

Tilianin attenuates MPP⁺-induced oxidative stress and apoptosis of dopaminergic neurons in a cellular model of Parkinson's disease

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Abstract. The flavonoid tilianin is derived from the leaves of *Dracocephalum moldavica* L. amiales and has been proven to serve a neuroprotective role in cerebral ischemia. Therefore, the aim of the present study was to determine whether tilianin could prevent oxidative stress and the apoptosis of dopaminergic neurons in Parkinson's disease (PD). The dopaminergic neuron MES23.5 cell line was treated with 1-methyl-4-phenylpyridinium (MPP⁺) to construct a PD cell model. Following pretreatment with tilianin, the Cell Counting Kit-8 assay was used to assess cell viability. The protein and mRNA expression levels of tyrosine hydroxylase were determined using immunofluorescence, reverse transcription-quantitative PCR (RT-qPCR) and western blotting. mRNA and protein expression levels of inflammatory cytokines IL-6, IL-1 β and TNF- α and oxidative stress-related enzymes manganese superoxide dismutase and catalase were also quantified using RT-qPCR and western blotting, respectively. Cell apoptotic rate was analyzed using the TUNEL assay and the expressions of apoptosis-related proteins Bcl-2, Bax and cleaved caspase-3 were detected by western blotting. MAPK signaling pathway-related protein expression levels were assessed via western blotting in MPP⁺-stimulated MES23.5 cells with or without tilianin pretreatment. Tilianin was demonstrated to exert no cytotoxic effects on MES23.5 cells and was able to prevent MPP⁺-induced reductions in cell viability. Pretreatment with tilianin also inhibited MPP⁺-induced inflammatory cytokine secretion, oxidative stress and apoptosis of MES23.5 cells. In addition, the protein expression levels of MAPK signaling pathway-related proteins were upregulated by MPP⁺, whereas pretreatment with tilianin downregulated these in a dose-dependent manner. The results of the present study indicated that tilianin may exert anti-inflammatory and antioxidant

effects and inhibit the MAPK signaling pathway, which may ameliorate injury to dopaminergic neurons induced by PD.

Introduction

Parkinson's disease (PD) is a common age-related neurodegenerative disease, with the age of onset ranging between 40 and 70 years (1,2). Currently, the prevalence of PD in individuals aged ≥ 60 years in China is 1-2% (3). The seventh national census released in May 2021 reported that $\sim 18.7\%$ of China's population is aged >60 years, which suggests that the number of new cases of PD will increase annually as a result of the aging population (4). PD significantly impacts the quality of life of those affected, whereby patients face losing their independence and becoming reliant on nursing care in the advanced stages of the disease (5). For this reason, research on the pathogenesis and treatment of PD has major social and economic implications. However, the lack of an in-depth understanding of PD has led to a limited range of therapeutic strategies (6). Levodopa is currently the mainstay symptomatic treatment for PD and can improve symptoms such as muscular rigidity and postural instability; however, it cannot stop disease progression (7). The side effects of levodopa include nausea, agitation, psychological symptoms and abnormal limb movement, with the efficacy of the drug decreasing over a period of 3-5 years. However, even with treatment, neuronal apoptosis continues to exacerbate the disease (8). Therefore, the main focus of PD research is to explore the etiology and pathogenesis of PD in order to develop alternative therapeutic methods.

Oxidative stress is a major cause of neuronal apoptosis in PD and, therefore, the discovery and development of successful antioxidant treatments is an important focus of current PD research (9). Traditional Chinese herbal medicines are considered to be natural sources of antioxidants, among which flavonoid compounds have been demonstrated to exhibit pharmacological properties as a result of their diverse bioactivity (10,11). Tilianin is a natural polyphenolic flavonoid isolated from *Dracocephalum moldavica* L. amiales and it has a variety of pharmacological properties, including neuroprotective, cardioprotective, antihypertensive, anti-atherosclerotic, antioxidant, anti-inflammatory and antidiabetic effects (12). Previous studies have reported the role of tilianin against oxidative stress in cerebral ischemia/reperfusion injury via inhibition of the p38 MAPK signaling pathway, as well as in

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tracheal epithelial cells via inhibition of the ERK signaling pathway (13,14). Based on these earlier studies, the aim of the present study was to explore whether tilianin could protect against the inflammation and oxidative stress that damages dopaminergic neurons in PD, and to determine the role of the MAPK signaling pathway in this mechanism.

