Maria/RS, Brazil. https://orcid.org/0000-0001-9671-9391

[6] Lutheran University of Brazil – ULBRA. Santa Maria/RS, Brazil. https://orcid.org/0000-0003-4371-4989

[7] Federal University of Santa Maria - UFSM. Santa Maria/RS, Brazil. https://orcid.org/0000-0002-4867-4990

[8] Federal University of Santa Maria - UFSM. Santa Maria/RS, Brazil. https://orcid.org/0000-0002-2944-7362

[9] Federal University of Santa Maria - UFSM. Santa Maria/RS, Brazil. https://orcid.org/0000-0003-3008-6899

[10] Federal University of Santa Maria - UFSM. Santa Maria/RS, Brazil. https://orcid.org/0000-0002-2960-7047

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ABSTRACT

Parkinson's disease (PD) remains an incurable condition, and strategies to mitigate oxidative stress and neurodegeneration have been widely investigated, including the use of plant-derived extracts and infusions rich in bioactive compounds, such as yerba mate (*Ilex paraguariensis*). Based on this premise, the present study evaluated the effects of an aqueous yerba mate extract in an in vitro model of PD. Differentiated SH-SY5Y cells exhibiting a neuronal phenotype were exposed to increasing concentrations of rotenone (5, 10, 40, and 100 µM), and the concentration that induced cytotoxicity after 24 h while maintaining 80–85% cell viability relative to the control was selected. Neuronal cultures were supplemented with aqueous yerba mate extract (0, 1, 5, 10, 20, and 100 µg/mL). The extract was characterized by the presence of alkaloids (caffeine and theobromine), tannins, and polyphenolic compounds. Treatment with yerba mate at 10 µg/mL in cultures exposed to 40 µM rotenone reduced cytotoxicity and apoptosis, as assessed by flow cytometry, and modulated the gene expression of antioxidant enzymes and the expression and protein levels of caspases-3 and -8. Although oxidative stress parameters in the yerba mate plus rotenone group remained higher than those observed in cultures treated with yerba mate alone, indicating partial attenuation of antioxidant effects in the presence of rotenone, protection against oxidative DNA damage was observed. Overall, these findings suggest that the aqueous yerba mate extract exerts modulatory effects on oxidative stress and cell death pathways in this in vitro model of PD.

Keywords: Antioxidants; Parkinson disease; Oxidative stress; Neuroprotection.

EFEITO NEUROPROTETOR DO EXTRATO AQUOSO DE ERVA MATE *(ILEX PARAGUARIENSIS)* EM MODELO *IN VITRO* DE DOENÇA DE PARKINSON

RESUMO

A doença de Parkinson (DP) permanece uma condição incurável, e estratégias voltadas à atenuação do estresse oxidativo e da neurodegeneração têm sido amplamente investigadas, incluindo o uso de extratos e infusões de origem vegetal ricos em compostos bioativos, como a erva-mate (*Ilex paraguariensis*). Com base nessa premissa, o presente estudo avaliou os efeitos do extrato aquoso de erva-mate em um modelo in vitro de DP. Células SH-SY5Y diferenciadas, com fenótipo neuronal, foram expostas a concentrações crescentes de rotenona (5, 10, 40 e 100 µM), sendo selecionada a concentração que induziu citotoxicidade após 24 h, mantendo 80–85% de viabilidade celular em relação ao controle. As culturas neuronais foram suplementadas com extrato aquoso de erva-mate (0, 1, 5, 10, 20 e 100 µg/mL). O extrato foi caracterizado pela presença de alcaloides (cafeína e teobromina), taninos e compostos polifenólicos. O tratamento com erva-mate a 10 µg/mL em culturas expostas a 40 µM de rotenona reduziu a citotoxicidade e a apoptose, avaliadas por citometria de fluxo, além de modular a expressão gênica de enzimas antioxidantes e a expressão e os níveis de proteína das caspases-3 e -8.Embora os parâmetros de estresse oxidativo no grupo erva-mate associado à rotenona tenham permanecido superiores aos observados nas culturas tratadas apenas com erva-mate, indicando atenuação parcial dos efeitos antioxidantes na presença da rotenona, foi observada proteção contra o dano oxidativo ao DNA. De modo geral, esses achados sugerem que o extrato aquoso de erva-mate exerce efeitos moduladores sobre o estresse oxidativo e as vias de morte celular neste modelo *in vitro* de DP.

Palavras-chave: Antioxidantes; Doença de Parkinson; Estresse oxidativo; Neuroproteção.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra [1]. Oxidative stress plays a critical role in its pathogenesis, contributing to neuronal damage and the progression of dopaminergic neurodegeneration [2] Evidence have suggested that some functional and beverage foods could present beneficial effects on PD mainly by present in their chemical matrix bioactive molecules such as caffeine [3] theobromine [4], and flavonoids [5].

This is the case with yerba mate (*Ilex paraguariensis*), a type of plant native to the sub-tropical region of South America [6]. Yerba mate leaves have a low commercial cost, and after the drying and grinding process, they are used to prepare Chimarrão (mate), a traditional drink consumed daily by the population in countries such as Brazil, Paraguay, Uruguay, and Argentina [7, 8]. In mate, the yerba mate is placed in a container and then hot water (around 75 °C (± 2 °C) is poured over the yerba, and it can be consumed quickly. Studies have shown that the aqueous extract of yerba mate has antioxidant and anti-inflammatory properties due to the presence of alkaloids, methylxanthine, carotenoids, minerals and vitamins, saponins, quercetin, and kaempferol, as well as flavonoids such as catechin, epicatechin, gallocatechin, and epigallocatechin [7,9,10]. Some studies using yerba mate hydroalcoholic extract have already demonstrated its neuroprotective effect and suggest that it may exert an antiparkinsonian effect, as seen in *in vivo* models, through the modulation of antioxidant enzymes and nitric oxide associated with the reduction of behaviours characteristic of PD, in addition to promoting improved memory and learning in rodents [7,9]. The neuroprotective capacity of the aqueous extract of yerba mate was observed in the face of the convulsive effect induced by Pethylenetetrazolium in rats [10].

Previous studies have chemically characterized aqueous yerba mate extracts and reported antioxidant and immunomodulatory effects in human peripheral blood mononuclear cells (PBMCs) and in coelomocytes from *Eisenia fetida* [11]. These findings provided the rationale for investigating the potential neuroprotective effects of this extract in neuronal models.

Although some studies have suggested neuroprotective properties of yerba mate, its effects remain insufficiently characterized when considering the form in which it is

most widely consumed, a traditional aqueous infusion -mate-. In addition, limited information is available regarding the dose–response profile and potential cytotoxicity of yerba mate. In this context, *in vitro* approaches allow the direct evaluation of the effects of yerba mate aqueous extract on neuronal cells under controlled experimental conditions. Therefore, the present study aimed to investigate the neuroprotective effects of an aqueous yerba mate extract in a rotenone-induced *in vitro* model of Parkinson's disease using SH-SY5Y cells

2. Materials and Methods

2.1 Cell culture and neuronal differentiation

Undifferentiated human neuroblastoma cells **(**SH-SY5Y**)** were obtained from the American Type Culture Collection (ATCC, CRL-2266). Cells were maintained in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS)**,** 1% penicillin/streptomycin**,** and amphotericin B**,** and cultured at 37 °C in a humidified atmosphere containing 5% CO_2**.**

For neuronal differentiation, SH-SY5Y cells were treated with retinoic acid (RA) under standard culture conditions. RA was dissolved in dimethyl sulfoxide (DMSO) to prepare a 100 mM stock solution, which was subsequently diluted in the culture medium to a final concentration of 10 µM**.** The culture medium was replaced at defined intervals throughout the 3-day differentiation period**,** following the protocol described by Shipley et al. (2016) [12]. Differentiation was confirmed by morphological criteria, including neurite outgrowth and reduced proliferative activity (Figure 1A)**,** consistent with a neuronal-like phenotype with dopaminergic features.

