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Humulus japonicus extract alleviates oxidative stress and apoptosis in 6−hydroxydopamine−induced PC12 cells

Feng Wang[1,3], Byoung Ok Cho[2,4], Jae Young Shin[2], Suping Hao[1], Seon Il Jang[1,2]✉

[1]*Department of Health Management, Jeonju University, Jeonju 55069, Korea*

[2]*Research Institute, Ato Q&A Co, LTD, Cheonjam−ro, Wansan−gu, Jeonju 55069, Korea*

[3]*Department of Life Sciences, Yuncheng University, Yuncheng, Shanxi Province 044000, PR China*

[4]*Institute of Health & Science, Jeonju University, Jeonju 55069, Korea*

ABSTRACT

Objective: To explore the possible neuroprotective activities of *Humulus japonicus* extract against Parkinson's disease (PD) in a cellular model.

Methods: PD was modeled in PC12 cells using 6-hydroxydopamine (6-OHDA). The cell activity, intracellular levels of reactive oxygen species (ROS), anti-oxidative and anti-apoptotic effects, and other related indicators and related signaling pathways were evaluated to elucidate the neuroprotective effects of *Humulus japonicus* extract.

Results: *Humulus japonicus* extract exhibited anti-oxidative and anti-apoptotic effects in 6-OHDA-stimulated PC12 cells. It also reduced oxidative stress-induced ROS accumulation; upregulated antioxidant enzymes, such as glutathione, catalase, heme oxidase-1, and 8-oxguanine glycosylase 1; promoted cell survival by decreasing BAX and increasing Bcl-2 and sirtuin 1 expression *via* the MAPK and/or Nrf2 signaling pathways.

Conclusions: *Humulus japonicus* extract has antioxidative and anti-apoptotic effects and could be developed as a promising candidate for preventing and treating oxidative stress-related neurodegenerative diseases.

KEYWORDS: *Humulus japonicus*; Parkinson's disease; Oxidative stress; Apoptosis; MAPK; PC12 cells; Nrf2

1. Introduction

The pathogenesis of Parkinson's disease (PD), a well-known neurodegenerative disease includes mitochondrial dysfunction, neuronal cell apoptosis, and oxidative stress[1−3]. Continuous oxidative stress can result in neuronal damage mediated by the excessive generation of reactive oxygen species (ROS). The harmful effects of ROS in the brain are related to the rich transition metal ions and polyunsaturated fatty acids, and the high metabolic rate of easily oxidized and excitatory neurotransmitters, such as glutamate and dopamine, which sensitize the brain cells to ROS-mediated damages[4]. It is reported that oxidative damage and apoptosis induced by 6-hydroxydopamine (6-OHDA) toxicity is a result of the MAPK signaling pathway activation[1,5].

Humulus japonicus (*H. japonicus*), an annual herb of the Cannabaceae family, is generally distributed in Asian countries, including China, Japan, and Korea. The anti-inflammatory, antitumor,

Significance

Humulus japonicus, a medicinal plant rich in bioactive constituents, is widely distributed in Asia. There are many reports on structure characterization, antibacterial, anti-inflammatory, and anti-aging effects of this plant extract. However, the effect of *Humulus japonicus* on neurodegenerative diseases is less reported. The present study showed that *Humulus japonicus* extract has neuroprotective effects in 6-hydroxydopamine-stimulated neurons cells. It may be further explored using additional cellular models and animal models for their protective activities in oxidative stress-related neurodegenerative diseases.

✉To whom correspondence may be addressed. E-mail: sonjjang@jj.ac.kr

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antibacterial, and antimicrobial properties of *H. japonicus* extracts have been previously reported[6-8]. In addition, *H. japonicus* exhibited protective effects against aging and neurodegenerative diseases, such as Alzheimer's disease[9,10]. In particular, *H. japonicus* extracts can effectively scavenge ROS, such as superoxide radicals, hydroxyl radicals, together with hydrogen peroxide[7,8,10]. Recent research has indicated that *H. japonicus* extract is an excellent natural source of antioxidants, which may help prevent or delay the progression of PD; the methanol extract of 100% *H. japonicus* significantly attenuated 6-OHDA-induced mitochondrial apoptosis in the SH-SY5Y cells, increased glutathione (GSH) levels, and decreased extracellular signal-regulated protein kinase 1 and 2 phosphorylation[11]. There have been many reports on the identification of the main active substances and chemical structure of *H. japonicus* extracts[7,10,11], and the main bioactive components were luteolin, luteolin-7-glucoside, luteolin-7-glycoside, apigenin, and quercetin, thus luteolin was used as a reference in this study. Furthermore, the main pathological feature of PD is substantial nigra-striatum dopaminergic neuronal dysfunction, which causes insufficient dopamine release, induces motor symptoms, and is accompanied by non-motor symptoms. As a selective catecholaminergic neurotoxin, 6-OHDA can induce degradation of dopaminergic and noradrenergic nerve endings, increase oxidative stress, and microglia activation, which is widely used in the production of Parkinson's models. However, the effect of *H. japonicus* extracts on 6-OHDA-induced PD in PC12 cells has not been clarified. Therefore, in our paper, we aimed at exploring the effect of 80% ethanolic *H. japonicus* extract on cytotoxicity, oxidative stress, apoptosis, and signaling pathways in 6-OHDA-induced PC12 neuronal cells and elucidating the underlying mechanism of its effect.