Materials and methods

Cell culture and treatment. The dopaminergic neuron MES23.5 cell line (cat. no. CVCL-J351; <http://www.biovector.net/product/2277098.html>) was acquired from the BioVector National Type Culture Collection, Inc. MES23.5 cells were maintained in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Merck KGaA), 2% Sato's solution containing 25 mg insulin, transferrin, selenium and sodium pyruvate solution (ITS-A; cat. no. 51300044; Thermo Fisher Scientific, Inc.), 0.315 mg/ml progesterone (cat. no. HY-N0437; MedChemExpress) and 20 mg putrescine (cat. no. HY-N2407; MedChemExpress), in addition to 100 U/ml penicillin/streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. To construct the PD model, MES23.5 cells were treated with 300 μmol/l 1-methyl-4-phenylpyridinium (MPP⁺; Sigma-Aldrich; Merck KGaA) at 37°C for 24 h, whereas untreated MES23.5 cells cultured for 24 h at 37°C in normal medium were used as a control. Tilianin (Chengdu Pufei De Biotech Co., Ltd.) at concentrations of 0, 1, 3, 10 and 30 μM were selected for cell pretreatment in order to assess its effects on cell viability (14).

Analysis of cell viability. Cell viability was analyzed using the Cell Counting Kit-8 (CCK-8) assay (Beijing Solarbio Science & Technology Co., Ltd.). Briefly, following MES23.5 cell treatment with tilianin and MPP⁺ (300 μmol/l) in a 96-well plate (2x10⁴ cells/well) at 37°C for 24, 48 and 72 h, the viability of the cells was determined using 10 μl CCK-8 assay for 2 h according to the manufacturer's protocol. The optical density at a wavelength of 450 nm was quantified using a microplate reader.

Immunofluorescence (IF) staining. Tyrosine hydroxylase (TH) expression was detected via IF staining. MES23.5 cells (1x10⁵ cells/well) were fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.5% Triton X-100 and then blocked with 5% goat serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 30 min. Following incubation with mouse anti-TH primary antibody (1:250; cat. no. ab137869; Abcam) at 4°C overnight, cells were further incubated with Alexa Fluor 350-conjugated goat anti-mouse secondary antibody (1:250; cat. no. A0412; Beyotime Institute of Biotechnology) for 15 min at 37°C. Subsequently, cells were washed three times with PBS and stained using 0.5 μg/ml DAPI (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 15 min. TH-positive cells were counted in three randomly selected visual fields using a fluorescence microscope (magnification, x200).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from MES23.5 cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The Hifair[®] III

1st Strand cDNA Synthesis SuperMix kit (Shanghai Yeasen Biotechnology Co., Ltd.) was used to reverse transcribe total RNA into cDNA using the reaction of at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min and 4°C for 5 min. qPCR was subsequently performed using a Hifair[®] III One Step RT-qPCR SYBR Green Kit (Shanghai Yeasen Biotechnology Co., Ltd.) using an ABI 7500 thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) The qPCR primers were as follows: TH forward, 5'-GCCGTCTCAGAGCAGGATAC-3' and reverse, 5'-ACCTCGAAGCGCACAAAGTA-3'; IL-6 forward, 5'-AAA GAGGCACTGGCAGAAAA-3' and reverse, 5'-CAGGGG TGGTTATTGCATCT-3'; IL-1β forward, 5'-TACGAATCT CCGACCACACTACAG-3' and reverse, 5'-TGGAGGTGG AGAGCTTTCAGTTCATATG-3'; TNF-α forward, 5'-AGG CACTCCCCAAAAGATG-3' and reverse, 5'-ATAGCAAAT CGGCTGACGGT-3' and GAPDH forward, 5'-CTACCCCA ATGTGTCCGTC-3' and reverse, 5'-GGCTCTCTTGCTCA GTGTC-3'. The following thermocycling conditions were used for qPCR: Pre-denaturation was performed at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 sec and annealing at 60°C for 30 sec, as well as elongation at 72°C for 10 min. After normalization using GAPDH as an internal standard gene, relative mRNA expression levels were quantified and analyzed using the 2^{-ΔΔC_q} method (15).

