

The effect of different polyphenols against neurotoxicity induced by quinolinic acid in U87-MG glial cells

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Efecto de diferentes polifenoles frente a la neurotoxicidad inducida por ácido quinolínico en células gliales U87-MG

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Abstract

Neurodegenerative disorders (ND) are very debilitating aging-related diseases and mitochondrial dysfunction, oxidative and nitrosative stress (ONS) have been demonstrated to be associated with its clinical manifestations. Mitochondria stand out as crucial organelles in the interplay between neurodegeneration and neuroinflammation, and polyphenols are promising mitochondria-targeting medicine. Phenolic compounds can regulate mitochondria controlling their redox state, function and apoptosis system. In this work, it was investigated the neuroprotective potential of *Araucaria angustifolia* (AAE) and *Camellia sinensis* (GT) extracts and six isolated compounds (resveratrol, gallic acid, ellagic acid, catechin, epicatechin and proanthocyanidins) in U87-MG glial cells. Further, the compounds that exhibited the best results were tested in a neurodegeneration model using quinolinic acid (QA). Among phenolic compounds, AAE and GT stood out, and maintained the glial viability around 100%, even in lower doses. Cells exposed to QA presented decreased viability, exacerbated reactive oxygen species (ROS) generation, reduced the mitochondrial membrane potential, and increased inflammatory response. U87-MG glial cells pre-treated with AAE or GT for 1 hour and then exposed to QA for 24 hours were able to prevent all these alterations induced by QA. Despite the similar results found with both GT and AAE, the last one was capable to prevent all the parameters tested in this work. In conclusion, we suggest that AAE could be a potential agent to prevent ND related to mitochondrial dysfunction associated with ONS.

Keywords: Neurodegeneration; Quinolinic acid; Polyphenols; Oxidative stress.

Resumo

Doenças neurodegenerativas (DN) são desordens altamente debilitantes relacionadas ao envelhecimento, e a disfunção mitocondrial, estresse oxidativo e nitrosativo (EON) têm sido associados com as manifestações clínicas desse grupo de patologias. As mitocôndrias se destacam como organelas cruciais na relação entre a neurodegeneração e a neuroinflamação, e nesse sentido, os polifenóis são considerados promissores enquanto fármacos que têm como alvo as mitocôndrias. Compostos fenólicos são capazes de regular as mitocôndrias através do controle de seu estado redox, função e sistema apoptótico. Neste trabalho investigou-se o potencial neuroprotetor dos extratos de *Araucaria*

angustifolia (AAE) e *Camellia sinensis* (GT), bem como de seis compostos isolados (resveratrol, ácido gálico, ácido elágico, catequina, epicatequina e proantocianidinas) em um modelo de neurodegeneração usando ácido quinolínico (AQ). Células gliais U87-MG foram pré-tratadas com AAE ou GT ou um dos compostos fenólicos por 1 hora e, então, expostas ao AQ por 24 horas. As células expostas ao AQ apresentaram diminuição da viabilidade, produção exacerbada de espécies reativas de oxigênio (EROs), redução do potencial de membrana mitocondrial e aumento da resposta inflamatória. Apesar dos resultados similares encontrados com o GT e o AAE, este último se destacou por ser capaz de prevenir todos os parâmetros testados neste trabalho. Em conclusão, sugere-se que o AAE apresenta-se como um agente em potencial para a prevenção de DN relacionadas com a disfunção mitocondrial associada ao EON.

Palavras-chaves: Neurodegeneração; Ácido quinolínico; Polifenóis; Estresse oxidativo.

Resumen

Enfermedades neurodegenerativas (EN) son trastornos altamente debilitantes relacionados con el envejecimiento y la disfunción mitocondrial, estrés oxidativo y nitrosativo (EON) están asociados a la aparición del cuadro clínico. Las mitocondrias se destacan como organelas cruciales en la interacción entre neurodegeneración y neuroinflamación, y polifenoles son considerados como medicamentos prometedores dirigidos a las mitocondrias. Compuestos fenólicos pueden regular las mitocondrias controlando su estado redox, función y sistema de apoptosis. En este trabajo, fue investigado el potencial neuroprotector de los extractos de *Araucaria angustifolia* (AAE) y *Camellia sinensis* (GT), y seis compuestos aislados (resveratrol, ácido gálico, ácido elágico, catequina, epicatequina e proantocianidinas) em um modelo de neurodegeneración utilizando ácido quinolínico (AQ). Células gliales U87-MG fueran pretratadas por 1 hora con AAE o GT o un de los compuestos aislados, y, después, expuestas al AQ por 24 horas. Las células expuestas al AQ presentaron viabilidad disminuida, alta producción de especies reactivas de oxígeno (EROs), reducción de la polarización mitocondrial y aumento de la respuesta inflamatoria. Apesar de los resultados similares encontrados para el GT y el AAE, este ultimo se destacó, siendo capaz de prevenir todos los parámetros testados en este trabajo. En conclusión, se sugiere que el AAE podrida ser un agente de prevención contra las EN relacionadas con la disfunción mitocondrial asociada al EON.

Palabras clave: Neurodegeneración; Ácido quinolínico; Polifenoles; Estrés oxidativo.