2. Materials and methods

2.1. Reagents

DoGenBio (Seoul, South Korea) provided the EZ-western Lumi Pico Alpha together with EZ-Cytox reagent assay kits. Roswell park memorial institute 1640 medium, fetal bovine serum, as well as horse serum were acquired from Gibco (Gibco, Grand Island, NY, USA). Penicillin and streptomycin were offered *via* Invitrogen (Carlsbad, CA, USA). The polyvinylidene fluoride membranes and the loading buffer for SDS-PAGE were acquired from Millipore (Burlington, MA, USA) and Biosesang (Seongnam, South Korea), respectively. Furthermore, Sigma-Aldrich (St. Louis, MO, USA) provided 6-OHDA, dimethyl sulfoxide, luteolin (purity ≥ 98%), protease inhibitors, and phosphatase inhibitors. Radio-immunoprecipitation assay buffer (RIPA buffer) and nuclear and cytoplasmic extraction reagents for NE-PER were obtained from Thermo Fisher Scientific (Rockford, IL, USA). Biosciences (Franklin Lakes, NJ, USA) and Bio-Rad (Hercules, CA, USA) respectively provided the Bio-Rad Protein Assay and Actin antibody. Primary antibodies against p-JNK, JNK, p-p38, p38, p-ERK, ERK, Nrf2, heme oxidase 1 (HO-1), 8-oxguanine glycosylase 1 (OGG1), BAX, Bcl-2, and sirtuin-1 (SIRT1), together with the secondary antibodies combined with HRP-IgG, could be acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell Signaling Technology Inc. (Beverly, MA, USA) offered the lamin B1. The GSH and catalase (CAT) assay kits (Cat# 707002 and 703002) were bought from Cayman Chemical (Ann Arbor, MI, USA). The probe of carboxy-H2DCFDA was obtained from Invitrogen.

2.2. Preparation of H. japonicus extracts

H. japonicus leaves employed in the current study were collected from Cheonjam Hill, Wansan-Gu, Jeoju-shi, Jeonbuk, Korea on June 25, 2020. The plant was certified and subsequently identified through Hong-Jun Kim, a professor at College of Oriental Medicine, Woosuk University (Wanju, Korea). The voucher sample (#2020-06-25-HJ) was stored in our laboratory. The leaves were cleaned using distilled water, dried for 16 h at 60 ℃, and then seeded (50 g) with 2 000 mL of 80% (*v/v*) ethanol in a 40 ℃ water bath for 2 d. The extract was filtered using a filter paper (ADVANTEC, Togo, Japan) with 0.45-mm pore size. After filtration, the extract was concentrated in a vacuum at 45 ℃, and ethanol was removed. The remaining solution was freeze-dried at −55 ℃ in a freeze dryer to acquire the dry powder, which was maintained at −20 ℃ until use in the subsequent studies.

2.3. Cell culture

The PC12 cell line (ATCC® CRL1721™) was acquired from rat pheochromocytoma, which was provided by ATCC (Manassas, USA). PC12 cells were treated with the nerve growth factor (4 ng/mL) to induce differentiation. PC12 cells were cultivated with 5% (*v/v*) fetal bovine serum, 10% (*v/v*) horse serum, 100 μg/mL streptomycin, and 100 units/mL penicillin under a temperature of 37 ℃ with 5% CO_2 humidified air. The media were replaced every other day, and the above cells were cultured for 7 d, during which time they diffused to a proper density (about 75%-80%).

2.4. Cell viability assay

The water-soluble tetrazolium salt method was employed to detect the cytotoxicity of *H. japonicus* extracts and 6-OHDA in the PC12 cells and determine the appropriate concentrations of *H. japonicus* extracts and 6-OHDA for subsequent experiments. Briefly, 2.5×10^5 cells/mL of PC12 cells were inoculated into the plates (96-well), cultivated for 1 d, and then treated with varying concentrations of *H. japonicus* extracts (up to 200 μg/mL) and 6-OHDA (up to 500 μM) for 20 h. Then, each well was added with EZ-Cytox (0.01 mL) and cultivated for an additional 4 h. At 450 nm, the absorbance was

detected by a microplate reader (Tecan, Männedorf, Switzerland). The cell survival rate was measured by comparing the optical density of the living cells in the untreated control with those in the treated cells.

2.5. Detection of intracellular ROS levels

In brief, 2×10^5 cells/mL of PC12 cells were cultivated in the plates (6-well) for 1 d and then pretreated with 25 and 50 μg/mL of *H. japonicus* extracts and 30 μM luteolin for 60 min. Cells were subsequently stimulated by 125 μM 6-OHDA and harvested 1 h later. Next, cells (1×10^6 cells/mL) were suspended in pre-warmed Dulbecco`s phosphate-buffered saline (DPBS) solution containing 5 μM H_2DCFDA and cultivated for 15 min in darkness at 37 ℃. The cells were then centrifuged at 130 ×*g* for 5 min, followed by supernatant removal and resuspension of cells in pre-warmed DPBS. This process was repeated twice to wash the cells. CytoFlex Beckman coulter flow cytometer (Brea, CA, USA) was utilized to analyze ROS levels, after excitation by a laser at 488 nm and measurement at 535 nm.

2.6. Intracellular antioxidant enzyme activity

Briefly, PC12 cells (2.5×10^5 cells/mL) were cultured in plates (6-well) for 16 h, pretreated with or without 25 and 50 μg/mL of *H. japonicus* extracts and 30 μM luteolin for 1 h, and subsequently treated with 6-OHDA (125 μM) for 24 h. Specific kits were employed to analyze the CAT and GSH activities according to the manufacturer's instructions (Cayman Chemical, USA).

