



Neuroprotective Effects of the Combination of Green Tea, Saffron, Docosahexaenoic Acid, and α -Lipoic Acid in an In Vitro Model of Parkinson's Disease

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterised by dopamine deficiency and the accumulation of α -synuclein (α -syn), which aggregates into pathological inclusions known as Lewy bodies and Lewy neurites, distributed across multiple brain regions, with a particular prevalence in dopaminergic neurons. Alongside hallmark motor symptoms, PD is often accompanied by non-motor manifestations that severely affect patients' quality of life. Levodopa remains the most effective therapy; however, it is associated with a wide range of side effects and shows little to no efficacy against non-motor symptoms. This study investigates the neuroprotective effects of a combination of four bioactive compounds—green tea, saffron, docosahexaenoic acid (DHA) and α -lipoic acid (ALA)—against the PD-related neurodegeneration. Their ability to cross the blood–brain barrier (BBB) while maintaining its integrity was evaluated using a validated in vitro model. Individual and combined effects of these compounds were assessed on mesencephalic dopaminergic cells exposed to 6-hydroxydopamine (6-OHDA), a widely used in vitro model of PD-like neurotoxicity. The results demonstrated that the combined treatment (Mix) significantly restored cell viability after 6-OHDA exposure and more effectively reduced oxidative and nitrosative stress, as well as lipid peroxidation, compared to single compounds. Furthermore, the Mix markedly decreased the production of pro-inflammatory cytokines (including tumour necrosis factor- α (TNF- α) and interleukins) and downregulated the expression of PTEN-induced kinase 1 (PINK1) and Parkin, two key markers of PD-related neurodegeneration. In conclusion, these results indicate that the Mix has a suggest a synergistic-like impact on various disease-causing pathways in PD, highlighting its promise as a multi-faceted neuroprotective approach.

Keywords Parkinson's disease · Nutraceutical · Green tea · Docosahexaenoic acid · Saffron

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Introduction

Parkinson's disease (PD) is a progressive and rapidly growing neurodegenerative disease, affecting 2–3% of the population over 65 years of age [1]. This condition was first described in 1817 by the London physician James Parkinson, who referred to it as "shaking paralysis" in his essay titled "An essay on the shaking palsy". He characterised PD as a complex disorder involving tremors, gait abnormalities and a stooped posture. In addition to these motor symptoms, PD is recognised to encompass a variety of Non-Motor Symptoms (NMS), such as sleep disorders, pain, fatigue, gastrointestinal dysfunction, urinary disturbances, cognitive decline and dysautonomia [2].

From a pathophysiological perspective, the disease is primarily linked to the degeneration of dopaminergic neurons in the Substantia Nigra pars compacta and the excessive accumulation of α -synuclein-positive Lewy bodies (LBs) in various brain regions. The aggregation of α -synuclein (α -syn) is particularly disruptive to synaptic transmission in dopaminergic neurons, leading to reduced dopamine synthesis and bioavailability [3]. The dopamine transporter (DAT) and dopamine receptors (D1–D5) are crucial in modulating a wide array of dopamine-mediated functions, which include, but are not limited to cognition, reward, satiety, voluntary motor movements, gastrointestinal motility, and secretion [4].

This pathophysiological profile leads to the onset of a highly complex syndrome, which includes both motor and non-motor neuropsychiatric manifestations. Notably, NMS often appear a decade or more before the onset of hallmark motor features such as bradykinesia, balance problems, tremor, and rigidity [5]. This prodromal phase is typically characterised by hyposmia, sleep disturbances such as excessive daytime sleepiness and Rapid Eye Movement Sleep Behaviour Disorder (RBD), gastrointestinal dysfunction, particularly constipation, and affective symptoms such as depression and anxiety. NMS are not only characteristic of the prodromal phase of the disease but also persist throughout life. Therefore, recognising this early phase is crucial for initiating neuroprotective therapies when neurodegeneration is already underway but not yet advanced enough to impair motor functions [2].

Unfortunately, to date, no disease-modifying pharmacological therapies exist. Current therapeutic strategies primarily aim to alleviate symptoms without affecting disease progression [6]. Therapeutic strategies focus on restoring circulating dopamine levels to counteract the onset of symptoms. The first-line treatment is L-DOPA, a dopamine precursor that crosses the BBB and is enzymatically converted to dopamine within the central nervous system (CNS). One limitation of this therapy is that a significant

proportion of the L-DOPA metabolism occurs peripherally; therefore, a DOPA decarboxylase inhibitor is often co-administered with L-DOPA to reduce peripheral metabolism and increase central bioavailability [7]. Despite its efficacy, long-term use of L-DOPA can lead to various complications, particularly due to reduced and fluctuating treatment response caused by the progressive deterioration of the dopaminergic system, as well as its short half-life, which results in fluctuating plasma concentrations of the active ingredient throughout the day. Such fluctuations contribute to the emergence of motor complications and ineffective symptom control, exposing the patient to daily variations in their clinical manifestation [8].

Alternatively, the bioavailability and efficacy of L-DOPA can be prolonged by blocking the enzymes responsible for its catabolism, using monoamine oxidase type B (MAO-B) or catechol-O-methyltransferase (COMT) inhibitors, which reduce the catabolism of dopamine, thereby lowering the required dosage and mitigating motor fluctuations [9, 10].

Regarding NMS, these are poorly responsive to L-DOPA therapy, and over time, they become increasingly disabling after a six-year cut-off to the patient's quality of life [11]. Current management of these symptoms is contingent and tailored to the progression of the disease and the individual patient's condition [12]. To date, each NMS is treated with a targeted drug and a single therapeutic approach able to manage their overall complexity is still lacking [13].

Given the current approaches' lack of efficacy in modifying the course of disease, the development of alternative strategies is imperative to support and improve disease management. In this context, phytochemicals, have demonstrated antioxidant and anti-inflammatory qualities that may counteract the oxidative stress and inflammation implicated in neurodegenerative conditions such as PD [14, 15]. For example, the green tea polyphenol epigallocatechin-3-gallate (EGCG) has been shown to strongly inhibit α -syn aggregation, thereby mitigating the toxicity associated with its accumulation [16]. In vivo studies have further demonstrated that tea polyphenols alleviate motor disorders in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) Parkinsonian models [17]. Indeed, pretreatment of SH-SY5Y cells with 0.1 μ M EGCG was shown to significantly attenuate 6-hydroxydopamine (6-OHDA)-induced cell death through the modulation of STAT3 activity [18]. Furthermore, in SK-N-AS cells treated with 6-OHDA to mimic an in vitro Parkinson's disease model, EGCG inhibited α -syn upregulation and significantly reduced caspase-3 immunoreactivity [19]. Taken together, these preclinical results support a role for tea polyphenols in counteracting pathogenic mechanisms of PD. This translational relevance is further strengthened by meta-analyses in human populations, which consistently show that tea consumption correlates with a lower incidence of Parkinson's disease [20].

Similarly, saffron dry extract has proven helpful as a preventive agent against oxidative stress and apoptosis induced by rotenone, a compound known to replicate several key features of PD by regulating the Keap1/Nrf2 signalling pathway. Furthermore, its therapeutic potential was studied in a rat model with PD induced by 6-OHDA, and subsequent histopathological analysis suggested its neuroprotective effect [21]. Among saffron bioactive constituents, safranal can inhibit fibrillation and aggregation of α -syn by forming hydrophobic protein interactions [22].

In addition, saffron, with its bioactive components, can safeguard dopaminergic cells by inhibiting reactive oxygen species (ROS) production, downregulating caspase-3 activation, lowering lipid peroxidation and neuroinflammation, and preventing α -syn accumulation and aggregation. These mechanisms collectively enhance the brain's antioxidant defences [23]. As far as non-motor symptoms are concerned, crocins and safranal possess antidepressant properties. Indeed, these two key components of saffron have been shown to inhibit the reuptake of norepinephrine and serotonin, respectively [24]. This is further substantiated at the clinical level: randomized controlled trials confirm its benefits on sleep parameters [25], while international guidelines endorse saffron extract as a safe and effective adjunct in the management of anxiety and depressive symptoms [26].

Beyond phytochemicals, dietary fatty acids, such as docosahexaenoic acid (DHA), have emerged as promising neuroprotective substances. They are natural anti-inflammatory compounds that can reduce the cytokine storm and prevent cytokine-induced neurodegeneration [27]. Indeed, in an experimental model of PD, DHA has been shown to protect dopaminergic neurons and to improve locomotor activity [28]. Furthermore, it was recently discovered that DHA, crossing the BBB, can bring about a recovery of the dopaminergic system after extensive 6-OHDA-induced injury, suggesting possible neurorepairing effects [29]. These pre-clinical findings have also been corroborated by clinical evidence, with randomised controlled trials showing that omega-3 fatty acids combined with vitamin E supplementation can improve both clinical outcomes and inflammatory parameters in patients with PD [30, 31].

Finally, alpha-lipoic acid (ALA) can also exert neuroprotective effects in PD. A study by Zheng et al. [32] demonstrated how ALA could improve motor deficits in PD models by regulating iron metabolism and mitigating ferroptosis through the SIRT1/Nrf2 signalling pathway. Furthermore, in a mouse model of rotenone-induced PD, it was observed that ALA partially restored locomotor activity and increased the antioxidant activity of brain tissue compared to animals treated with rotenone alone [33]. Of note, since neuropathic pain represents a common and disabling non-motor manifestation of PD, ALA's documented clinical efficacy as an

antioxidant strategy for managing this condition further supports its therapeutic relevance [34].

Considering the growing body of evidence supporting the neuroprotective effects of these natural antioxidant and anti-inflammatory substances, the purpose of this study is to examine the molecular mechanisms by which a novel combination of green tea extract, saffron extract, DHA, and α -lipoic acid confers neuroprotection in a cellular model of PD. The goal was to assess their protective benefits by simulating a condition of neurotoxicity triggered by a Parkinson's inducer (6-OHDA).

Materials and Methods

Agent Preparation

Green tea (*Camellia sinensis* L., Kuntze, 60% catechins-40% EGCG used in a range of concentrations from 25 to 200 μ M) [35, 36], saffron (Saffron dry extract (d.e.) 0.3% used in a range of concentrations from 25 to 200 μ M) [37, 38], Docosanoic acid (DHA, used in a range of concentrations from 25 to 150 μ M) [38], and α -Lipoic Acid (used at 50 μ M) [39] donated by Kolinpharma S.p.A (Kolinpharma S.p.a., Lainate, Milan, Italy) were examined separately or combined after preparing it directly in Dulbecco's Modified Eagle Medium (DMEM, Merck Life, Milan, Italy). The administration of all substances to the cells was conducted with meticulous precision, ensuring the implementation of a molar concentration of the active ingredient utilised to titrate the raw material. Consequently, the concentration references are focused on EGCG for green tea and crocin for saffron. The green tea extraction process involved using water and ethyl acetate to extract the desired compounds from *C. sinensis* L. leaves. Subsequent analysis of green tea revealed the presence of total catechins and EGCG, with concentrations of approximately 60% and 40%, respectively. Conversely, saffron dry extract was derived from the stigma using an extraction technique that employed aqueous and alcoholic phases under specific pressure and temperature conditions. The active components of this extract were then analysed by titration, revealing safranal at 0.34%, picrocrocins at 0.37%, and crocins at 0.43%. The drug extract ratio is approximately 1:10–20. The different concentrations of 6-OHDA [40] were made in the same medium as the other agents, starting from a stock solution of 5 mM purchased by Bio-Techne SRL (Milano, Italy).