Western blotting. Total protein was extracted from MES23.5 cells using RIPA lysis buffer (Shanghai Yeasen Biotechnology Co., Ltd.). Protein quantification was performed using a BCA kit (Shanghai Yeasen Biotechnology Co., Ltd.). Total protein (20 μg protein/lane) was separated by SDS-PAGE on a 12% gel. The separated proteins were subsequently transferred onto a PVDF membrane and blocked with 5% skimmed milk for 1 h at room temperature. The membranes were incubated overnight at 4°C with primary antibodies against TH (cat. no. ab137869, 1:250; Abcam), manganese superoxide dismutase (MnSOD) (cat. no. ab68155, 1:1,000; Abcam), catalase (cat. no. ab209211, 1:2,000; Abcam), Bcl-2 (cat. no. ab182858, 1:2,000; Abcam), Bax (cat. no. ab32503, 1:1,000; Abcam), cleaved caspase-3 (cat. no. ab32042; 1:500; Abcam), phosphorylated (p)-ERK1/2 (cat. no. ab201015, 1:1,000; Abcam), ERK1/2 (cat. no. ab184699, 1:10,000; Abcam), p-p38 (cat. no. 4511, 1:1,000; Cell Signaling Technology, Inc.), p38 (cat. no. 8690, 1:1,000; Cell Signaling Technology, Inc.), p-JNK (cat. no. 4668, 1:1,000; Cell Signaling Technology, Inc.) and JNK (cat. no. 9252, 1:1,000; Cell Signaling Technology, Inc.). Subsequently, the membranes were incubated with mouse anti-rabbit IgG secondary antibodies conjugated to HRP (1:5,000; cat. no. sc-2357, Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Protein bands were visualized using Enhanced Chemiluminescence Detection Reagent (MilliporeSigma). ImageJ software Version 1.49 (National Institutes of Health) was used to analyze the chemiluminescent signals. Membranes were probed with anti-GAPDH antibody (Abcam, cat. no. ab9485, 1:2,500) as a loading control.

Analysis of oxidative stress. A Reactive Oxygen Species (ROS) Assay Kit (cat. no. C1300-1; Applygen Technologies, Inc.) was used to detect ROS production using a fluorescence microscope (magnification, x40) according to the manufacturer's protocol.

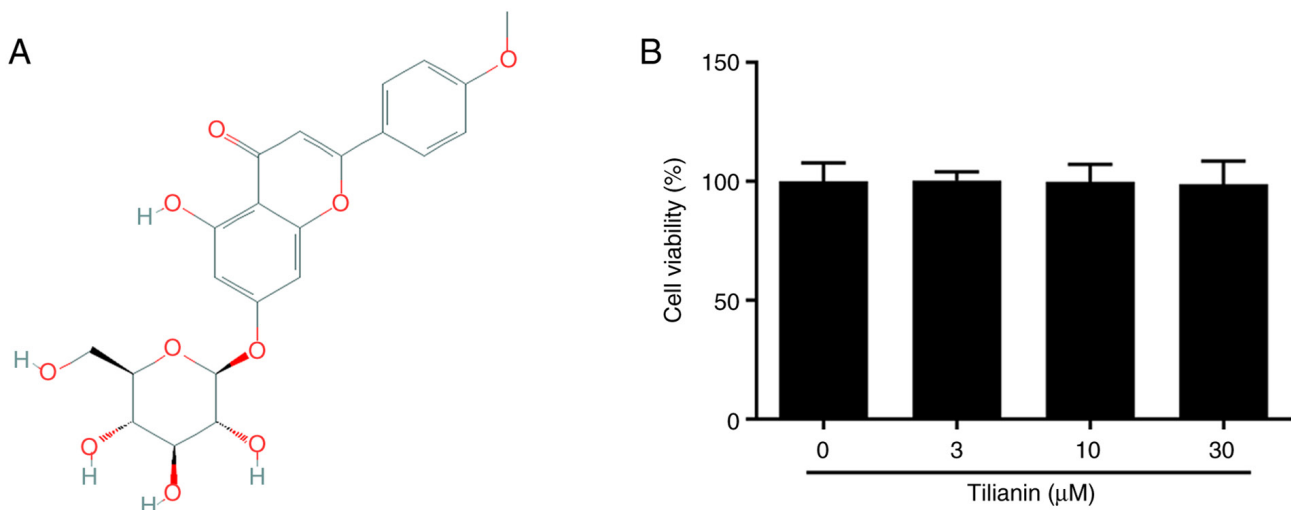


Figure 1. Effects of tilianin on the viability of MES23.5 cells. (A) Chemical structure of tilianin. (B) Effects of tilianin at different concentrations on cell viability were examined using the Cell Counting Kit-8 assay. Results are presented as the mean \pm SD.

