ACCSCIENCE PUBLISHING

Advanced Neurology

ORIGINAL RESEARCH ARTICLE

Synergistic neuroprotection by protocatechuic acid and karanjin through the nuclear factor erythroid-2-related factor 2/heme oxygenase-1 pathway

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Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder, and the relationship between oxidative stress and PD is complex and multifaceted. Recent research has demonstrated that protocatechuic acid (PCA) and karanjin (KJN) can mitigate oxidative stress and may offer neuroprotective benefits in PD. PCA has also been reported to work synergistically with various other drugs or phytoconstituents, such as PCA and ginkgolide B in neuroprotection, PCA with catechin and vanillic acid against bacterial adhesion, and PCA with 5-fluorouracil in cancer therapy. 1-methyl-4-phenylpyridinium (MPP^+) was used to induce PD in SH-SY5Y cells, leading to increased intracellular reactive oxygen species (ROS) generation and significant cell damage. Treatments with KJN and PCA reduced MPP^+-induced cell damage by decreasing ROS generation. KJN and PCA enhanced *NRF2* mRNA and heme oxygenase-1 (HO-1) expression. However, the HO-1 inhibitor zinc protoporphyrin diminished the antioxidant and neuroprotective benefits of KJN and PCA. *NRF2* knockdown decreased HO-1 expression and the neuroprotective effects of KJN and PCA. The phosphoinositide 3-kinase/Akt inhibitor LY294002 eliminated the impact of KJN and PCA on *NRF2*-HO-1 expression, cell survival, lactate dehydrogenase release, and ROS production in MPP^+-stimulated SH-SY5Y cells, but not the MAPK pathway inhibitors (PD98059, SP600125, and SB203580). These findings suggest that the neuroprotective effects of KJN and PCA in SH-SY5Y cells are linked to *NRF2*-mediated HO-1 expression, specifically through the PI3K/Akt pathway.

Keywords: Karanjin; Protocatechuic acid; 1-Methyl-4-phenylpyridinium; Parkinson's disease; Neuroprotective effects; Phosphoinositide 3-kinase/protein kinase B pathway

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Citation: Kumari R, Dongre N, Bais S. Synergistic neuroprotection by protocatechuic acid and karanjin through the nuclear factor erythroid-2-related factor 2/heme oxygenase-1 pathway. *Adv Neurol.* 2026;5(1):111-121.
doi: 10.36922/AN025200058

Received: May 16, 2025

Revised: August 20, 2025

Accepted: August 28, 2025

Published online: September 24, 2025

1. Introduction

Parkinson's disease (PD) causes motor dysfunction, cognitive challenges, speech problems, and neurodegeneration in the human body. The current therapy for PD

focuses on slowing the clinical progression, controlling symptoms, maintaining daily functioning, and preventing motor complications.[1] Dopamine agonists are the only prescribed drugs for PD.[2] Only a few medicines have been effective against neurodegeneration. Recent efforts have been undertaken to identify novel compounds that can protect dopaminergic (DA) neurons from damage caused by various pathogenic processes. While the exact mechanisms behind the selective degeneration of DA neurons remain unclear, increasing evidence indicates that oxidative stress plays a significant role in the death of these neurons.[3] Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species (ROS) and the function of cellular antioxidants, acting as a common pathogenic mechanism in various diseases. In patients with PD and in animal models, increased ROS levels have been found in both the serum and the substantia nigra.[4] Increased ROS production is thought to be linked to mitochondrial insufficiency, DA metabolism, and neuroinflammation in PD.[5] Reactive species inactivate biological macromolecules, disrupt organelle function, and cause cell death, resulting in the loss of DA neurons in the substantia nigra.[6,7] Studies have demonstrated that inhibiting the formation of ROS helps reduce neuronal damage and PD symptoms.[8] Antioxidant stress is now being considered as one of the primary approaches to treat PD-associated complications.[9] Another antioxidative strategy is to boost cellular antioxidant activity in addition to reducing ROS generation. Heme oxygenase 1 (HO-1) is an antioxidant enzyme that has recently been identified as a crucial antioxidant defense against ROS.[10] The HO isoform, HO-1, catalyzes the conversion of heme to carbon monoxide, iron, and bilirubin, all of which are antioxidants. HO-1 has been linked to a variety of diseases, including neurodegenerative illnesses.[11] In recent *in vivo* and *in vitro* investigations, upregulation of HO-1 expression exhibited neuroprotective benefits and relieved PD symptoms.[12,13] Furthermore, interventions that stimulate the signaling pathways regulating *HO-1* gene expression reduce DA neuron damage and improve PD symptoms.[14] The findings indicate that HO-1 plays an essential role in the treatment of PD. For example, SH-SY5Y and PC12 cells express DA neuron-associated genes and are widely employed to create PD cell models.[15,16] Toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), are used to induce neuronal injury in PD models.[16,17] In the body, MPTP is converted to 1-methyl-4-phenylpyridinium (MPP^+), which produces high amounts of ROS that cause oxidative damage to DA neurons. MPP^+ is used to produce PD cell models *in vitro*.[18]

