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Antioxidative stress effects of *Humulus japonicus* extracts on neuronal PC12 cells

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Abstract

The objective of the current study was to assess the antioxidant activity of different solvents containing *Humulus japonicus* extract (HJE) and their antioxidant effects on neurons. *H. japonicus* ethanol extracts (HEEs) together with *H. japonicus* water extracts (HWEs) were generated via extraction of *H. japonicus* leaves with 30%, 50%, and 80% ethanol solutions under a hot water temperature of 121 °C. HEEs showed higher levels of bioactive compounds, such as total flavonoids and polyphenols, with stronger antioxidant activities in contrast to HWEs. HEE (80%) showed the highest content of bioactive compounds and antioxidant activity. Next, we used a H_2O_2-induced rat model of pheochromocytoma PC12 cells to investigate the antioxidant activity and cellular signal regulation of 80% HEEs. The results showed that HJEs resisted oxidative stress via MAPK and/or AKT-NRF2 and NF-kappa B signaling pathways and upregulated antioxidant enzymes such as SOD, catalase, HO-1, and OGG1, thereby reducing intracellular ROS levels. Further, HJE promoted neuronal survival by upregulating sirtuin 1 expression. This study provides evidence supporting the potential benefits of HJE in preventing oxidative stress in PC12 cells, suggesting its application as a promising functional food additive or therapeutic agent for the prevention and the treatment of oxidative stress-related neurodegenerative diseases.

Keywords: *Humulus japonicus;* antioxidant capacity; oxidative stress; MAPK, PC12 cell.

Practical application: HJE can be utilized as the natural product source for the pharmaceutical industry or as functional food.

1 Introduction

Oxidative stress plays a major role on neurodegenerative diseases (Barnham et al., 2004), and is regarded as a therapeutic target for preventing and treating neuronal degeneration (Choi et al., 2012). Oxidative stress occurs when ROS accumulate in the cells due to insufficient neutralization or excessive production. ROS induce serious molecular injury to the main cellular components, for instance, via oxidation of lipids, proteins, sugars and DNA (Meo et al., 2020). Among various ROS, H_2O_2 is one of the most significant, which has physiological significance. Superoxide dismutase (SOD) catalyzes the production of H_2O_2 in cells and thus facilitates the diffusion of various molecules through the biofilm at a relatively low concentration, resulting in serious injury to the cell macromolecules (Martindale & Holbrook, 2002).

Humulus japonicus (HJ) is an annual medicinal herb belonging to the Cannabis family, and is widely distributed throughout temperate Asia (Wang et al., 2021). *H. japonicus* has been used in traditional Chinese medicine to treat tuberculosis, diarrhea, and pneumonia. HJ leaves in Korea have been used to treat tuberculosis, dysentery, and hypertension. HJ extract can prevent the onset of neurodegenerative diseases, for example AD, in a mouse model treated with a 100% methanol extract (Park et al., 2017), and in a 6-hydroxydopamine-induced Parkinson's disease model treated with a 100% methanol and ethanol extract (Ryu et al., 2017; Lee et al., 2019). However, few studies compared the complexity of different extraction methods of HJ, and most studies focused on the identification and functional analysis of HJ bioactive components. In addition, a simple and safe process of preparation of crude extracts can facilitate manufacture. Therefore, in this study, *H. japonicus* ethanol extracts (HEEs) and *H. japonicus* water extracts (HWEs) were synthesized with ethanol solutions containing a variety of concentrations and hot water, respectively. The bioactive components including total flavonoids and total phenolic acids in HJEs were screened. HJE antioxidant activities were assessed in terms of ABTS and DPPH scavenging activity, and redox mechanisms. Next, we selected the optimal extraction method, and used H_2O_2 to induce a rat pheochromocytoma PC12 cell model to investigate the antioxidant activity and signal regulation pathways associated with HJE.

