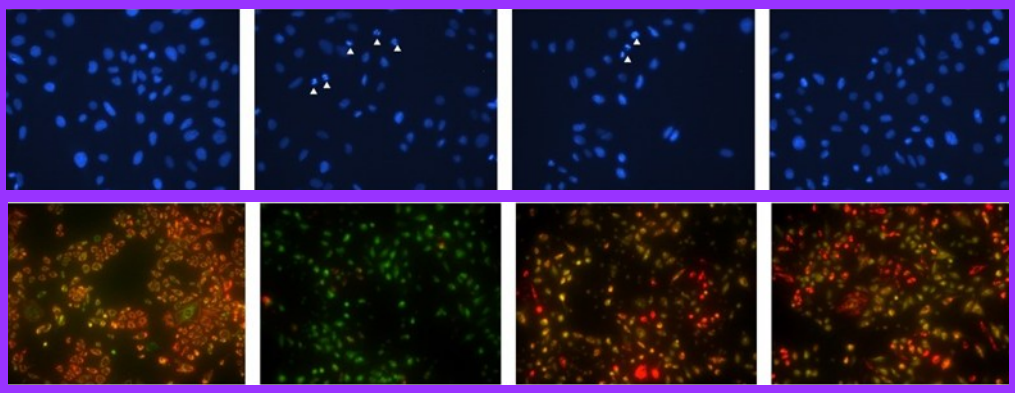

BJP

Bangladesh Journal of Pharmacology

Research Article

2-Methoxy-6-acetyl-7-methyljuglone, a natural naphthoquinone from *Polygonum cuspidatum*, protects against 6-OHDA-induced neurotoxicity in PC12 cells

A Journal of the Bangladesh Pharmacological Society (BDPS) **Bangladesh J Pharmacol 2026; 21: 20-27**
Journal homepage: www.bdpsjournal.org; www.banglajol.info
Abstracted/indexed in Academic Search Complete, Agroforestry Abstracts, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Global Health, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index
ISSN: 1991-0088

2-Methoxy-6-acetyl-7-methyljuglone, a natural naphthoquinone from *Polygonum cuspidatum*, protects against 6-OHDA-induced neurotoxicity in PC12 cells

Yuanfeng Fu, Weifeng Xu, Dongmeng Liu, Jiaolin Bao, and Ren-Bo Ding

Key Laboratory of Tropical Biological Resources of Ministry of Education, School of Pharmaceutical Sciences, Collaborative Innovation Center of Life and Health, Hainan University, Haikou-570228, China.

Article Info

Received: 1 January 2026
Accepted: 19 February 2026
Available Online: 2 March 2026
DOI: 10.3329/bjp.v21i1.86818

Cite this article:
Fu Y, Xu W, Liu D, Bao J, Ding RB. 2-Methoxy-6-acetyl-7-methyljuglone, a natural naphthoquinone from *Polygonum cuspidatum*, protects against 6-OHDA-induced neurotoxicity in PC12 cells. Bangladesh J Pharmacol. 2026; 21: 20-27.

Abstract

This study investigated the neuroprotective effects of 2-methoxy-6-acetyl-7-methyljuglone (MAM, a compound isolated from *Polygonum cuspidatum*) in 6-hydroxydopamine (6-OHDA)-injuried pheochromocytoma (PC12) cells. 6-OHDA treatment significantly induced cell death, apoptosis and mitochondrial membrane potential loss. MAM pretreatment significantly rescued PC12 cell viability, inhibited the 6-OHDA-induced apoptosis and depolarization in mitochondrial membrane potential. Results also demonstrated that 6-OHDA significantly induced ERK and NF-κB phosphorylation, while MAM pretreatment markedly reduced the 6-OHDA-elevated ERK and NF-κB phosphorylation levels. This suggested that MAM exerted neuroprotective effects likely by suppressing ERK-related oxidative stress and NF-κB-mediated neuroinflammation, thereby protecting neuronal cells from 6-OHDA-induced apoptosis. Collectively, these results suggested that MAM exerted a neuroprotective effect in 6-OHDA-induced Parkinson's disease cell model by suppressing ERK and NF-κB-mediated apoptotic mechanisms.

Introduction

Parkinson's disease is the second most common and complex neurodegenerative disease which greatly impact patient's daily life (Poewe et al., 2017). It brings heavy economic burdens on patients and society. Therefore, it is necessary to put further effort in develop-ping new drugs that can effectively prevent or treat Parkinson's disease.

Oxidative stress is increasingly recognized as a key contributor of dopaminergic neuron degeneration in the pathogenesis of multiple neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Andersen, 2004; Lin and Beal, 2006). Mitochondria are extremely vulnerable to oxidative stress, and excessive reactive oxygen species (ROS) can cause mitochondrial damage and dysfunction, leading to mitochondrial-dependent apoptosis in neurons. This ultimately results in the degeneration and death of dopaminergic neurons in Parkinson's disease (Kung et al., 2021).

Neuroinflammation also plays an important role in the pathogenesis of Parkinson's disease. Studies have found that increased activation of microglia, T lymphocyte infiltration, and upregulation of proinflammatory cytokine IL-1β, and TLR4 gene expression in the brains of Parkinson's disease patients (Kouli et al., 2020). Microglia serve as a crucial component which is invol-

ved in inflammatory response for the central nervous system. Microglia may release ROS in an overactivated state, causing neuronal damage (Block et al., 2007). Furthermore, hypertrophied astrocytes were observed in the affected brain regions of Parkinson's disease patients, suggesting that astrocytes are involved in the immune response to Parkinson's disease (Yamada et al., 1992). Therefore, inhibiting the activation of neuroinflammation can be used to treat Parkinson's disease.