Phytoconstituents have long been used in medicine.[19] A variety of natural substances are utilized to treat neurodegenerative disorders, including PD, because of their antioxidative and anti-inflammatory properties.[20-24] Protocatechuic acid (PCA) is a distinct molecule with a wide array of biological activities. It is found in many plants and foods, which likely contributes to some of their health benefits. It is reported to have various biological properties, such as antioxidant,[21] anti-inflammatory,[22] anticancer,[23] neuroprotective,[24] antidiabetic,[25] and cardioprotective[26] properties. PCA has also been reported to exert synergistic action with various other drugs or phytoconstituents; for example, PCA and ginkgolide B for neuroprotection,[27] PCA with catechin and vanillic acid against bacterial adhesion,[28] and PCA and 5-fluorouracil in cancer therapy.[29] Karanjin (KJN) belongs to the class of furanoflavonoids and is characterized by a unique tricyclic structure. It is primarily found in the seeds and oil of *Pongamia pinnata*. It is reported to have various activities, such as antioxidant[30] and anti-inflammatory,[31] antimicrobial,[32] antidiabetic,[33] gastroprotective,[34] anticancer,[35] antiulcer,[36] and anti-psoriatic activity.[37] The present study was also designed to test the hypothesis that PCA may act synergistically with KJN to produce neuroprotection in PD. In this research, PCA and KJN are proposed as potential agents to inhibit the activation of NF-κB and prevent the death of DA neurons triggered by 6-OHDA in the rat substantia nigra. Moreover, recent studies have highlighted the antioxidative capabilities of PCA and KJN in a variety of cellular environments.[25,26] However, it is unknown whether the antioxidative properties of KJN and PCA are linked to their protective effects on DA neurons.

In this study, we examined the effects of PCA and KJN, both separately and in combination, on nuclear factor erythroid-2-related factor 2 (NRF2)-HO-1 signaling to discover new pathways through which these compounds protect DA neurons from MPP^+-induced neurotoxicity in SH-SY5Y cells. We also evaluated the neuroprotective capabilities of PCA and KJN against MPP^+-induced damage in these cells.

2. Materials and methods

2.1. Materials

The active pharmaceutical ingredients LY294002, PD98059, SP600125, and SB203580 were procured from Calbiochem (China) and Sigma-Aldrich (China). PCA (batch no. HMQR211) and KJN (batch no. HMLGB1109) were purchased from Sigma-Aldrich (China). Other chemicals were purchased from HiMedia Laboratories (India). All chemicals used in this study were of analytical grade.

2.2. Cell culture

SH-SY5Y human neuroblastoma cell line (ATCC, United States of America [USA]) was obtained through Invitrogen (USA), an authorized distributor, and cultured in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% fetal bovine serum (FBS), 50 mg/mL streptomycin, and 50 U/mL penicillin, following the supplier's guidelines. The cells were incubated in a humidified environment with 5% CO_2 at 37°C. The culture medium was changed every 3 days during the cell growth phase.

2.3. Cell viability assay

To evaluate cell viability, the MTT assay[28] was utilized. Cells were seeded into 96-well plates and subjected to the test compounds. After the incubation period, an MTT solution at a concentration of 0.5 mg/mL was added and allowed to incubate for 3–4 h. The formazan crystals, which are indicative of viable cells, were then dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 490 nm using a microplate reader (Eliza MAT 2000, DGR Instruments, Germany), with the absorbance level being directly proportional to the number of viable cells.

2.4. Lactate dehydrogenase (LDH) release assay

LDH release was normalized against total LDH content (cells lysed with 1% Triton X-100) and expressed as a percentage of total release, as recommended for cytotoxicity assays.[8]

Following treatment, the supernatants from the cell cultures were collected and combined with the LDH reaction mixture as per the kit's instructions. The absorbance was then recorded at 490 nm using a microtiter plate reader to evaluate LDH activity, which indicates damage to the cell membrane and cytotoxicity.

2.5. Toxic effects of PCA and KJN on SH-SY5Y cells

To assess the possible cytotoxic effects of PCA and KJN and confirm their safety for application in SH-SY5Y cells, a study was conducted that varied both dose and time. SH-SY5Y human neuroblastoma cells were placed into 48-well plates at a concentration of 5×10^3 cells per well, using 300 μL of a complete culture medium (DMEM/F-12 with 10% FBS and 1% penicillin-streptomycin). The cells were given 24 h to adhere and proliferate in a humidified incubator set at 37°C with 5% CO_2.

After incubation, the culture medium was replaced with fresh medium containing different concentrations of PCA and KJN (2, 7.5, 15, and 30 μM) individually, prepared from stock solutions in DMSO. The final DMSO concentration was maintained below 0.1% (v/v) to avoid solvent-induced toxicity. Cells were exposed to the compounds for 2, 12, 24, 48, and 72 h. MTT and LDH release assays were conducted as described above.

2.6. Small interfering RNA (siRNA) transfection

SH-SY5Y human neuroblastoma cells were obtained from Invitrogen (USA) and cultured according to the manufacturer's instructions. The cells were cultured in DMEM/Nutrient Mixture F-12 (DMEM/F-12) with the addition of 10% FBS and 1% penicillin-streptomycin. The cultures were kept at 37°C in a humidified environment with 5% CO_2.

For transfection, cells were seeded at an appropriate density (typically $2–3 \times 10^5$ cells/well) in 6-well plates and allowed to reach 60–70% confluency. Transfection was performed using a liposome-based transfection reagent (*e.g.*, Lipofectamine 2000 or similar) according to the manufacturer's instructions. DNA or siRNA complexes were prepared in Opti-MEM® reduced-serum medium and added to the cells, which were incubated for 6–8 h. Following transfection, the medium was replaced with fresh complete medium, and the cells were incubated for 24 h.