2.2. Experimental design and treatment

After differentiation, cells were allocated into the following experimental groups: control (untreated), yerba mate aqueous extract, rotenone, and combined treatment (yerba mate extract + rotenone). The experimental design is summarized in Figure 1B**.**

Initially, concentration–response assays were performed to determine the experimental concentrations of yerba mate aqueous extract (1, 5, 10, 20, and 100 µg/mL) and rotenone (1, 5, 10, 40, and 100 µM). The concentrations selected for subsequent experiments were determined from concentration–response assays.

Differentiated SH-SY5Y cells were exposed to treatments for 24 h or 72 h, depending on the subsequent analyses. All experiments were performed in triplicate and repeated in three independent biological experiments (n = 3). A schematic representation of the experimental design is shown in Figure 1B.

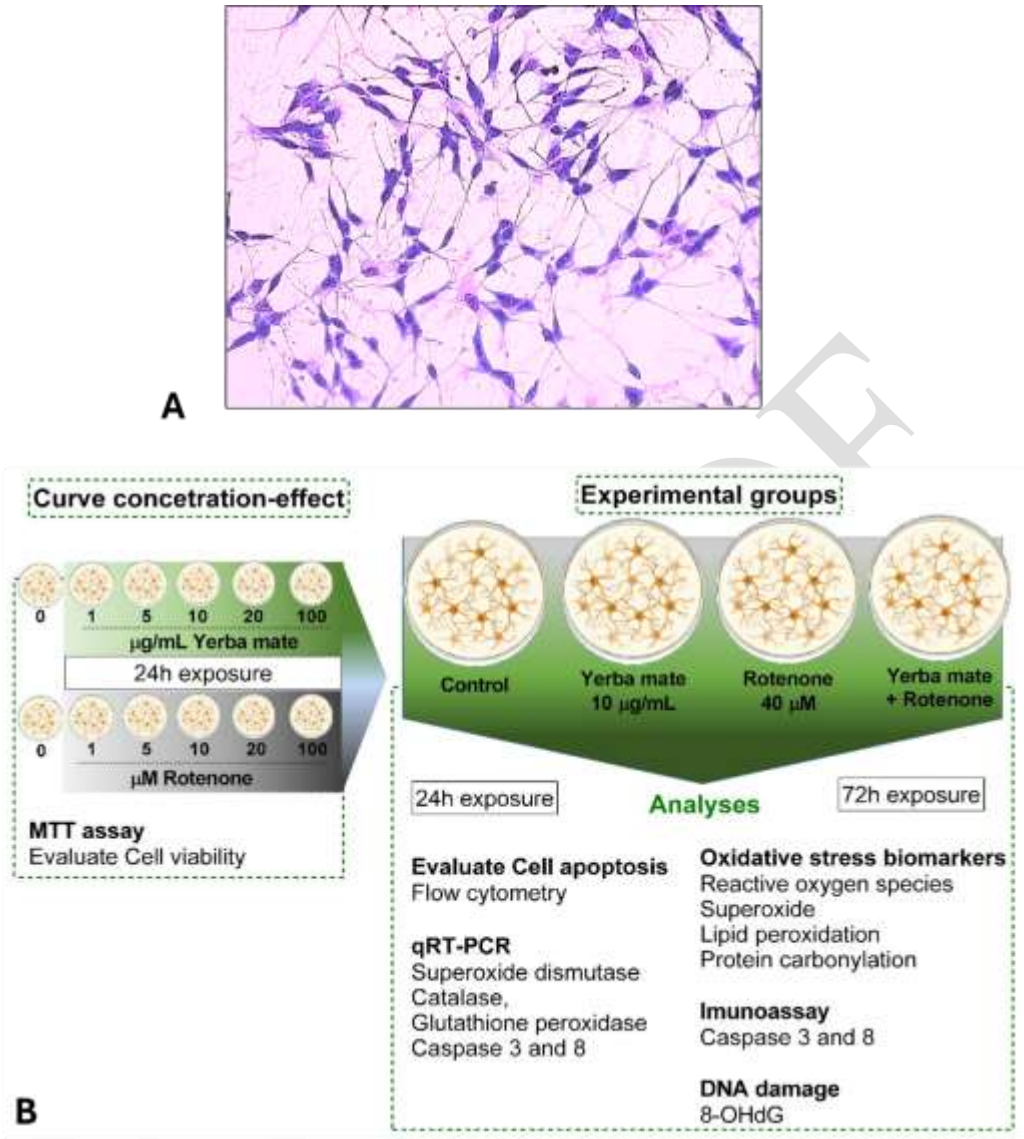

Figure 1. Overview of SH-SY5Y differentiation and experimental design. (A) Representative photomicrograph of SH-SY5Y cells after differentiation with all-trans retinoic acid (RA, 10 µM), showing neurite outgrowth and neuronal-like morphology used as criteria for differentiation. (B). Schematic representation of the experimental design. Gene expression of antioxidant enzymes and caspases was evaluated by qRT-PCR.

2.3. Yerba mate aqueous extract and chemical characterization

The quantification of caffeine and theobromine in the extracts was performed by high-performance liquid chromatography (HPLC) with ultraviolet detection at 272 nm. Analyses were carried out using a Shimadzu Prominence LC-20A chromatographic system equipped with a LC-20AT quaternary pump, SIL-20A autosampler, DGU-20A5 online degasser, CBM-20A system controller, and a SPD-20AV diode array detector. Analyte separation was achieved using a reverse-phase ODS-3 column (150 × 4.6 mm; 5 μm; Phenomenex Prodigy ODS-3 100A, Torrance, CA, USA). A caffeine stock solution (250 μg/mL) was prepared and stored at 5 °C, and calibration standards were obtained by serial dilution in the mobile phase to generate analytical curves with at least five concentration points. The lowest calibration level allowed the detection of 0.005% of the analyte, considering a 1 g sample diluted in 100 mL (LOD = 0.05 mg/g). Prior to analysis, hot-water aqueous extracts were filtered through 0.45 μm membranes and transferred to autosampler vials. Chromatographic conditions included a flow rate of 1.0 mL/min, with mobile phase A consisting of 0.1% phosphoric acid in water and mobile phase B consisting of acetonitrile. Calibration standards were injected periodically throughout the analyses, showing high reproducibility, with correlation coefficients (R) ≥ 0.9999. The chromatographic analysis was performed based on a previously published method described by Alves et al. (2019) [11], with adaptations to the experimental design of the present study. Following quantification, the extracts were adjusted to the experimental concentrations and prepared for application in cell-based assays.