2 Materials and methods

2.1 Materials

Dimethyl sulfoxide, DPPH, quercetin, Folin & Ciocalteu's phenol reagent, vitamin C, gallic acid, ABTS, H_2O_2 solution (30%), protease inhibitors, and phosphatase inhibitors were provided by Sigma-Aldrich (St. Louis, MO, USA). Junsei Chemical Co., Ltd. (JC,

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Japan) supplied ferric chloride hexahydrate, trichloroacetic acid, and sodium carbonate. Alomone Labs (Jerusalem BioPark, Israel) provided the native mouse NGF 2.5S protein. Horse serum (HS), fetal bovine serum (FBS), together with Roswell Park Memorial Institute (RPMI) 1640, were obtained from Gibco (Grand Island, NY, USA). Streptomycin/penicillin together with EZ-western Lumi Pico Alpha and EZ-Cytox reagents were acquired from Invitrogen (Carlsbad, CA, USA) and DoGenBio (Seoul, Korea), respectively. Bio-Rad Laboratories (Hercules, CA, USA) supplied the Bradford's assay reagent. Polyvinylidene fluoride membranes together with SDS-PAGE loading buffer (5 ×) were acquired from Millipore (MA, USA) and Biosesang, Seongnam, South Korea, respectively. Thermo Scientific (Rockford, IL, USA) supplied the NE-PER cytoplasmic and nuclear extraction reagents as well as the radio-immunoprecipitation assay buffer (RIPA buffer). β-actin (Biosciences, Franklin Lakes, NJ, USA) antibodies and antibodies against Akt, p-Akt (Cell Signaling Technology Inc., Beverly, MA, USA), HO-1, p-NF-κB, NF-κB/P65, OGG1, SIRT 1, and NRF2, and secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) combined with HRP were used in this study. Amersham Biosciences (Piscataway, NJ, USA) provided the enhanced chemiluminescence detection system kit. Catalase together with SOD was provided by Cayman Chemical (Ann Arbor, MI, USA). The carboxy-H_2DCFDA probe was obtained from Invitrogen (Carlsbad, CA, USA).

2.2 Extraction of HJ

HJ Water Extract (HWE)

HJ leaves used in the current study were obtained from Cheonjam Hill, Wansan-Gu, Jeoju-shi, Jeonbuk, Korea on June 25, 2020. The plant was identified and certified by Professor Hong-Jun Kim at the College of Oriental Medicine, Woosuk University (Wanju, Korea). The voucher specimen (#2020-06-25-HJ) was maintained in our laboratory (Professor Seon Il Jang). The distilled water was utilized to clean leaves and then dried at a temperature of 60 °C for one day, followed by the addition of 1000 mL of water to 50 g of dried leaves and subsequent heating at 121 °C in an automatic autoclave for 30 min. The mixture was cooled and the extract was filtered using a filter paper (ADVANTEC, Togo, Japan) with a pore size of 0.45 μm. The filtered extract was frozen completely in a freezer at -50 °C, freeze-dried at at -55 °C to obtain dry powder, and maintained under a temperature of -20 °C for further experiments. The HWE yield was 13.31%.

HJ ethanol extract (HEE)

Dry leaves (50 g) were extracted in 2000 mL of 30%, 50%, and 80% (v/v) ethanol for two days in a water bath at 40 °C. The extract was filtered using filter paper with a pore size of 0.45 μM. The filtered extract was vacuum-concentrated at 45 °C to remove ethanol, and the remaining solution was freeze-dried in a - 55 °C freeze dryer to obtain dry powder, which was subsequently maintained at - 20 °C for the following experiments. The yields of 30, 50 and 80% HEEs were 12.04%, 14.24% and 11.08%, respectively.