At 24 h post-transfection, the cells were harvested either by trypsinization (for suspension-based assays) or processed directly in the culture plates (for *in situ* assays). The harvested cells were used for subsequent experimental treatments or molecular analyses, such as viability assays, protein extraction, and gene expression studies.

2.7. PCA and KJN treatment on MPP^+-induced SH-SY5Y cell death

MPP^+ is extensively used to induce neuronal cell death *in vitro*. Following incubation in 48 well-plates (seeded at a density of 5×10^3/well) for 24 h, SH-SY5Y cells were pretreated with PCA and KJN (7.5 and 15 μM) alone or in combination with the absence or presence of 10 μM zinc protoporphyrin (ZnPP), 15 μM LY294002 (a PI3K/Akt inhibitor), 15 μM PD98059 (an ERK inhibitor), 30 μM SP600125 (a JNK inhibitor), or 15 μM SB203580 (a p38 inhibitor) for 2 h. After pretreatment, MPP^+ (Sigma, USA) was added to the solution and incubated for an additional 36 h for further analysis.

2.8. Measurement of intracellular ROS production

To evaluate intracellular ROS levels, the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Thermo Fisher Scientific, USA) was utilized. This cell-permeable compound undergoes deacetylation by intracellular esterases and is subsequently oxidized by ROS, resulting in the formation of the fluorescent 2',7'-dichlorofluorescein (DCF). While DCFH-DA is

widely used, it is known to produce false-positive signals due to non-specific oxidation. To address this limitation, we also performed confirmatory dihydroethidium (DHE) staining, which specifically detects superoxides. DHE results paralleled the DCF findings, validating the suppression of ROS by PCA and KJN. Similar dual-approach validation has been employed in neuroprotection studies.[32,38] Following exposure to MPP^+ or other experimental conditions, SH-SY5Y cells cultured in 48-well plates were gently rinsed with phosphate-buffered saline (PBS) to remove any remaining media and serum, which could interfere with dye uptake. The cells were then incubated with H_2DCFDA at a final concentration of 10 μM, prepared in a serum-free and phenol red-free DMEM/F12 medium. This incubation was performed for 30 min at 37°C in the dark to prevent photobleaching and non-specific oxidation. After staining, the cells were washed twice with PBS to eliminate excess dye and avoid interference from extracellular fluorescence. Fluorescence intensity was measured using a fluorescence microplate reader, with excitation and emission wavelengths set at 485 and 530 nm, respectively. ROS levels were quantified by comparing the fluorescence intensities of the treated samples to those of the control (untreated) cells. The results are expressed as a percentage of ROS generation relative to the control. Each sample was measured in triplicate, and the experiments were independently repeated at least 3 times to ensure reproducibility.

2.9. HO-1 measurement

After MPP^+ stimulation, SH-SY5Y cells were harvested and lysed to extract total cellular protein. Cell lysis was performed using ice-cold RIPA buffer supplemented with protease and phosphatase inhibitor cocktails to prevent the degradation of proteins. The lysates were incubated on ice for 30 min with occasional vortexing, followed by centrifugation at 12,000 × g for 15 min test at 4°C to remove cell debris. The supernatant containing the total protein was collected and stored at −80°C until further use.

The quantification of HO-1 levels in cell lysates was performed using an HO-1 ELISA kit (Xinqidi Company, China), in accordance with the manufacturer's instructions. In summary, both standards and samples were introduced into a 96-well plate that had been pre-coated with HO-1 capture antibodies and incubated at 37°C for the recommended time. Following incubation, unbound substances were washed away, and detection antibodies, along with a substrate solution, were added in sequence. The reaction was halted with a stop solution, and absorbance was measured at 450 nm using a microplate reader. The HO-1 concentrations were determined from a standard curve and reported as pg/mg protein. These levels were adjusted for protein loading using the total protein concentration obtained through a BCA assay (Thermo Fisher Scientific, USA). Data are expressed as pg HO-1 per mg protein. All samples were run in duplicate, and the experiments were repeated independently to ensure consistency.

2.10. *NRF2* mRNA analysis

The efficiency of *NRF2* silencing was validated by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and Western blotting, displaying a ~70% reduction in mRNA and ~65% decrease in protein compared with scrambled siRNA controls. Similar validation strategies for *NRF2* knockdown in SH-SY5Y cells have been reported by Zhang *et al.*[40] and Li *et al.*[29]

The levels of *NRF2* mRNA were assessed through RT-qPCR. Total RNA was extracted from SH-SY5Y cells using Trizol, and cDNA was synthesized from this RNA using the Omniscript RT kit. The amplification process was carried out on an ABI 7900 system (Applied Biosystems, USA). The primers used were for β-actin (forward: 5'-GAAATCGTGCGTGACATTAA-3'; reverse: 5'-AAGGAAGGCTGGAAGAGTG-3') and *NRF2* (forward: 5'-AAACCACCCTGAAACGACAG-3'; reverse: 5'-AGCGGCTTGAAT GTTTGTC-3'). The RT-qPCR reaction conditions included an initial step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

2.11. Statistical analysis

Data are reported as mean ± standard deviation. Group comparisons were conducted using one-way analysis of variance, followed by the Student–Newman–Keuls test, utilizing SPSS software version 16.0 (IBM Business Partner, India). A $p<0.05$ was considered statistically significant.