2.7. Whole protein extraction and Western blotting assay

Briefly, PC 12 cells (5×10^5 cells/mL) were cultured in the culture plates (90 mm × 20 mm) for 1 d. The cells were then treated with 25 and 50 μg/mL of *H. japonicus* extracts and 30 μM luteolin and incubated for 60 min. Then, 125 μM of 6-OHDA was added to stimulate the cells for 30 min or 24 h. Whole-cell lysates corresponding to each treatment were prepared in RIPA buffer supplemented with phosphatase and protease inhibitors, which were subsequently centrifuged at 14000 ×*g* for 15 min to obtain the supernatants involving the overall protein extract. After quantitative analysis of proteins utilizing the Bio-Rad protein detection assay, each sample was subjected to 12% or 10% SDS-PAGE (90 min, 110 V). The proteins were isolated and then transferred to a membrane of polyvinyl fluoride (1 h, 100V). Then, the membrane was cultivated in 5% of bovine serum albumin (GenDEPOT Corporation, USA) for 60 min and subsequently incubated with various primary antibodies including p-JNK (SC-293136, dilution 1:500), JNK (SC-7345, dilution 1:500), p-p38 (SC-166182, dilution 1:500), p38 (SC-81621, dilution 1:500), p-ERK1/2 (SC-81492, dilution 1:500), ERK1/2 (SC-135900, dilution 1:500), HO-1 (ab13243, dilution 1:500), OGG1/2 (SC-376935, dilution 1:1 000), BAX (SC-7480, dilution 1:200), β-actin (SC-47778, dilution 1:1 000) and Bcl-2 (SC-492, dilution 1:200) at 4 ℃ overnight. After the above incubation, Tris-buffered saline involving 0.01 percent of Tween-20 (TBST) was utilized to wash membranes thrice (5 min each time), which were incubated deeply through the corresponding HRP-combined secondary antibody (m-IgGκ BP-HRP, SC-56102, dilution 1:5 000) at 20 ℃ for 120 min. The membranes were subsequently cleaned five times utilizing TBST (5 min each), and the chemiluminescence reagent of an EZ-western Lumi Pico Alpha kit together with an imaging system (Alliance version 15.11; UVITEC Cambridge, UK) was employed to acquire the images. In this study, a stripping buffer was employed, which allows the existence of multiple proteins on one membrane. ImageJ version 1.52 (National Institutes of Health, Bethesda, MD, USA) was applied for determining the band density. To ensure that the protein could be calculated in accordance with the same standard, the membranes were isolated to produce anti-β-actin antibodies and calibrated applying the protocol mentioned previously.

2.8. Extraction of cytosol and nuclear protein and Western blotting assay

Briefly, PC 12 cells (5×10^5 cells/mL) were cultivated in plates (90 mm×20 mm) for 24 h, treated with luteolin (30 μM) and 25 and 50 μg/mL of *H. japonicus* extracts, and then incubated for 1 h. Subsequently, 6-OHDA (125 μM) was added, and the cells were stimulated for 30 min. Based on the guidelines of the manufacturer, the cytoplasmic and nuclear extraction reagents of NE-PER™ were employed for the extraction of cytoplasmic and nuclear proteins from the cells under each treatment condition. In brief, the cells were gathered, suspended in cold DPBS, which was ultimately centrifuged at 500 ×*g* for 3 min to obtain cell pellets. DPBS was then removed, and 0.1 mL reagent of ice-CER I was added. The suspension was then vigorously shaken and cultured for 10 min on ice. Next, 0.0055 mL reagent of CER Ⅱ was added, and the acquiring suspension was shaken and then cultivated for 1 min on ice. The suspension of cell was centrifuged at 16000 ×*g* for 5 min, and the supernatant involving cytoplasmic protein was immediately transferred into a fresh test tube and kept under a temperature of −80 ℃. After centrifugation, the insoluble precipitate was resuspended in 0.05 mL reagent of ice-cold NER including phosphatase and protease inhibitors, incubated for 40 min on ice, and shaken every 10 min for 15 s. The supernatant containing nuclear extracts was subsequently centrifuged at 16000 ×*g* for 10 min and transferred immediately into a new pre-chilled tube, which was next maintained at −80 ℃ until use. Protein quantification and Western blotting were performed as described in Section 2.7, and the primary antibodies including SIRT1 (SC-74465, dilution 1:500), Nrf2 (SC-365949, dilution 1:500), β-actin (SC-47778, dilution

1:1000), and lamin B1 (Rabbit mAb#12586, dilution 1:1000) were used. The second antibodies were mouse-IgGκ BP-HRP (SC-56102, dilution 1:5000) and mouse anti-rabbit IgG-HRP (SC-2357 dilution 1:5000).

2.9. Statistical analysis

The SPSS program (version 22 SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The data were expressed as mean ± SD. Statistically significant differences between groups were determined by one-way ANOVA, followed by *post-hoc* test. A *P*-value less than 0.05 was considered significantly different.

3. Results

3.1. Neuroprotective effect in 6-OHDA-stimulated PC12 cells

The cytotoxic activities of various concentrations of *H. japonicus* extracts were evaluated after 24 h of treatment. At concentrations between 0 and 100 μg/mL, cell viability was slightly reduced, but there was no cytotoxicity (Figure 1A). Compared with the control group, 125, 250, and 500 μM 6-OHDA treatment significantly reduced cell viability (Figure 1B). *H. japonicus* extracts at 25 and 50 μg/mL evidently enhanced cell viability in 6-OHDA-stimulated cells (Figure 1C).

3.2. Intracellular ROS level in 6-OHDA-stimulated PC12 cells

The level of ROS in 6-OHDA-stimulated cells was increased by 324.44% when compared with the control. *H. japonicus* extracts were able to reduce 6-OHDA-stimulated ROS generation in PC12 cells by 28.60% and 59.71%, respectively ($P < 0.05$) (Figure 2A). These results indicate that the antioxidant activity of *H. japonicus* extracts can reduce oxidative stress in 6-OHDA-stimulated PC12 cells and protect against oxidative stress-induced neuronal injury.