Chemical characterization was carried out in independent triplicates. The total content of phenolic compounds in each extract was determined spectrophotometrically using the Folin Ciocalteu method, measuring absorbance at 730 nm. For results, a gallic acid calibration curve was used, and contents were expressed as equivalent milligrams (mg) of total phenolics per milliliter (mL) of extract (mg/mL). Total alkaloid content was quantified by precipitation reaction using Dragendorff's reagent (Sigma-Aldrich Co., St. Louis, MO, USA. Absorbance was measured at 435 nm.

2.4. Cell viability assay

Cell viability was assessed using the MTT assay [11]. After treatments, culture medium was removed, and cells were incubated with MTT solution (5 mg/mL) for 1 h at 37 °C. The resulting formazan crystals were solubilized with DMSO, and absorbance was measured at 560 nm using a microplate reader.

2.5. Flow cytometry analysis

Apoptosis was evaluated using an Annexin V-FITC/Propidium Iodide staining kit (BD Pharmingen™) according to the manufacturer's instructions. Samples were analyzed using a BD Accuri™ C6 flow cytometer. Sample preparation was according to the manufacturer's instructions. Cells were incubated with annexin-V reagents for 15 minutes at room temperature (22–25ºC) in the dark, then immediately analyzed by flow cytometry. The generated flow diagrams were analyzed using FlowJo software. Viable cells were identified as negative for Annexin-V and PI; early apoptotic cells were positive for Annexin-V only; late apoptotic cells were positive for Annexin-V and PI; and necrotic cells were positive for PI only. Early and late apoptotic cells (Annexin V–positive cells, with or without propidium iodide staining) were grouped and analyzed together as total apoptosis.

2.6. Oxidative stress biomarkers

2.6.1. Quantification of ROS levels

To quantify ROS levels, a cell-permeant fluorescent compound called 2–7-dichlorofluorescein diacetate (DCFDA) was used [13]. In this assay, DCFDA is hydrolyzed to DCFH, trapped within the cell. This non fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by cell oxidative factors. After the designated treatment period, the neuronal cells were treated with DCFDA (10 μM) for 60 minutes at 37 °C. The fluorescence was measured via excitation at 488 nm and emission at 525 nm on a microplate reader.

2.6.2. Quantification of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured according to the modified method of Jentzsch et al. [17]. All reagents were mixed, i.e., 1× TBA acid diluent, SDS lysis solution, TBA reagent, and 1× BHT solution. Each MDA-containing sample and standard was assayed in duplicate. All the reagents and samples were incubated in a water bath at 95°C for 1h. Next, the samples were cooled and centrifuged at 1000 rpm for 10 min, and absorbance was read at 532 nm.

2.6.3. Protein carbonyl

Protein carbonylation is measured by the method of Morabito et al. [18], where damage is measured by determining the formation of carbonyl groups based on the reaction with dinitrophenylhydrazine. The sample was diluted by 1:80 using Tris-HCl buffer, treated with 200 µL DNPH, and incubated in the dark at room temperature for 60 min. Next, 500 µL of denaturation buffer (3% SDS), 2000 µL of ethanol, and 2000 µL of hexane were added to the sample and centrifuged at 3000 rpm for 15 min. The supernatant obtained was removed, and the pellet was resuspended in 1000 µL of denaturation buffer and incubated in a water bath (40 – 50°C) for 20 min until it dissolved completely. Next, 100 µL of each sample was transferred to a 96-well plate in triplicate, and absorbance was read at 370 nm.

2.6.4. Superoxide quantification

Superoxide levels were quantified by performing an assay that produced formazan salt through a reaction between nitroblue tetrazolium (NBT) chloride and superoxide [16]. Briefly, the cells were seeded in a 96-well plate, treated with 10 µL of NBT solution (10 mg/mL) diluted in 1× PBS, homogenized, incubated at 37°C for 3h, and centrifuged. Next, 75 µL of supernatant was removed, and the same volume of DMSO was added to each well. After incubation for 20 min at 37°C, 75 µL of cell suspension was transferred to another 96-well plate, and absorbance was measured at 550 nm.

2.6.5 DNA damage

DNA damage was determined by 8-deoxyguanosine (8-OHdG) using an ELISA immunoassay kit obtained from Abcam (Cambridge, MA, USA) according to the manufacturer's instructions. All levels of these variables were corrected by mg/protein

2.6 Quantitative real time PCR (qRT-PCR)

Total RNA was extracted using Trizol, following the manufacturer's instructions (Ludwing-Biotec, Rio Grande do Sul, Brazil). The concentration of the extracted RNA was measured using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer 260/280 nm. The RNA (1 μg/μL) was treated with 0.2 μL DNAase (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C for 5 min, followed by heating at 65°C for 10 min. The cDNA was then generated by reverse transcription using 1 μL of Iscript cDNA and 4 μL of Iscript Mix (Bio-Rad Laboratories, California, United States). The reaction consisted of the following steps: heating at 25°C for 5 min, at 42°C for 30 min, and at 85°C for 5 min, followed by incubation at 5°C for 60 min. Real-time PCR was performed as previously described by Barbisan et al. [17], using a QuantiFast SYBR® Green PCR Kit and a Rotor Gene® (Qiagen, Hilden, Germany). The primers were designed and validated based on the corresponding human gene reference sequences available in the NCBI database (PubMed), using the official Gene ID for each target gene. The specific sequences of the human primers used are described in Table 2.

Table 2. List of primers

Name-Gene ID	Primers	
	Sense (5′-3′)	Antisense (5′-3′)
ACTB Gene ID: 60	TGTGGATCAGCAAGCAGGAGTA	TGCGCAAGTTAGGTTTTGTCA
Superoxide dismutase 2 Gene ID: 6648	GCCCTGGAACCTCACATCAA	GGTACTTCTCCTCGGTGACGTT
Catalase Gene ID: 847	GATAGCCTTCGACCCAAGCA	ATGGCGGTGAGTGTCAGGAT
Glutathione peroxidase Gene ID: 2876	GGTTTTCATCTATGAGGGTGTTTCC	GCCTTGGTCTGGCAGAGACT
Caspase 3 Gene ID: 836	TTTGAGCCTGAGCAGAGACATG	TACCAGTGCGTATGGAGAAATGG
Caspase 8 Gene ID: 841	AAGGAGCTGCTCTTCCGAATT	CCCTGCCTGGTGTCTGAAGT

2.7. Caspase immunoassays

The caspase 3 and 8 analyses were performed using the Quantikine Human Caspase Immunoassay to measure caspase in the cell culture supernatants, according to the manufacturer's instructions. All reagents and working standards were prepared, and the excess microplate strips were removed. The assay diluent RD1W was added (50 µL) to each well. Further, 100 µL of standard control for our sample was added per well, after which the well was covered with the adhesive strip and incubated for 1h and 30 min at room temperature. Each well was aspirated and washed twice for a total of three washes. The antiserum of each molecule analyzed here was added to each well covered with a new adhesive strip and incubated for 30 min at room temperature. The aspiration/wash step was repeated, and 200 µL of substrate solution was added to each well and incubated for 20 minutes at room temperature. Finally, the 50 µL stop solution was added to each well, and the optical density was determined within 30 minutes using a microplate reader set to 450 nm.