Accumulating evidence in recent years have shown that a variety of traditional Chinese Medicine components exhibit neuroprotective activities, luteolin-7-O-glucoside suppressed the 6-OHDA-induced depolarization of mitochondrial membrane potential and nuclear damage, and reduced the activities of caspase-3 and acetylcholinesterase, indicating its neuroprotective activity (Rehfeldt et al., 2022). Schisandrol A could significantly improve the motor disorder and striatal neural injury, alleviated the depression-like symptoms and memory dysfunction in Parkinson's disease mice-induced by 6-OHDA (Yan et al., 2019). Further studies suggest that the neuroprotective effects of such herbal compounds are commonly attributed to their antioxidant and anti-inflammatory properties. Therefore, screening for compounds with antioxidant and anti-inflammatory properties from traditional Chinese Medicine presents a feasible strategy for developing novel Parkinson's disease therapeutics.

Polygonum cuspidatum Sieb.et Zucc, a plant of the Polygonaceae family, is a traditional Chinese medicine that exhibits the potential to alleviate symptoms of Alzheimer's disease and prevention of AIDS, etc (Ke et al., 2023). For instance, resveratrol, a polyphenolic compound extracted from the root of *P. cuspidatum*, has been demonstrated by numerous studies to exert neuroprotective effects in various Parkinson's disease models, and the underlying mechanisms are primarily associated with its antioxidant, anti-inflammatory, and anti-apoptotic activities (Dos Santos et al., 2022). Emodin is also a main active constituent of *P. cuspidatum*. Both *in vitro* and *in vivo* studies have demonstrated its pharmacological activities, including neuroprotective, anti-inflammatory, antioxidant and anti-cancer (Sharifi-Rad et al., 2022).

2-Methoxy-6-acetyl-7-methyljuglone (MAM), a natural naphthoquinone compound isolated from traditional Chinese herb *P. cuspidatum* and has the characteristic of antioxidant and anti-inflammatory (Chern et al., 2014). However, whether MAM showed a protective effect on Parkinson's disease model is still unknown. In this study, aims are to investigate the neuroprotective property of MAM on an *in vitro* Parkinson's disease model with 6-OHDA-injured PC12 cells.

Materials and Methods

Cell Culture

Rat PC12 cells were purchased from the American Type Culture Collection (USA). The cells were cultured in F-12K medium supplemented with 15% (v/v) heat-inactivated horse serum, 2.5% (v/v) FBS, penicillin (100 U/mL), and streptomycin (100 ug/mL) and were maintained at a humidified 5% (v/v) CO_2 atmosphere at 37℃ in an incubator. All cell culture materials were purchased from Gibco Invitrogen (USA).

Extract and isolation

Five kilograms of *P. cuspidatum* was pulverized and extracted three times with 95% ethanol. The combined extract was evaporated under vacuum to a concrete. The concrete was subsequently suspended in water, and then was extracted step by step with petroleum ether and ethyl acetate. The ethyl acetate extraction was subjected to silica gel column chromatography, eluting with a gradient of chloroform-methanol (100:0 to 95:5, v/v). The fraction containing MAM was purified by re-crystallization to yield the pure compound. Purity of MAM was determined by HPLC using an Agilent SBC-18 column (4.6 × 250 mm, 5 µm), with purity exceeding 98%. (Figure S1). The standard of MAM was purchased from Yuanye Bio-Technology Co., Ltd, China.

MTT assay

3-(4,5-Dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (USA). PC12 cells were plated at a density of 1 × 10^4 cells/100 µL per well in 96-well plates and maintained in F-12K medium for 24 hours. Subsequently, PC12 cells were treated with different concentrations of drug. After drug treatment, the supernatant was discarded from each well. Then MTT solution (prepared in fresh 0.5% (v/v) heat-inactivated horse serum; final concentration 0.5 mg/mL) was added to each well, and the cells were incubated at 37℃ for 4 hours. The medium was then discarded, and 150 µL of DMSO was added to each well and place the plate on a shaker to dissolve the violet formazan crystals in intact cells. The cell viability was measured by the MTT assay. The absorbance at 570 nm was measured by a Wallac-Victor3 V microplate reader (Perkin-Elmer, The Netherlands) (van Meerloo et al., 2011).

JC-1 assay

The mitochondrial membrane potential (φm) was monitored by the fluorescent dye JC-1. JC-1 is a lipophilic cationic dye that is able to permeate through the mitochondrial membrane. PC12 cells were treated with different concentrations of drug. After drug treatment, the medium was discarded. Cells were washed with

Box 1: Hoechst 33342 staining

Principle

Hoechst 33342 is a cell-permeant, blue-fluorescent DNA stain that binds to the minor groove of AT-rich regions in double-stranded DNA and often used for visualizing apoptosis.

Requirements

12-Well plate; Fluorescence microscope with a camera (Carl Zeiss, USA); Hoechst 33342; Phosphate buffer solution; PC12 cell; RNase

Procedure

Step 1: PC12 cells were seeded in a 12-well plate at a density of 1×10^5 cells per well in 2 mL of F-12K medium.

Step 2: After drug treatment, the medium was discarded and cells were washed with ice-cold phosphate buffer solution, fixed with 1% (v/v in PBS) formaldehyde.