Cell Cultures

N27 mesencephalic dopaminergic cells were cultured in Roswell Park Memorial Institute medium (RPMI, Merck, Milan, Italy) supplemented with 10% fetal bovine serum

(FBS; Merck, Milan, Italy), 1% L-glutamine, penicillin (10 U/mL) and streptomycin (10 U/mL; GIBCO). Cells were seeded at a density of 0.5×10^6 in a 75-cm² flask (Corning, New York, NY) and incubated at 37 °C in 5% CO₂ and 95% humidity saturated conditions [41]. For the experiments, 1×10^4 cells were plated on a 96-well plate to study cell viability by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and ROS production by the colorimetric test; 1×10^6 cells were plated on a 6-well plate to quantify TNF α , interleukin (IL)-1 β , IL-6 and to investigate the molecular pathways involved in PD development such as Parkin and PTEN-induced kinase 1 (PINK1) by ELISA Kit after the induction of damage with 6-OHDA.

The astrocytic cell line CCF-STTG1 was grown in flask in RPMI medium (Merck, Milan, Italy) supplemented with 10% FBS (Merck, Milan, Italy) with the addition of 2 mM Hepes (Merck, Milan, Italy), 2 mM L-Glutamine (Merck, Milan, Italy) and 1% penicillin–streptomycin (P/S, Merck, Milan, Italy) and maintained in incubator at 37 °C with 95% humidity and 5% CO₂ [42]. In order to recreate the BBB in vitro, 4×10^4 astrocytes/cm² were seeded on a 24-well [43].

HUVECs, were grown in 0.1% gelatin-coated flask and cultured with Endothelial Growth Medium-2 (EGM-2) medium containing 2% fetal bovine serum (FBS), 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-1000, 0.1% heparin (all produced by Lonza, Walkersville, MD, USA) and maintained at 37 °C and 5% CO₂ as previously described [43]. Passage 3 to 6 cells were used for all experiments. To create an in vitro BBB 3D model, 1×10^5 HUVEC cells/cm² were plated in the apical compartment of 6.5 mm Transwell® with a polyester membrane with a pore size of 0.4 μ m [43].

Experimental Protocol

To understand whether green tea, saffron, DHA and ALA could modulate the mechanisms leading to the development of Parkinson's disease (PD), they were tested individually and in combination on mesencephalic dopaminergic cells (N27 cell line) that had been induced for PD with 6-OHDA at a concentration of 150 μ M for 6 h. This concentration was selected after a dose–response study on the N27 cell line, which analysed the effects on cell viability of varying concentrations of 6-OHDA. The experiments were divided into two phases: a first set of cell viability experiments assessed the optimal concentration of green tea (ranging from 25 to 200 μ M) [35–37], saffron (ranging from 25 to 200 μ M) [36, 37] and DHA (ranging from 25 to 150 μ M) [39], preparing for subsequent steps through a dose–response study by treating N27 cells with all substances for 24 h. Subsequently, the selected optimal concentrations of green tea, saffron, and

DHA, as well as in combination with ALA at 50 μ M [39], were tested on N27 cells alone to analyse cell viability, permeability through the blood–brain barrier (evaluated over a time course from 4 to 24 h), and blood–brain barrier integrity by assessing transepithelial electrical resistance (TEER) and specific tight junction levels (Claudin 5 and Marvel). After this evaluation, further experiments were performed to define the 6-OHDA concentration that best mimics PD conditions in vitro. N27 cells were treated with different concentrations of 6-OHDA (ranging from 25 to 200 μ M) for 1, 6, 12, and 24 h [44]. In the second phase of experiments, PD was induced in N27 mesencephalic dopaminergic cells by a 6-h treatment with 150 μ M 6-OHDA to assess the effects of the combination (Mix) on the modulation of mechanisms underlying PD development, comparing the results with those of single substances. This involved analysing cell viability, ROS and NO production, lipid peroxidation, and pro-inflammatory cytokine production (TNF α , IL-1 β , and IL-6). Additionally, an analysis was conducted on two significant deregulated markers during PD progression, namely Parkin and PINK1, using specific ELISA kits.

MTT Cell Viability Assay

Cell viability following stimulation was evaluated at the conclusion of the experiment using the MTT test, as described in the literature [45]. Cell viability was assessed by measuring absorbance at 570 nm with a correction at 690 nm using a spectrometer (Infinite 200 Pro MPlEx, Tecan) after the cells were cultured for 2 h at 37 °C in DMEM without phenol red, 0% FBS, and 1% MTT probe. Comparisons with 100% viable control cells were used to get the results.

Blood–Brain Barrier (BBB) In Vitro Model

Endothelial cells (HUVEC) and astrocytes were co-cultured using techniques documented in the literature [43]. In brief, 6.5-mm Transwell® inserts (Corning Costar, Sigma-Aldrich, Milan, Italy) were inverted, and 4×10^4 astrocytes/cm² were seeded on the basolateral side and allowed to adhere for 4 h to construct the basement membrane. After that, the Transwell® were positioned correctly, and the cells were allowed to grow for 48 h. Following the seeding of 1×10^5 HUVEC cells/cm² in the apical compartment, the inserts were put into a 24-well plate. After 7 days of culture, Transwell® were processed and permeability studies were performed [46].

During the differentiation phase, transepithelial electrical resistance (TEER) was monitored using an EVOM3 volt/ohm meter (World Precision Instruments, Sarasota, FL, USA) equipped with STX2 chopstick electrodes. Measurements were taken until the monolayer reached a TEER value of at least 70 Ω -cm², indicating the formation of a tight and

functional barrier prior to stimulation [36]. Subsequently, cells were incubated for 15 min at 37 °C in a humidified atmosphere containing 5% CO₂, and TEER measurements were repeated to confirm the stability of the barrier before beginning the experimental procedures.

To evaluate the ability of the tested compounds to cross the blood–brain barrier (BBB), the drugs were added to the apical compartment for incubation periods ranging from 4 to 24 h. Permeability was assessed using a 0.04% fluorescent tracer (Santa Cruz Biotechnology, CA, USA). The medium collected from the basolateral side of the Transwell® inserts was then analyzed to determine the apparent permeability coefficient (Papp, cm/s) for green tea, saffron, DHA, and lipoic acid, both individually and in combination. The Papp values were calculated according to the following equation [47]:

$$P_{app} = \frac{dQ}{dt} \times \frac{V_{Donor}}{m_0 \times A}$$

dQ: amount of substance transported (nmol or µg),
dt: incubation time (s),
m₀: amount of substrate applied to the donor compartment (nmol or µg),
A: Transwell® membrane surface area (cm²),
V Donor: volume of the donor compartment (cm³).
×: multiplication operation

The inclusion of cell-free negative controls enabled the elimination of potential interference caused by the Transwell membrane. Each experiment was conducted in triplicate and independently repeated five times.

Claudin 5 Assay Kit

The Claudin 5 ELISA Kit (MyBiosource, San Diego, CA, USA) was used to measure the quantity of claudin 5 in BBB cell lysates in accordance with the manufacturer's instructions [48]. A spectrometer (Infinite 200 Pro M Plex, Tecan, Männedorf, Switzerland) was used to analyse the absorbance at 450 nm. The data was compared to the standard curve (0–2500 pg/mL) to determine the concentration, which was then expressed in ng/mL. In comparison to the untreated control sample, the data are presented as mean ± SD (%).

Marveld Assay Kit

The TRIC/marveld in BBB cell lysates was measured using a TRIC ELISA kit (MyBiosource, San Diego, CA, USA) in accordance with the manufacturer's instructions [36]. A spectrometer (Infinite 200 Pro M Plex, Tecan, Männedorf, Switzerland) was used to measure the absorbance at 450 nm. To calculate the concentration in ng/mL, the data

are compared to the standard curve, which is 0.625–20 ng/mL. In comparison to the untreated control sample, the data are presented as mean ± SD (%).

ROS Production

The amount of superoxide anion (O₂^{•−}) released following stimulations was measured using this method [39]. Following treatment, all samples, treated and untreated, were incubated with 100 µl of cytochrome C and 100 µl of superoxide dismutase for 30 min in an incubator (all reagents were from Sigma-Aldrich). A spectrometer (Infinite 200 Pro M Plex, Tecan) detected the absorbance at 550 nm. O₂^{•−} was expressed as nanomoles of reduced cytochrome C per microgram of protein relative to the untreated control, as a percentage (%).

Lipid Peroxidation Assay

Lipid peroxidation in cells was estimated using the thiobarbituric acid assay (TBARS), in accordance with published methods [49]. Briefly, 100 µL of sample or standard was added to a 5 mL vial, followed by 100 µL of SDS solution. In each vial, 4 ml of Dye Reagent was added and boiled for 1 h. At the end of incubation, samples were cooled on ice for 10 min, centrifuged for 10 min at 1600×g at 4 °C, and then 150 µL from each sample was added to a 96-well plate. Absorbance was measured at 530–540 nm to determine the degree of lipid peroxidation.

Nitric Oxide (NO) Production Assay

To measure NO production, a kit was utilised to assess its production in response to various stimuli, according to manufacturer instructions [50]. After exposure to the respective treatments, the absorbance of the samples was measured using a spectrometer (Infinite 200 Pro M Plex, Tecan, Männedorf, Switzerland) at 520 nm to 550 nm. The results were reported as a normalised percentage (%) relative to untreated samples, using the standard curve established with standard nitrate.

Tumour Necrosis Factor-alpha (TNF-α) ELISA Kit

TNFα concentration was determined using a TNFα Elisa kit (Merck Life Science, Rome, Italy) following the experimental protocol [51]. A spectrophotometer measured the colourimetric intensity at 450 nm (Multiplex M, Tecan). Results were calculated by generating a calibration curve (range 24.58 pg/ml to 6000 pg/ml) and expressed as a percentage relative to the control.

Interleukin (IL)–1 β ELISA Kit

Quantification of IL-1 β was assessed using the IL-1 β ELISA kit (R&D Systems, MN) following the manufacturer's instructions [52]. Absorbance was measured with a microplate reader at 450 nm with correction at 570 nm. IL-1 β was quantified by correlating sample readings to the created standard curve (range 12.5 pg/ml to 800 pg/ml) and expressed as a percentage relative to the control.