1. Introduction

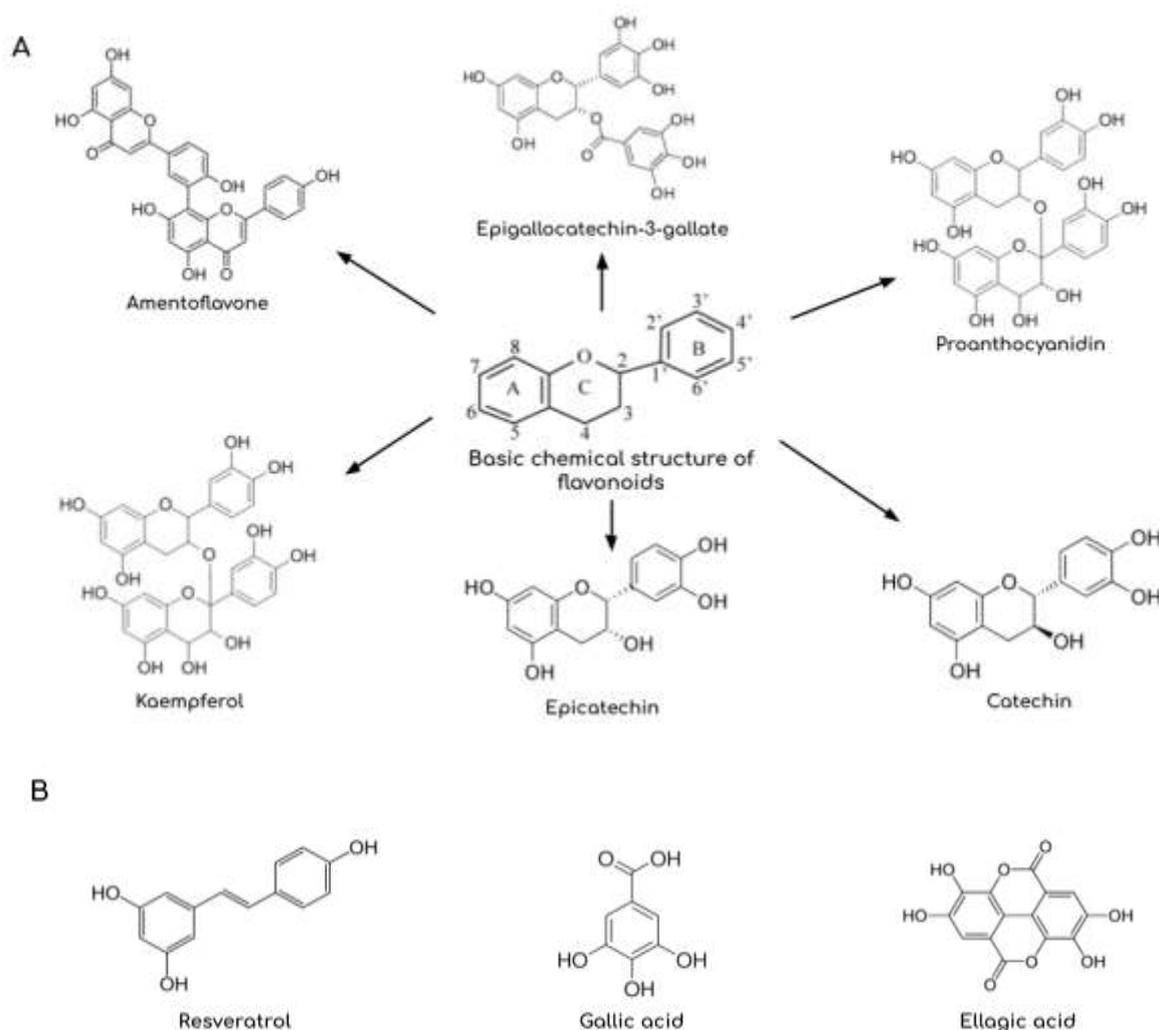
Our brain is a very complex structure. Because of its complexity, a range of disorders may arise even from small miscommunications between neurons. Neurodegenerative diseases (ND) are a heterogeneous group of disorders characterized by the progressive degeneration of the structure and function of nerve cells over time, leading to their death. There are many known ND, such as Alzheimer's, Parkinson's and Huntington's disease, Dementia, and Amyotrophic Lateral Sclerosis (Erkkinen et al., 2018; Norris et al., 2020). These diseases have different etiology and may affect the central nervous system (CNS) or peripheral nervous system (PNS) (Chen et al., 2020). Furthermore, patients can experience a lack of muscle control or coordination of voluntary movements (ataxia) and cognitive decline (Slanzi et al., 2020). The origin of these disorders is not clear, but the interaction between genetics, aging, and environmental risk factors appears to be a consensus in the scientific community (Renaud & Martinoli, 2019). New findings also suggest that stress-related disorders (eg.: post-traumatic stress disorders, and acute stress reaction) may be associated with the risk of developing ND (Song et al., 2020). The risk of developing ND increases with age, and regions with growing elderly populations, such as Europe, tend to be more affected by ND in the coming decades (Deuschl et al., 2020).

Tryptophan (TRP)-kynurenine (KYN) metabolic pathway is the main degradation route of TRP, an essential amino acid involved in brain homeostasis, which is also the precursor of central and peripheral serotonin, kynurenine, and its metabolites (Colle et al., 2020). Alterations found in the kynurenine pathway (KP), not only because of some metabolites produced but also due to the enzymatic actions, play a role in many conditions, including ND. The main catabolite of the KP is quinolinic acid (QA), a selective agonist of N-methyl-D-aspartate (NMDA) receptors. QA activates these receptors and promotes an intracellular increase of Ca²⁺, eventually leading to a range of synaptic alterations. The excessive activation of NMDA receptors induces neurotoxicity, which can cause neuronal death (Biernacki et al., 2020). This neurotoxicity is associated with mitochondrial dysfunction, exacerbated generation of reactive oxygen species (ROS), and/or proinflammatory (leading to chronic neuroinflammation) metabolites (Török et al., 2020; Visentin et al., 2020), which contribute to the widespread problems faced in ND.

Although treatment may help to relieve some of the physical and mental symptoms, researchers still need to find a way to slow down ND progression. Moreover, the cure for these disorders is currently unknown. Therefore, it is crucial to develop new approaches for the prevention and treatment of ND. In this regard, studies involving polyphenols have been gaining attention due to their health-promoting effects (Hano & Tungmunnithum, 2020).