Cell apoptosis assay. The TUNEL Apoptosis Detection (FITC) Kit (Shanghai Qcbio Science & Technologies Co., Ltd.) was used to observe the apoptotic rate of MES23.5 cells. Briefly, the cells were fixed with 4% paraformaldehyde at room temperature for 30 min and permeabilized with 0.1% Triton X-100 at room temperature for 3 min. The permeabilized samples were then treated with DNase I at 37°C for 30 min to prepare the positive control slides. Following incubation with 50 μ l TUNEL working solution at 37°C for 1 h in the dark, the slides were immersed in DAPI solution (2 μ g/ml, diluted with PBS) for 5 min at room temperature. The samples were sealed with VECTASHIELD® Antifade Mounting Medium (Vector Laboratories, Inc.; Maravai LifeSciences) and then five regions of apoptotic cells were randomly selected for viewing under a fluorescence microscope (magnification, $\times 20$). The green fluorescence at 520 ± 20 nm was observed using a standard filter. The blue fluorescence of DAPI was observed at 460 nm.

Statistical analysis. Data analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc.). Data are presented as the mean \pm SD. Each experiment was conducted in triplicate. Differences among multiple groups were analyzed using one-way ANOVA with a post hoc Bonferroni multiple comparison test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of tilianin on the viability of MES23.5 cells. The chemical structure of tilianin is shown in Fig. 1A. Cell viability analysis demonstrated that different concentrations of tilianin did not affect the viability of MES23.5 cells (Fig. 1B). Therefore, these results suggested that tilianin exerted no cytotoxic effects on MES23.5 cells.

Effect of tilianin on MPP⁺-induced loss of cell viability and TH deficiency. MPP⁺-stimulated MES23.5 cells exhibited reduced viability. However, pretreatment with tilianin improved the viability of MPP⁺-induced cells in a dose-dependent manner

(Fig. 2A). Moreover, the results of the IF analysis demonstrated that MPP⁺ decreased the TH protein expression levels in MES23.5 cells compared with those in the control group, whereas tilianin pretreatment increased the TH levels in MPP⁺-induced cells in a dose-dependent manner (Fig. 2B). Similar results were observed using RT-qPCR and western blotting (Fig. 2C and D). These results indicated that tilianin may effectively protect MES23.5 cells from MPP⁺-induced reduction in viability and TH deficiency.

Effect of tilianin on the MPP⁺-induced inflammatory response and oxidative stress. Further experiments revealed that MPP⁺ elevated the mRNA expression levels of the pro-inflammatory cytokines IL-6, IL-1 β and TNF- α in MES23.5 cells, whereas tilianin pretreatment reduced their mRNA expression levels in a dose-dependent manner (Fig. 3A-C). It was also observed that MPP⁺-induced ROS production decreased with increasing tilianin concentrations (Fig. 3D). Moreover, the protein expression levels of MnSOD and catalase were found to be increased in MPP⁺-stimulated cells, whereas these expression levels were decreased in the tilianin pretreatment groups (Fig. 3E). These results demonstrated that tilianin may ameliorate the MPP⁺-induced inflammatory response and oxidative stress in MES23.5 cells.

Effect of tilianin on MPP⁺-induced cell apoptosis. As shown in Fig. 4A and B, an increase in the number of TUNEL-positive (apoptotic) MES23.5 cells was observed in the MPP⁺ group, whereas the number of apoptotic cells was reduced by tilianin pretreatment in a dose-dependent manner. Furthermore, the protein expression levels of the antiapoptotic gene Bcl-2 were shown to be downregulated by MPP⁺ stimulation, whereas tilianin pretreatment upregulated Bcl-2 expression levels in MPP⁺-stimulated MES23.5 cells (Fig. 4C). By contrast, the protein expression levels of the apoptosis markers Bax and cleaved caspase-3 were upregulated in MPP⁺-stimulated MES23.5 cells, but were downregulated following tilianin pretreatment. Therefore, these results indicated that tilianin may prevent the MPP⁺-induced apoptosis of MES23.5 cells.