3.3. GSH and CAT activity in 6-OHDA-stimulated PC12 cells

The levels of CAT and GSH were reduced to 92.55% and 43.15% of the control values, respectively after treatment with 6-OHDA

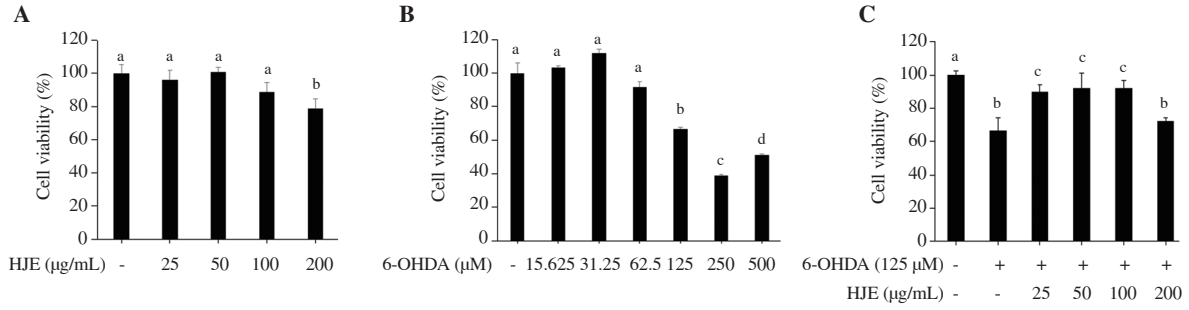

Figure 1. The effect of *Humulus japonicus* extract (HJE) on viability of PC12 cells. The data are expressed as mean ± SD ($n = 3$) and analyzed by one-way ANOVA followed by *post-hoc* test. Different letters indicate significant difference at $P < 0.05$.

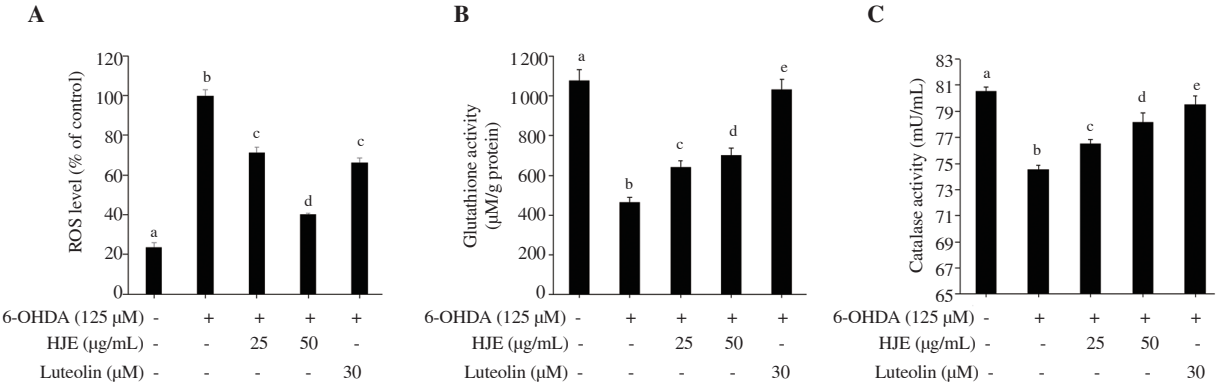

Figure 2. The effect of HJE on (A) intracellular ROS level, (B) glutathione, and (C) catalase in 6-OHDA-stimulated PC12 cells. The data are expressed as mean ± SD ($n = 3$) and analyzed by one-way ANOVA followed by *post-hoc* test. Different letters indicate significant difference at $P < 0.05$.

alone. Nevertheless, pretreatment with 25 and 50 μg/mL *H. japonicus* extracts increased CAT and GSH levels to 95.03%, 97.10%, 59.48%, and 65.21% of control values, respectively in 6-OHDA-stimulated cells ($P < 0.05$) (Figure 2B and 2C).

3.4. OGG1 and HO-1 expression in 6-OHDA-stimulated PC12 cells

Our results indicated that compared with untreated cells, 6-OHDA markedly decreased HO-1 and OGG1 expression ($P < 0.05$), while 25 and 50 μg/mL *H. japonicus* extracts significantly increased HO-1 and OGG1 expression, which was similar to that of luteolin treatment in 6-OHDA-induced cells ($P < 0.05$) (Figure 3A and 3B).

3.5. Expression of BAX, Bcl-2, and BAX/Bcl-2 ratio in 6-OHDA-stimulated PC12 cells treated with H. japonicus extracts

As shown in Figure 4A, the BAX expression was increased remarkably after 6-OHDA stimulation for 24 h ($P < 0.05$). Nevertheless, 30 μM luteolin and 25 and 50 μg/mL *H. japonicus* extracts pronouncedly reduced 6-OHDA-stimulated BAX expression ($P < 0.05$). In addition, 6-OHDA downregulated the Bcl-2 expression significantly in PC12 cells. However, 30 μM luteolin and 25 and 50 μg/mL *H. japonicus* extracts reversed the 6-OHDA-induced changes ($P < 0.05$) (Figure 4B). As illustrated in Figure 4C, BAX/Bcl-2 was upregulated evidently after 6-OHDA stimulation ($P < 0.05$). By

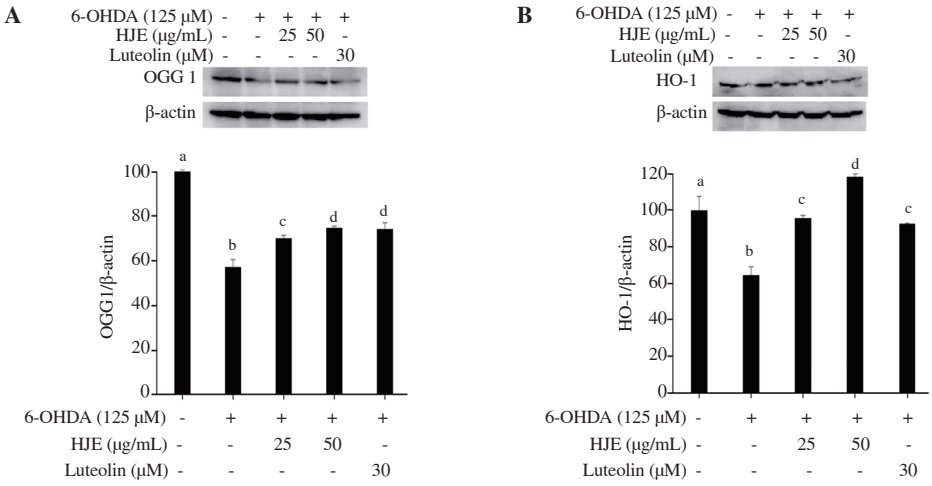

Figure 3. The effect of HJE on the protein expression of (A) OGG1 and (B) HO-1 in 6-OHDA-stimulated PC12 cells. The protein expression levels were determined by Western blotting assay and the band densities were analyzed using ImageJ analysis software. The data are expressed as mean ± SD ($n = 3$) and analyzed by one-way ANOVA followed by *post-hoc* test. Different letters indicate significant difference at $P < 0.05$. HO-1: heme oxidase-1; OGG1: 8-oxguanine glycosylase 1.