Step 3: Stain with 10 µg/mL Hoechst 33342 with 5 µg/mL RNase in phosphate buffer solution for 15 min at room temperature.

Step 4: Subsequently, cells were washed 2-3 times with ice-cold phosphate buffer solution, 3-5 min each time.

Step 5: Images were recorded with a fluorescence microscope

Reference

Crowley et al., 2016b

phosphate buffer solution and then incubated with 2.5 µg/mL JC-1 for 10 min. After staining, carefully remove the supernatant. Subsequently, cells were washed twice with cell staining buffer, and finally add 1 mL of culture medium to each well. The fluorescence of JC-1 was captured by fluorescence microscopy (Chazotte, 2011).

Cell cycle assay

Cells in the proliferative cycle exhibit DNA content ranging from 2N to 4N depending on their phase (G0/G1 phase, S phase and G2/M phase). Apoptotic cells undergo nuclear DNA fragmentation, breaking down into numerous small fragments. Following ethanol fixation, cell membrane permeabilization allows low-molecular-weight DNA fragments to escape through the cell membrane, leaving only large DNA fragments. These apoptotic cells, having partial DNA loss, form a distribution zone with DNA content <2N upon DNA staining, termed the "sub-G1 phase". Therefore, apoptosis could be assessed by the proportion of cells in the sub-G1 phase. After exposure to various concentrations of drug, cells were harvested and permeabilized with 1 mL ice-cold 70% ethanol at 20℃ overnight. Then centrifuge at 400 x g for 5 min to remove the supernatant. After centrifugation to remove ethanol, cells were washed with phosphate buffer solution and then resuspended in 500 µL phosphate buffer solution supplemented with 5 µL DNase free RNase (10 mg/mL, final concentration 100 µg/mL), incubated at 37℃ for 20 min. Then centrifuge at 400 x g for 5 min to collect cell pellet. Subsequently, cells were resuspended in 95 µL phosphate buffer solution supplemented with 5 µL propidium iodide solution (5 µg/mL, final concentration 0.25 µg/mL), incubated in the dark for 20 min. Flow cytometry analysis using a FACS caliber (BD FACS CantoTM) (Pozarowski and Darzynkiewicz, 2004).

Annexin V/ propidium iodide double staining assay

Annexin V-PI double staining was employed to further detect apoptosis and quantitatively assess the neuroprotective effect of MAM on 6-OHDA-induced damage in PC12 cells. Apoptosis rate was measured by flow cytometric analysis according to the protocol provided by the annexin V-FITC apoptosis detection kit. Cells were centrifuged at 1,000 x g/min for 5 min, and the pellet was washed with phosphate buffer solution. After centrifugation to remove supernatant, cells were resuspended in 100 µL binding buffer to adjust the density to 1×10^6 cells/mL. Then, cells were incubated with annexin V-FITC and propidium iodide for 15 min at room temperature in the dark. Finally, add 400 µL of binding buffer and filter through a 70 µm cell strainer. The apoptosis rate was detected by flow cytometer (Crowley et al., 2016a).

Western blotting

After drug treatment, PC12 cells were harvested and lysed with RIPA lysis buffer containing 1 mM PMSF and 1% protease inhibitor cocktail and incubated for 30 min on ice. The cell lysates were centrifuged at 12,500 x g for 20 min at 4℃, and the supernatant was separated, and the protein concentrations was measured using the BCA protein assay kit. Protein samples (30 µg) were separated by SDS-PAGE and transferred to polyvinyllidene difluoride membrane (Bio-Rad, USA). Subsequently, the membrane was blocked with 5% (v/v) non-fat milk for 1 hour at room temperature. The blots were analyzed with primary antibodies ((1:1000, Cell Signaling Technology, USA), and peroxidase conjugated secondary antibodies (1:2000, Cell Signaling Technology) were used to detect the proteins of interest with an ECL advanced western blotting detection kit (Amersham, UK). Densitometric measurement of band intensity was performed with the Quantity One (Bio-Rad) (Sule et al., 2023).

Statistical analysis

The data were expressed as means ± standard errors of the mean (SEMs) and analyzed by GraphPad Prism V4.0 (GraphPad Software, Inc., USA). Statistical significance was evaluated with One-way analysis of variance (ANOVA) and Dunnett's test. The value of statistical significance was set at $p<0.05$.

Results

Effect on cell viability

PC12 cells were exposed to 0.1-10 μM MAM for 24 hours had no significant cytotoxicity in PC12 cells (data not shown). The results showed that exposure of PC12 cells to 500 μM 6-OHDA (model group) for 12 hours significantly induced cell death when compared to the untreated control cells (Figure 1). Pretreatment with MAM (0.5, 1, 2, 3, and 4 μM) could significantly increase cell viabilities in 6-OHDA-treated PC12 cells comparing to model group, and the cell viabilities of PC12 cells increased most when pretreating with 2 μM MAM. Taken together, these results suggested that MAM exhibited a good neuroprotective capacity to protect against 6-OHDA-induced neurotoxicity in PC12 cells.