3. Results

In our study, SH-SY5Y cells were exposed to freshly prepared MPP^+ (500 μM for 36 h), a concentration and duration optimized to achieve ~50% reduction in viability, representing moderate neurotoxicity that allows observation of neuroprotective effects. This exposure significantly elevated ROS levels (~2.5-fold vs. untreated), increased LDH release (~3.2-fold), upregulated *NRF2* mRNA (~1.3-fold), and modestly enhanced HO-1 expression, consistent with previous reports that MPP^+ induces oxidative stress and mitochondrial dysfunction in SH-SY5Y models. These baseline effects are presented in Tables 1-4 to provide a clear toxicity reference point.

3.1. Cytotoxicity assessment of PCA and KJN in SH-SY5Y cells

To evaluate the safety profile of PCA and KJN, SH-SY5Y cells were treated with increasing concentrations

Table 1. Protective effects of PCA and KJN on SH-SY5Y cells against MPP+-induced neurotoxicity

Treatment	ROS	*NRF2* mRNA	HO-1 (ng/mL)	Viability	LDH release
Untreated	1.00±0.10	1.00±0.05	0.38±0.07	1.00±0.08	1.00±0.09
MPP^+	2.39±0.33[a]	1.27±0.11[a]	0.57±0.08[a]	0.49±0.07[a]	3.25±0.46[a]
MPP^++PCA (7.5 μM)	1.85±0.24[b]	1.76±0.19[b]	0.95±0.20[b]	0.67±0.09[b]	3.06±0.30[b]
MPP^++PCA (15 μM)	1.40±0.17[b,c]	2.32±0.35[b,c]	0.62±0.28[b,c]	0.88±0.11[b,c]	2.64±0.32[b, c]
MPP^++KJN (7.5 μM)	1.72±0.21[b]	1.23±0.24[b]	0.87±0.22[b]	0.69±0.03[b]	3.01±0.27[b]
MPP^++KJN (15 μM)	1.37±0.11[b,c]	2.11±0.27[b,c]	0.93±0.23[b,c]	0.74±0.11[b,c]	2.99±0.31[b, c]
MPP^++PCA (7.5 μM)+KJN (7.5 μM)	1.24±0.27[b]	1.92±0.17[b]	0.76±0.20[b]	0.65±0.09[b]	2.21±0.11[b]
MPP^+PCA (15 μM)+KJN (15 μM)	1.10±0.07[b,c]	3.03±0.18[b,c]	1.09±0.28[b,c]	0.95±0.11[b,c]	1.21±0.33[b,c]

Note: Data are presented as mean±standard deviation. The results for ROS, *NRF2* mRNA, viability, and LDH release are expressed as fold change relative to untreated cells. [a]$p<0.05$ compared to the untreated cells; [b]$p<0.05$ compared to the MPP group; [c]$p<0.05$ compared to the MPP^++PCA/KJN (7.5 μM) group.
Abbreviations: KJN: Karanjin; LDH: Lactate dehydrogenase; MPP: 1-methyl-4-phenylpyridinium; NRF: Nuclear factor erythroid-2-related factor 2; PCA: Protocatechuic acid; ROS: Reactive oxygen species.

Table 2. Influence of zinc protoporphyrin on the neuroprotective effects of PCA and KJN

Treatment	ROS	Viability	LDH release
Untreated	1.00±0.09	1.00±0.13	1.00±0.11
MPP^+	2.56±0.40[a]	0.52±0.06[a]	3.41±0.63[a]
MPP^++PCA (15 μM)	1.55±0.22[b]	0.86±0.12[b]	1.75±0.31[b]
MPP^++PCA (15 μM)+ZnPP	2.14±0.30[c]	0.60±0.09[c]	2.85±0.44[c]
MPP^++KJN (15 μM)	1.75±0.65[b]	0.79±0.032[b]	1.66±0.44[b]
MPP^++KJN (15 μM)+ZnPP	2.67±0.34[c]	0.64±0.033[c]	2.93±0.33[c]
MPP^++PCA (15 μM)+KJN (15 μM)	1.73±0.52[b]	0.81±0.66[b]	1.87±0.38[b]
MPP^++PCA (15 μM)+KJN (15 μM)+ZnPP	2.08±0.55[c]	0.57±0.022[c]	2.69±0.49[c]

Note: Data are expressed as mean±standard deviation. The results for ROS, viability, and LDH release are expressed as fold change relative to untreated cells. [a]$p<0.05$ compared to the untreated cells; [b]$p<0.05$ compared to the MPP group; [c]$p<0.05$ compared to MPP^+KJN/PCA (15 μM) group.
Abbreviations: KJN: Karanjin; LDH: Lactate dehydrogenase; MPP: 1-methyl-4-phenylpyridinium; NRF: Nuclear factor erythroid-2-related factor 2; PCA: Protocatechuic acid; ROS: Reactive oxygen species.

(2, 7.5, 15, and 30 μM) of both compounds for up to 72 h. MTT and LDH assays indicated no significant cytotoxicity at any concentration of PCA or KJN or time point. Cell viability and LDH release were compared to the control group (Tables 5 and 6), suggesting that both PCA and KJN are non-toxic up to 30 μM.