2.9. Statistical Analysis

All assays were performed in independent experiments in triplicate. Statistical analyses were performed using GraphPad Prism 9 software. In the curve concentration effect, the results were compared using one-way ANOVA, followed by Tukey *post-hoc*. In the tests that evaluated the neuroprotective effect of yerba mate extract on rotenone, the two-way ANOVA test was used, followed by the Newman-Keuls *post-hoc*. Significant differences were considered where the $p \leq 0.05$.

3. Results

Dose–response assays and gene expression analyses were performed after 24 h, whereas oxidative stress biomarkers, DNA damage and caspase protein levels were evaluated after 72 h.

3.1 Yerba mate chemical characterization

The chemical characterization of the yerba mate aqueous extract demonstrated high concentrations of bioactive compounds (Table 1). The total polyphenol content was 510.9 ± 42.2 mg/mL, indicating a high abundance of phenolic compounds in the extract. Total alkaloid content was 128.6 ± 1.0 mg/g of extract. Among these alkaloids, methylxanthine analysis identified caffeine and theobromine at concentrations of 89.9 ± 3.4 µg and 19.9 ± 3.1 µg per 0.002 g of extract, respectively. Regarding tannins, hydrolyzed tannins were markedly higher (770.9 ± 71.2 mg GAE/mL) than condensed tannins (15.9 ± 4.2 mg GAE/mL).

Overall, the chemical profile indicates that the yerba mate aqueous extract is rich in polyphenols, alkaloids, tannins, and methylxanthines, compounds widely associated with antioxidant and biological activities.

Table 1. Bioactive compounds concentration in the yerba mate aqueous extract

Compounds	Yerba mate
Total polyphenols (mg/mL)	510.9 ± 42.2
Alkaloids (mg/g)	128.6 ± 1.0
Tannins hydrolysed (mg EAG/mL)	770.9 ± 71.2
Tannins condensed (mg EAG/mL)	15.9 ± 4.2
Caffeine (extract µg/ 0.002 g)	89.9 ± 3.4
Theobromine (extract µg/0.002 g)	$^{19.9}$ ± 3.1

Data are presented as mean ± SD from three replicates.

3.2. Curve dose response effect

Initial analyses performed at 24 h were used to establish cytotoxicity and select the experimental concentrations. The cell response to rotenone treatments at (1, 5, 10, 40, and 100 µM) was measured to confirm the toxicity of these concentrations. All rotenone concentrations significantly reduced cell viability compared to the control group ($p < 0.0001$ for all comparisons). As expected, rotenone treatments decreased cell viability in a dose-dependent way with a rate of mortality greater than 50% at the highest concentrations (40 and 100 µM) (Figure 2A).

The effect of different concentrations (1, 5, 10, 20, and 100 µM/mL) of yerba mate aqueous extract on cell viability was verified (Figure 2B). There was no statistical difference between the control groups, showing that yerba mate did not cause toxicity at any concentration in SH-SY5Y cells.

Based on this, the concentration chosen to mimic PD in the cells was 40 µM rotenone, and since the yerba mate aqueous extract did not demonstrate any toxic effect on the cells, the concentration chosen was 10 µg/mL. Flow cytometry analysis was performed to confirm the cytoprotective effect of the yerba mate aqueous extract, showing a significant difference among treatments ($p < 0.001$). As shown in Figure 2C, rotenone exposure markedly increased total apoptotic cell frequency compared to the control group (224.9%). In contrast, cells treated with yerba mate alone showed apoptotic levels comparable to the control (101.1%). When combined, yerba mate partially attenuated the

rotenone-induced increase in apoptosis, although values remained higher than control (152.4%).

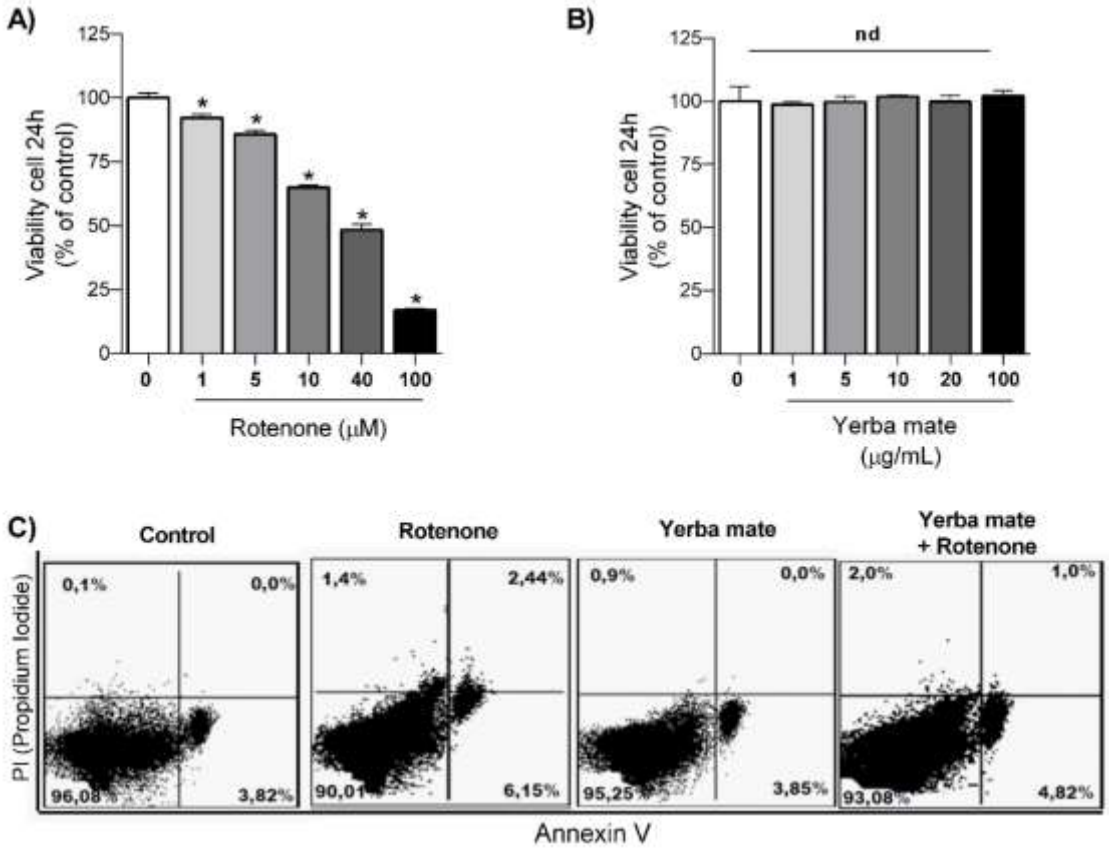

Figure 2. Toxicological curve for determining rotenone and yerba mate aqueous extract concentrations through cell viability by the reduction test (MTT). (A) Evaluation of the SH-SY5Y cells viability exposed for 24h to different concentrations of rotenone (1, 5, 10, 40, 100 µM). (B) Evaluation of the viability of SH-SY5Y cells exposed for 24h to different concentrations (0, 1, 5, 10, 30, 100 µg/mL) of yerba mate aqueous extract. The results were analyzed by one-way ANOVA and expressed as mean and ± standard deviation (SD), (n=3). Statistical difference was considered when $p \leq 0.05$, and * indicate a difference from the control group. Flow cytometry assay (C) Flow cytometry analysis of apoptosis using Annexin V–FITC and propidium iodide (PI). Early and late apoptotic cells (Annexin V–positive cells, with or without PI staining) were grouped and quantified together as total apoptosis.