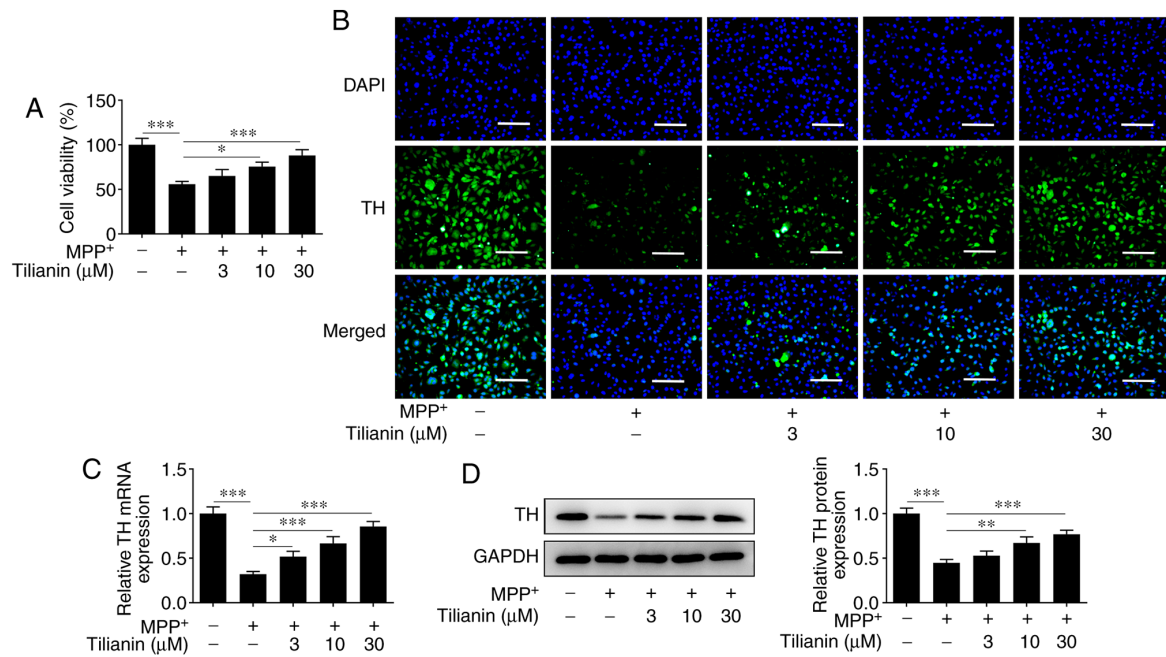


Figure 2. Effects of tilianin on MPP⁺-induced reduction in cell viability and TH deficiency. (A) Effect of tilianin at different concentrations on the viability of cells stimulated with MPP⁺ was examined using the Cell Counting Kit-8 assay. (B) Immunofluorescence assay of TH expression. Scale bar, 100 μm. TH expression was detected by (C) reverse transcription-quantitative PCR and (D) western blotting. Results are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. TH, tyrosine hydroxylase; MPP⁺, 1-methyl-4-phenylpyridinium.