Phenolic compounds are described as secondary metabolites (non-nutrient products) which have at least one phenolic group with one or more hydroxyl groups. They can be found in fruits, vegetables, seeds, and many other plant structures. Some of its functions include protection (against microbial infection, herbivores attack, ultraviolet radiation), reproduction (attractants for pollinators and seed-dispersing animals), and adaptation to harsh environments in general (Del Rio et al., 2013; Di Ferdinando et al., 2014). Polyphenols are classified into two main groups called flavonoids and non-flavonoids (Figure 1). Flavonoids are divided into six subclasses known as flavonols, flavones, isoflavones, flavanones, anthocyanins, and flavan-3-ols, while non-flavonoids are subdivided into lignans, stilbenes, hydroxybenzoic acids, hydroxycinnamic acids, and tannins (Pei et al., 2020). The beneficial effects observed in dietary phenolic compounds can be explained based on their antioxidant, anti-inflammatory, and neuroprotective properties (Gorzynik-Debicka et al., 2018; Russo et al., 2020). Polyphenols also play an important role as mitochondrial regulators, improving its function (especially the electron transport chain mechanism), modulating the redox state, and inhibiting the apoptosis triggered by this organelle (Naoi et al., 2019).

Figure 1. (A) Basic chemical structure of flavonoids and representative metabolites. (B) Non-flavonoids representatives.



Source: Authors.

This study aimed to evaluate whether the natural extracts from *Araucaria angustifolia*, *Camellia sinensis*, and selected isolated compounds (resveratrol, gallic acid, ellagic acid, catechin, epicatechin, and proanthocyanidins) have neuroprotective effects, in a model of neurodegeneration induced by quinolinic acid using U87-MG glial cells. As glial cells display a pivotal role in maintaining the homeostasis of CNS and PNS, providing support and protection for neurons, new strategies targeting these cells are crucial to minimizing ND occurrence.

2. Materials and methods

2.1 Plant materials and phenolic compounds

Araucaria angustifolia extract (AAE) was obtained from non-sterile seeds collected from mature female strobili of *A. angustifolia* in Caxias do Sul, Rio Grande do Sul, Brazil (29°9'34.90"S, 51°8'45.34"W). Licenses for collecting (Biodiversity Authorization and Information System-SISBIO authorization n° 36668- 2) and accessing (Brazilian Institute of Environment and Renewable Natural Resources-IBAMA n° 02001.001127/2013-94; National Genetic Patrimony Management System-SISGEN n° A69831D) *A. angustifolia* strobilus were previously obtained, according to the national guidelines. A voucher sample (No. 40710/40711) has been deposited in the herbarium of the University of Caxias do Sul, RS, Brazil. AAE was obtained using 5 g of bracts in 100 mL of distilled water using reflux (100 °C, 15 min). After the extraction, AAE was filtered in Millipore equipment (pore size, 0.45 µm; SFGS 047LS, Millipore Corp.) and lyophilized (LIOBRAS model L-101) under vacuum pressure to yield a powder, which was stored protected from light until use. The chemical matrix was determined by HPLC (HP 1100 UV/VIS detector; Santa Clara, CA, USA). The following compounds were identified in the extract: 1,3,4,5-tetrahydroxy-cyclohexane carboxylic acid, 3-O-methyl-D-chiro inositol, 4-nitrophenyl-b-Dglucopyranoside, 40-methoxytectorigenin, 3-glucoside-dihydro quercetin, amentoflavone 4',4'',7,7''-tetramethyl ether, dodecanoic acid, hexadecanoic acid (Branco et al., 2015; Branco et al., 2019). The extract (1 mg/mL) was diluted in ultrapure water (Milli-Q®) and filtered with a syringe filter (0.22 µm pore size).

Camellia sinensis from China (batch: HR20170602), was acquired from Active Pharmaceutica, Palhoça, SC, Brazil. Green tea (GT) (1 mg/mL) was prepared as an infusion by dilution in ultrapure water (Milli-Q®) at 100°C for 30 minutes, allowing the temperature to decrease over time. Then, it was filtered with a syringe filter (0.22 µm pore size). GT main compounds are catechins and ellagitannins (Tomas-Barberan & Yang, 2018; Musial et al., 2020).

Resveratrol (Sigma-Aldrich R5010) was diluted in dimethyl sulfoxide 0,1% v/v (DMSO). Ellagic acid (Sigma-Aldrich E2250) was diluted in a 1 mM NaOH solution. Gallic acid, catechin, epicatechin (all from Sigma-Aldrich; G7384, C1788, and E1753, respectively), and proanthocyanidins (acquired from Active Pharmaceutica, batch: GRS201512002) were diluted in cell medium right before the experiments.

2.2 Cell culture and experimental design

U87-MG human glioma cell line (BCRJ, 0356, Rio de Janeiro, Brazil) was cultured in DMEM low glucose medium (Sigma-Aldrich D5523) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Laborclin 630111) and 1% penicillin (100 UI/mL)-streptomycin (100 µg/mL). Cells were maintained in a humidified atmosphere (5% CO₂ and 95% air at 37°C) until assays. Natural extracts from *A. angustifolia* (3, 5, 25 and 50 µg/mL), and *C. sinensis* (1, 10, 50 and 100 µg/mL) were administered for 1 hour. Isolated compounds, resveratrol (10 and 25 µM), gallic and ellagic acids (1, 10 and 20 µM), catechin and epicatechin (10, 250 and 500 µM), and proanthocyanidins (10, 50 and 100 µg/mL) were administered for 1 hour. After the treatments with polyphenols or isolated compounds, cells were exposed to QA (Sigma-Aldrich P63204) at concentrations of 250, 700, and 750 µM, for 24 hours, to mimic neurodegeneration (Limana Da Silveira et al., 2018; Pierozan et al., 2018; Sundaram et al., 2014). Treatments were carried out in 75 cm² flasks and DMEM low glucose medium was used as control.

2.3 Cell viability

MTT (thiazolyl blue tetrazolium bromide; Sigma-Aldrich M2128) assay (Denizot & Lang, 1986) was carried out to determine cell viability (1×10^5 cells per well in 96-well plate). U87-MG cells were treated with different concentrations of natural extracts, isolated compounds, and QA, as previously described. Afterward, the treatments were removed and the MTT solution (proportion of 1:2) was added. The samples were incubated for 24 hours at the temperature of 37°C, in the absence of light. Subsequently, MTT solution was removed and the resulting formazan violet product was dissolved in 100 μ L dimethylsulfoxide (DMSO), stirred for 15 minutes, and the absorbance was measured using a microplate reader (Victor-X3, multilabel counter, Perkin Elmer, Finland) at 517 nm. Results were expressed in percentage compared to non-treated control cells.