Test doses were defined based on dose–response assays, considering cytotoxicity thresholds, maintaining sufficient cell viability for downstream analyses, and selecting biologically relevant but non-lethal concentrations. Thus, 40 µM rotenone was selected to induce significant sublethal toxicity, while 10 µg/mL yerba mate aqueous extract was chosen for its lack of cytotoxicity and evidence of cytoprotective effects.

The results presented subsequently refer to analyses performed after 72 h of exposure and focus on oxidative stress, genomic damage, gene expression, and apoptosis.

3.3. Evaluation of oxidative stress biomarkers

Initially, in the quantification of ROS (Figure 3A), it was observed that cells exposed to rotenone produced greater amounts than the control group ($p < 0.0001$) and the yerba mate group ($p < 0.0001$). Overall, rotenone markedly increased oxidative stress compared to control, whereas yerba mate supplementation partially attenuated this effect, without fully restoring control levels. There was no statistical difference between the control and yerba mate groups ($p= 0.3751$).

In the evaluation of superoxide production (Figure 3B), an increase in levels was observed in the group exposed to rotenone when compared to the control ($p < 0.0001$) and the yerba mate group ($p < 0.0001$). The group exposed to yerba mate + rotenone produced a higher amount of superoxide than the control ($p < 0.0001$) and the yerba mate group ($p < 0.0001$), and a lower amount when compared to the rotenone group ($p < 0.0001$). There was no statistical difference between the control and yerba mate groups ($p = 0.5245$).

When evaluating lipid peroxidation (Figure 3C), it was observed that cells exposed to the rotenone group produced more TBARS compared to the control group ($p = 0.0013$) and yerba mate group ($p < 0.0001$). Furthermore, the yerba mate group showed less lipid peroxidation compared to the control group ($p < 0.0001$). The yerba mate + rotenone compared exposure group also had a higher TBARS production when compared to the control ($p = 0.0001$) and yerba mate group ($p < 0.0001$), and a lower production when compared to the rotenone group ($p = 0.0005$).

Protein damage was verified through carbonylation (Figure 3D). Higher amounts of protein carbonyls were observed in the group exposed only to rotenone and in the yerba

mate + rotenone group, when compared to the control group ($p < 0.0001$ and $p < 0.0001$, respectively). Cells in the groups exposed only to yerba mate and in the yerba mate + rotenone group had lower concentrations of protein carbonyls, when compared to the rotenone group ($p < 0.0001$ and $p < 0.0001$, respectively). Furthermore, the yerba mate + rotenone group showed greater protein carbonylation when compared to the group exposed only to yerba mate ($p = 0.0001$). There was no statistical difference between the yerba mate and control groups ($p = 0.9694$).

In this study, DNA damage refers specifically to oxidative DNA lesions, quantified by 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels. (Figure 3E). It was observed that the cells exposed to rotenone had a higher amount of 8-OHdG, when compared to the control ($p = 0.0121$), yerba mate ($p = 0.0125$), and yerba mate + rotenone ($p = 0.0314$). The cells exposed only to the aqueous extract of yerba mate and also to the group treated with yerba mate + rotenone showed no difference from the control ($p > 0.9999$ and $p = 0.8917$, respectively). There was no statistically significant difference between the yerba mate group and the yerba mate + rotenone group.

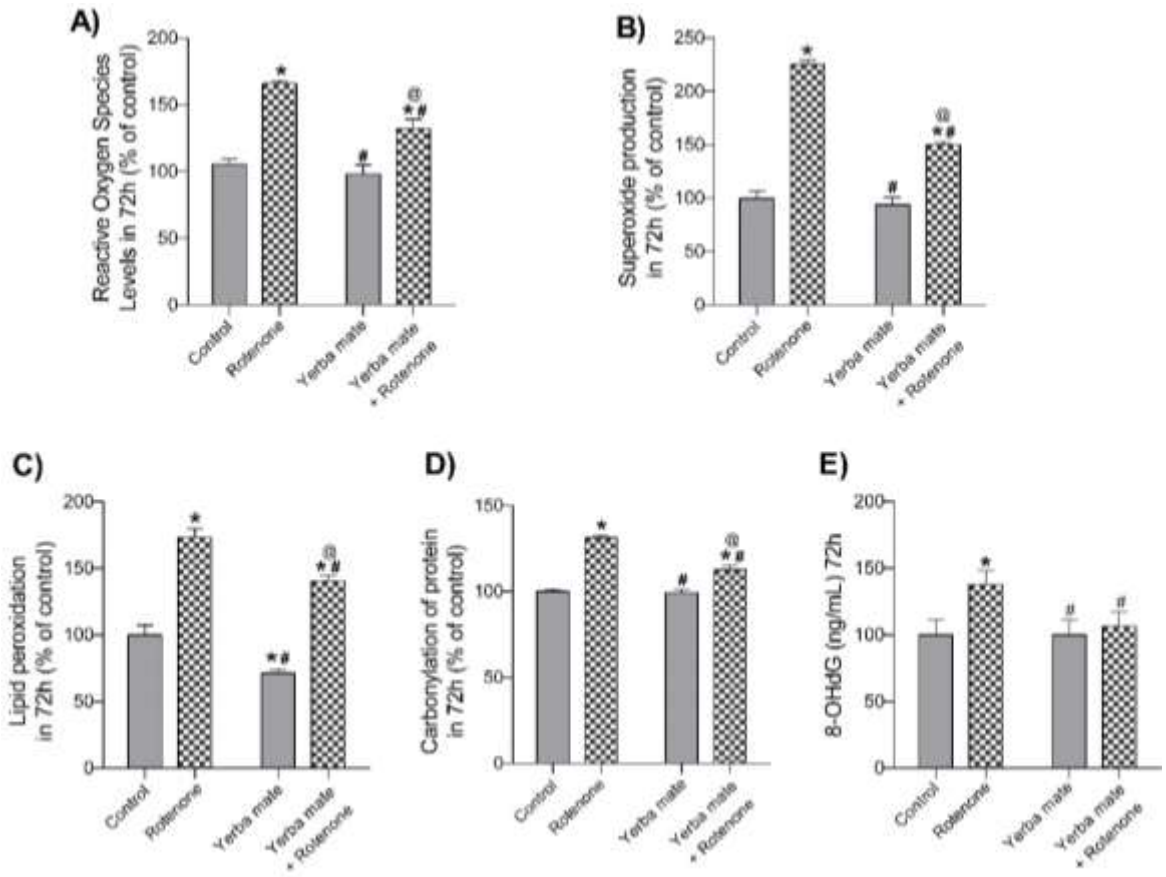

Figure 3. Evaluation of oxidative stress biomarkers and oxidative damage to DNA in the SH-SY5Y cells exposed to 10 μg/mL of yerba mate aqueous extract and 40 μM rotenone. (A) Quantification reactive oxygen species (ROS) levels by dichlorofluorescein diacetate reduction (DCFH-DA). (B) Determination of superoxide levels. (C) Lipid peroxidation was evaluated by quantifying thiobarbituric acid reactive species (TBARS). (D) Estimating protein carbonylation and (E) oxidative damage to the DNA was quantified through 8-hydroxy-2'-deoxyguanosine (8-OHdG). All results were expressed as mean and ± standard deviation (SD), (n=3), and were considered statistically significant at $p \leq 0.05$. *Indicate a difference from the control # indicates a difference from the rotenone group, and @ indicates a difference compared to the yerba mate group.