IL-6 ELISA Kit

The concentration of IL-6 in basolateral co-culture media was evaluated using the IL-6 ELISA kit (eBioscience, USA) [53]. Absorbance was read at 450 nm, and the data were analysed by relating the sample readings to the generated standard curve (range 3.1 pg/ml to 300 pg/ml), expressed as a percentage relative to the control.

PTEN-induced Kinase 1 (PINK1) ELISA Kit

PINK1 activity was determined in cell lysates using an ELISA kit (PINK1 ELISA Kit, MyBiosource, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, cells were lysed with 1 $\times$ cold PBS and centrifuged at 1500 $\times g$ for 10 min at 4 °C. 100 μ l of each sample was added to a well and incubated at 37 °C for 90 min; then, the solution was removed, and 100 μ l of detection solution A was added to each well and incubated for 45 min at 37 °C. After this time, the wells were washed, and 100 μ l of detection solution B was added to each well, which was then incubated for another 45 min. Next, 90 μ l of substrate solution was added to each well, and the plate was incubated for 20 min at 37 °C in the dark. 50 μ l of Stop Solution was used to terminate the reaction. The absorbance was recorded with a spectrometer (Infinite 200 Pro MPlex, Tecan) at 450 nm and concentrations expressed in ng/mL by comparing the data with the standard curve (range 0.625 ng/ml–20 ng/ml).

Parkin ELISA Kit

Parkin activity was determined in cell lysates using an ELISA kit (Parkin ELISA Kit, MyBiosource, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, 100 μ L of each sample was added, and the mixture was incubated at 37 °C for 90 min. Afterwards, the material was removed, and 100 μ L of detection solution A was added and incubated for 45 min at 37 °C. After this time had elapsed, the wells were washed and 100 μ L of detection solution B was added to each well and then incubated for another 45 min. Next, 90 μ L of substrate solution was added to each well, and the plate was incubated for 20 min at 37 °C in the dark. 50 μ L of Stop Solution was used to halt

the reaction. The absorbance was analysed with a spectrometer (Infinite 200 Pro MPlex, Tecan) at 450 nm, with the concentration expressed in ng/mL by comparing the data to the standard curve (range 0.625 ng/ml–20 ng/ml).

Statistical Analysis

Data were collected from at least four independent experiments, conducted in triplicate for each protocol, and analysed using Prism GraphPad statistical software. Results are presented as means $\pm$ SD (standard deviation), and statistical evaluation was performed using one-way ANOVA followed by Bonferroni post hoc testing. A p-value of less than 0.05 was considered statistically significant.

Results

Concentration-dependent Screening for Working Concentration Selection

To determine the most effective concentration of each selected compound and to prevent potential cytotoxic effects, cell viability in N27 cells was assessed using the MTT assay. As shown in Fig. 1, all tested concentrations resulted in a statistically significant increase in cell viability compared with the control group. Specifically, Fig. 1A illustrates cell viability with green tea, in which all concentrations tested enhanced viability compared to the control. In particular, the most pronounced effect was observed in cells treated with 100 μ M green tea, which resulted in a 48% increase compared to 200 μ M, a 68% increase compared to 50 μ M, and an 82% increase compared to 25 μ M. Regarding DHA, the concentrations tested did not induce cytotoxicity compared to the control (Fig. 1B). More specifically, the most effective concentration was 25 μ M, which increased cell viability by approximately 16%, 38%, and 90% compared to 150 μ M, 100 μ M, and 50 μ M, respectively. In addition, the study revealed that all saffron concentrations tested also improved cell viability compared with the control group (Fig. 1C). The most pronounced effect was observed following treatment with saffron at a concentration of 25 μ M, which resulted in an increase in viability by 78% (compared to 200 μ M), 34% (compared to 100 μ M), and 25% (compared to 50 μ M). Based on these results, the following concentrations were selected for subsequent assays: green tea 100 μ M, saffron 25 μ M and DHA 25 μ M, together with the ALA concentration of 50 μ M reported in the literature [39]. Indeed, as shown in Fig. 1D, the single substances at selected concentrations were tested alone and in combination to evaluate N27 cell viability. As expected, single substances increased cell viability compared to the control, but this effect was amplified when combined. Notably, their combination (Mix)

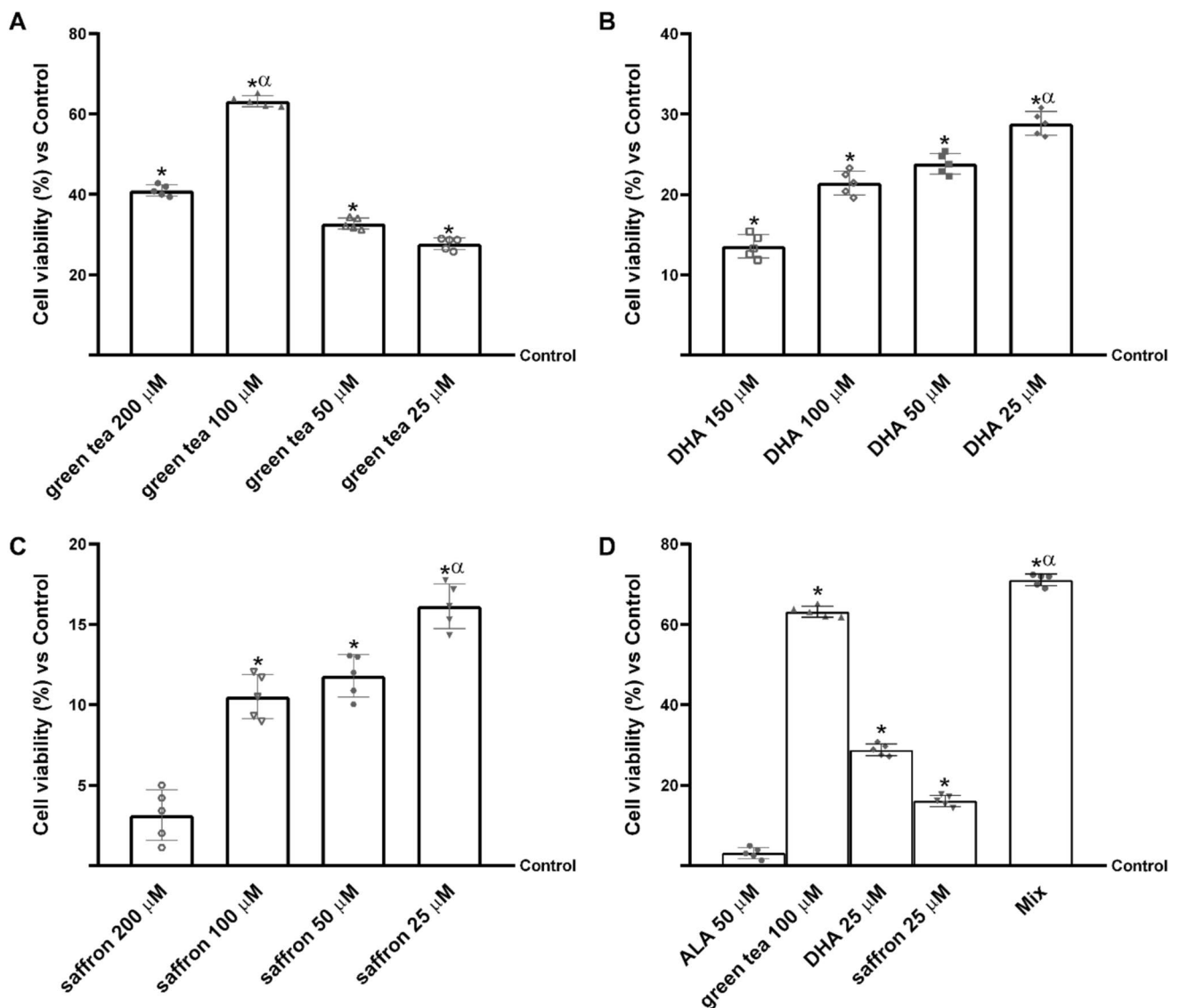


Fig. 1 Dose–response study on cell viability in N27 mesencephalic dopaminergic cells tested using the MTT assay. In (A), the dose–response study after green tea administration (200–25 μ M); in (B), the dose–response study after DHA administration (10–1 μ M); in (C), the dose–response study after saffron administration (200–25 μ M); and in (D), the cell viability analysis of the combina-

tion was evaluated. Mix=green tea 100 μ M+saffron 25 μ M+DHA 25 μ M+ALA 50 μ M. The results are expressed as mean $\pm$ SD (%) of five independent experiments normalised to the control (0 line corresponding to 100% cell viability), each performed in triplicate and expressed as percentage increase. * $p < 0.05$ vs Control; α $p < 0.05$ vs other concentration

potentiated the effects of single compounds by about 94% compared to ALA 50 μ M, 11% compared to green tea 100 μ M, 59% compared to DHA 25 μ M and 77% compared to saffron 25 μ M.

Evaluation of Integrity and Permeability in an In Vitro Model of the BBB

The integrity of the BBB was examined in vitro through TEER examination, claudin 5 and marveld analysis. All single substances maintained epithelial integrity, as

indicated by TEER analysis, with a peak at 12 h. Additionally, the Mix effect was consistently greater compared to the control, and Mix also had a stronger effect from 4 to 12 h compared to the single compounds (Fig. 2A). The levels of claudin 5 and marveld were measured to confirm the integrity of the BBB after treatment with the substances under examination. The examination of claudin-5 levels revealed that stimulation with Mix had a stronger effect than all individual compounds (Fig. 2B). After a marveld level examination, a similar result was observed in Fig. 2C, with Mix having a stronger impact than all single