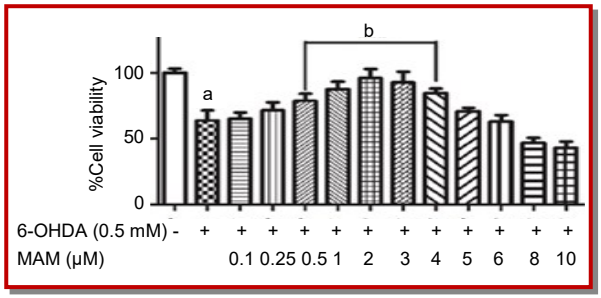

Figure 1: Effects of MAM on cell viability in 6-OHDA-injured PC12 cells. The PC12 cells were pretreated with MAM (0.1-10 μM) for 12 hours before 6-OHDA treatment. Subsequently, MAM was withdrawn from medium and the PC12 cells were then exposed to 500 μM 6-OHDA for another 12 hours to induce neurotoxicity. Data are mean ± SEM (n=6). Compared with the control group, [a]p<0.001; compared with the 6-OHDA model group, [b]p<0.001

Effect on cytotoxicity and mitochondrial membrane potential

PC12 cells were pretreated with MAM before 6-OHDA damage. When observing PC12 cells under a fluorescence microscope, the stained cell nucleus exhibited blue fluorescence (Figure 2A). The nucleus of untreated control cells appeared round or elliptical, with no observation of nuclear condensation and fragmentation. In contrast, cell nucleus with bright blue fluorescence was observed in 6-OHDA treated PC12, which meant nuclear condensation and fragmentation were occurring, indicating the presence of apoptotic bodies (as indicated by white arrows). MAM pretreatment significantly reduced the number of apoptotic bodies, demonstrating that MAM effectively inhibited cell apoptosis induced by 6-OHDA in PC12 cells.

Mitochondria play an important role in regulating cell cycle, apoptosis, and cell death. Cell apoptosis causes increased permeability of mitochondrial membrane, which in turn leads to loss of mitochondrial membrane potential. Therefore, depolarization of mitochondrial membrane potential can serve as a marker of cell apoptosis.

In healthy cells with high mitochondrial membrane potential, JC-1 exists as aggregates and yields red fluorescence. Whereas in depolarized mitochondria, JC-1 exists as monomers and yields green fluorescence. Therefore, changes in mitochondrial membrane potential can be measured by the green/red fluorescence ratio. As shown in Figure 2B, compared with the control group, 6-OHDA-treated cells displayed reduced red fluorescence and enhanced green fluorescence, indicating a significant decrease in mitochondrial membrane potential in PC12 cells. When cells were pretreated with MAM, the 6-OHDA-induced decline in mitochondrial membrane potential was reversed (Figure 2B). Collectively, these results suggested that

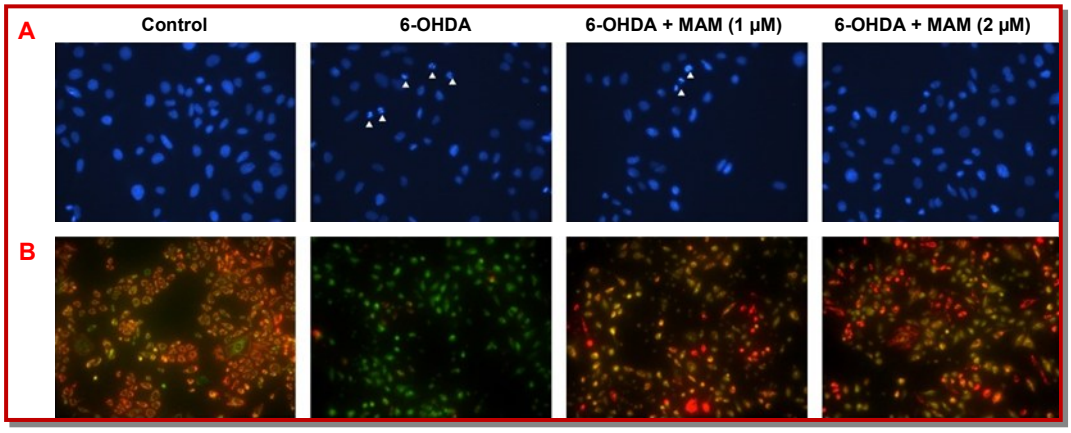

Figure 2: Effects of MAM on the number of apoptotic bodies and mitochondrial membrane potential loss in 6-OHDA-injured PC12 cells. Hoechst 33342-stained PC12 cells demonstrated the anti-apoptotic effect of MAM against 6-OHDA-induced neurotoxicity. White arrows indicate the apoptotic bodies within the cell nucleus (A). JC-1-stained PC12 cells demonstrated the protective effect of MAM against 6-OHDA-induced mitochondrial membrane potential loss (B). Images acquired by fluorescence microscope (200x)