3.4. Gene expression of antioxidant enzymes

Regarding SOD2 gene expression (Figure 4A), exposure to rotenone significantly downregulated SOD2 levels compared to the control group ($p < 0.0001$), whereas treatment with yerba mate alone significantly increased SOD2 expression relative to control ($p < 0.0001$). No significant difference was observed between the control group

and the yerba mate + rotenone group ($p = 0.0983$). When compared to the rotenone group, both yerba mate and yerba mate + rotenone treatments significantly increased SOD2 expression ($p < 0.0001$ and $p = 0.0002$, respectively), with higher expression observed in the yerba mate group compared to the combined treatment ($p < 0.0001$).

For CAT gene expression (Figure 4B), rotenone exposure significantly reduced CAT levels compared to the control group ($p < 0.0001$). In contrast, cells treated with yerba mate alone or in combination with rotenone showed increased CAT expression relative to control ($p < 0.0001$ and $p = 0.0034$, respectively) and compared to the rotenone group ($p < 0.0001$ for both comparisons). Notably, CAT expression was higher in the yerba mate group than in the yerba mate + rotenone group ($p < 0.0001$).

Regarding GPx gene expression (Figure 4C), rotenone treatment significantly reduced GPx levels compared to the control group ($p < 0.0001$). Conversely, exposure to yerba mate alone or combined with rotenone significantly increased GPx expression relative to control ($p < 0.0001$ for both) and to the rotenone group ($p < 0.0001$ for both comparisons). Higher GPx expression was observed in the yerba mate group compared to the yerba mate + rotenone group ($p < 0.0001$).

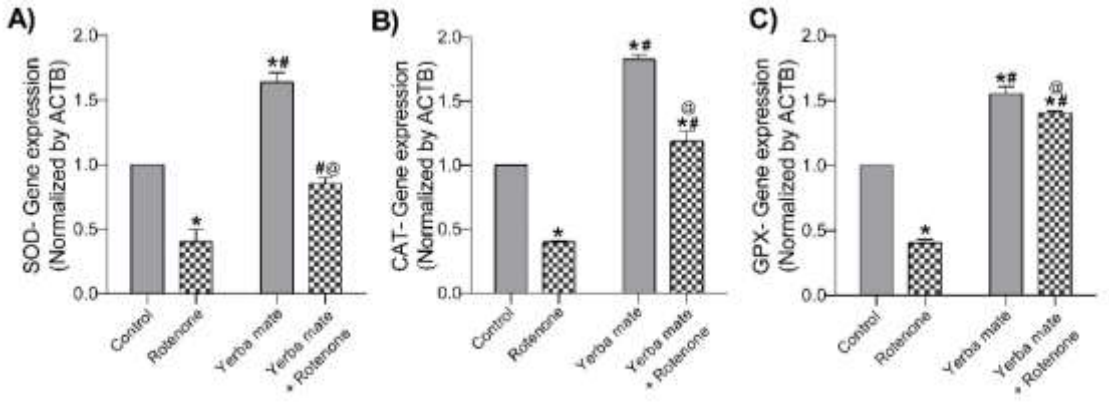

Figure 4. Gene expression of antioxidant enzymes of SH-SY5Y cells exposed for 24h to 10 μg/mL aqueous extract of yerba mate and 40μM rotenone. (A) Expression of the genes of the enzyme superoxide dismutase 2 (SOD2), (B) Catalase (CAT), and (C) Glutathione peroxidase (GPx). All results were expressed as mean and ± standard deviation (SD), (n=3), and were considered statistically significant at $p \leq 0.05$. *Indicate a difference from the control # indicates a difference from the rotenone group and @ indicates a difference compared to the yerba mate group.

3.5. Investigation of apoptosis in SH-SY5Y cells

The investigation of apoptosis began by evaluating caspase-3 gene expression (Figure 5A). Regarding apoptotic signaling, rotenone exposure increased both caspase-3 and caspase-8 expression compared to control, whereas yerba mate supplementation attenuated this response.

Increased expression of caspase 3 was observed in the rotenone and yerba mate + rotenone groups, compared to the control group ($p < 0.0001$ and $p = 0.0239$, respectively). To the group treated with aqueous extract of yerba mate showed a decline in the expression of caspase 3, when compared to the rotenone group ($p < 0.0001$). In contrast, cells exposed only to the aqueous extract of yerba mate showed a decrease in caspase 3 expression when compared to the rotenone and yerba mate + rotenone groups ($p < 0.0001$ and $p = 0.0447$, respectively). There was no statistical difference between the yerba mate and control groups ($p = 0.9677$).

Similar results were observed for caspase 8 (Figure 5B), where rotenone and the yerba mate + rotenone treatment increased the gene expression of this protein compared

to the control group ($p < 0.0001$ and $p < 0.0001$, respectively) and compared to the yerba mate group ($p < 0.0001$ and $p < 0.0001$, respectively). There was no statistical difference between the yerba mate and control groups ($p = 0.8511$). However, the yerba mate group showed lower caspase 8 expression compared to the yerba mate + rotenone group ($p < 0.0001$). In the immunoassay, an increase in caspase-3 levels (Figure 5C) was observed in the rotenone and yerba mate + rotenone groups when compared to the control group ($p < 0.0001$ and $p < 0.0001$, respectively) and yerba mate ($p < 0.0001$ and $p < 0.0001$, respectively). There was no difference between the control and yerba mate groups ($p = 0.7848$), however, the yerba mate group remained with lower levels when compared to the yerba mate + rotenone group ($p < 0.0001$). Similarly, caspase-8 levels (Figure 5D) were higher in the rotenone and yerba mate + rotenone groups when compared to the control group ($p < 0.0001$ and $p < 0.0001$, respectively) and yerba mate ($p < 0.0001$ and $p < 0.0001$, respectively). Even so, the yerba mate + rotenone and yerba mate groups showed lower caspase-8 levels compared to the rotenone-exposed group ($p < 0.0001$ and $p < 0.0001$, respectively). There was no statistical difference between the control and yerba mate groups ($p = 0.4515$).