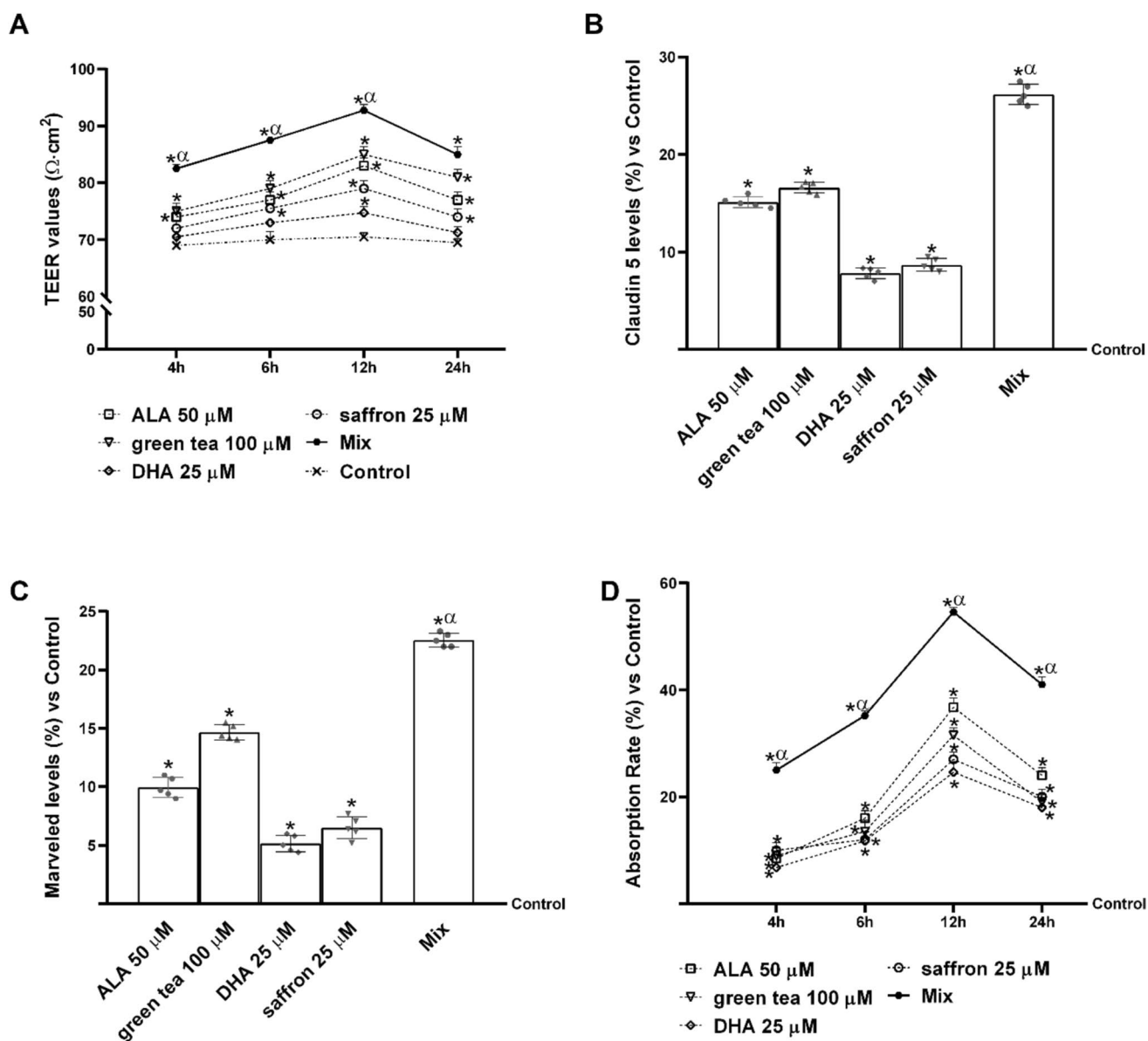


Fig. 2 Analysis of the integrity and permeability of the BBB. In (A) the TEER values measured using EVOM3, the breakpoint between the axes corresponds to the threshold value below which the experimental model does not mature; in (B) claudin 5 levels measured through a specific ELISA kit; in (C) Marvel levels measured through a specific ELISA kit; in (D) absorption analysis through

the BBB by fluorescent probe. Mix=green tea 100 μM +saffron 25 μM +DHA 25 μM +ALA 50 μM . The results are expressed as mean $\pm$ SD (%) of five independent experiments normalised to the control (0 line), each performed in triplicate and expressed as percentage increase. * $p < 0.05$ vs Control; α $p < 0.05$ vs single substances

substances. These data confirm Mix's ability to preserve and enhance the barrier integrity.

We also investigated the ability of the tested substances to cross the BBB. As demonstrated in Fig. 2D, all evaluated compounds demonstrated the capacity to permeate the BBB within the observation period of 4 to 24 h, with a peak absorption observed at 12 h. The Mix exhibited a statistically higher absorption rate than the single substances,

indicating a synergistic effect that enhanced BBB permeability when the compounds were administered in combination. This beneficial effect persisted up to 24 h compared to single substances. The increase in absorption rate was approximately 41.5% compared to green tea 100 μM , approximately 55% compared to DHA 25 μM , approximately 47% compared to saffron 25 μM and approximately 32.6% compared to ALA 50 μM .

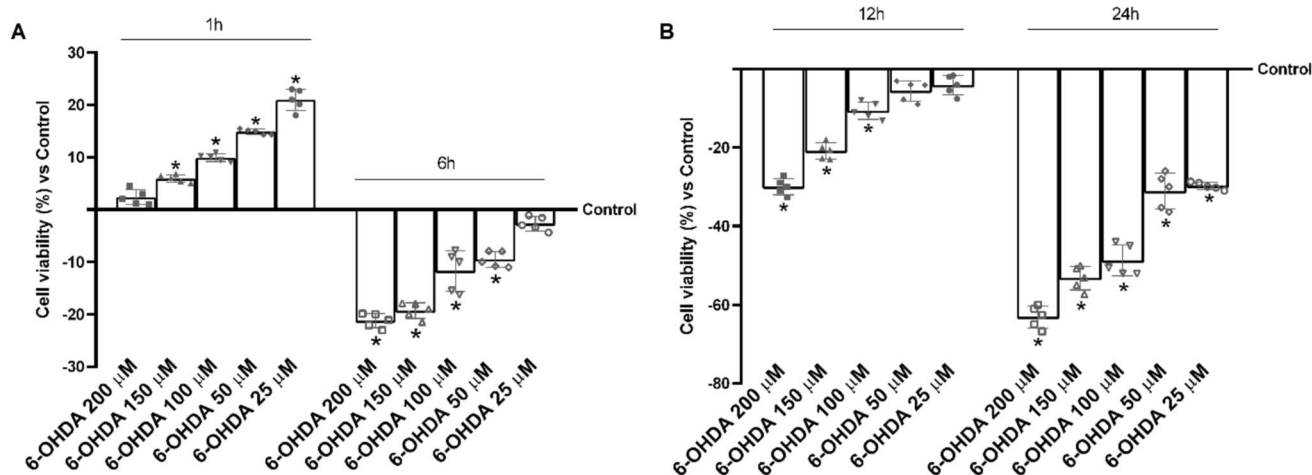


Fig. 3 Evaluation and validation of Parkinson's Disease in vitro model after treatment with 6-OHDA. MTT results are expressed as mean $\pm$ SD (%) of 5 normalised biological replicates vs. control. *

$p < 0.05$ vs. control (0 line corresponding to 100% cell viability), each performed in triplicate and expressed as the percentage increase

Evaluation and Validation of Parkinson's Disease In Vitro Model

To test the neuroprotective potential of green tea, saffron, DHA and lipoic acid in a pathological context, it was necessary to mimic 6-OHDA-induced neurotoxicity in N27 cells (Fig. 3). From the time-dependent (1h to 24h) dose–response study (200 μ M to 25 μ M), the cytotoxic effects of 6-OHDA as a Parkinson's inducer were confirmed. No cytotoxic effects were observed during the first hour of treatment with 6-OHDA at any tested concentration. Nonetheless, starting from 6h, detrimental effects were detected at concentrations of 50 μ M and above. However, only the 150 μ M concentration consistently induced damage similar to that occurring in PD over the entire treatment period, particularly at 6h of treatment (about -16% vs. 200 μ M; about 33% vs. 100 μ M; about 53% vs. 50 μ M; and about 89% vs. 25 μ M). As supported by the literature in N27 dopaminergic cells, 6-OHDA induces dose-dependent toxicity: 150 μ M for 24h causes significant cell death [54], whereas shorter exposures, such as 100 μ M for 6h, trigger oxidative stress without massive cell loss [55]. Therefore, the selected concentration of 150 μ M for 6h provides an intermediate condition, inducing substantial yet controlled dopaminergic injury suitable for modeling PD-related cellular mechanisms in vitro. Consequently, 6-OHDA was used in subsequent experiments at a concentration of 150 μ M for 6h to mimic in vitro the molecular mechanisms of PD-related alterations.

Evaluation of Neuroprotective effects in PD Condition

Cell viability, ROS production, lipid peroxidation, and NO production were assessed in N27 cells subjected to PD conditions induced by pre-treatment with 6-OHDA for 6h (Fig. 4). A pure neuronal model was used to isolate direct neuronal responses; in accordance with the literature, this approach enables the assessment of intrinsic inflammatory and oxidative processes by excluding the confounding effects of glial cells [56, 57]. As shown in Fig. 4A, the treatment with 6-OHDA at 150 μ M significantly reduced cell viability compared to the control. In contrast, administration of single substances and their combination effectively reversed the cellular damage previously induced by 6-OHDA (all substances and Mix $p < 0.05$ vs 6-OHDA). Furthermore, the Mix demonstrated the greatest effect in repairing the damage, leading to a statistically significant increase in cell viability compared with green tea and saffron (Table 1). Figure 4B shows increased oxidative stress in the cellular PD model, with significantly elevated ROS levels in 6-OHDA-treated cells compared to control. In contrast, after treatment with each single substance, ROS production was significantly reduced compared with 6-OHDA-induced damage. Moreover, the Mix demonstrated a greater and synergistic effect in reducing ROS production compared to the individual compounds ($p < 0.05$ vs all substances) (Table 1). Regarding lipid peroxidation activity, 6-OHDA-induced damage resulted in a 58% increase compared to the control. Treatment with the individual substances led to a