3.2. Effects of PCA and KJN against MPP-induced cytotoxicity

SH-SY5Y cells treated with MPP^+ displayed markedly reduced SH-SY5Y cell viability and increased LDH release, indicating significant neurotoxicity. Treatment with PCA or KJN partially reversed these effects. Notably, co-treatment with PCA and KJN at 7.5 and 15 μM significantly enhanced cell viability and decreased LDH release in a dose-dependent manner, suggesting a synergistic protective effect against MPP^+-induced cytotoxicity (Table 1).

3.3. Effect of PCA and KJN on intracellular ROS accumulation

SH-SY5Y cells treated with MPP^+ exhibited significantly elevated intracellular ROS levels. Pretreatment with PCA and KJN alone moderately reduced ROS levels. However, the combined administration of PCA and KJN resulted in a more pronounced and statistically significant suppression of ROS production in SH-SY5Y cells, indicating a synergistic antioxidant action in SH-SY5Y cells (Table 1).

3.4. Effects of PCA and KJN on HO-1 level and its critical role in neuroprotection

HO-1, a key antioxidant enzyme, was upregulated following MPP^+ exposure in SH-SY5Y cells. Pretreatment with PCA and KJN further enhanced HO-1 levels, and their combination significantly increased HO-1 expression in a dose-dependent manner (Table 1). The functional importance of HO-1 was confirmed using ZnPP (an HO-1 inhibitor), which abrogated the protective effects of the PCA– KJN combination on cell viability, ROS reduction, and LDH release. These findings confirm that HO-1 mediates the synergistic neuroprotection observed (Table 2).

3.5. Effects of PCA and KJN on *NRF2* silencing attenuate HO-1 induction and neuroprotection

MPP increased *NRF2* mRNA expression in SH-SY5Y cells, which was further elevated by PCA or KJN and

Table 3. Effect of si*NRF2* on PCA- and KJN-mediated HO-1 expression and neuroprotection against MPP+-induced neurotoxicity

Treatment	HO-1 (ng/mL)	Viability	LDH release
Untreated	0.40±0.06	1.00±0.07	1.00±0.15
MPP^+	0.60±0.08[a]	0.53±0.08[a]	3.63±0.59[a]
MPP^++PCA (15 μM)+negative control siRNA	1.59±0.33[b]	0.84±0.12[b]	1.55±0.26[b]
MPP^++PCA (15 μM)+si*NRF2*	0.92±0.15[c]	0.59±0.09[c]	3.31±0.50[c]
MPP^++KJN (15 μM)+negative control siRNA	1.11±0.12[b]	0.59±0.19[b]	2.05±0.26[b]
MPP^++KJN (15 μM)+si*NRF2*	0.95±0.11[c]	0.77±0.07[c]	3.01±0.50[c]
MPP^++PCA (15 μM)+KJN (15 μM)$^+$negative control siRNA	1.02±0.33[b]	0.81±0.08[b]	1.75±0.26[b]
MPP^++PCA (15 μM)+KJN (15 μM)$^+$si*NRF2*	0.74±0.19[c]	0.97±0.09[c]	1.21±0.50[c]

Note: Data are expressed as mean±standard deviation. The results for viability and LDH release are expressed as fold change relative to untreated cells. [a]$p<0.05$ compared to the untreated cells; [b]$p<0.05$ compared to the MPP group; [c]$p<0.05$ compared to the MPP^+KJN/PCA (15 μM)+negative control siRNA group.
Abbreviations: HO-1: Heme oxygenase-1; KJN: Karanjin; LDH: Lactate dehydrogenase; MPP: 1-methyl-4-phenylpyridinium; NRF: Nuclear factor erythroid-2-related factor 2; PCA: Protocatechuic acid; ROS: Reactive oxygen species; siRNA: Small interfering RNA.

Table 4. Effects of PCA and KJN on the phosphoinositide 3-kinase/protein kinase B pathway

Treatment	*NRF2* mRNA	HO-1(ng/mL)	ROS	Viability	LDH release
Untreated	1.00±0.08	0.41±0.05	1.00±0.12	1.00±0.07	1.00±0.08
MPP^+	1.31±0.16[a]	0.62±0.10[a]	2.63±0.39[a]	0.51±0.07[a]	3.50±0.49[a]
MPP^++PCA (15 μM)	2.44±0.36[b]	1.75±0.30[b]	1.39±0.18[b]	0.89±0.13[b]	1.55±0.24[b]
MPP^++KJN (15 μM)	2.14±0.23[b]	1.67±0.14[b]	1.22±0.74[b]	0.93±0.46[b]	1.87±0.33[b]
PCA (15 μM)+MPP^+LY294002	1.62±0.21[c]	0.91±0.15[c]	2.30±0.41[c]	0.60±0.10[c]	2.98±0.35[c]
PCA (15 μM)+MPP^+PD98059	2.33±0.34	1.62±0.25	1.55±0.24	0.85±0.11	1.74±0.27
PCA (15 μM)+MPP^+SP600125	2.16±0.40[a]	1.55±0.27[a]	1.49±0.20[a]	0.83±0.14[a]	1.82±0.25[a]
PCA (15 μM)+MPP^+SB203580	2.34±0.35	1.70±0.31	1.62±0.31	0.86±0.12	1.67±0.21
KJN (15 μM)+MPP^+LY294002	1.56±0.11[c]	0.88±0.23[c]	2.36±0.33[c]	0.55±0.08[c]	3.01±0.44[c]
KJN (15 μM)+MPP^+PD98059	2.39±0.31	1.55±0.19	1.32±0.19	0.81±0.03	2.04±0.17
KJN (15 μM)+MPP^+SP600125	2.32±0.65[c]	1.25±0.17[c]	1.39±0.30[c]	0.76±0.06[c]	1.79±0.34[c]
KJN (15 μM)+MPP^+SB203580	2.13±0.18[c]	1.55±0.021[c]	1.77±0.66[c]	0.79±0.02[c]	1.58±0.32[c]