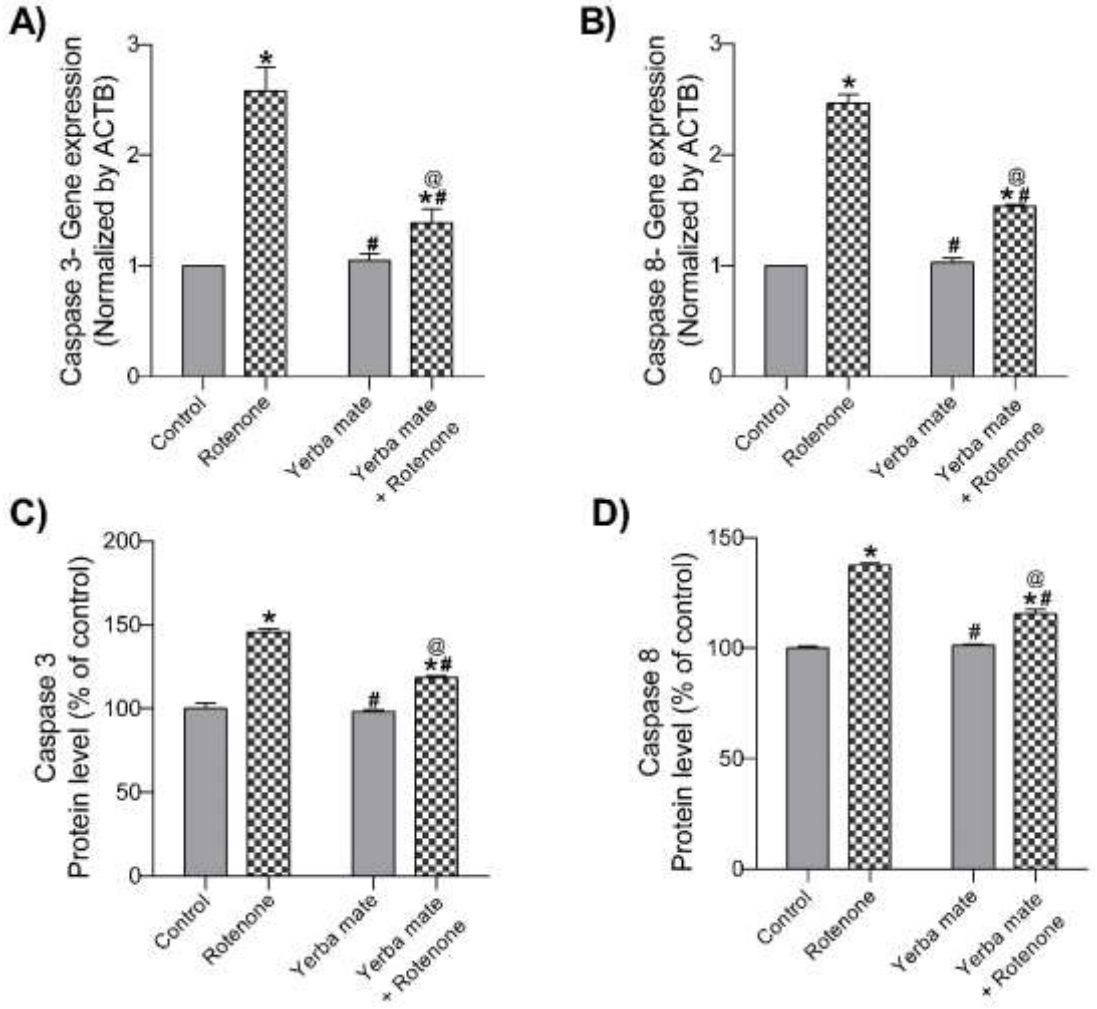

Figure 5. Evaluation of apoptosis in SH-SY5Y cells exposed to 10 µg/mL aqueous extract of yerba mate and 40 µM rotenone through (A-B) Gene expression of caspase 3 and 8 in 24h and (C-D) immunoassay to quantify in 72h, caspase 3 and 8 protein levels. These results were expressed as mean and ± standard deviation (SD), (n=3), and were considered statistically significant at $p \leq 0.05$. *Indicates a difference from the control, # indicates a difference from the rotenone group and @ indicates a difference compared to the yerba mate group.

4. Discussion

This study demonstrated that the aqueous extract of yerba mate exerts neuroprotective effects in a rotenone-induced in vitro model of Parkinson's disease using SH-SY5Y cells. The protective response was characterized by attenuation of oxidative stress, reduction of oxidative DNA damage (8-OHdG), and modulation of apoptotic

pathways. Importantly, these effects were partial in the presence of the neurotoxic insult, indicating mitigation rather than complete neutralization of rotenone-induced cellular damage.

The SH-SY5Y cell line differentiated into dopaminergic-like neurons is a well-established *in vitro* model for investigating Parkinson's disease-related mechanisms, particularly those associated with mitochondrial dysfunction and oxidative stress [18].

Exposure to rotenone, a mitochondrial complex I inhibitor, induces mitochondrial dysfunction, oxidative stress, and activation of apoptotic pathways, mimicking key aspects of Parkinsonian neurodegeneration and providing a suitable platform for evaluating neuroprotective strategies [18,23].

The way yerba mate was used in this study (aqueous extract) was based on the traditional preparation method commonly used by people living in the Pampa Biome (Rio Grande do Sul, Brazilian State, Argentina, and Uruguay). Nowadays, its consumption traditionally occurs through three different beverages: Chimarrão, Tereré, and Mate tea [19]. The present findings are consistent with previous reports describing antioxidant and immunomodulatory properties of aqueous yerba mate extracts in non-neuronal models, including human PBMCs and coelomocytes from *Eisenia fetida* [11]. These earlier observations support the hypothesis that the bioactive compounds present in yerba mate may exert protective effects across different cellular systems.

To assess the neuroprotective effect of the yerba mate extract, SH-SY5Y cells were exposed to rotenone, a molecule that inhibits mitochondrial complex 1. This effect elevates superoxide anion levels, thereby inducing oxidative stress and cell death via apoptosis. For this reason, rotenone has been used as an experimental model for PD [20, 21]. However, it has been reported that many natural products can attenuate the effects of rotenone, thereby exhibiting neuroprotective activity [6-22].

In the present study, concentration–response assays were conducted initially to define the experimental concentrations. Rotenone induced a clear dose-dependent reduction in cell viability, and a concentration of 40 µM was selected for its ability to produce substantial cytotoxicity while maintaining sufficient viable cells for downstream analyses. The aqueous extract of yerba mate did not exhibit cytotoxicity at the tested concentrations, supporting its cellular safety in this model. The concentration of 10

µg/mL was selected based on its observed cytoprotective profile in flow cytometry, including reduced apoptotic and necrotic cell populations [18].

In this study, prolonged exposure (72 h) allowed the detection of cumulative oxidative damage and apoptotic signaling. Under these conditions, yerba mate supplementation attenuated several markers of oxidative stress and cell death, suggesting a protective effect against sustained mitochondrial impairment.

Oxidative stress plays a central role in rotenone-induced neurotoxicity. Accordingly, cells exposed to rotenone exhibited increased levels of reactive oxygen species, superoxide anion, lipid peroxidation, and protein carbonylation. Supplementation with the aqueous extract of Yerba mate attenuated these alterations, although oxidative parameters in the yerba mate + rotenone group remained higher than those observed in untreated controls. This finding indicates a partial antioxidant protection under conditions of sustained mitochondrial impairment.

Generally, the antioxidant defense system protects cells from damage induced by ROS through the activities of antioxidant enzymes such as SOD, CAT, and GPx. To this end, the gene expression of these antioxidant enzymes was evaluated, and it was found that yerba mate aqueous extract preserved SOD2 gene expression at the control level, while CAT and GPx genes showed overexpression relative to the control and the rotenone group. There is consistent theoretical support for the modulation of gene expression by bioactive molecules found in functional foods and beverages [23,24,25].

Given the central role of redox homeostasis in cell survival, it is plausible that bioactive compounds present in yerba mate influence the transcription of genes involved in antioxidant defense. Previous studies have reported modulation of antioxidant enzyme–related gene expression in response to dietary bioactive compounds, including superoxide dismutase and glutathione peroxidase [27–28].