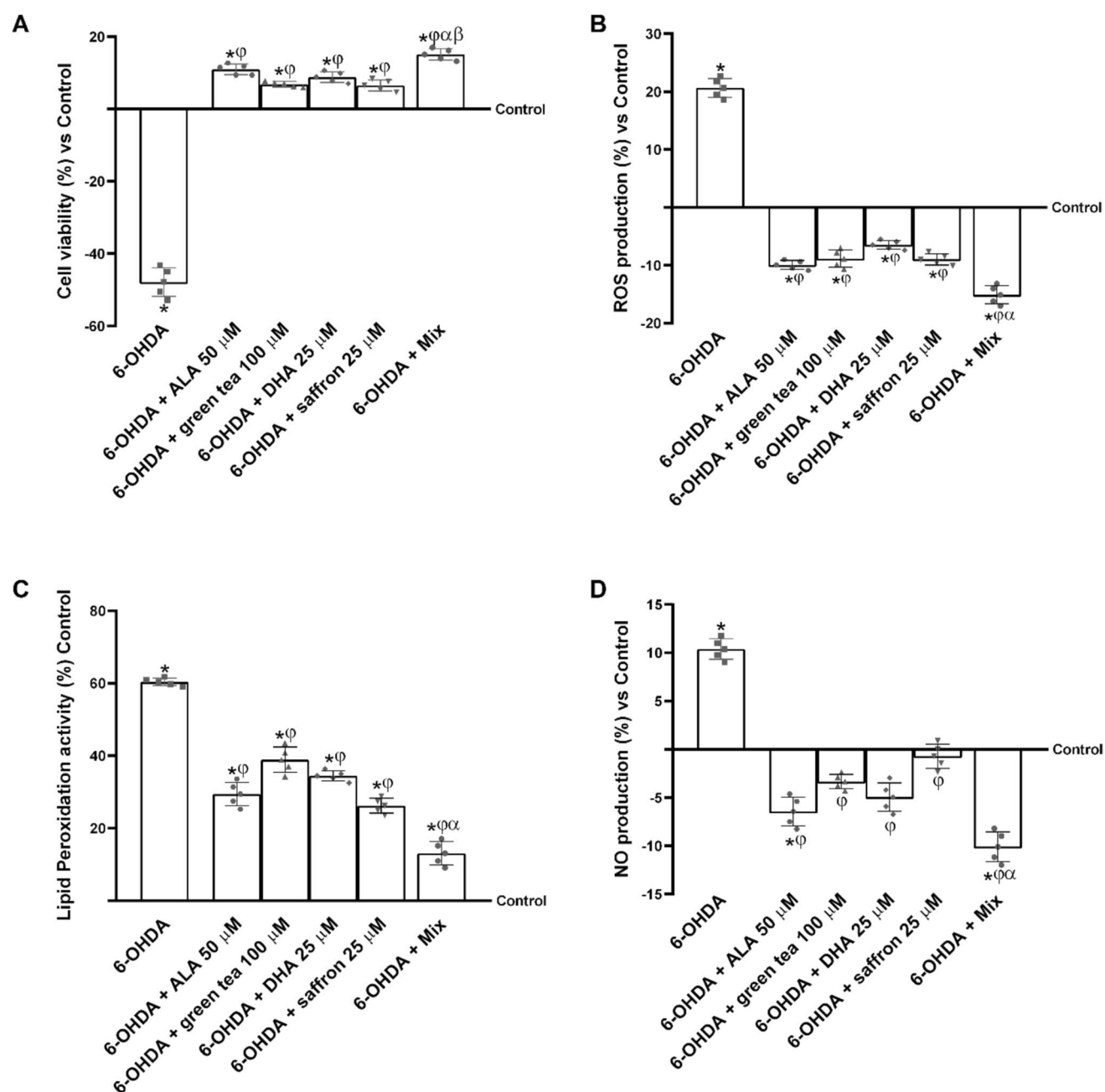


Fig. 4 Evaluation of neuroprotective effects in PD conditions induced by treatment with 6-OHDA. In (A), cell viability was assessed using the MTT test; in (B), ROS production was measured through Cytochrome C reduction; in (C), lipid peroxidation was detected using a specific kit; and in (D), NO production was measured by a particular assay kit. The results are expressed as mean $\pm$ SD (%) of five normalised biological replicates versus control.

Mix = green tea 100 μ M + saffron 25 μ M + DHA 25 μ M + ALA 50 μ M; 6-OHDA = 150 μ M. In (A), * p < 0.05 versus control (0 line corresponds to 100% cell viability); ϕ p < 0.05 versus 6-OHDA; α p < 0.05 versus green tea; β p < 0.05 versus saffron. In (B–C–D), * p < 0.05 versus control (0 line corresponds to 100% cell viability); ϕ p < 0.05 versus 6-OHDA; α p < 0.05 versus all single substances

statistically significant reduction in lipid peroxidation levels relative to the 6-OHDA group (p < 0.05). Notably, treatment with the Mix induced a statistically significant, synergistic decrease compared with the effects observed with the single compounds (p < 0.05 vs all single compounds) (Fig. 4C

– Table 1). Furthermore, the treatment with 6-OHDA determined an increase in NO production. As demonstrated in Fig. 4D, treatment with all single substances significantly attenuated the damage induced by 6-OHDA. At the same time, the Mix resulted in a statistically significant and

Table 1 Modulation (%) Mix vs single compounds

	Cell viability (increment)	ROS prod (reduction)	Lipid perox (reduction)	NO prod (reduction)
Mix vs ALA	+31%	−34%	−55%	−36%
Mix vs Green tea	+57%	−41%	−66%	−67%
Mix vs DHA	+41%	−57%	−62%	−51%
Mix vs Saffron	+57%	−40%	−50%	−93%
Mix vs 6-OHDA	+1.32-fold	−1.7-fold	−78%	−1.97-fold

Percentage modulation in the efficacy of the Mix treatment compared to each compound for all evaluated parameters: cell viability, ROS production, lipid peroxidation and NO production (as shown in Fig. 4) $p < 0.05$ versus all single compounds

synergistic reduction compared to the individual compounds (Table 1). These findings demonstrate that the Mix exerts a synergistic effect in mitigating 6-OHDA-induced damage, particularly in terms of ROS and NO production, and lipid peroxidation.

To confirm the neuroprotective effect of the Mix, the main inflammatory markers involved in PD pathogenesis were investigated. As shown in Fig. 5, TNF α (Fig. 5A), IL-1 β (Fig. 5B) and IL-6 (Fig. 5C) production, which increased following 6-OHDA exposure, was significantly reduced by all single substances. In addition, Mix demonstrated a greater, synergistic effect in reducing cytokines production compared to the individual substances (Table 2). Regarding IL-6, the Mix restored cytokine levels to near baseline levels (Fig. 5C).

Finally, the PINK1/Parkin pathway was evaluated and analysed as the primary molecular mechanism involved in PD progression. As demonstrated in Fig. 6A and B, the results indicated an activation of these molecular mechanisms following stimulation with 6-OHDA. Single-substance treatments led to a statistically significant reduction in PINK1 and Parkin levels when compared with the 6-OHDA-induced damage group. Notably, the Mix exhibited superior efficacy compared to the individual compounds (Table 3).

Discussion

Parkinson's disease (PD) is a neurodegenerative disorder characterised by the progressive loss of dopaminergic neurons in the substantia nigra [58]. It is the second most common neurodegenerative disease worldwide, with a prevalence of approximately 200 per 100,000 individuals, expected to double by 2040 due to population ageing. Although current therapies effectively address motor symptoms, targeted approaches for non-motor symptoms remain limited, despite their significant impact on quality of life.

In the absence of disease-modifying treatments, complementary strategies to manage symptom progression are increasingly explored. Phytochemicals have gained attention

for their antioxidant and anti-inflammatory properties and their protective effects in chronic and neurodegenerative diseases [59]. Among these, green tea is one of the most extensively studied nutraceuticals in PD. Epidemiological data indicate that the daily consumption of two or more cups of tea is associated with a reduced risk of PD, primarily due to its catechin content. A large Finnish cohort study reported a lower PD incidence among individuals consuming more than three cups of tea per day [60], findings later confirmed by meta-analyses including approximately 350,000 participants, showing a 26% risk reduction with the consumption of two or more cups daily [20].

Polyunsaturated fatty acids (PUFAs), particularly omega-3 fatty acids such as docosahexaenoic acid (DHA), have also demonstrated neuroprotective effects, including improvements in motor and cognitive function, anti-neuro-inflammatory activity via cytokine downregulation, antioxidant effects, and anti-apoptotic mechanisms [61].

Alpha-lipoic acid (ALA), beyond its established neuroprotective role [33, 62], has been investigated for the treatment of neuropathic pain [63], a relevant non-motor symptom of PD characterised by persistent discomfort and paresthesia. Although its pathophysiology remains unclear, the accumulation of neurotoxic metabolites such as homocysteine and methylmalonic acid has been implicated [64]. Evidence from preclinical and clinical studies supports the efficacy of ALA in alleviating neuropathic pain, likely due to its antioxidant capacity [65–67].

Finally, saffron has emerged as a potential adjunctive treatment for non-motor symptoms of PD, particularly sleep disturbances and mood disorders. Randomised controlled trials have shown improvements in sleep quality and duration [25], and recent Canadian guidelines recognise saffron extract as an effective and safe option for anxiety and depressive symptoms [26].

Based on these findings, the present study evaluated the effects of combining green tea, saffron, DHA, and ALA, with the aim of developing a novel strategy capable of targeting the molecular mechanism underlying the onset and progression of PD. The study aimed to assess whether

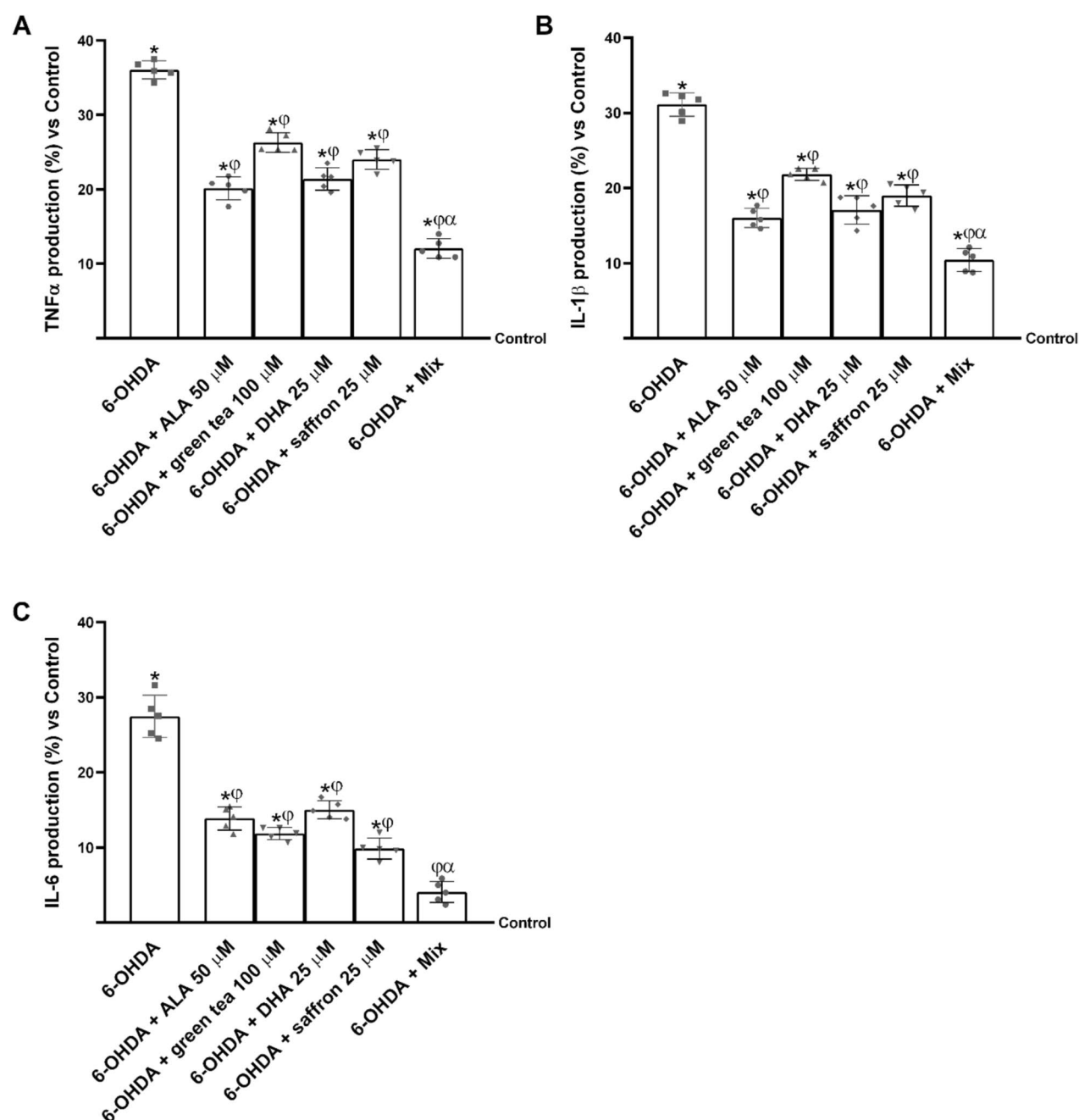


Fig. 5 Evaluation of neuroprotective effects in a PD condition induced after treatment with 6-OHDA. In panel (A), TNF α production analysis was performed using a specific ELISA kit; in panel (B), IL-1 β production analysis was performed using a specific ELISA kit; in panel (C), IL-6 production analysis was performed using a