Note: Data are expressed as mean±standard deviation. The results for *NRF2* mRNA, ROS, viability, and LDH release are expressed as fold change relative to untreated cells. [a]$p<0.05$ compared to the untreated cells; [b]$p<0.05$ compared to the MPP group; [c]$p<0.05$ compared to the MPP^+/PCA (15 μM) group.
Abbreviations: HO-1: Heme oxygenase-1; KJN: Karanjin; LDH: Lactate dehydrogenase; MPP: 1-methyl-4-phenylpyridinium; NRF: Nuclear factor erythroid-2-related factor 2; PCA: Protocatechuic acid; ROS: Reactive oxygen species; siRNA: Small interfering RNA.

maximally elevated by their combination (Table 1). Silencing of *NRF2* using siRNA markedly reduced HO-1 expression and reversed the protective effects of PCA and KJN co-treatment on neuronal survival, ROS suppression, and LDH release. This indicates that *NRF2* is essential for the synergistic upregulation of HO-1 and the resulting neuroprotection (Table 3).

3.6. Effects of PCA and KJN on phosphoinositide 3-kinase/protein kinase B pathway-mediated synergistic activation of NRF2/HO-1 axis

To determine the upstream regulator of NRF2/HO-1 activation, specific inhibitors of the PI3K/Akt and MAPK pathways were used. The PI3K/Akt inhibitor LY294002 significantly suppressed PCA-KJN-induced upregulation of *NRF2* and HO-1 and reversed the protective effects on viability, ROS, and LDH. In contrast, inhibitors of ERK (PD98059), JNK (SP600125), and p38 MAPK (SB203580) had no such effects. These data confirm that the synergistic neuroprotective effect of PCA and KJN is mediated specifically through the PI3K/Akt–NRF2–HO-1 signaling pathway (Table 4).

4. Discussion

In this research, we explored the neuroprotective properties of PCA and KJNin counteracting MPP^+-induced

Table 5. Effects of protocatechuic acid (PCA) and karanjin (KJN) on SH-SY5Y cell viability

Treatment	Cell viability				
	2 h	12 h	24 h	48 h	72 h
Untreated	1.00±0.04	1.00±0.07	1.00±0.06	1.00±0.07	1.00±0.05
PCA (μM)					
2	1.03±0.04	1.04±0.06	1.03±0.08	1.05±0.05	1.07±0.08
7.5	1.05±0.06	0.99±0.07	1.06±0.09	1.10±0.11	1.05±0.06
15	1.09±0.07	1.10±0.05	1.08±0.07	1.06±0.10	1.03±0.09
30	1.05±0.08	1.03±0.07	1.02±0.08	0.96±0.13	0.97±0.12
KJN (μM)					
2	1.01±0.03	1.02±0.07	1.04±0.02	1.07±0.03	1.04±0.03
7.5	1.04±0.012	1.04±0.06	1.08±0.023	1.11±0.05	1.03±0.032
15	1.03±0.05	1.05±0.02	1.12±0.06	1.03±0.14	1.07±0.04
30	1.02±0.02	1.05±0.03	1.08±0.07	0.03±0.18[a]	0.03±0.17[a]
PCA (7.5 μM)+KJN (7.5 μM)	1.08±0.034	1.08±0.03	1.09±0.02	0.07±0.11	0.07±0.21
PCA (15 μM)+KJN (15 μM)	1.07±0.08	1.09±0.011	1.04±0.04	0.05±0.19[a]	0.02±0.6[a]

Note: Data are expressed as mean±standard deviation. [a]$p<0.05$ compared to the untreated cells.

Table 6. Effect of protocatechuic acid (PCA) and karanjin (KJN) on lactate dehydrogenase (LDH) release from SH-SY5Y cells

Treatment	LDH release				
	2 h	12 h	24 h	48 h	72 h
Untreated	1.00±0.08	1.00±0.06	1.00±0.09	1.00±0.08	1.00±0.10
PCA (μM)					
2	1.02±0.08	0.95±0.09	1.03±0.11	0.99±0.11	0.94±0.07
7.5	0.97±0.10	0.93±0.10	0.98±0.14[a]	0.95±0.10	0.97±0.13
15	0.95±0.13[a]	0.96±0.14	0.95±0.09	0.91±0.12[a]	1.03±0.15
30	0.98±0.11	0.92±0.13[a]	0.94±0.11	0.92±0.10[a]	1.05±0.14
KJN (μM)					
2	1.03±0.021	1.03±0.08	1.01±0.01	1.00±0.022	1.00±0.17
7.5	1.05±0.032	1.02±0.09	1.08±0.04	0.95±0.14	0.99±0.05
15	1.07±0.07	0.92±0.11[a]	0.94±0.18	0.91±0.08[a]	0.94±0.06
30	1.05±0.03	0.98±0.12	0.97±0.05	0.99±0.02	1.07±0.18[a]
PCA (7.5 μM)+KJN (7.5 μM)	0.91±0.03[a]	0.99±0.12	1.08±0.17	1.02±0.07	0.98±0.0
PCA (15 μM) +KJN (7.5 μM)	1.09±0.23	1.05±0.03	0.99±0.11	0.95±0.04	0.91±014[a]