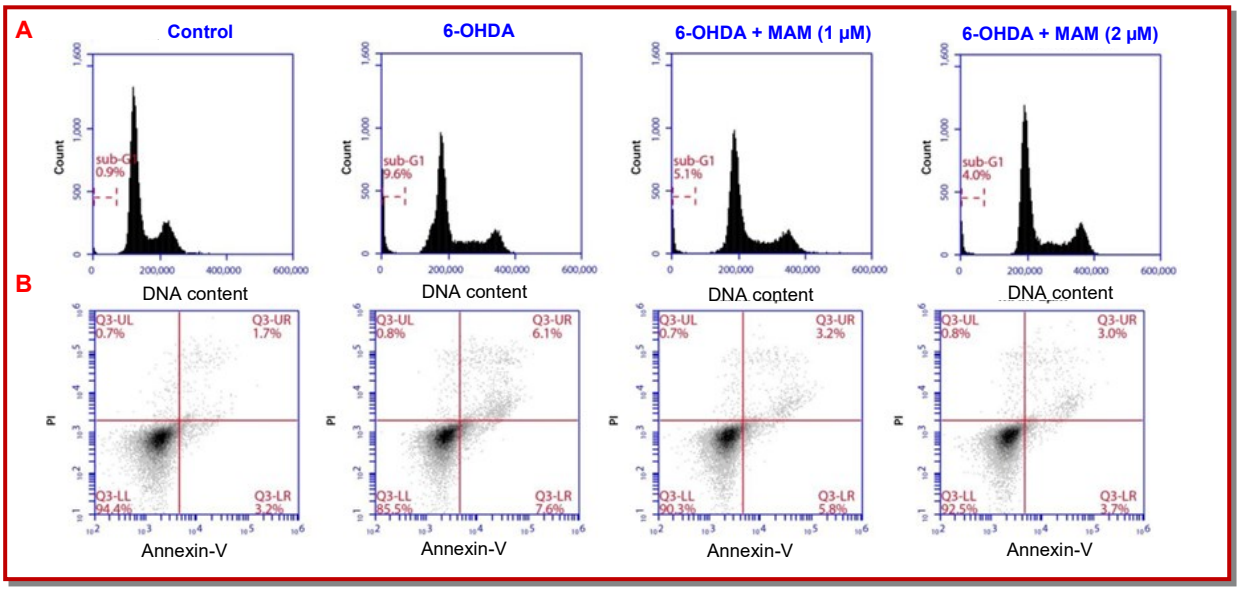

Figure 3: Effects of MAM on apoptosis in 6-OHDA-damaged PI-stained (A) and annexin V-PI-stained (B) PC12 cells

MAM inhibited apoptosis by ameliorating the damaging effects of 6-OHDA on the mitochondrial membrane potential of PC12 cells.

Effect on apoptosis

The property of MAM in inhibiting apoptosis was further verified by this assay. As shown in Figure 3A, cells treated with 6-OHDA exhibited a higher proportion of sub-G1 phase proportion (9.6%) compared to that of 0.9% in the control group. Whereas, following treatment with 0.5 and 1 μM MAM, the percentages of sub-G1 phase decreased from 9.6% to 5.1% and 4.0%, respectively, compared to the 6-OHDA-treated group (Figure 3A). These findings indicated that MAM alleviated 6-OHDA-induced cell apoptosis in PC12 cells as evidenced by reducing the proportion of sub-G1 phase cells.

As shown in Figure 3B, compared with the control group, the apoptosis rate in the 6-OHDA-treated group significantly increased (from 4.9 to 13.7%). However, pretreatment with MAM (0.5 and 1 μM) reduced the percentage of apoptotic cells (Figure 3B). Under pretreatment conditions of 0.5 and 1 μM MAM, the apoptosis rates decreased to 9.0% and 6.7%, respectively, indicating that MAM significantly inhibited 6-OHDA-induced apoptosis in PC12 cells.

Effect on 6-OHDA-elevated ERK and NF-κB phosphorylation

Western blot analysis was employed to further investigate the potential molecular mechanisms underlying the protective effects of MAM. As shown in Figure 4, compared with the control group, the phosphorylation level of ERK was significantly elevated in cells treated with 6-OHDA. The phosphorylated ERK level was markedly reduced in the MAM (0.5, 1, and 2 μM) pre-treated groups compared with the 6-OHDA-treated group.

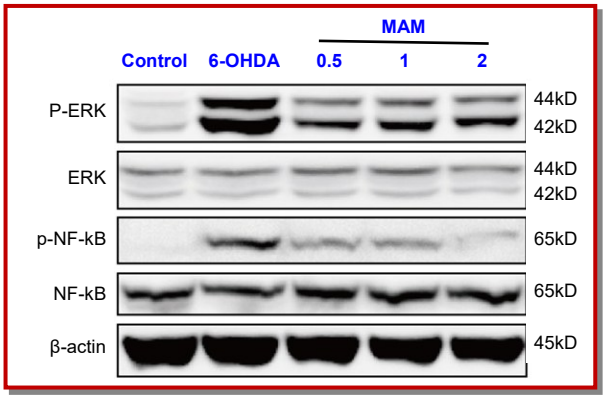

Figure 4: Effects of MAM on phosphorylation levels of ERK and NF-κB in 6-OHDA-damaged PC12 cells using Western blot analysis

Furthermore, compared to the control group, 6-OHDA-induced PC12 cells exhibited elevated NF-κB phosphorylation level, indicating the occurrence of neuroinflammation. MAM pretreatment significantly reduced the 6-OHDA-elevated NF-κB phosphorylation levels in PC12 cells.