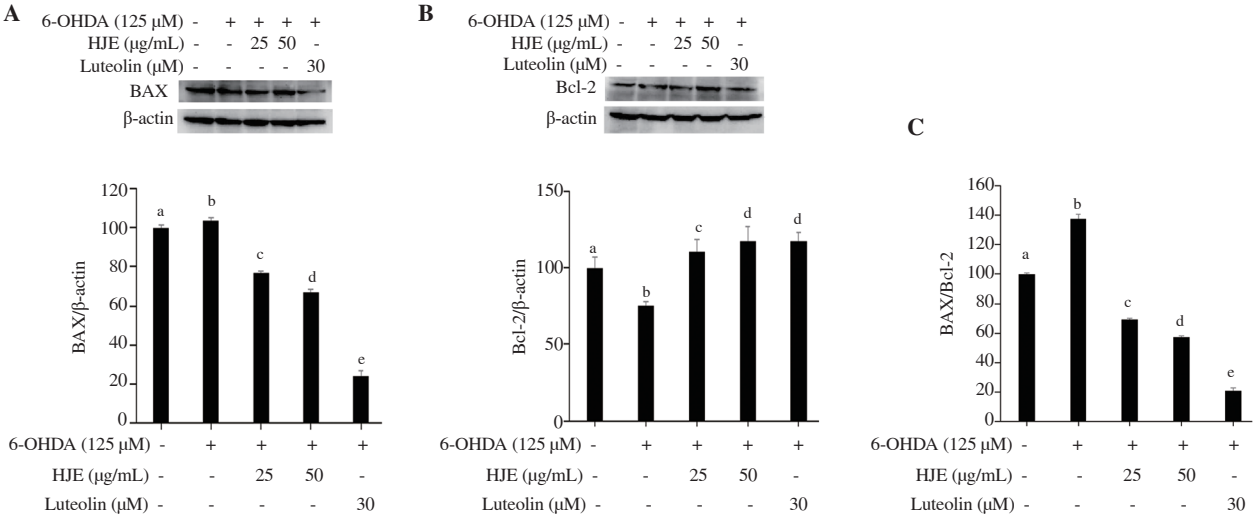

Figure 4. The effects of HJE on (A) BAX, (B) Bcl-2, and (C) BAX/Bcl-2 expression levels in 6-OHDA-stimulated PC12 cells. The protein expression levels were determined by Western blotting assay and the band densities were analyzed using ImageJ analysis software. The data are expressed as mean ± SD ($n = 3$) and analyzed by one-way ANOVA followed by *post-hoc* test. Different letters indicate significant difference at $P < 0.05$.

comparison, BAX/Bcl-2 ratio was obviously downregulated in the cells pretreated with 30 μM luteolin or 25 and 50 μg/mL *H. japonicus* extracts.

3.6. Effect of H. japonicus extracts on the MAPK signaling pathway in 6-OHDA-stimulated PC12 cells

To explore the anti-apoptotic mechanism of *H. japonicus* extracts, we measured the regulatory effect of *H. japonicus* extracts on the MAPK signaling pathway. As illustrated in Figure 5, treatment with 6-OHDA (125 μM) facilitated the ERK, JNK, and p38 phosphorylation to p-ERK, p-JNK, and p-p38, respectively. However, pretreatment with *H. japonicus* extracts exhibited a remarkable decrease in the p-ERK, p-JNK, and p-p38 expression ($P < 0.05$).

3.7. Effect of H. japonicus extracts on the Nrf2 signaling pathway in 6-OHDA-stimulated PC12 cells

The cytosolic Nrf2 level was significantly decreased, whereas the level of nuclear Nrf2 was evidently enhanced in 6-OHDA-stimulated cells. Pretreatment with 25 and 50 μg/mL *H. japonicus* extracts remarkably upregulated the nuclear and cytosolic Nrf2 levels (Figure 6A and 6B).