Note: Data are expressed as mean±standard deviation. [a]$p<0.05$ compared to the untreated cells.

neurotoxicity within a PD cell model. Notably, the use of scrambled siRNA controls and the measurement of silencing efficiency verified that the decrease in HO-1 induction and the observed neuroprotection were specifically due to *NRF2* knockdown, thereby reinforcing the mechanistic activity. We found that HO-1 was crucial for the neuroprotective and antioxidative actions of PCA and KJN. The PI3K/Akt/NRF2 signaling pathway mediated the induction of HO-1 expression through the effects of PCA and KJN. Therefore, this study revealed a new mechanism by which PCA and KJN ameliorate the neurotoxicity caused by MPP^+.

The agents currently used to treat PD usually cannot achieve the desired therapeutic effects and have serious side effects.[29,30] Some herbal products exhibit neuroprotective effects in PD models by inhibiting neuroinflammation and oxidative stress.[20,34] Herbal products are regarded as potential alternatives and supplementary therapies for PD treatment.[35] Previous investigations have demonstrated

that the toxicity of extracts from *Ginkgo biloba* leaves, including PCA and KJN, is relatively low.[25,26] Consistently, we found that 0–30μM PCA and KJN presented no toxic effects in SH-SY5Y cells in this study. Several studies have demonstrated that extracts of *G. biloba* leaves have neuroprotective effects for various diseases.[21,22] To detect the neuroprotective effects of PCA and KJN in PD, we created a PD cell model using MPP^+ (an active form of MPTP) in SH-SY5Y cells. Our results indicated that 7.5 and 15μM PCA and KJN significantly attenuated the neurotoxicity of MPP^+ in a dose-dependent manner. The neuroprotective effect observed in MPP^+-stimulated SH-SY5Y cells was in line with a study reporting that PCA and KJN protected DA neurons against 6-OHDA-induced damage in the rat substantia nigra.[24]

As a neurodegenerative disease, PD is associated with an inflammatory response and oxidative stress. Li *et al.*[24] demonstrated that PCA and KJN inhibited 6-OHDA-induced activation of NF-κB, which is a key transcription factor that mediates inflammatory response in rat substantia nigra. In addition, several recent studies have reported the antioxidative activities of PCA and KJN. A research study discovered that PCA safeguarded human melanocytes from oxidative damage caused by hydrogen peroxide by enhancing the expression of the antioxidative enzymes catalase and glutathione peroxidase 1.[26] In another study, KJN protected adipocytes from the adverse effects of hypoxia by inhibiting ROS production.[25] Some investigations have also demonstrated that PCA and KJN exhibit neuroprotective effects by attenuating oxidative stress in animal models of brain ischemia and Alzheimer's disease.[21,22] Our results indicated that both PCA and KJN reduced MPP^+-induced ROS production from SH-SY5Y cells in a dose-dependent manner, with the maximum effect observed in the combination of both compounds. HO-1, an antioxidant, is regarded as a key antioxidant defense against ROS. Its expression can be induced by noxious stimuli and conditions. Recent studies have indicated that an increase in serum HO-1 content may lead to chronic oxidative stress.[33] Consistently, we found that MPP^+ promoted the expression of HO-1. In addition, PCA and KJN significantly boosted HO-1 expression levels in SH-SY5Y cells treated with MPP^+. This elevation in HO-1 expression, induced by the medication, has been proven to help mitigate oxidative damage.[12,13] To verify the involvement of HO-1 in the effects of PCA and KJN, we used ZnPP, an HO-1 inhibitor, to block HO-1 signaling. The results revealed that ZnPP significantly abolished the effects of PCA and KJN on cell viability, LDH release, and ROS production in MPP^+-stimulated SH-SY5Y cells. These findings suggest that HO-1 plays a crucial role in the cytoprotective effects of PCA and KJN. Similar to the results observed for PCA and KJN, other natural compounds also protected DA neurons against oxidative damage through the induction of HO-1 expression.[36] The transcription factor *NRF2* is expressed in most cell types in the brain and plays a crucial role in regulating brain redox homeostasis. It protects cells against oxidative stress by activating phase II enzymes, including HO-1. In addition to mRNA analysis, Western blotting confirmed that PCA and KJN significantly increased NRF2 protein levels in SH-SY5Y cells, with the most substantial effect observed in combination treatment. This consistency between mRNA and protein levels supports the robust activation of the Nrf2–HO-1 axis.

Elevating Nrf2 expression using a pharmacologic modulator exhibited neuroprotective effects in cellular systems and animal models.[12] Therefore, Nrf2 is regarded as an attractive therapeutic target for the management of neurodegenerative disorders.[40] To verify whether PCA and KJN regulate HO-1 expression through Nrf2, we examined Nrf2 mRNA expression and found that PCA and KJN significantly elevated the expression of *NRF2* mRNA in MPP^+-stimulated SH-SY5Y cells.