Discussion

Current Parkinson's disease medications only control symptoms without slowing or halting disease progression. Oxidative stress and neuroinflammation play pivotal roles in Parkinson's disease pathogenesis. A variety of natural compounds have demonstrated good potential in Parkinson's disease treatment due to their anti-inflammatory and antioxidant properties. Nobile-

tin has been reported to protect dopaminergic neurons from 1-methyl-4-phenylpyridinium (MPP^+)-induced neurotoxicity and attenuate microglia-mediated brain inflammation by suppressing IL-1β expression in MPP^+-treated rat brain tissues (Jeong et al., 2014). Quercetin has been found to reverse 6-OHDA-induced ROS production and mitochondrial membrane potential loss in PC12 cells, and relieve the progressive motor impairment in 6-OHDA-induced Parkinson's disease rats (Wang et al., 2021). Oxyflavone A, a natural plant compound used for Parkinson's disease treatment, has been shown to significantly improve 6-OHDA-induced neurotoxic damage (Shao et al., 2024). Specifically, oxyflavone A significantly rescued cells from 6-OHDA-induced impairments in viability and mitochondrial membrane potential. In Parkinson's disease mice, treatment with oxyflavone A markedly alleviated motor dysfunction and increased concentrations of neurotransmitters, such as dopamine, dopamine metabolite (DOPAC), and homovanillic acid. These findings demonstrate the feasibility of pursuing antioxidant and anti-inflammatory compounds as therapeutic strategies for Parkinson's disease.

MAM is isolated from *P. cuspidatum* and has demonstrated to possess various pharmacological activities. Among these, its antitumor effect is the most studied pharmacological activity of MAM. MAM could decrease the survival rate of various cancer cells, including lung cancer (Sun et al., 2016; Sun et al., 2019), breast cancer, melanoma (Sun et al., 2016), colorectal cancer (Sun et al., 2017) and glioblastoma (Yu et al., 2020). Moreover, MAM also exhibits antiviral activity, inhibiting human rhinovirus 3C protease activity with an IC_{50} value of 4.6 μM (Singh et al., 2001). Its inhibitory potency is significantly stronger than that of two other compounds (emodin and methoxyemodinm) in *P. cuspidatum* (Singh et al., 2001). Previous studies have shown that MAM possesses both antioxidant and anti-inflammatory properties. Pretreatment with MAM (24 hours) protected PC12 cells from the cytotoxicity induced by the oxidant tert-butyl hydroperoxide (t-BHP)(Li et al., 2011). Furthermore, MAM exerts protective effects against acute ischemic stroke. It enhanced survival rates in stroke mice and improved cerebral infarction, neurological deficits, and neurological dysfunction (Chern et al., 2014). MAM also demonstrated a protective effect on blood-brain barrier integrity by reducing ROS production, inhibiting inflammatory cell infiltration, and downregulating NF-κB-associated proinflammatory factors iNOS and COX-2 (Chern et al., 2014). Therefore, to investigate the neuroprotective effects of MAM on Parkinson's disease and its underlying mechanisms, this study established an *in vitro* Parkinson's disease model by inducing PC12 cell damage with the neurotoxin 6-OHDA, followed by a series of evaluations of MAM's protective effects.

Parkinson's disease is characterized by the death of dopaminergic neurons in the substantia nigra of the midbrain. Studies have demonstrated that apoptosis is the primary mechanism of dopaminergic neuronal death in Parkinson's disease (Erekat, 2022). Hoechst 33342 staining revealed nucleus condensation and fragmentation in 6-OHDA-treated PC12 cells, indicating the occurrence of apoptosis, which was inhibited by MAM pretreatment. The dissipation of mitochondrial membrane potential is a hallmark of apoptosis. Therefore, the changes of mitochondrial membrane potential were examined. Results showed that 6-OHDA induced the depolarization of mitochondrial membrane potential, while MAM effectively mitigated this phenomenon. Further flow cytometry analysis of apoptosis revealed that MAM pretreatment significantly decreased the apoptosis rates, suggesting MAM may protect dopaminergic neurons by inhibiting apoptosis. Based on these findings, western blotting was used to explore the potential molecular mechanisms underlying MAM's neuroprotective effects.

Oxidative stress and neuroinflammation are key pathogenic mechanisms of Parkinson's disease. Increased ROS production causes oxidative stress, which in turns activates stress-mediated MAPK/ERK cascade and leads to apoptosis-related neuronal cell death in Parkinson's disease (Cheung and Slack, 2004). Mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinases (ERK) signaling cascade is a key serine-threonine protein kinase pathway present in all eukaryotes. This signaling mediates multiple critical intracellular processes, particularly participating in stress-mediated cellular processes such as apoptosis, survival and differentiation (Plotnikov et al., 2011). In various neurodegenerative diseases, including (Bhat and Zhang, 1999) Parkinson's disease and Alzheimer's disease, the MAPK/ERK signaling cascade plays a crucial role in regulating neuronal cell death (Cheung and Slack, 2004). For example, hydrogen peroxide treatment induced ERK phosphorylation in oligodendrocyte-like CG4 cells, while ERK pathway inhibitors mitigated hydrogen peroxide-induced cell death. Furthermore, in a 6-OHDA-induced Parkinson's disease model, studies demonstrated that ERK pathway inhibitors suppressed 6-OHDA-induced ERK phosphorylation, suggesting that ERK phosphorylation plays a key role in 6-OHDA-induced neurotoxicity (Kulich and Chu, 2001).