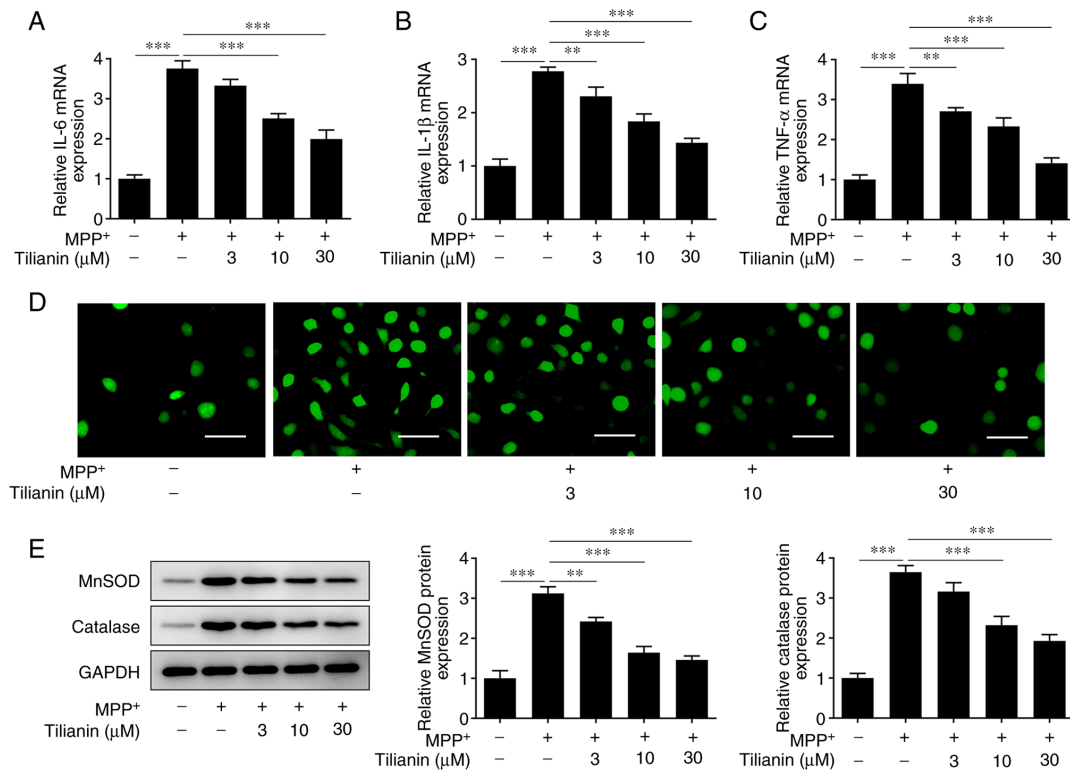


Figure 3. Effects of tilianin on MPP⁺-induced inflammatory response and oxidative stress. (A) IL-6, (B) IL-1β and (C) TNF-α expression in cells stimulated with MPP⁺, with or without tilianin preconditioning, was detected by reverse transcription-quantitative PCR. (D) Reactive oxygen species levels were detected using a commercial assay kit. Scale bar, 50 μm. (E) Western blot analysis of MnSOD and catalase expression levels. Results are presented as the mean ± SD. **P<0.01, ***P<0.001. MPP⁺, 1-methyl-4-phenylpyridinium; MnSOD, manganese superoxide dismutase.

Effect of tilianin on the MAPK signaling pathway in MPP⁺-stimulated MES23.5 cells. Western blotting demonstrated that the expression levels of MAPK signaling

pathway-related proteins (p-p38, p-ERK1/2 and p-JNK) were high in MPP⁺-stimulated MES23.5 cells, and these protein expression levels were downregulated in a dose-dependent

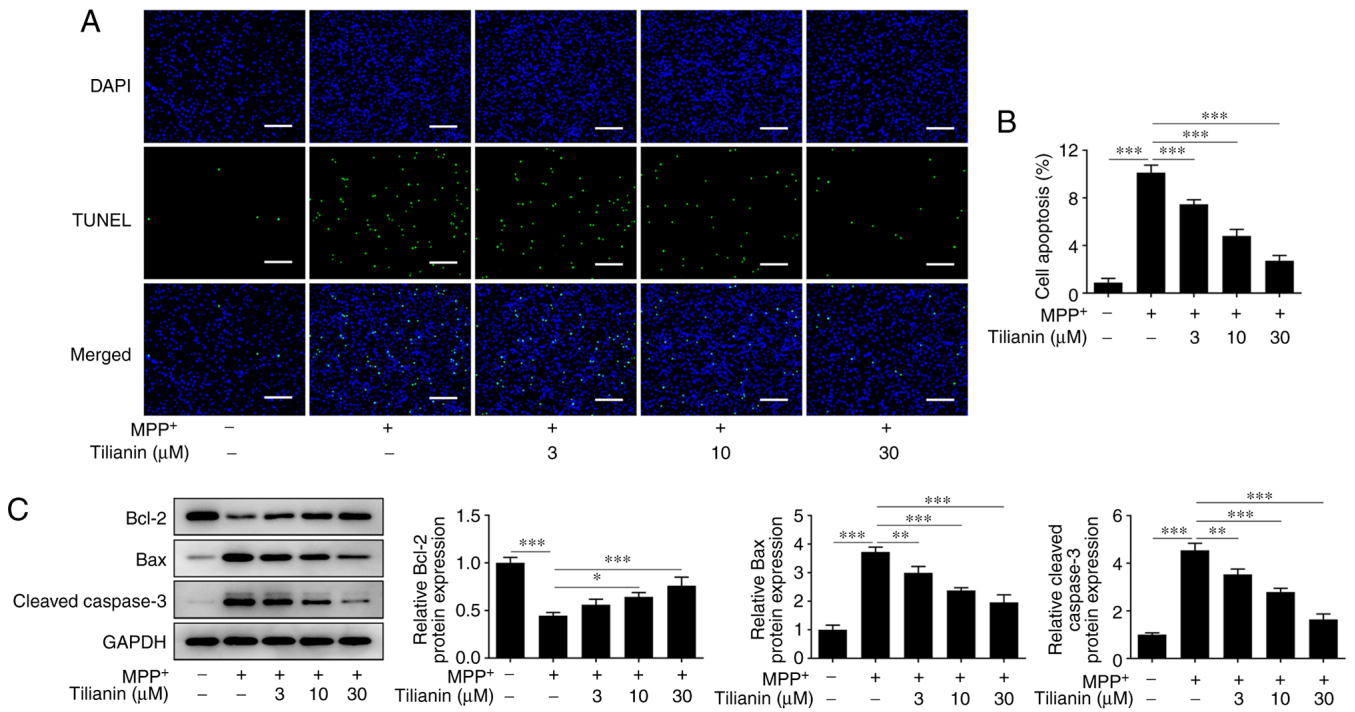


Figure 4. Effects of tilianin on MPP⁺-induced cell apoptosis. (A and B) TUNEL assay of cell apoptosis in untreated cells, and in cells treated with MPP⁺ or MPP⁺ and different concentrations of tilianin. Scale bar, 100 μm. (C) Western blot analysis of the expression levels of Bcl-2, Bax and cleaved caspase-3. Results are presented as the mean ± SD. *P<0.05, **P<0.01, ***P<0.001. MPP⁺, 1-methyl-4-phenylpyridinium.