2.4 Flow Cytometry

Intracellular reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were measured by cytometry flow. After treatments, as previously described, cells were trypsinized and centrifuged at 800 rpm for 5 minutes. Then, the samples were resuspended in PBS and centrifuged at 800 rpm for 5 minutes, this procedure was performed twice. The fluorescence intensity of 10.000 cells was quantified by the flow cytometer instrument BD FACScalibur Flow Cytometer: 4-color (BD Biosciences). Results were collected by the software CellQuest Pro (BD Biosciences) and analyzed using the software FlowJo (TreeStar Inc.).

2.4.1 Intracellular ROS analysis

2',7'-dichlorofluorescein diacetate (DCFH-DA) is a non-fluorescent reagent that penetrates the cell-matrix and turns to highly fluorescent 2',7'-dichlorofluorescein (DCF) upon oxidation. To measure intracellular ROS levels, cells were dyed with DCFH-DA (10 mM; Sigma-Aldrich, Darmstadt, Germany) and incubated at 37°C in the absence of light, for 30 minutes. This methodology was modified from Frozza et al., 2017. Absorbance was measured at 488 nm excitation and 533 nm emission. Results were expressed in percentage (%) of control.

2.4.2 MMP ($\Delta\psi_m$)

MPP is an important parameter of mitochondrial function and an indicator of cell health. For this research, alterations in the MMP were evaluated using amphiphilic cationic fluorochrome 3,3'-dihexyloxycarbocyanine iodide (DiOC6(3); Molecular Probes Inc., USA). Cells were dyed with DiOC6(3) at the concentration of 175 nM for 30 minutes and analyzed using the flow cytometry technique. For this assay, the FL1 filter was used. Results were expressed in percentage (%) of control.

2.5 Cell lysis

After the treatments and according to Branco et al., 2015, cells were trypsinized and centrifuged at 4°C and 800rpm for 5 minutes. The pellet was resuspended in 1 mL of PBS and centrifuged two more times. The supernatant was discarded and the remaining pellet was frozen at -80°C until the lysis. RIPA buffer (Sigma-Aldrich R0278) was used in the presence of protease inhibitors (Sigma-Aldrich S8830), according to the manufacturer's instructions. Subsequently, the samples were incubated in ice for 10 minutes and sporadically agitated in the vortex. Then, cells were centrifuged at 8000 xg for 10 minutes at 4°C. The supernatant was stored at -80°C until analyzes were performed.

Total protein levels were determined according to Lowry et al., 1951. Results were expressed in mg/mL, and it was used to express the results of nitric oxide assessment.

2.6 Intracellular Nitric Oxide Levels

To identify inflammation markers in the samples, nitric oxide (NO) levels were indirectly determined using Griess reaction assay, as described by Green et al., 1981. Griess reagent was added to the cell lysate at the proportion of 1:1. The blend was homogenized and incubated at room temperature and in the absence of light for 10 minutes. Sodium nitroprusside was used as a standard curve. Absorbance was measured using a microplate reader (Victor-X3, multilabel counter, Perkin Elmer, Finland) at 550 nm. Results were expressed in nmol of nitrite/mg of protein.

2.7 Statistical analysis

Data from this quantitative study were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Results were deemed significant if the *p*-value is less than 0.05. The software GraphPad Prism 7 for Windows (GraphPad Software, San Diego, CA, USA) was used for statistical analysis. The experiments were performed in triplicate.

3. Results

3.1 Quinolinic acid can reduce glial viability in a dose-dependent manner

The first experimental approach was to determine whether QA was cytotoxic to U87-MG glial cells and what would be the best dose to carry out posterior experiments (Figure 2A). QA 250 μ M and 700 μ M reduced cell viability by approximately 29% and 43%, respectively. Despite all doses proving to be cytotoxic, QA 750 μ M was the most lethal and reduced cell viability by nearly 62%. Since the highest concentration of QA caused massive mortality in cells, it was discarded and the dose of 700 μ M was selected for further assays.

3.2 Differential effects by polyphenol-rich extracts in glial viability

Cells were treated with the natural extracts from *Araucaria angustifolia* or *Camellia sinensis* for 1 h to verify their possible effects on glial cells viability (Figure 2B). AAE 3 μ g/mL increased cell viability by 26%, while higher AAE concentrations did not show differences from control. GT 1 and 10 μ g/mL increased cell viability by about 24% and 21%. On the other hand, GT 50 and 100 μ g/mL significantly decreased cell viability by approximately 19% and 11%, respectively.