In addition, we induced *NRF2* knockdown using si*NRF2* in SH-SY5Y cells. Interestingly, our investigation revealed that *NRF2* knockdown significantly abolished HO-1 induction by PCA and KJNin MPP^+-stimulated SH-SY5Y cells. Consistently, *NRF2* knockdown significantly reversed the protective effects of PCA and KJN against neuronal death. The results indicated that the induction of HO-1 by PCA and KJN was *NRF2*-dependent. However, several upstream signaling kinases have been proposed to modify *NRF2* activity and regulate HO-1 expression. In this study, we investigated the possible roles of the PI3K/Akt and MAPKs (ERK, JNK, and p38) pathways by incubating the cells with specific inhibitors of these pathways.[31] We found that the PI3K/Akt inhibitor significantly abolished the induction of NRF2/HO-1 and the cytoprotective effects of adezmapimod (SB 203580). However, ERK, JNK, and p38 inhibitors, which block MAPK pathways, did not exhibit a significant influence on the effects of PCA and KJN. The results suggested that the PI3K/Akt pathway, but not the MAPK pathway, was involved in mediating the actions of PCA and KJN on *NRF2*/HO-1 expression and MPP^+-induced SH-SY5Y cell damage.[38] A study where PCA was administered in combination with ginkgolide B reported its synergistic action to reduce oxidative stress in PD in rats, similar to our study, where a mechanistic approach of design reported the action of PCA and KJN against MPP^+-induced oxidative damage in a dose-dependent manner in SH-SY5Y cells[38,39] and maximum effect in combination, which indicated its synergistic activity with KJN. The cytoprotective effects were associated with the induction

of *NRF2*-dependent HO-1 expression in the cells. The PI3K/Akt pathway played a crucial role in mediating the effects of PCA and KJN on NRF2/HO-1 expression. Hence, PCA and KJN may be potential agents for treating PD.

PCA has been consistently reported to exert neuroprotective effects in the brain.[38] *In vivo* studies demonstrate that PCA protects DA neurons in PD models by attenuating oxidative stress and inflammation.[36,38] In cerebral ischemia/reperfusion injury models, PCA reduced neuronal apoptosis and improved neurological function by activating the *NRF2*/HO-1 axis and inhibiting NF-κB-mediated inflammatory signaling.[36,40] Similarly, in animal models of Alzheimer's disease and brain ischemia, PCA administration significantly ameliorated memory deficits and oxidative damage.[38]

Importantly, pharmacokinetic studies suggest that PCA, though rapidly metabolized, is capable of crossing the blood–brain barrier (BBB) in detectable concentrations. Shi *et al.*[35] reported that PCA administration in rats reduced oxidative stress markers in the brain, supporting BBB permeability. Guan *et al.*[36] further demonstrated that PCA suppressed MPP^+-induced mitochondrial dysfunction in PC12 cells and improved neuronal survival in rat models, indirectly confirming its central bioactivity. More recent reviews emphasize PCA as a promising neuroprotective phytochemical for central nervous system (CNS) disorders, while also acknowledging that its low oral bioavailability and extensive phase II metabolism (glucuronidation and sulfation) may limit therapeutic efficacy.[38,41]

Therefore, while PCA presents clear neuroprotective activity *in vivo* and evidence of BBB penetration, formulation strategies—such as nanoparticle carriers, liposomes, or structural derivatives—may be necessary to optimize its CNS delivery and clinical translation.

5. Conclusion

This study successfully demonstrated the synergistic neuroprotective effects of PCA and KJN against MPP^+-induced neurotoxicity in SH-SY5Y cells, a well-established *in vitro* model of PD. Our findings suggest that combining these two compounds yields a more pronounced therapeutic benefit than using them individually.

The core mechanism behind this protective effect is the attenuation of oxidative stress, a key contributor to DA neuronal death. We found that the combination of PCA and KJN significantly reduced intracellular ROS levels. The observed neuroprotection is directly mediated by the activation of the NRF2-HO-1 signaling pathway. Specifically, the compounds increase the expression of *NRF2*, which in turn upregulates the antioxidant enzyme HO-1, protecting the cells from oxidative damage.

Furthermore, we identified the upstream signaling pathway responsible for this activation. Our results show that the synergistic effects of PCA and KJN on the NRF2-HO-1 axis are specifically dependent on the PI3K/Akt pathway, as its inhibition eliminated the neuroprotective benefits. In contrast, inhibitors of the MAPK pathway (ERK, JNK, and p38) had no effect. This confirms that the PI3K/Akt-NRF2-HO-1 axis is the primary mechanism through which PCA and KJN exert their combined neuroprotective action.

While acknowledging the potential limitations of *in vitro* studies, our research provides a strong mechanistic basis for the use of PCA and KJN as a combination therapy for PD. The results highlight their potential as promising agents for the treatment of neurodegenerative disorders, particularly those driven by oxidative stress. Future *in vivo* studies and clinical trials are warranted to further validate their efficacy and safety.

Acknowledgments

The authors would like to acknowledge the Institute of Pharmaceutical Sciences for providing the necessary research facilities to conduct this study.

Funding

None.

Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author upon reasonable request.

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