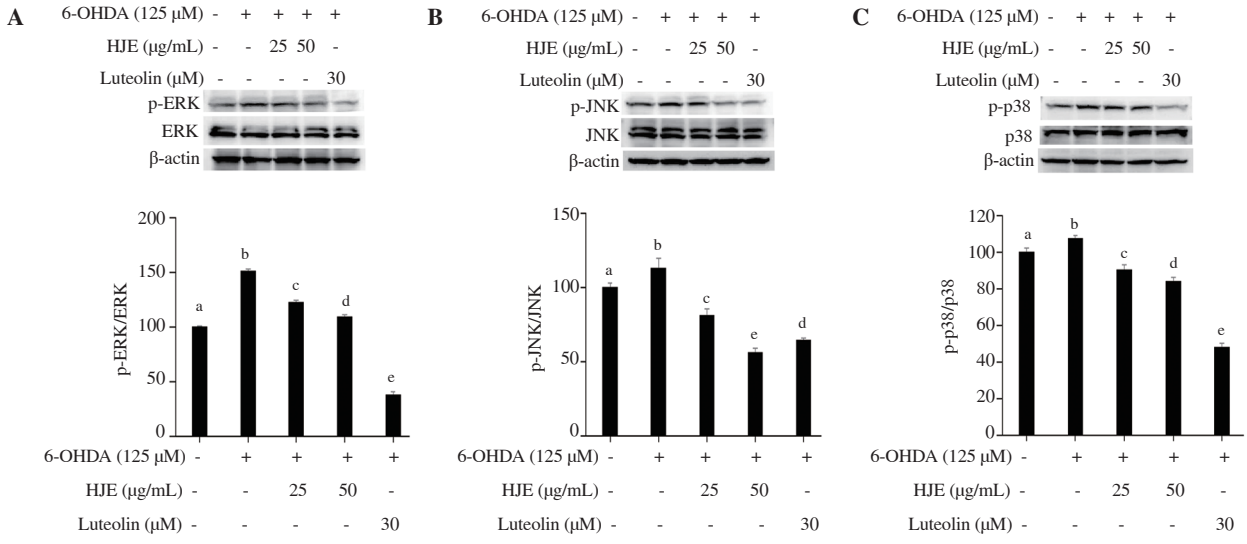

Figure 5. The effect of HJE on the MAPK signaling pathways in 6-OHDA-stimulated PC12 cells. The protein expression levels of (A) ERK, (B) JNK, and (C) p38 were determined by Western blotting assay and the band densities were analyzed using ImageJ analysis software. The data are expressed as mean ± SD ($n = 3$) and analyzed by one-way ANOVA followed by *post-hoc* test. Different letters indicate significant difference at $P < 0.05$.

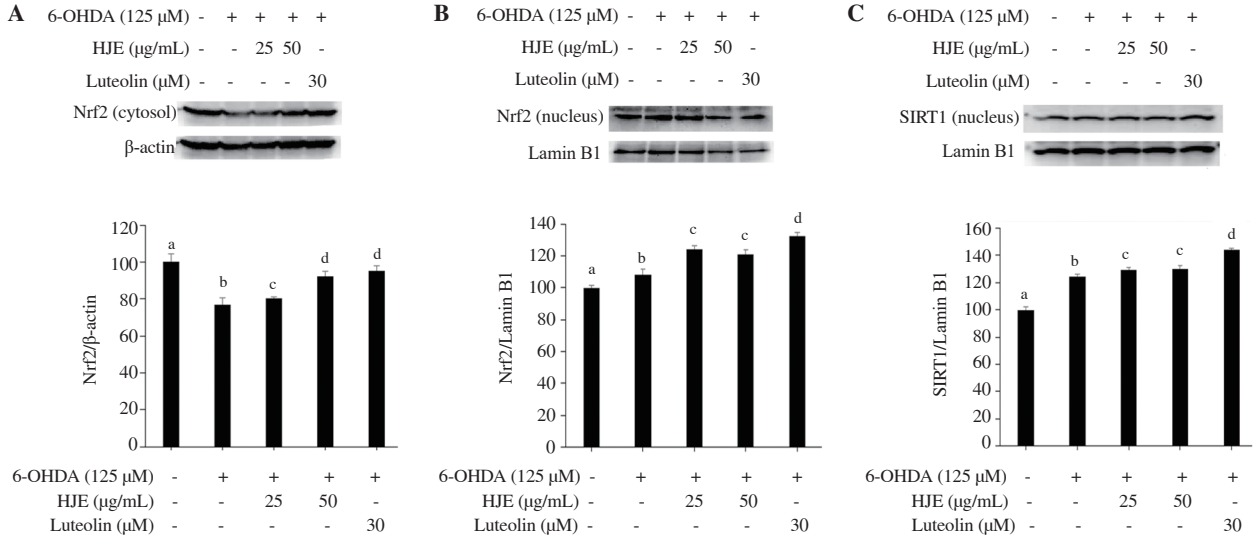

Figure 6. The effects of HJE on (A) the cytosolic Nrf2, (B) nuclear Nrf2, and (C) nuclear SIRT1 levels in 6-OHDA-induced PC12 cells. The protein expression levels were determined by Western blotting and the band densities were analyzed using ImageJ analysis software. The data are expressed as mean ± SD ($n = 3$) and analyzed by one-way ANOVA followed by *post-hoc* test. Different letters indicate significant difference at $P < 0.05$.

3.8. Effect of H. japonicus extracts on SIRT1 expression in 6–OHDA–stimulated PC12 cells

The results indicated that the 6-OHDA evidently promoted SIRT1 expression. Pretreatment with *H. japonicus* extracts (25 and 50 μg/mL) continued to significantly increase the expression of SIRT1 (Figure 6C).

4. Discussion

Previous studies have hypothesized that medicinal plants, such as ginseng, *Withania somnifera* (ashwagandha), wolfberry, *Ginkgo biloba*, and *Bacopa monnieri*, and natural compounds, such as resveratrol and curcumin, possess neuroprotective effects and successfully prevent or alleviate the neurological symptoms reported in clinical researches or *in vivo*. The high levels of ROS have been shown to facilitate irreparable oxidative damage to cellular function and structure. GSH can prevent damage to important cellular components associated with ROS, for example, heavy metals, free radicals, peroxides, and lipid peroxides[12]. The body can protect itself from ROS by utilizing mechanisms of antioxidant enzymes. These antioxidant enzymes decrease hydrogen peroxide and lipid hydrogen peroxide levels; therefore, they possess an essential effect in preventing lipid peroxidation and maintaining the functions and structures of cell membranes. Antioxidant enzymes, including HO-1 and CAT, inactivate ROS *via* a variety of enzymatic reactions, thus contributing to cell protection[12]. CAT is one of the enzymes with the highest turnover rate in all of the enzymes; One CAT molecule can convert millions of hydrogen peroxide molecules into water and oxygen per second[13]. In humans, OGG1 is a DNA glycosylase encoded by the *OGG1* gene. OGG1 is a bifunctional glycosylase, it can not only lyse the glycosidic bond of mutagenic lesions but also lead to strand breaks in the DNA skeleton. OGG1 initiates the first step in the DNA base excision repair pathway. However, oxidant stress in the cell transiently halts enzymatic activity of substrate-bound OGG1. If the expression of OGG1 is decreased in cells, it may increase the occurrence of mutations, thus increasing carcinogenesis[14]. HO-1 is a kind of enzyme, which catalyzes the heme decomposition into iron, biliverdin as well as carbon monoxide. *HO–1*, a target gene of Nrf2, is demonstrated to prevent various diseases, including atherosclerosis, hypertension, sepsis, pain, kidney injury, and acute lung injury[15,16]. HO-1 can protect cells by decreasing levels of superoxide along with other ROS[17].