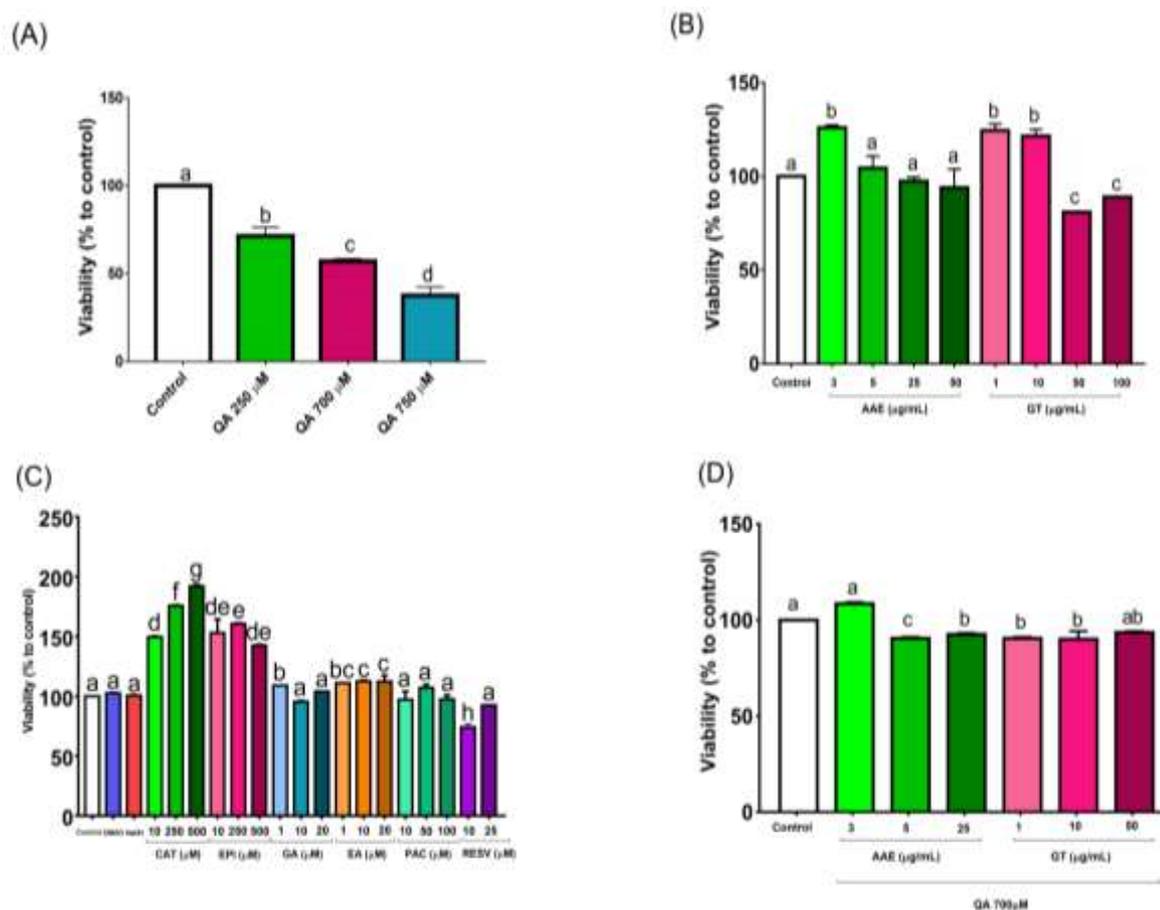
3.3 Concentration-response curves of isolated polyphenols in glial cells

Following the exposure to the isolated compounds (catechin, epicatechin, gallic acid, ellagic acid, proanthocyanidin or resveratrol) it was observed that CAT 10, 250, and 500 μ M increased cell viability by about 49%, 75%, and 92%, respectively. EPI 10, 250, and 500 μ M increased cell viability by approximately 53%, 60%, and 42%, respectively. Regarding the phenolic acids, GA 1 μ M increased cell viability by 9%, whereas EA 1, 10, and 20 μ M increased by about 20%, 46%, and 56%, respectively. In addition, RESV 10 μ M significantly reduced viability by around 26%. Conversely, PAC, at all tested concentrations were not able to change cell viability, when compared to control (Figure 2C).

3.4 Polyphenol-rich extracts can prevent QA-induced cytotoxicity in glial cells

After a cautious screening and by taking into consideration the synergic effects highlighted by molecules present in natural crude extracts, AAE (3, 5, and 25 μ g/mL) and GT (1, 10, and 50 μ g/mL) were selected to investigate their cytoprotective effects against QA 700 μ M, the concentration which was able to induce almost 50% of cells mortality. Once more AAE 3 μ g/mL had the best results and increased cell viability by 8%. Both AAE 5 and 25 μ g/mL reduced viability by 10% and 8%. GT 1 and 10 μ g/mL reduced cell viability about 10%, whereas GT 50 μ g/mL caused a reduction of approximately 7%, displaying very similar results (Figure 2D).

Figure 2. Viability assay of U87-MG glial cells exposed to quinolinic acid (A), *Araucaria angustifolia* and *Camellia sinensis* extracts (B), isolated compounds (C), and co-exposition of extracts (1 h) plus QA (24 h) (D). Values were standardized by negative control (medium) or positive control (DMSO 0,1% or NaOH 1 mM). Legend: DMSO: dimethyl sulfoxide; NaOH: sodium hydroxide; QA: Quinolinic Acid; AAE: *Araucaria angustifolia* extract; GT: green tea (*Camellia sinensis* extract); CAT: catechin; EPI: epicatechin; GA: gallic acid; EA: ellagic acid; PAC: proanthocyanidin RESV: resveratrol. Different letters represent statistical significance by analysis of variance ANOVA followed by Tukey post hoc test ($p < 0.05$).