specific ELISA kit. Mix = green tea 100 μ M + saffron 25 μ M + DHA 25 μ M + ALA 50 μ M; 6-OHDA = 150 μ M. The results are expressed as mean $\pm$ SD (%) of 5 normalised biological replicates compared to the control. * $p < 0.05$ vs. control (0 line); ϕ $p < 0.05$ vs. 6-OHDA; α $p < 0.05$ vs. all single substances

their synergistic action could provide enhanced neuroprotection compared to single-compound treatments, particularly by modulating oxidative stress, inflammation and key pathways involved in PD.

The optimal concentration of all substances to be combined was first verified on mesencephalic dopaminergic cells under PD-like conditions after 6-OHDA exposure, a well-established PD model. Under these conditions, all the individual compounds, especially in combination, were able

Table 2 Reduction (%) Mix vs single compounds

	TNF- α	IL-1 β	IL-6
Mix vs ALA	−37%	−38.5%	−71%
Mix vs Green tea	−55%	−55%	−67%
Mix vs DHA	−43%	−43%	−73%
Mix vs Saffron	−48%	−48%	−55.5%
Mix vs 6-OHDA	−66%	−68%	−85.5%

Percentage modulation in the efficacy of the Mix treatment compared to each compound for all evaluated parameters: TNF- α , IL-1 β and IL-6 productions (as shown in Fig. 5) $p < 0.05$ versus all single compounds

to improve the well-being of these cells, avoiding adverse effects from the substances under study. Furthermore, the study evaluated the ability of all tested substances, individually and in combination, to reach the target site after crossing the BBB, a crucial prerequisite for central nervous system efficacy. The BBB regulates the passive diffusion of water, specific gases and lipid-soluble molecules essential for the functionality and neuroprotection of the CNS [68]. Thus, assessing the permeability of the tested compounds was crucial. To this end, a validated model [46] has been developed to replicate the structure of the BBB, facilitating investigation of the passage of individual compounds and the Mix through absorption analysis. Results supported the hypothesis that the Mix is safe, capable of crossing the BBB and maintaining activity within the CNS for up to 24 h without altering permeability or disrupting barrier integrity.

Subsequently, experiments were carried out to assess the effects of individual substances and Mix in the presence of a PD inducer such as 6-OHDA [44]. It has been highlighted that oxidative stress, inflammation, and disease-specific markers are key contributors to neurodegenerative processes underlying both motor and non-motor symptoms [69]. This study demonstrated that these markers are positively modulated by the Mix, supporting its high neuroprotective potential. These findings are consistent with the existing literature on the individual effects of each compound; however, when combined, they exhibit synergistic activity that enhances their overall efficacy. Indeed, the Mix appeared to amplify their individual effects, suggesting a superior efficacy than single substances, and this suggests that the combined formulation may represent a promising complementary strategy for managing the mechanisms underlying PD.

First, elevated levels of ROS and NO are observed in the cellular model of Parkinson's disease. Treatment with the Mix has been shown to counteract both oxidative and nitrosative stress, demonstrating a strong antioxidant effect. Moreover, in PD, excessive and prolonged microglial activation can lead to uncontrolled release of inflammatory cytokines, contributing to pathological processes and

Table 3 Increment (%) mix vs single compounds

	PINK1	Parkin
Mix vs ALA	+71%	+62%
Mix vs Green tea	+67%	+66%
Mix vs DHA	+73%	+58%
Mix vs Saffron	+58.5%	+51%

Percentage increase in the efficacy of the Mix treatment compared to each compound for the Pink1/Parkin pathway (as shown in Fig. 6), $p < 0.05$ versus all single compounds

toxicity. Patients with PD have elevated levels of various brain and cerebrospinal fluid cytokines, such as TNF α , IL-1 β , IL-2, IL-4, and IL-6, resulting in impaired normal brain cell function. Microglia-derived cytokines are increasingly being investigated as potential biomarkers for PD [70]. Given this evidence, the present study sought to evaluate the Mix's capacity to curb the production of pro-inflammatory cytokines. The results confirmed the ability of individual substances and their combination to reduce TNF α , IL-1 β , and IL-6 production, compared with the damage induced by 6-OHDA. Again, the Mix demonstrated statistically superior anti-inflammatory efficacy compared with its components, reinforcing the hypothesis of a synergistic effect.

The PINK 1 and Parkin proteins, whose mutations are associated with familial forms of PD, are significant factors in mitophagy, the process responsible for the selective degradation of damaged mitochondria. These proteins accumulate at the contact sites between the endoplasmic reticulum and mitochondria, thereby modulating communication between these two organelles. Alterations in the tethering between the endoplasmic reticulum and mitochondria are a common hallmark of many neurodegenerative diseases, such as PD [71].

PINK 1 is a kinase, part of a group of enzymes that modify the behaviour of their target proteins by binding them to a phosphate group via the phosphorylation process. Under healthy conditions, PINK 1 levels are kept low. However, under conditions of mitochondrial stress, PINK 1 migrates to the outer mitochondrial membrane, where it accumulates and undergoes autophosphorylation, resulting in activation. This leads to ubiquitin phosphorylation, which subsequently binds to Parkin, thereby promoting its phosphorylation by PINK1 on its ubiquitin-like domain (UBL). Ubiquitin is a marker that identifies proteins for degradation and promotes the removal of damaged mitochondria. As a result, the enzymatic activation process of Parkin occurs, accompanied by ubiquitin binding to nearby proteins [72]. In the present study, 6-OHDA increased PINK1 and Parkin levels compared with the control group, indicating activation of mitophagic pathways in response to mitochondrial injury. This finding suggests that in vitro models exposed to mitochondrial toxins trigger the accumulation of PINK 1

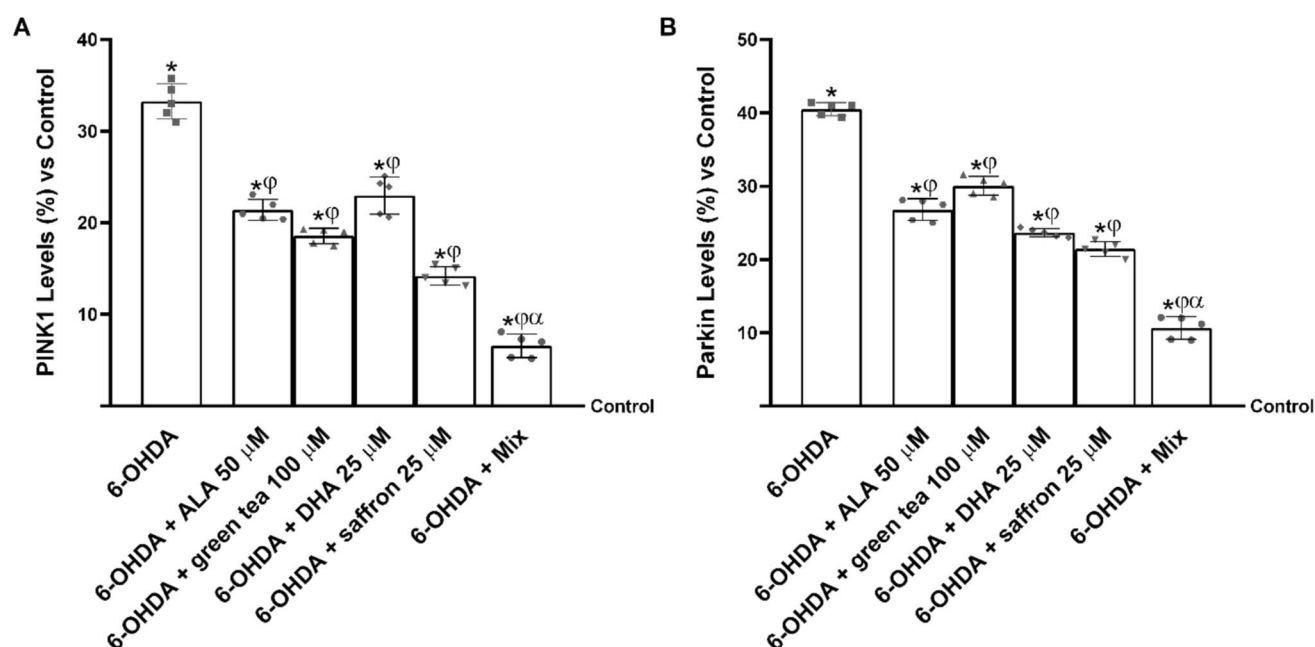


Fig. 6 Evaluation of neuroprotective effects in PD conditions induced after treatment with 6-OHDA. In (A), PINK1 levels were measured using a specific ELISA kit; in (B), Parkin levels were measured using a specific ELISA kit. Mix = green tea 100 μ M + saffron 25 μ M + DHA

25 μ M + ALA 50 μ M; 6-OHDA = 150 μ M. The results are presented as mean $\pm$ SD (%) of 5 normalised biological replicates versus control. * $p < 0.05$ versus control (0 line); ϕ $p < 0.05$ versus 6-OHDA; α $p < 0.05$ versus all single substances

on mitochondria, activating Parkin and ubiquitin, resulting in the removal of damaged mitochondria [73]. In contrast, the analysis of PINK 1 and Parkin levels revealed that both single substances, as well as their combination, could protect mitochondria from 6-OHDA-induced damage. This was evidenced by decreased PINK 1 and Parkin levels in response to the damage, although this decrease was not observed in physiological controls. Once again, the Mix proved to be more effective than the individual compounds in restoring PINK 1/Parkin levels more efficiently and confirming its neuroprotective potential against PD-specific pathophysiological markers.