Nuclear factor kappa B (NF-κB) is a transcription factor that play a pivotal role in modulating the inflammatory response, which transactivates proinflammatory genes associated with cytokine or chemokine modulation and apoptosis (Hayden and Ghosh, 2004). Dysregulated NF-κB activation contributes to acute and chronic inflammatory disorders (Hayden and Ghosh, 2004). Studies have shown that NF-κB activation is associated with dopamine-induced apoptosis in Parkinson's disease model, suggesting NF-κB involvement in substantia

nigra degeneration in Parkinson's disease (Panet et al., 2001). In Parkinson's disease patients, the proportion of immunoreactive NF-κB-positive dopaminergic neurons in substantia nigra pars compacta was over 70-fold higher than in controls, suggesting NF-κB nuclear translocation may participate in the pathological process of Parkinson's disease (Hunot et al., 1997). This study further investigated whether the neuroprotective effects of MAM in Parkinson's disease are mediated by inhibiting the abnormal activation of MAPK/ERK and NF-κB signaling, which is closely associated with oxidative stress and neuroinflammation. Experimental results demonstrated that 6-OHDA significantly induced ERK and NF-κB phosphorylation, while whereas MAM pretreatment markedly reduced the 6-OHDA-elevated ERK and NF-κB phosphorylation levels. This suggests that MAM exerted neuroprotective effects likely by suppressing ERK-related oxidative stress and NF-κB-mediated neuroinflammation, thereby protecting neuronal cells from 6-OHDA-induced apoptosis. This study also has certain limitations in further validating molecular mechanisms, including elucidating how MAM regulates oxidative stress and neuroinflammation in depth, as well as exploring other regulatory signaling pathways in addition to ERK and NF-κB signaling.

This study has some limitations such as the molecular mechanisms of the neuroprotective effect of MAM remain insufficiently explored, and the efficacy of MAM *in vivo* has not yet been demonstrated through animal experiments.

Conclusion

MAM exerted neuroprotective effects by inhibiting 6-OHDA-induced cell apoptosis, depolarization in mitochondrial membrane potential and neuroinflammation through regulating ERK and NF-κB phosphorylation, highlighting its potential as a therapeutic agent for Parkinson's disease prevention and treatment.

Financial Support

This work was supported by National Natural Science Foundation of China (No. 32560887, 82560768), Hainan Province Science and Technology Special Fund (No. ZDYF2025SHFZ022), Hainan University Collaborative Innovation Center Research Fund (No. XTCX2022JKB01) and Hainan Nanhai New Star Project (No. 202309009)

Ethical Issue

The development, acquisition, authentication, cryopreservation, and transfer of cell lines between laboratories were followed according to the guidelines published in British Journal of Cancer, 2014

Conflict of Interest

Authors declare no conflict of interest

Acknowledgement

The authors thank members of the Ding and Bao laboratory for helpful advice and discussion.

References

Andersen JK. Oxidative stress in neurodegeneration: Cause or consequence? Nat Med. 2004; 10 Suppl: S18-25.

Bhat NR, Zhang P. Hydrogen peroxide activation of multiple mitogen-activated protein kinases in an oligodendrocyte cell line: Role of extracellular signal-regulated kinase in hydrogen peroxide-induced cell death. J Neurochem. 1999; 72: 112 -19.

Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: Uncovering the molecular mechanisms. Nat Rev Neurosci. 2007; 8: 57-69.

Chazotte B. Labeling mitochondria with JC-1. Cold Spring Harb Protoc. 2011; 2011: 065490.

Chern CM, Wang YH, Liou KT, Hou YC, Chen CC, Shen YC. 2-Methoxystypandrone ameliorates brain function through preserving BBB integrity and promoting neurogenesis in mice with acute ischemic stroke. Biochemical pharmacology. 2014; 87: 502-14.

Cheung EC, Slack RS. Emerging role for ERK as a key regulator of neuronal apoptosis. Science's STKE: Signal transduction knowledge environment. 2004; 2004: Pe45.

Crowley LC, Marfell BJ, Scott AP, Waterhouse NJ. Quantitation of apoptosis and necrosis by annexin V binding, propidium iodide uptake, and flow cytometry. Cold Spring Harb Protoc. 2016a; 2016: 087288.

Crowley LC, Marfell BJ, Waterhouse NJ. Analyzing cell death by nuclear staining with hoechst 33342. Cold Spring Harb Protoc. 2016b; 2016: 087295.

Dos Santos MG, Schimith LE, Andre-Miral C, Muccillo-Baisch AL, Arbo BD, Hort MA. Neuroprotective effects of resveratrol in *in vivo* and *in vitro* experimental models of Parkinson's disease: A systematic review. Neurotox Res. 2022; 40: 319-45.

Erekat NS. Apoptosis and its therapeutic implications in neurodegenerative diseases. Clin Anat. 2022; 35: 65-78.

Hayden MS, Ghosh S. Signaling to NF-kappaB. Genes & development. 2004; 18: 2195-224.

Hunot S, Brugg B, Ricard D, Michel PP, Muriel MP, Ruberg M, Faucheux BA, Agid Y, Hirsch EC. Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with Parkinson disease. Proc Natl Acad Sci USA. 1997; 94: 7531-36.

Jeong KH, Jeon M-T, Kim HD, Jung UJ, Jang MC, Chu JW, Yang SJ, Choi IY, Choi M-S, Kim SR. Nobiletin protects dopaminergic neurons in the 1-methyl-4-phenylpyridinium-

treated rat model of Parkinson's disease. J Med Food. 2014; 18: 409-14.

Ke J, Li MT, Xu S, Ma J, Liu MY, Han Y. Advances for pharmacological activities of *Polygonum cuspidatum*: A review. Pharm Biol. 2023; 61: 177-88.

Kouli A, Camacho M, Allinson K, Williams-Gray CH. Neuroinflammation and protein pathology in Parkinson's disease dementia. Acta Neuropathol Commun. 2020; 8: 211.

Kulich SM, Chu CT. Sustained extracellular signal-regulated kinase activation by 6-hydroxydopamine: Implications for Parkinson's disease. J Neurochem. 2001; 77: 1058-66.