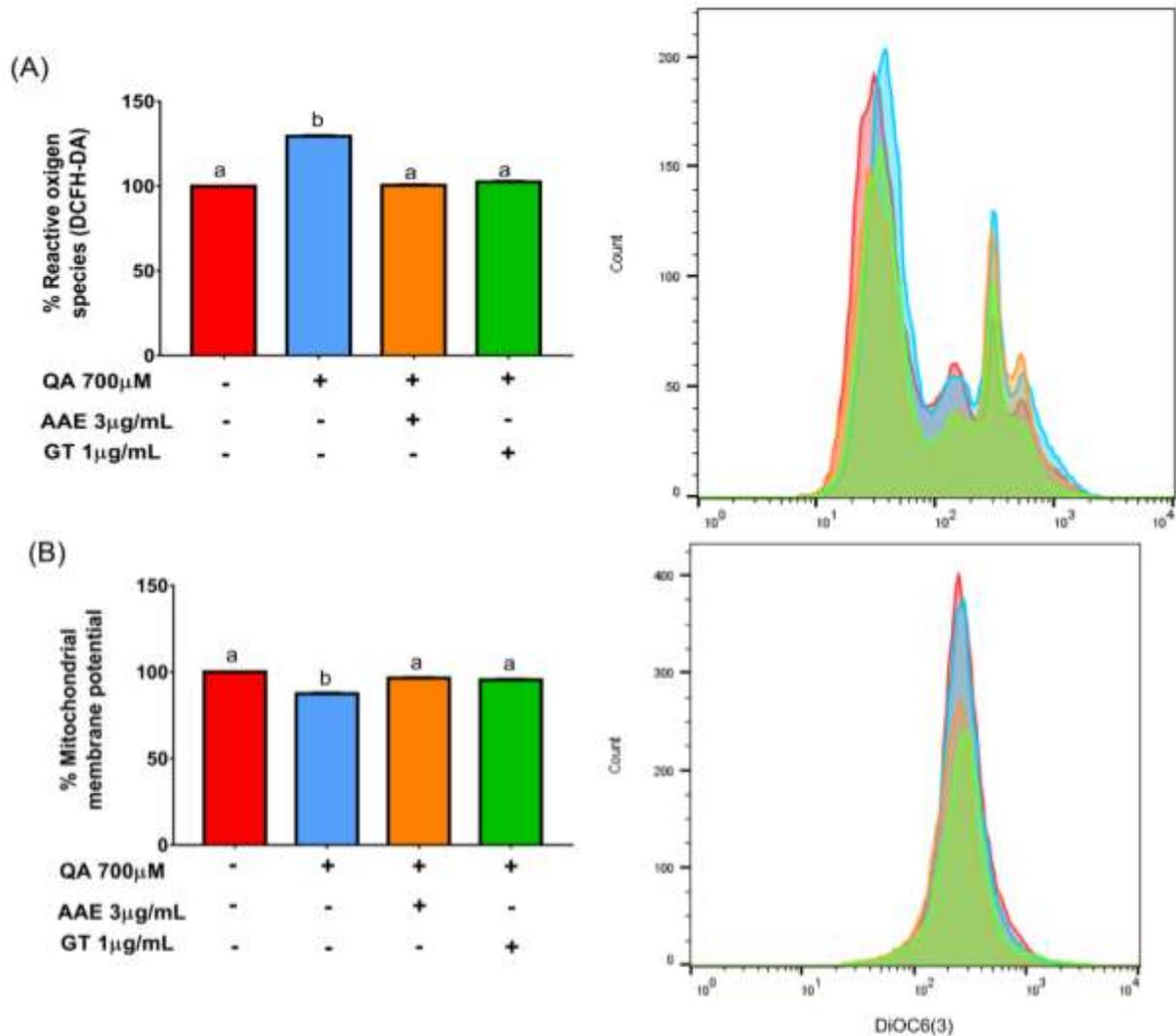
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3.5 Efficiency of extracts on mediation intracellular ROS and MMP

The results of intracellular ROS measurement and MMP assessments are shown in Figure 3. Since AAE 3 μ g/mL and GT 1 μ g/mL showed the best results in MTT viability assay, these doses were selected for the continuity of the work. QA 700 μ M generated around 30% of intracellular ROS to non-exposed cells (control). Both AAE 3 μ g/mL and GT 1 μ g/mL were able to prevent ROS generation by about 29 and 27%, respectively (Figure 3A). As shown in Figure 3B, QA 700 μ M reduced the mitochondrial membrane potential by approximately 12%. Both AAE 3 μ g/mL and GT 1 μ g/mL prevented this effect and were capable of bringing it to basal levels.

Although both extracts had similar results, AAE was slightly better in preventing reactive species generation and maintaining MMP, both impaired by QA exposition. Therefore, AAE 3 μ g/mL was selected to perform further experiments.

Figure 3. (A) Reactive oxygen species production and (B) Mitochondrial membrane potential of U87-MG glial cells treated with AAE and GT (1 h) and exposed to QA (24h). Legend: QA: Quinolinic acid; AAE: *Araucaria angustifolia* extract; GT: green tea (*Camellia sinensis* extract). Different letters represent statistical significance by analysis of variance ANOVA followed by Tukey post hoc test ($p < 0.05$).

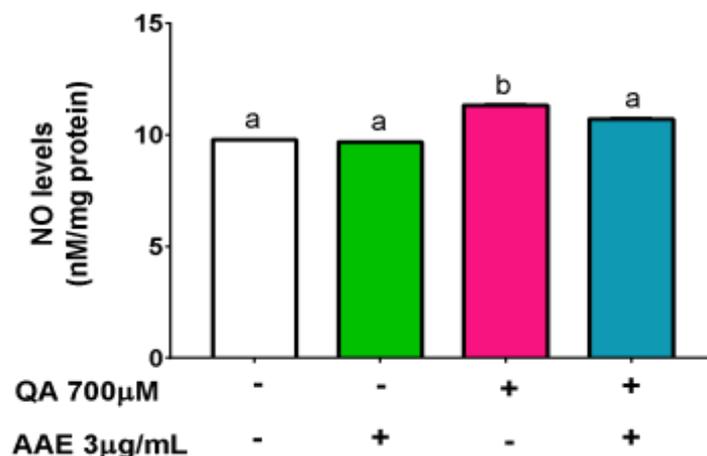


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3.6 Nitric oxide levels

Neuroinflammation is known as a phenomenon involved in ND. Then, to indirectly access inflammation response, the nitric oxide assay was carried out (Figure 4). It is possible to observe that AAE 3 μ g/mL didn't show a statistical difference compared to control. Although the exposure to QA 700 μ M for 24 hours induced an inflammatory response in U87-MG glial cells (increased nitric oxide levels around 15%), the pre-treatment with AAE 3 μ g/mL for 1 hour was able to prevent this response by approximately 9%.

Figure 4. Nitric oxide levels of U87-MG glial cells treated with AAE (1 h) and exposed to QA (24h). Legend: QA: Quinolinic acid; AAE: *Araucaria angustifolia* extract. Different letters represent statistical significance by analysis of variance ANOVA followed by Tukey post hoc test ($p < 0.05$).



Source: Authors.