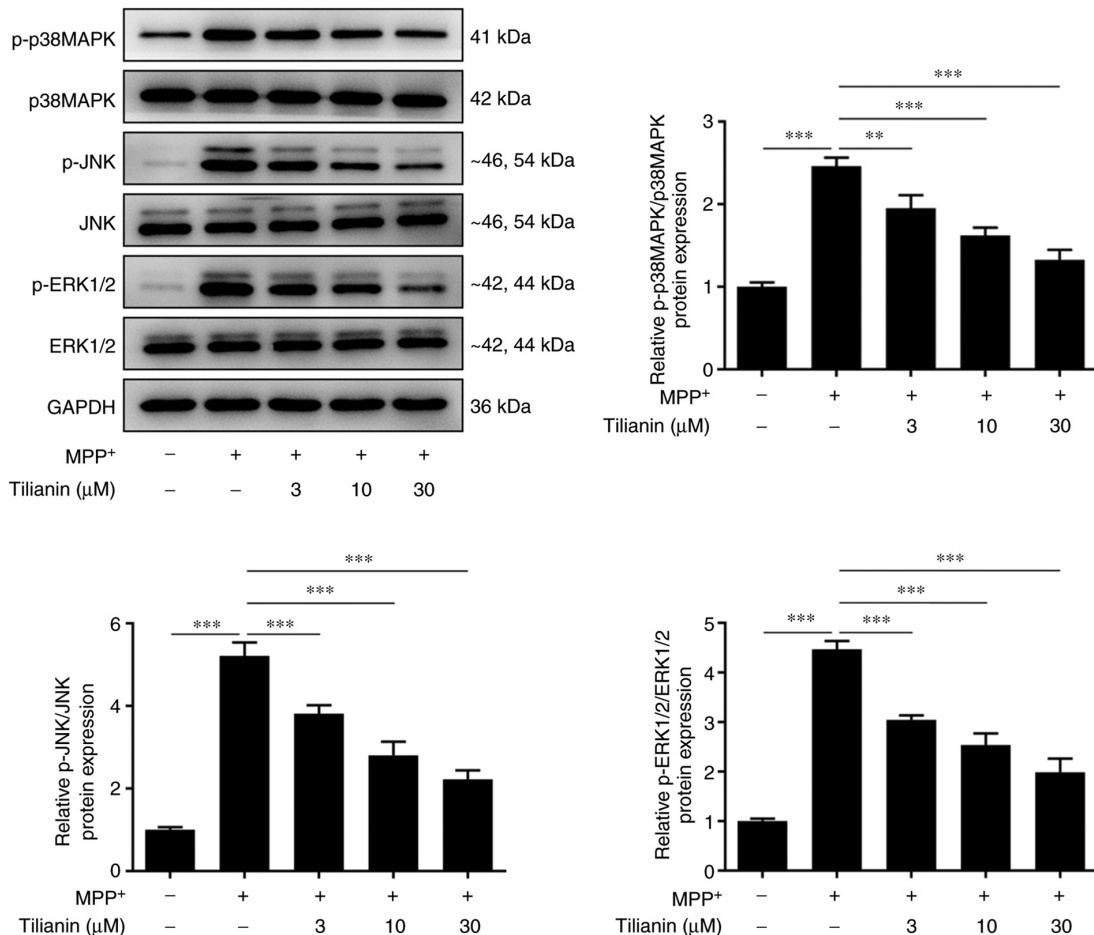


Figure 5. Effects of tilianin on the MAPK pathway in MPP⁺-stimulated MES23.5 cells. The expression levels of p-p38MAPK, p38MAPK, p-ERK1/2, ERK1/2, p-JNK and JNK in cells stimulated with MPP⁺ with or without tilianin preconditioning were detected by western blotting. Results are presented as the mean ± SD. **P<0.01, ***P<0.001. MPP⁺, 1-methyl-4-phenylpyridinium; p, phosphorylated.

manner when cells were pretreated with tilianin (Fig. 5). These results suggested that tilianin may inhibit MPP⁺-induced activation of the p38, ERK1/2 and JNK signaling pathways in MES23.5 cells.