Kung HC, Lin KJ, Kung CT, Lin TK. Oxidative stress, mitochondrial dysfunction, and neuroprotection of poly-phenols with respect to resveratrol in Parkinson's disease. Biomedicines 2021; 9: 918.

Li YB, Lin ZQ, Zhang ZJ, Wang MW, Zhang H, Zhang QW, Lee SM, Wang YT, Hoi PM. Protective, antioxidative and antiapoptotic effects of 2-methoxy-6-acetyl-7-methyljuglone from *Polygonum cuspidatum* in PC12 cells. Planta Med. 2011; 77: 354-61.

Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 2006; 443: 787-95.

Panet H, Barzilai A, Daily D, Melamed E, Offen D. Activation of nuclear transcription factor kappa B (NF-kappaB) is essential for dopamine-induced apoptosis in PC12 cells. J Neurochem. 2001; 77: 391-98.

Plotnikov A, Zehorai E, Procaccia S, Seger R. The MAPK cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation. Biochim Biophys Acta. 2011; 1813: 1619-33.

Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkmann J, Schrag AE, Lang AE. Parkinson disease. Nat Rev Dis Primers. 2017; 3: 17013.

Pozarowski P, Darzynkiewicz Z. Analysis of cell cycle by flow cytometry. Methods Mol Biol. 2004; 281: 301-11.

Rehfeldt SCH, Silva J, Alves C, Pinteus S, Pedrosa R, Laufer S, Goettert MI. Neuroprotective effect of luteolin-7-O-glucoside against 6-OHDA-induced damage in undifferentiated and RA-differentiated SH-SY5Y cells. Int J Mol Sci. 2022; 23: 2914.

Shao M, Zhao C, Pan Z, Yang X, Gao C, Kam GH, Zhou H, Lee SM. Oxyphylla A exerts antiparkinsonian effects by ameliorating 6-OHDA-induced mitochondrial dysfunction and dyskinesia *in vitro* and *in vivo*. Chem Biol Interact. 2024; 403: 111224.

Sharifi-Rad J, Herrera-Bravo J, Kamiloglu S, Petroni K, Mishra AP, Monserrat-Mesquida M, Sureda A, Martorell M, Aidarbekovna DS, Yessimsiitova Z, Ydyrys A, Hano C, Calina D, Cho WC. Recent advances in the therapeutic potential of emodin for human health. Biomed Pharmacother. 2022; 154: 113555.

Singh SB, Graham PL, Reamer RA, Cordingley MG. Discovery, total synthesis, HRV 3C-protease inhibitory activity, and structure-activity relationships of 2-methoxystypandrone and its analogues. Bioorg Med Chem Lett. 2001; 11: 3143-46.

Sule R, Rivera G, Gomes AV. Western blotting (immunoblotting): History, theory, uses, protocol and problems. Biotechniques 2023; 75: 99-114.

Sun W, Bao J, Lin W, Gao H, Zhao W, Zhang Q, Leung CH, Ma DL, Lu J, Chen X. 2-Methoxy-6-acetyl-7-methyljuglone (MAM), a natural naphthoquinone, induces NO-dependent apoptosis and necroptosis by H_2O_2-dependent JNK activation in cancer cells. Free Radic Biol Med. 2016; 92: 61-77.

Sun W, Wu X, Gao H, Yu J, Zhao W, Lu JJ, Wang J, Du G, Chen X. Cytosolic calcium mediates RIP1/RIP3 complex-dependent necroptosis through JNK activation and mitochondrial ROS production in human colon cancer cells. Free Radic Biol Med. 2017; 108: 433-44.

Sun W, Yu J, Gao H, Wu X, Wang S, Hou Y, Lu JJ, Chen X. Inhibition of lung cancer by 2-methoxy-6-acetyl-7-methyljuglone through induction of necroptosis by targeting receptor-interacting protein 1. Antioxid Redox Signal. 2019; 31: 93-108.

van Meerloo J, Kaspers GJ,Cloos J. Cell sensitivity assays: The MTT assay. Methods Mol Biol. 2011; 731: 237-45.

Wang WW, Han R, He HJ, Li J, Chen SY, Gu Y, Xie C. Administration of quercetin improves mitochondria quality control and protects the neurons in 6-OHDA-lesioned Parkinson's disease models. Aging (Albany NY). 2021; 13: 11738-51.

Yamada T, Kawamata T, Walker DG, McGeer PL. Vimentin immunoreactivity in normal and pathological human brain tissue. Acta Neuropathol. 1992; 84: 157-62.

Yan T, Sun Y, Gong G, Li Y, Fan K, Wu B, Bi K, Jia Y. The neuroprotective effect of schisandrol A on 6-OHDA-induced PD mice may be related to PI3K/AKT and IKK/IκBα/NF-κB pathway. Exp Gerontol. 2019; 128: 110743.

Yu J, Zhong B, Jin L, Hou Y, Ai N, Ge W, Li L, Liu S, Lu JJ, Chen X. 2-Methoxy-6-acetyl-7-methyljuglone (MAM) induced programmed necrosis in glioblastoma by targeting NAD(P)H: Quinone oxidoreductase 1 (NQO1). Free Radic Biol Med. 2020; 152: 336-47.

Author Info

Ren-Bo Ding and Jiaolin Bao (Principal contact)
e-mail: dingrenbo@hainanu.edu.cn; baojiaolin@hainanu.edu.cn
First two authors contributed equally