4. Discussion

Neurodegenerative diseases can have many clinical manifestations, including memory and cognitive impairment, movement and speech dysfunction, besides many other features. Exposure to stress conditions leads to an imbalance in cellular homeostasis and mitochondrial dynamics that culminate in neuronal cell damage and/or death and chronic inflammation (Devi et al., 2021; Rathnayake et al., 2019). The chronic activation of microglial macrophages in the brain during the aging process can lead to the overproduction of pro-inflammatory cytokines, which contribute to the perpetuation of the neuroinflammation cycle, important in the development of ND (McGrattan et al., 2019). Quinolinic acid is an important metabolite of the KP that is used as a substrate to the essential co-factor nicotinamide adenine dinucleotide (NAD⁺), and for this reason, under physiological condition, is kept at low concentrations in the organism (Sas et al., 2018). However, during neuroinflammation, the KP is dysregulated, and this imbalance leads to overproduction and accumulation of QA. This metabolite then becomes neurotoxic, causing the death of brain cells (Sundaram et al., 2014).

In this study, we evaluated the ROS production, mitochondrial dysfunction, and inflammatory response of U87-MG glial cells exposed to QA. This KP catabolite was able to promote mitochondrial dysfunction, increase ROS production, and induce an inflammatory response in the cells. As corroborated by other authors using different study models (Limana Da Silveira et al., 2018; Pierozan et al., 2018; Sundaram et al., 2014), this environment mimics the biochemical alterations observed in ND.

Because of the involvement of oxidative stress and inflammation in neurodegeneration, ND are difficult to treat (Rekatsina et al., 2020). Moreover, the uncertainty about the efficacy of current medications reveals the urgency to better understand the mechanisms behind the development of these disorders, to improve the quality of life of patients and to develop more effective therapies for different prognoses (Bulck et al., 2019; Stephenson et al., 2018). Therefore, novel therapeutic approaches using polyphenols are being investigated. Although their short and long-term health effects are not fully understood, the benefits of consuming polyphenols may be linked to the regulation of metabolism and cell proliferation (Cory et al., 2018). In recent years, many studies indicate that these compounds, found in dietary plant sources, can have a neuroprotective effect, mainly due to their radical scavenging and anti-inflammatory activities (Potì et al., 2019).

To evaluate the neuroprotective effects exhibited by polyphenols, two plant extracts and six isolated compounds were selected for this work. As observed in the MTT assay, *Araucaria angustifolia* and *Camellia sinensis* extracts were able to improve cell viability. Although none of the AAE doses were cytotoxic, the lower concentration (3 $\mu\text{g/mL}$) showed the best results. Similar evidence was found in a previous study from Branco et al. (2019), where the authors explored the benefits of AAE in a neuropsychiatric and/or neurodegenerative disorders model associated with mitochondrial dysfunction using dopaminergic cells. Researchers found that AAE, even in a low dose (5 $\mu\text{g/mL}$ for 24 h), was able to promote cellular survival (pre- and post-treatment) and positively modulated mitochondrion, stimulating this cell to better control their redox state. These findings may be explained in part due to the components present in the chemical matrix of this extract (Branco et al., 2015). Differently from the AAE, higher doses (50 and 100 $\mu\text{g/mL}$) of the GT used in this study slightly reduced cell viability. Because the concentrations of GT 1 and 10 $\mu\text{g/mL}$ had very similar results, the lower concentration was selected. In both extracts, AAE and GT, it was possible to observe that lower concentrations exhibited similar effects, however beneficial effects displayed by AAE were superior to those observed by GT.

At the same time, isolated compounds, many of them present in the chemical matrix of both extracts, were tested to verify their effectiveness in the glial microenvironment. All the concentrations of EPI and CAT were hyper stimulants. Since the cell cycle must be tightly coordinated, this represents a dangerous situation for cells because this high viability may interfere negatively (Dalton, 2015) and boost mitochondrial metabolism in an exaggerated way (Golpich et al., 2017). Even though EA and GA had reasonably protected cells against the effects of QA, these results were mild compared to the extracts from *Araucaria angustifolia* and *Camellia sinensis*. Despite the recognized biological activity of the isolated compounds used in this work, the synergism between the compounds found in the chemical matrix of the AAE and GT extracts (Branco et al., 2019; Colon & Nerín, 2016) may be the reason why they stood out as cytoprotective agents.

In that regard, the extracts from *Araucaria angustifolia* and *Camellia sinensis* were chosen to carry out the intracellular ROS and mitochondrial membrane potential assays using, respectively, the concentrations of 3 and 1 $\mu\text{g/mL}$. It is known that aging processes, as well as genetics and environmental risk factors, contribute to the imbalance of the oxidative-redox system, leading to an overproduction of ROS. Deficiencies in the antioxidant defenses lead to oxidative stress which finally culminates in oxidative damage, reaching lipids, nucleic acids, and proteins (Basílio et al., 2021; Bhat et al., 2015; Islam, 2017; Nunnari & Suomalainen, 2012). Moreover, because of its characteristics (high oxygen and energy demand, rich-lipid components), the brain is very susceptible to oxidative damage (Salim, 2017).