The present study was conducted using a monoculture neuronal model that does not incorporate microglial or astrocytic interactions. While this approach was essential to isolate direct neuronal pathways, it represents a methodological limitation regarding the complexity of the brain microenvironment. Consequently, future research employing multicellular models or co-culture systems will be instrumental in validating these findings within a more integrated biological context. Although the present study shows significant neuroprotective effects in an in vitro model of Parkinson's disease, further research is necessary to confirm these findings in vivo. Future investigations should evaluate the efficacy of this combination in established animal models, including assessments of motor function, dopaminergic neuron survival, and markers of oxidative stress and neuroinflammation, after pharmacokinetic and toxicological evaluations.

Additionally, pharmacokinetic interactions, bioavailability, blood–brain barrier permeability, and systemic safety, particularly in the liver and kidneys, should be examined to support the translational potential of this formulation.

Conclusion

To our knowledge, this is the first study to show that combining green tea, saffron, DHA, and ALA can effectively protect mesencephalic dopaminergic neurons from oxidative, inflammatory, and neurodegenerative damage caused by 6-OHDA. These results suggest that a nutraceutical formulation with these substances may contribute to targeting key pathogenic mechanisms underlying both motor and non-motor symptoms of Parkinson's disease. Since this is an in vitro study, further clinical research is needed to validate these results and establish the therapeutic potential of this combination in PD patients.

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conceptualisation, original draft preparation, and visualisation. Francesca Romana Ranieri contributed to conceptualisation, original draft preparation, and visualisation. Francesca Parini contributed to methodology, formal analysis and original draft preparation. Francesca Uberti contributed to conceptualisation, validation, resources, data curation, original draft preparation, visualisation, supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data Availability The data presented in this study are available upon reasonable request to the corresponding author since some industrial properties are included in the article.

Declarations

Clinical trial number Not applicable.

Registration details Not applicable.

Competing of interest The authors declare no competing interests.

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References

- Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkman J, Schrag AE, Lang AE (2017) Parkinson disease. *Nat Rev Dis Primers* 3:17013. <https://doi.org/10.1038/nrdp.2017.13>
- Titova N, Chaudhuri KR (2018) Non-motor Parkinson disease: new concepts and personalised management. *Med J Aust* 208(9):404–409. <https://doi.org/10.5694/mja17.00993>
- Lehrer S, Rheinstein PH (2022) α -synuclein enfoldes tyrosine hydroxylase and dopamine β -hydroxylase, potentially reducing dopamine and norepinephrine synthesis. *J Proteins Proteom* 13(2):109–115. <https://doi.org/10.1007/s42485-022-00088-z>
- Morris HR, Spillantini MG, Sue CM, Williams-Gray CH (2024) The pathogenesis of Parkinson's disease. *Lancet* 403(10423):293–304. [https://doi.org/10.1016/S0140-6736\(23\)01478-2](https://doi.org/10.1016/S0140-6736(23)01478-2)
- Boelens Keun JT et al (2021) Dietary approaches to improve efficacy and control side effects of levodopa therapy in Parkinson's disease: a systematic review. *Adv Nutr* 12(6):2265–2287. <https://doi.org/10.1093/advances/nmab060>
- Balestrino R, Schapira AHV (2020) Parkinson disease. *Eur J Neurol* 27(1):27–42. <https://doi.org/10.1111/ene.14108>
- Haddad F et al (2017) Dopamine and levodopa prodrugs for the treatment of Parkinson's disease. *Molecules* 23(1):40. <https://doi.org/10.3390/molecules23010040>
- Voon V et al (2017) Impulse control disorders and levodopa-induced dyskinesias in Parkinson's disease: an update. *Lancet Neurol* 16(3):238–250. [https://doi.org/10.1016/S1474-4422\(17\)30004-2](https://doi.org/10.1016/S1474-4422(17)30004-2)
- Cartabellotta A, Berti F, Patti AL, Quintana S, Eleopra R (2018) Linee guida per la diagnosi e il trattamento della malattia di Parkinson. *Evidence* 10(4):e1000181. <https://doi.org/10.4470/E1000181>
- Bloem BR, Okun MS, Klein C (2021) Parkinson's disease. *Lancet* 397(10291):2284–2303. [https://doi.org/10.1016/S0140-6736\(21\)00218-X](https://doi.org/10.1016/S0140-6736(21)00218-X)
- Chen YC, Chen RS, Weng YH, Huang YZ, Chen CC, Hung J, Lin YY (2021) The severity progression of non-motor symptoms in Parkinson's disease: a 6-year longitudinal study in Taiwanese patients. *Sci Rep* 11(1):14781. <https://doi.org/10.1038/s41598-021-94255-9>
- Chaudhuri KR, Healy DG, Schapira AH, National Institute for Clinical Excellence (2006) Non-motor symptoms of Parkinson's disease: diagnosis and management. *Lancet Neurol* 5(3):235–245. [https://doi.org/10.1016/S1474-4422\(06\)70373-8](https://doi.org/10.1016/S1474-4422(06)70373-8)
- Lyons KE, Pahwa R (2011) The impact and management of nonmotor symptoms of Parkinson's disease. *Am J Manag Care* 17(Suppl 12):S308–S314
- Parasram K (2018) Phytochemical treatments target kynurenine pathway induced oxidative stress. *Redox Rep* 23(1):25–28. <https://doi.org/10.1080/13510002.2017.1343223>
- Farkhondeh T, Pourbagher-Shahri AM, Ashrafzadeh M, Folgado SL, Rajabpour-Sanati A, Khazdair MR, Samarghandian S (2020) Green tea catechins inhibit microglial activation which prevents the development of neurological disorders. *Neural Regen Res* 15(10):1792–1798. <https://doi.org/10.4103/1673-5374.280300>
- Burré J, Sharma M, Südhof TC (2015) Definition of a molecular pathway mediating α -synuclein neurotoxicity. *J Neurosci* 35(13):5221–5232. <https://doi.org/10.1523/JNEUROSCI.4650-14.2015>
- Chen M et al (2015) Tea polyphenols alleviate motor impairments, dopaminergic neuronal injury, and cerebral α -synuclein aggregation in MPTP-intoxicated parkinsonian monkeys. *Neuroscience* 286:383–392. <https://doi.org/10.1016/j.neuroscience.2014.12.003>
- Wang L, Xu S, Xu X, Chan P (2009) (-)-Epigallocatechin-3-gallate protects SH-SY5Y cells against 6-OHDA-induced cell death through STAT3 activation. *J Alzheimers Dis* 17(2):295–304. <https://doi.org/10.3233/JAD-2009-1048>
- Özduran G, Becer E, Vatansever HS, Yücecan S (2022) Neuroprotective effects of catechins in an experimental Parkinson's disease model and SK-N-AS cells: evaluation of cell viability, anti-inflammatory and anti-apoptotic effects. *Neurol Res* 44(6):511–523. <https://doi.org/10.1080/01616412.2021.2024715>
- Qi H, Li S (2014) Dose-response meta-analysis on coffee, tea and caffeine consumption with risk of Parkinson's disease. *Geriatr Gerontol Int* 14(2):430–439. <https://doi.org/10.1111/ggi.12123>
- Leone S et al (2018) Phytotherapeutic use of the *Crocus sativus* L. (Saffron) and its potential applications: a brief overview. *Phytother Res* 32(12):2364–2375. <https://doi.org/10.1002/ptr.6181>
- Esmaealzadeh D et al (2023) Pharmacological effects of Saffron: an updated review. *Iran J Basic Med Sci* 26(10):1131–1143. <https://doi.org/10.22038/IJBMS.2023.69824.15197>
- Inoue E et al (2021) Saffron ameliorated motor symptoms, short life span and retinal degeneration in Parkinson's disease fly models. *Gene* 799:145811. <https://doi.org/10.1016/j.gene.2021.145811>
- Siddiqui A et al (2022) Anti-depressant properties of crocin molecules in saffron. *Molecules* 27(7):2076. <https://doi.org/10.3390/molecules27072076>
- Sadat Rafiei SK et al (2023) Saffron and sleep quality: a systematic review of randomized controlled trials. *Nutr Metab Insights*