The current study revealed that *H. japonicus* extracts possess protective activity against neurotoxicity caused by 6-OHDA in PC12 cells, which was potentially attributed to its antioxidant and anti-apoptotic properties. We observed that pretreatment with *H. japonicus* extracts significantly decreased ROS production and increased the levels of endogenous antioxidants, such as GSH and CAT. In addition, *H. japonicus* extracts pretreatment inhibited the 6-OHDA-induced mitochondrial apoptotic pathway activation in PC12 cells, indicating that *H. japonicus* extracts may be potentially beneficial for the protection of neuronal cells. As is known to all, this work clarified the neuroprotective effects of *H. japonicus* and the mechanisms involved in 6-OHDA-stimulated PC12 cells.

The MAPK signaling pathway is highly conserved and contains various threonine/serine kinases, including p38 subfamilies, JNK, and ERK. Pretreatment with *H. japonicus* extracts evidently decreased 6-OHDA stimulated MAPK activation and the cytotoxicity in PC12 cells. MAPKs reportedly translate developmental and environmental signals (stress and growth factors) into programmed and adaptive responses (apoptosis, inflammation, and differentiation)[18]. MAPKs play a key role in regulating cell proliferation, pro-apoptotic protein expression, and ROS production[18,19]. ERK1/2 is involved in the activation of multiple pathways, including MAPK signaling that regulates cell transcription, translation, proliferation, growth, and survival. c-JNK and p38 are activated *via* specific stressors and regulate apoptosis in multiple tissues[20]. Hence, the levels of ERK, p38, and JNK phosphorylation were detected in the treated PC12 cells. In 6-OHDA-stimulated PC12 cells, *H. japonicus* extracts strongly inhibited the MAPK signaling pathway, which could reduce ROS production and improve oxidative stress, thereby facilitating cell proliferation and enhancing cell viability. However, the outcomes of our study are not fully in accordance with the results reported by Ryu *et al.*, who found that 100% methanol extract of *H. japonicus* significantly reduced the phosphorylation of ERK1/2 in 6-OHDA-stimulated SH-SY5Y cells, without significant effects on p38 and JNK[11]. The discrepancy in findings could be attributed to different 6-OHDA concentrations used, *i.e.*, 50 μM in the study of Ryu *et al.* and 125 μM in the present study; thus, the induced stress level varied. In addition, the observed inconsistency could be on account of the involvement of distinct signaling pathways in the neuronal cell lineages used.

The signaling pathway of ARE/Nrf-2/Keap1 is the major protective pathway against endogenous and exogenous ROS[15,16]. The Keap1 and Nrf2 complexes destruction is owing to the interaction between Keap1 and bioactive molecules, which allows Nrf2 nuclear translocation, where it combines adenylate and uridine acid-rich elements (AREs) and can trigger the HO-1 expression[17]. Antioxidant enzymes are induced *via* Nrf2 activation through the MAPK signaling pathway. Furthermore, 6-OHDA-treated PC12 cells can activate MAPK signaling and decrease Nrf2 expression in the cytoplasm, thus allowing the transfer of Nrf2 to the nucleus, where it combines with the AREs and can trigger the antioxidant expression such as HO-1 and GSH; this implies that this effect suppresses the defense system of endogenous antioxidant and results in increased ROS generation, causing the death of cell and

DNA injury. Nevertheless, cells pretreated with 25 and 50 μg/mL *H. japonicus* extracts enhanced cytosolic and nuclear Nrf2 levels. Thus, pretreatment with *H. japonicus* extracts allows Nrf2 to translocate into the nucleus, thereby leading to a reduction in the production of ROS. However, further in-depth mechanistic studies are needed to fully elucidate the mechanism of action.

It is noteworthy that the molecular mechanism of neuroprotection contains the regulation of mitochondrial apoptosis cascade, which can be regulated finely by the imbalance between Bcl-2-related BAX and Bcl-2. BAX and Bcl-2 proteins are situated upstream of certain mitochondrial genes and are the significant regulators of the permeability of mitochondrial membrane. The excessive expression of these proteins regulates the cytochrome c release together with downstream caspase-3 protease activation, thus mediating cell death and survival. BAX (pro-apoptotic) can be transferred to the mitochondrial membrane in response to stimulation by death signals, forming homodimers or multimers that develop permeable transition pores in the mitochondrial membrane, thus disrupting differences in membrane ion and protein concentrations and releasing cytochrome c, as well as other pro-apoptotic factors. In the presence of dATP, cytochrome c binds to Apaf-1 *via* the cysteine protease structural domain, forming apoptotic vesicles and attracting the cysteine protease-9 precursor, which undergoes further oligomerization, and then activates caspase-3, initiating the caspase cascade reaction. Overexpressed Bcl-2 protein (anti-apoptotic) can form heterodimers with BAX, inhibit BAX translocation and dimerization, close permeability transition channels, and inhibit downstream caspase-3 activation by blocking cytochrome c release, thus effectively inhibiting the onset of apoptosis. Furthermore, Bcl-2 can bind to Apaf-1 and inhibit its function, preventing the activation of pro-cysteine-9 and mediating its anti-apoptotic effect. In addition, the overexpression of Bcl-2 results in nuclear GSH accumulation, thus altering nuclear redox homeostasis, which prevents intracellular calcium ion flow and inhibits the cytochrome c release from the membrane of mitochondria; this, in turn, inhibits caspase-3 activation and blocks the apoptosis process.