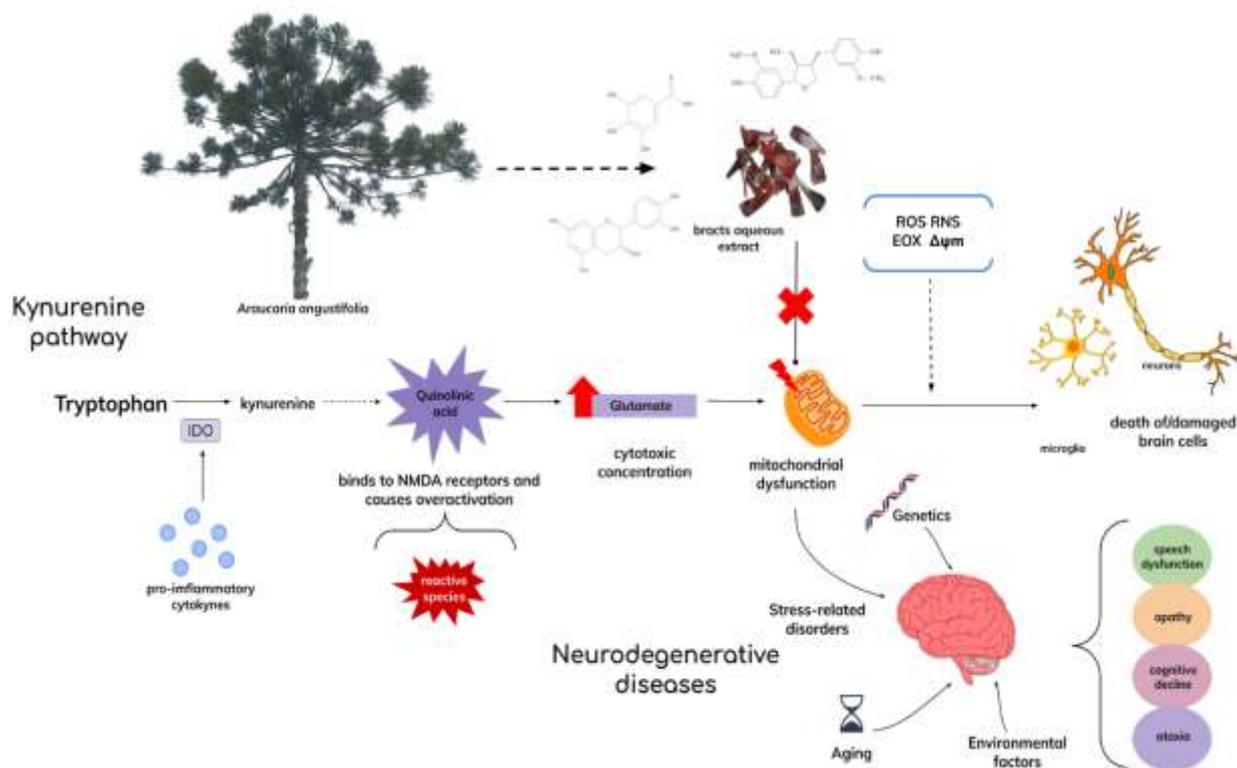
Beyond being responsible for energy production, the mitochondria are also involved in modulating the intrinsic apoptosis pathway under stress conditions, a vital response for maintaining cell health and viability (Johnson et al., 2021). Unfortunately, the mitochondrion is also the main site for ROS production, especially in complexes I and III. Reactive species are believed to be linked with the development of diseases related to mitochondrial dysfunction, such as ND (Bulck et al., 2019; Nunnari & Suomalainen, 2012; Visentin et al., 2020; Wu et al., 2019). Neuroinflammation, a common ND outcome, is mediated by activated brain-residing called glial cells (Rathnayake et al., 2019). Between them, microglia cells stand out. These cells have phagocytic activity and are responsible for neuronal development and maintenance. When activated, they produce inflammatory substances and cytotoxic molecules, such as nitric oxide (NO) and ROS (Subhramanyam et al., 2019). One of the ways to activate microglial cells is via QA exposition (Feng et al., 2017). In this study, QA increased ROS production and reduced the mitochondrial membrane potential, both characteristics found in ND (Bader & Winklhofer, 2020; Chu, 2019). On the other hand, AAE was capable of preventing these outcomes. These results show its potential as a neuroprotective agent for ND therapy since the current medication does not target oxidative stress and mitochondrion.

The induction and release of pro-inflammatory cytokines, such as interleukins, tumor necrosis factor, and interferon, activates the KP (Zádor et al., 2021). All these pro-inflammatory factors activate the indoleamine (IDO) enzyme, which in turn

limits tryptophan availability, causing overproduction of reactive species (ROS and RNS) and induce production of kynurenine metabolites, including QA (Visentin et al., 2020). Increased levels of QA in the brain are very detrimental since this metabolite binds and activates NMDA receptors and stimulates the neuronal release of glutamate. High levels of glutamate and the constant activation of excitatory neuronal cells are neurotoxic once they cause an influx of Ca^{2+} in the mitochondrion through the ion-channel complex, culminating in mitochondrial dysfunction and neuronal loss (Castro-Portuguez & Sutphin, 2020; Maddison & Giorgini, 2015).

The activation of pro-inflammatory markers and inflammatory response typically occurs when the organism encounters pathogens. Interestingly enough, sterile inflammation can occur when these processes take place even though no pathogen is identified (Zindel & Kubes, 2020). Indeed, sterile inflammation has been linked with aging-related ND (Stephenson et al., 2018). NO is an example of NRS and is known as an inflammatory marker (Rathnayake et al., 2019). In this study, levels of NO were measured to indirectly identify a possible inflammatory response activation. It was found that QA induced an inflammatory response in U87-MG glial cells, by increasing nitric oxide levels. On the other hand, the pre-treatment with AAE was able to prevent this response. Under physiological conditions, NO controls several cognitive and homeostatic functions in the CNS (Ghasemi et al., 2018). However, the accumulation of NO leads to nitrosative stress and can trigger neuroinflammation, as well as compromise cellular integrity and viability and cause mitochondrial dysfunction (Radi et al., 2014; Tse, 2017). New therapies targeting NO pathways seem to be promising in the treatment of ND. In addition to modulating this target, AAE was also able to prevent ROS generation and mitochondrial dysfunction caused by QA. These events are summarized in Figure 5.

Figure 5. The activation of the kynurenine pathway culminates in mitochondrial dysfunction which is one of many risk factors involved in the development of ND. Because of its rich antioxidant matrix, AAE can prevent many processes linked with ND.



Source: Authors.

While these findings are fascinating, the present study should be interpreted considering its limitations. As it was performed using an *in vitro* experimental model, further *in vivo* evaluations with a wide range of biochemical and molecular targets are necessary to complement all the effects reported here.

5. Final Considerations

ND are very debilitating conditions with currently no known cure. There is consensus among scientists that inflammation, oxide-nitrosative stress, and mitochondrial dysfunction are common pathways in their development. This work aimed to evaluate the possible neuroprotective effects of AAE, GT, and six isolated compounds in a model of neurodegeneration using U87-MG glial cells exposed to QA. AAE stood out and showed the best results among all the polyphenols tested, being a candidate as a drug or supplement to prevent ND. Due to these promising results, more mitochondria-focused assays are crucial to elucidate the mechanisms behind the relationship between the AAE and these organelles in the ND context.

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