- 16:11786388231160317. <https://doi.org/10.1177/11786388231160317>
26. Sarris J et al (2022) Clinician guidelines for the treatment of psychiatric disorders with nutraceuticals and phytochemicals: the World Federation of Societies of Biological Psychiatry (WFSBP) and Canadian Network for Mood and Anxiety Treatments (CANMAT) Taskforce. *World J Biol Psychiatry* 23(6):424–455. <https://doi.org/10.1080/15622975.2021.2013041>
27. Li P, Song C (2020) Potential treatment of Parkinson's disease with omega-3 polyunsaturated fatty acids. *Nutr Neurosci*. <https://doi.org/10.1080/1028415X.2020.1735143>
28. Ozkan A et al (2016) The protective mechanism of docosahexaenoic acid in mouse model of Parkinson: the role of heme oxygenase. *Neurochem Int* 101:110–119. <https://doi.org/10.1016/j.neuint.2016.10.012>
29. Kerdiles O et al (2025) Additive neurorestorative effects of exercise and docosahexaenoic acid intake in a mouse model of Parkinson's disease. *Neural Regen Res* 20(2):574–586. <https://doi.org/10.4103/NRR.NRR-D-23-00595>
30. Taghizadeh M et al (2017) The effects of omega-3 fatty acids and vitamin E co-supplementation on clinical and metabolic status in patients with Parkinson's disease: a randomized, double-blind, placebo-controlled trial. *Neurochem Int* 108:183–189. <https://doi.org/10.1016/j.neuint.2017.03.014>
31. Tamtaji OR et al (2019) The effects of omega-3 fatty acids and vitamin E co-supplementation on gene expression related to inflammation, insulin and lipid in patients with Parkinson's disease: a randomized, double-blind, placebo-controlled trial. *Clin Neurol Neurosurg* 176:116–121. <https://doi.org/10.1016/j.clineuro.2018.12.006>
32. Zheng Q et al (2023) Alpha lipoic acid ameliorates motor deficits by inhibiting ferroptosis in Parkinson's disease. *Neurosci Lett* 810:137346. <https://doi.org/10.1016/j.neulet.2023.137346>
33. Kulikova O et al (2023) Neuroprotective efficacy of a nanomicellar complex of carnosine and lipoic acid in a rat model of rotenone-induced Parkinson's disease. *Antioxidants* 12(6):1215. <https://doi.org/10.3390/antiox12061215>
34. Viana MDM et al (2022) Alpha-lipoic acid as an antioxidant strategy for managing neuropathic pain. *Antioxidants* 11(12):2420. <https://doi.org/10.3390/antiox11122420>
35. Abdul-Latif R et al (2021) Natural antioxidants in the management of Parkinson's disease: review of evidence from cell line and animal models. *J Integr Med* 19(4):300–310. <https://doi.org/10.1016/j.joim.2021.03.007>
36. Mulè S et al (2024) The combined effect of green tea, saffron, resveratrol, and citicoline against neurodegeneration induced by oxidative stress in an in vitro model of cognitive decline. *Oxid Med Cell Longev* 2024:7465045. <https://doi.org/10.1155/2024/7465045>
37. Chalatsa I et al (2019) The *Crocus sativus* compounds trans-crocin 4 and trans-crocetin modulate the amyloidogenic pathway and tau misprocessing in Alzheimer disease neuronal cell culture models. *Front Neurosci* 13:249. <https://doi.org/10.3389/fnins.2019.00249>
38. Lindskog M et al (2006) Neuroblastoma cell death in response to docosahexaenoic acid: sensitization to chemotherapy and arsenic-induced oxidative stress. *Int J Cancer* 118(10):2584–2593. <https://doi.org/10.1002/ijc.21555>
39. Molinari C et al (2019) Role of combined lipoic acid and vitamin D3 on astrocytes as a way to prevent brain ageing by induced oxidative stress and iron accumulation. *Oxid Med Cell Longev*. <https://doi.org/10.1155/2019/2843121>
40. Li Q et al (2023) Artemisinin confers neuroprotection against 6-OHDA-induced neuronal injury in vitro and in vivo through activation of the ERK1/2 pathway. *Molecules* 28(14):5527. <https://doi.org/10.3390/molecules28145527>
41. Selvakumar GP et al (2018) Glia maturation factor dependent inhibition of mitochondrial PGC-1 α triggers oxidative stress-mediated apoptosis in N27 rat dopaminergic neuronal cells. *Mol Neurobiol* 55(9):7132–7152. <https://doi.org/10.1007/s12035-018-0882-6>
42. Jogalekar MP, Serrano EE (2019) Total RNA isolation from separately established monolayer and hydrogel cultures of human glioblastoma cell line. *Bio-protocol* 9(14):e3305–e3305. <https://doi.org/10.21769/BioProtoc.3305>
43. Molinari C et al (2020) The role of BDNF on aging-modulation markers. *Brain Sci* 10(5):285. <https://doi.org/10.3390/brainsci10050285>
44. Nourmohammadi S et al (2022) Thymol protects against 6-hydroxydopamine-induced neurotoxicity in in vivo and in vitro model of Parkinson's disease via inhibiting oxidative stress. *BMC Complement Med Ther* 22(1):40. <https://doi.org/10.1186/s12906-022-03524-1>
45. Uberti F et al (2017) Iron absorption from three commercially available supplements in gastrointestinal cell lines. *Nutrients* 9(9):1008. <https://doi.org/10.3390/nu9091008>
46. Kulczar C et al (2017) Development of a direct contact astrocyte-human cerebral microvessel endothelial cells blood–brain barrier coculture model. *J Pharm Pharmacol* 69(12):1684–1696. <https://doi.org/10.1111/jphp.12803>
47. Mulè S et al (2025) Molecular insights into the nociceptive modulation by palmitoylethanolamide and *Equisetum arvense* extract: an in vitro study across the blood–brain barrier. *Nutrients* 17(12):1998. <https://doi.org/10.3390/nu17121998>
48. Suwanto S, Sasmono RT, Sinto R, Ibrahim E, Suryamin M (2017) Association of endothelial glycocalyx and tight and adherens junctions with severity of plasma leakage in dengue infection. *J Infect Dis* 215(6):992–999. <https://doi.org/10.1093/infdis/jix041>
49. Han F, Li S, Zhang R, Yang W, Xu C, Xu X et al (2021) Interleukin-6 promotes ferroptosis in bronchial epithelial cells by inducing reactive oxygen species-dependent lipid peroxidation and disrupting iron homeostasis. *Bioengineered* 12(1):5279–5288. <https://doi.org/10.1080/21655979.2021.1964158>
50. Lattuada D et al (2015) Fimbrial cells exposure to catalytic iron mimics carcinogenic changes. *Int J Gynecol Cancer* 25(3):389–398. <https://doi.org/10.1097/IGC.0000000000000379>
51. Molinari C et al (2021) Preventing C2C12 muscular cells damage combining magnesium and potassium with vitamin D3 and curcumin. *J Tradit Complement Med* 11(6):532–544. <https://doi.org/10.1016/j.jtcme.2021.05.003>
52. Uberti F et al (2021) A combination of α -lipoic acid (ALA) and palmitoylethanolamide (PEA) blocks endotoxin-induced oxidative stress and cytokine storm: a possible intervention for COVID-19. *J Diet Suppl*. <https://doi.org/10.1080/19390211.2021.1966152>
53. Huang K et al (2018) Effect of PM2.5 on invasion and proliferation of HeLa cells and the expression of inflammatory cytokines IL-1 and IL-6. *Oncol Lett* 16(6):7068–7073. <https://doi.org/10.3892/ol.2018.9516>
54. Gao L, Zhou W, Symmes B, Freed CR (2016) Re-cloning the N27 dopamine cell line to improve a cell culture model of Parkinson's disease. *PLoS One* 11(8):e0160847. <https://doi.org/10.1371/journal.pone.0160847>
55. Choi DH, Cristóvão AC, Guhathakurta S, Lee J, Joh TH, Beal MF et al (2012) NADPH oxidase 1-mediated oxidative stress leads to dopamine neuron death in Parkinson's disease. *Antioxid Redox Signal* 16(10):1033–1045. <https://doi.org/10.1089/ars.2011.3960>
56. Nourmohammadi S, Yousefi S, Manouchehrabadi M, Farhadi M, Azizi Z, Torkaman-Boutorabi A (2022) Thymol protects against 6-hydroxydopamine-induced neurotoxicity in in vivo and in vitro model of Parkinson's disease via inhibiting oxidative stress. *BMC Complement Med Ther* 22(1):40. <https://doi.org/10.1186/s12906-022-03524-1>

57. Cardo LF, Monzón-Sandoval J, Li Z, Webber C, Li M (2023) Single-cell transcriptomics and in vitro lineage tracing reveals differential susceptibility of human iPSC-derived midbrain dopaminergic neurons in a cellular model of Parkinson's disease. *Cells* 12(24):2860. <https://doi.org/10.3390/cells12242860>
58. Kin K, Yasuhara T, Kameda M, Date I (2019) Animal models for Parkinson's disease research: trends in the 2000s. *Int J Mol Sci* 20(21):5402. <https://doi.org/10.3390/ijms20215402>
59. Muscolo A, Mariateresa O, Giulio T, Mariateresa R (2024) Oxidative stress: the role of antioxidant phytochemicals in the prevention and treatment of diseases. *Int J Mol Sci* 25(6):3264. <https://doi.org/10.3390/ijms25063264>
60. Singh NA, Mandal AK, Khan ZA (2016) Potential neuroprotective properties of epigallocatechin-3-gallate (EGCG). *Nutr J* 15:60. <https://doi.org/10.1186/s12937-016-0179-4>
61. Li P, Song C (2022) Potential treatment of Parkinson's disease with omega-3 polyunsaturated fatty acids. *Nutr Neurosci* 25(1):180–191. <https://doi.org/10.1080/1028415X.2020.1735143>
62. Zheng Q, Ma P, Yang P, Zhai S, He M, Zhang X et al (2023) Alpha lipoic acid ameliorates motor deficits by inhibiting ferroptosis in Parkinson's disease. *Neurosci Lett* 810:137346. <https://doi.org/10.1016/j.neulet.2023.137346>
63. Viana MDM, Lauria PSS, Lima AA, Oprezka LCF, Marcelino HR, Villarreal CF (2022) Alpha-lipoic acid as an antioxidant strategy for managing neuropathic pain. *Antioxidants* 11(12):2420. <https://doi.org/10.3390/antiox11122420>
64. Cattaneo C, Jost WH (2023) Pain in Parkinson's disease: pathophysiology, classification and treatment. *J Integr Neurosci* 22(5):132. <https://doi.org/10.31083/j.jin2205132>
65. Agathos E, Tentolouris A, Eleftheriadou I, Katsaouni P, Nemtzas I, Petrou A et al (2018) Effect of α -lipoic acid on symptoms and quality of life in patients with painful diabetic neuropathy. *J Int Med Res* 46(5):1779–1790. <https://doi.org/10.1177/0300060518756540>
66. El-Nahas MR, Elkannishy G, Abdelhafez H, Elkhamisy ET, El-Sehrawy AA (2020) Oral alpha lipoic acid treatment for symptomatic diabetic peripheral neuropathy: a randomized double-blinded placebo-controlled study. *Endocr Metab Immune Disord Drug Targets* 20(9):1531–1534. <https://doi.org/10.2174/1871530320666200506081407>
67. Papanas N, Ziegler D (2014) Efficacy of α -lipoic acid in diabetic neuropathy. *Expert Opin Pharmacother* 15(18):2721–2731. <https://doi.org/10.1517/14656566.2014.972935>
68. Zhang S, Gan L, Cao F, Wang H, Gong P, Ma C et al (2022) The barrier and interface mechanisms of the brain barrier, and brain drug delivery. *Brain Res Bull* 190:69–83. <https://doi.org/10.1016/j.brainresbull.2022.09.017>
69. Mazzotta GM, Conte C (2024) Alpha-synuclein toxicity and non-motor Parkinson's. *Cells* 13(15):1265. <https://doi.org/10.3390/cells13151265>
70. Araújo B, Caridade-Silva R, Soares-Guedes C, Martins-Macedo J, Gomes ED, Monteiro S et al (2022) Neuroinflammation and Parkinson's disease: from neurodegeneration to therapeutic opportunities. *Cells* 11(18):2908. <https://doi.org/10.3390/cells11182908>
71. Barazzuol L, Giamogante F, Brini M, Cali T (2020) PINK1/Parkin mediated mitophagy, Ca²⁺ signalling, and ER-mitochondria contacts in Parkinson's disease. *Int J Mol Sci* 21(5):1772. <https://doi.org/10.3390/ijms21051772>
72. Daou S, Sicheri F (2017) Vivid views of the PINK1 protein. *Nature* 552(7683):38–39. <https://doi.org/10.1038/d41586-017-07691-x>
73. Han R, Liu Y, Li S, Li XJ, Yang W (2023) PINK1–PRKN mediated mitophagy: differences between in vitro and in vivo models. *Autophagy* 19(5):1396–1405. <https://doi.org/10.1080/15548627.2022.2139080>

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