The ratio of BAX/Bcl-2 can be utilized as a regulator to describe the cell sensitivity to apoptosis. Our study showed that the BAX/Bcl-2 ratio was significantly increased in 6-OHDA-induced cells, indicating that 6-OHDA promoted mitochondrial apoptosis. However, this ratio was significantly reduced following treatment with *H. japonicus* extracts, suggesting that *H. japonicus* extracts inhibited mitochondrial apoptosis and promoted cell survival. The cell survival-promoting effect of *H. japonicus* extracts could be attributed to its bioactive composition, including various plant polyphenols, flavonoids, and their derivatives. *H. japonicus* extracts perform a neuromodulatory role through the activation of various intracellular signaling pathways that are necessary for neuroprotection. It is reported that protein kinase C (PKC)/ERK1/2 can decrease the expression of Bcl-2, BCL-w (BCL-2-like protein), B-cell lymphoma-extra large, as well as other anti-apoptotic proteins, and the Akt-ERK1/2 can suppress the pro-apoptotic activity of cell death agonist related to Bcl-2 and Bcl-2-interacting mediator and subsequently activate caspases 9 and 3[21,22]. Neuronal cells regulate apoptosis by activating different intracellular signaling pathways. In this study, *H. japonicus* extracts suppressed the levels of phosphorylated ERK1/2, JNK, and p38 and reduced the BAX/Bcl-2 ratio, indicating that *H. japonicus* extracts improve the viability of 6-OHDA-stimulated cells by regulating apoptosis *via* the MAPK pathways. However, the precise role of these pathways in mediating the 6-OHDA-induced anti-apoptotic response of PC12 cells needs to be further elucidated.

Adenosine affords protection against neuronal apoptosis *via* NAD (+)-dependent histone deacetylase SIRT1[23], a mammalian homolog of the yeast protein. Reportedly, upregulated activator SIRT1 activity *via* agents, such as resveratrol, can extend the lifespan of several species[24] Moreover, the aging process has been associated with SIRT1[10]. Sirtuin is an evolutionarily conserved protein that in the enzymatic reactions, NAD (+) was utilized as a co-substrate. In the human Sirtuin family, there are seven proteins (namely, SIRT1-7), in which SIRT1 is the most conserved and characteristic. In the brain, particularly in the hypothalamus, SIRT1 has an essential effect on the regulation of the circadian rhythm and system energy homeostasis. Additionally, SIRT1 has been confirmed to protect against neurodegeneration by regulating the transcription factors involved in stress resistance and neuronal protection[25] and modulating the function of several significant transcription factors, including forehead box protein O, NF-κB, and p53[23,26]. Moreover, SIRT1 has been shown to reduce amyloid β deposition *via* activation of a non-amyloid pathway[27,28]. In the present study, *H. japonicus* extracts increased SIRT1 expression in 6-OHDA-treated neuronal PC12 cells, thereby attenuating intracellular ROS production and promoting cell survival and/or delaying cellular senescence, similar to the life-extending effects of calorie restriction[29]. Thus, *H. japonicus* extracts could promote cell survival *via* SIRT1 regulation. Resveratrol, the identified polyphenol in red wine and grapes, targets SIRT1 and exerts beneficial effects on the lifespan[24,30]. *H. japonicus* extracts demonstrate antioxidant and anti-apoptotic effects by modulating the AMPK-SIRT1 pathway[10]. However, the underlying mechanisms need to be confirmed in future studies.

Following liquid chromatography with tandem mass spectrometry, an abundance of polyphenols and 36 compounds were detected in an 80% ethanol extract of *H. japonicus*[31]. Likewise, the 70% ethanol fractionation of *H. japonicus* is rich in polyphenols and contains 14 known compounds and 9 putative compounds by high-property liquid chromatography combined with nuclear magnetic resonance together with quadrupole-time-of-flight mass spectrometry. The major compounds identified in fractionated *H. japonicus* included

luteolin-7-*O*-glucoside as well as apigenin-7-*O*-glucoside. The luteolin and apigenin are known to significantly inhibit monoamine oxidase B enzyme activity[32]. Based on these results, further researches are essential to determine effective bioactive compounds against PD and optimize the extraction of the said bioactive compounds.

Herein, our findings revealed that *H. japonicus* extracts prevented PD. *H. japonicus* extracts significantly attenuated 6-OHDA-caused death in PC12 cells by enhancing antioxidant molecules, such as CAT and GSH, thus inhibiting intracellular ROS accumulation and the MAPK signaling pathways. Furthermore, the protective effect of *H. japonicus* extracts on the 6-OHDA-induced mitochondrial apoptosis was associated with the downregulated BAX and upregulated Bcl-2 protein expression. In addition, *H. japonicus* extracts promoted neuronal cell survival by enhancing SIRT1 expression.

Although the present research has proved that *H. japonicus* extracts could alleviate the 6-OHDA-induced PD symptoms mainly through antioxidative and anti-apoptotic pathways, *in vivo* and clinical studies should be conducted to further verify its effect. In addition, this study mainly studied the crude ethanol extract of *H. japonicus* and did not verify the structure of specific active ingredients and the effect of the single ingredient.

In conclusion, *H. japonicus* extracts exhibited anti-oxidative and anti-apoptotic effects in 6-OHDA-stimulated PC12 cells *via* upregulated antioxidant enzymes, and suppressed MAPK and/or Nrf2 signaling pathways. *H. japonicus* extracts may be a promising candidate for preventing and treating oxidative stress-related neurodegenerative diseases.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

SIJ and BOC designed, directed, and supervised the project and reviewed the manuscript. FW processed the experimental data, performed the analysis, and wrote the manuscript. JYS edited manuscript, helped supervise the project and contributed to the draft interpretation of the results. SH collected the data and performed the data analysis.

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