Discussion

The main pathological manifestations of PD are the degeneration and loss of dopaminergic neurons in the substantia nigra compacta in the midbrain; the formation of intraneuronal inclusions, known as Lewy bodies, in the residual dopamine neurons; and the appearance of dystrophic synapses (16-18). The exact etiology of the degeneration and death of dopaminergic neurons in PD remains unclear, but may involve numerous factors, including genetics, environmental factors, aging and oxidative stress (19,20). The exacerbation of oxidative stress is considered to be an important contributor to dopaminergic neuron injury in the pathogenesis of PD (21,22).

Oxidative stress signaling pathways can transmit messages outside of cells and, therefore, cells rely on internal signaling pathways to transduce signals intracellularly (23). As a downstream target of oxidative stress, the MAPK signaling pathway transmits extracellular oxidative stress signals inside the cell (24). The MAPK family is a group of conserved serine/threonine protein kinases, which are major signaling molecules in signal transduction and can form signaling pathways that are crucial for signal transmission networks in eukaryotic cells (25). Among MAPK family members, the ERK1/2 signal transduction pathway regulates cell proliferation and differentiation (26), whereas JNK and p38, collectively named the MAPK stress signaling pathway, serve an important role in stress responses, such as inflammation and apoptosis (27).

Tilianin, the main active component in *D. moldavica* L., acts as a cardioprotective agent in myocardial ischemia/reperfusion injury in rats via improving mitochondrial dysfunction, inhibiting oxidative stress and, thereby, alleviating cardiomyocyte apoptosis (28). Previous studies have reported the role of tilianin against oxidative stress in cerebral ischemia/reperfusion injury through inhibiting p38 expression, whereas in tracheal epithelial cells tilianin has been demonstrated to inhibit the ERK signaling pathway, which also reduces oxidative stress (13,14). In addition, Jiang *et al* (29) reported that tilianin ameliorated memory impairment and neurodegeneration by inhibition of neuronal apoptosis and inflammation in the hippocampus of rats with permanent occlusion of the bilateral common carotid artery via increasing p-CaMKII/ERK/CREB signal transduction. In addition, a previous study has demonstrated that tilianin exhibits low cytotoxicity (30). In the present study, different concentrations of tilianin did not affect the viability of MES23.5 cells, whereas tilianin pretreatment improved the viability in MPP⁺-stimulated MES23.5 cells in a dose-dependent manner. All tilianin pretreatment groups exhibited lower pro-inflammatory cytokine mRNA expression levels, and downregulated ROS levels and MnSOD and catalase protein expression levels. MPP⁺-induced apoptosis of dopaminergic neurons was also effectively alleviated by tilianin in a dose-dependent manner. Furthermore, a dose-dependent decline in the expression of MAPK signaling pathway-related proteins (p-p38, p-ERK1/2 and p-JNK) was

observed in MPP⁺-stimulated MES23.5 cells following tilianin preconditioning.

Dopaminergic neuron injury leading to dysfunctional dopamine synthesis is central to the onset of PD (31). TH, a rate-limiting enzyme in dopamine biosynthesis that is mainly expressed in the brain and the adrenal gland, is used by neurons to synthesize dopamine following ingestion of tyrosine (32,33). Overall, the function and expression of TH are important in dopamine synthesis. In the present study, TH mRNA and protein expression levels were decreased following MPP⁺ stimulation; however, tilianin preconditioning significantly enhanced the mRNA and protein expression levels of TH in MPP⁺-stimulated MES23.5 cells. This result suggested that tilianin may prevent the degradation of TH in PD. The present study mainly explored the effects of tilianin on PD *in vitro* and the results revealed that tilianin attenuated MPP⁺-induced oxidative stress and apoptosis of dopaminergic neurons in a cellular model of PD. However, the effects of tilianin on animals or humans with PD were not investigated, and the protective role of tilianin in PD *in vivo* will be further investigated and verified in future studies.

In conclusion, the data in the present study demonstrated that tilianin may serve an anti-inflammatory and antioxidant role by inhibiting the MAPK signaling pathway, which may suppress dopaminergic neuron injury in PD. Therefore, tilianin may hold promise as a novel therapeutic agent in the treatment of PD. However, data from further *in vivo* experiments and clinical trials are needed to support the conclusions of the present study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL and SX designed the study, performed the experiments and drafted and revised the manuscript. JL analyzed the data and SX performed the literature search. Both authors confirmed the authenticity of the raw data and have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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