The modulating effect of the genes of the antioxidant system can be attributed to the bioactive substances in the yerba mate aqueous extract, such as polyphenols, tannins, and alkaloids, including caffeine and theobromine. As in other studies, caffeine is higher than theobromine in the extract and in the dry matter [29]. Each substance acts according to its chemical structure, triggering different mechanisms, such as transferring hydrogen atoms or individual electrons, or exerting a reducing or chelating effect on metals [30]. It

can be said that, even with increased production of superoxide anion radicals and ROS, the yerba mate aqueous extract neutralized them, protecting cells against peroxidation and protein damage. In general, although the changes in oxidative parameters remained higher in the yerba mate + rotenone group than in the control group, they also differed from those in the rotenone group, indicating partial protection. Furthermore, the difference observed between the yerba mate and yerba mate + rotenone groups suggests that the presence of the cytotoxic agent compromised the full antioxidant action of the extract, demonstrating that its beneficial effects are maintained, but attenuated, under conditions of induced oxidative stress.

In addition to the gene modulation observed here, the neutralization of reactive species generated in excess by rotenone exposure may have been partially offset by the substances in the extract, such as caffeine and theobromine. This information may be concrete based on studies that associate the antioxidant potential of yerba mate mainly with caffeine and theobromine [29,30].

The protection against lipid peroxidation in SH-SY5Y cells by the yerba mate aqueous extract is a significant result, as lipid peroxidation alters the fluidity and permeability of cell membranes, affecting normal cell function and potentially triggering apoptosis or necrosis, both of which are closely related to the development of many diseases. Evidence suggests that the phenolic hydroxyl groups present in food-based plant polyphenols effectively prevent or minimize lipid peroxidation of the cell membrane [31].

Our results related to the SOD 2, CAT and GPx genes expression agree with other findings, in which the yerba mate aqueous extract positively modulated the expression of antioxidant enzyme genes [10]. Considering the observed changes in gene expression, especially in the presence of rotenone, it is possible that the increase in gene expression was induced by the elevated levels of superoxide anion resulting from mitochondrial complex 1 inhibition by rotenone. However, it was impossible to confirm whether this modulation in gene expression could lead to changes in the expression of the proteins of these antioxidant enzymes.

Even though the enzyme activities were not evaluated, the SOD 2 expression at the control level in the yerba mate + rotenone group, together with the increase in ROS, predicts that the superoxide anion may be being degraded, generating H_2O_2, thus

justifying the increase in the expression of the CAT and GPx genes, as an attempt by the cell to preserve itself, preventing the triggering of oxidative stress. Although CAT and GPx enzyme activity was not observed, the increase in the expression of their respective genes suggests that the genetic modulation exerted by yerba mate aqueous extract maintained the integrity of the cell's DNA, as observed in the analysis of 8-OHdG.

Being a biomarker that shows oxidative damage to nucleic acids, more precisely in cell and mitochondrial DNA [29], reduction in 8-OHdG at the control level indicates that yerba mate aqueous extract exerted neuroprotection.

However, since caspase-3 activation is a step before DNA fragmentation in apoptosis [33], caspase activation was verified to further confirm yerba mate's neuroprotective activity. Treatment with the aqueous extract of yerba mate reduced both the expression of caspase 3 genes and the quantified protein levels. It was also observed that the yerba mate extract reduced the expression and levels of caspase-8.

Caspases play a central role in apoptotic signaling. In the present study, rotenone increased the expression and levels of caspase-3 and caspase-8, whereas yerba mate supplementation attenuated these responses, supporting partial modulation of apoptotic pathways [34]. Therefore, elevated caspase levels in the extracellular medium may indicate cell damage or an inflammatory state.

A previous PD human post-mortem study reported that dopaminergic neurons in the substantia nigra showed a higher percentage of caspase-8 activation than controls [35]. On the other hand, there are prior investigations, such as those conducted by Sulthana et al. [36], that rotenone induces apoptosis by activation of the caspase route in SH-SY5Y cells, which can be attenuated by molecules such as PIASA, a newly designed peptide. In this context, it is plausible that yerba mate extract exerts a neuroprotective effect on neurons exposed to rotenone by modulating caspase gene expression and their release into the extracellular medium.

This assumption is notably supported by the analysis of apoptotic cell frequency (both viable and non-viable) conducted by flow cytometry. These results, which mainly relate to caspases, are promising, as studies show that inhibitors of these enzymes are neuroprotective. The difference observed between the groups treated only with yerba mate and with yerba mate associated with rotenone indicates that the presence of the

cytotoxic agent partially compromises the anti-apoptotic action of the extract, maintaining its beneficial effects, but with less magnitude. It has been that caspase-3/7 and caspase-8 are the main regulators of microglial activation, which can release neurotoxic pro-inflammatory factors [35].

The distinction between the effects observed in the yerba mate group and those in the yerba mate + rotenone group reinforces the interpretation that the extract confers resilience to neuronal cells, even though its anti-apoptotic efficacy is reduced under toxic conditions. This pattern is consistent with a protective, but not curative, role in the context of neurodegenerative stress.

It is worth noting that, according to some studies, the average consumption of mate is close to 0.5 L per day in South American countries [7]. Some studies related to the consumption of yerba mate and cancer are associated with the high temperature at which the drink is consumed and some other associated factors such as smoking, and not due to its components [7]. Finally, the limitations inherent to *in vitro* experimental models must be acknowledged. While SH-SY5Y cells provide valuable mechanistic insight, they do not recapitulate the complexity of the *in vivo* neuronal environment, including cell–cell interactions and systemic metabolism. Nonetheless, the present findings offer a relevant experimental basis supporting a potential neuroprotective role under conditions of oxidative and mitochondrial stress.

5. Conclusions

The aqueous extract of yerba mate showed beneficial effects on SH-SY5Y cells exposed to rotenone, attenuating oxidative stress and the activation of apoptotic pathways, according to the modulation of the expression of SOD, CAT, GPx and caspases-3 and -8. However, the differences observed between the Yerba mate and Yerba mate + rotenone groups indicate that, although the protective effects are maintained in the presence of the neurotoxic agent, their magnitude is partially reduced, suggesting neuroprotective potential that deserves further investigation.

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NEUROPROTECTIVE EFFECT OF AQUEOUS YERBA MATE (*ILEX PARAGUARIENSIS*) EXTRACT IN A PARKINSON'S DISEASE IN VITRO MODEL

Author Contributions	
Elize Musachio:	Visualization, Writing – original draft.
Tábada Samantha Marques Rosa:	Conceptualization, Writing – original draft.
Verônica Farina Azzolin:	Investigation, Methodology.
Moisés Henrique Mastella:	Methodology, Data curation.
Izabella Paz Danezi Felin:	Data curation, Formal analysis.
Marta Medeiros Frescura Duarte:	Investigation, Methodology.
Maria Denise Schimith:	Investigation, Methodology.
Aron Ferreira da Silveira:	Resources, Investigation.
Ivana Beatrice Mânica da Cruz:	Project administration, Funding acquisition.
Fernanda Barbisan:	Supervision, Writing – review & editing.
All authors approved the final version of the manuscript.	
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Corresponding Author:	Giuliana Martina Bordin Universidade Positivo Rua Professor Pedro Viriato Parigot de Souza, 5300. Curitiba/PR, Brazil. Zip code: 81280-330 giulianabordin@gmail.com
Editor-in-Chief:	Adriane Cristina Bernat Kolankiewicz. PhD
Editor:	Matias Nunes Frizzo. PhD