2.3 Total phenolic and flavonoid content

Total Phenolic Content (TPC)

The TPC was measured as described by Peterson et al. (2001) with minor modification. In brief, utilizing DMSO as a solvent, the HJ extract powder (100 mg/mL) was generated in Eppendorf tubes between at 0 and 1000 μg/mL, using a series of dilutions (containing distilled water) of the standard (gallic acid) to synthesize the extract. Folin-Ciocalteu's phenol reagent (0.1 mL) was mixed with 0.1 mL of each standard and extract, followed by the addition of 4% Na_2CO_3 (1 mL) after 5 minutes. The above mixture was placed at RT and under pressure for half an hour. All the analyses were performed in triplicate. The absorbance was read at 750 nm using an ELISA reader (Tecan, Mannedorf, Switzerland). Gallic acid was treated similar to the standard for the calculation of gallic acid equivalent, which was used as a standard to quantify the total phenol content of the HJ extract.

Total Flavonoid Content (TFC)

The TFC was determined as described by Ordonez et al. (2006) by mixing 0.5 mL of 10% aluminum chloride with 0.5 mL of 1 mg/mL HEE or HWE aliquot completely, and left at RT for five minutes. The absorbance of the supernatant was read at 405 nm. All the analyses were performed in triplicate. The total flavonoid content was measured using a calibration curve (r^2 = 0.996) with quercetin as a standard, and measured in quercetin equivalents of mg/gram of dry weight (mg·QUE/g).

2.4 Antioxidant activities

DPPH

The free radical-scavenging activity of DPPH was measured using the strategy of Blois (1958) with minor modification. HJ extract powder was dissolved in methanol. The concentrations of HJ extract solutions were 1000, 500, 250, 125, 62.5, and 31.25 μg/mL, and 9 mL of ethanol was mixed with 3 mM solution of DPPH (1 mL) to obtain a 0.3 mM solution of ethanol and DPPH. The tissue culture plate (96-well) was treated with 100 μL of HJ extract solution and 100 μL solution of ethanol and DPPH, followed by incubation for half an hour in the dark. The absorbance of the mixture at 540 nm measured with an ELISA reader. The DPPH scavenging activity of the HJ extract was measured using the Equation 1 below:

$$\text{DPPH radical scavenging activity}(\%) = \left(1 - \frac{\text{sample absorbance}}{\text{absorbance of blank}}\right) \times 100\% \quad (1)$$

The 50% inhibitory concentration (IC_{50}) of the HJ extract was measured using the regression curve between DPPH scavenging activity and concentration.

ABTS

The radical scavenging activity of ABTS was measured using a strategy proposed by Re et al. (1999) with minor modification.

In brief, the working solution of ABTS was prepared from an ABTS stock solution by mixing equal volumes of potassium persulfate solution (2.6 mM) and ABTS solution (7.4 mM), and then cultured in the dark for 12–24 hours between 2 and 8 °C, and subsequently mixed with distilled water at 732 nm prior to measuring the absorbance up to 0.7 ± 0.02. ABTS working solution (950 µL) was added to 31.25-1000 µg/mL of 50 µL of HJ in the Eppendorf tubes in the dark, and placed at a temperature of 20 °C for half an hour. The absorbance was measured at 732 nm. The activity is expressed as a percentage as follows (Equation 2):

$$\text{ABTS radical scavenging activity}(\%) = \left(1 - \frac{\text{sample absorbance}}{\text{absorbance of blank}}\right) \times 100\% \quad (2)$$

Reducing power

The analytical technique was modified from the procedure described by Oyaizu (1986). A 7-member serial dilution of HJ extracts was prepared within the range of 1000 to 31.25 µg/mL by mixing 0.25 mL and 1% of potassium ferricyanide, 0.2 mL and 0.2 M of PBS (with a pH of 6.6), and 0.25 mL of HJ extract solution, and then incubated in a 50 °C water bath for 20 minutes, followed by subsequent cooling. The mixture was immediately treated with 0.25 mL of 10% trichloroacetic acid, and then centrifuged for 10 minutes at 20 °C and 1200 × g, followed by the addition of 100 µL of 0.1% ferric chloride, 500 µL supernatant, and 500 µL distilled water and incubation for 10 minutes. The absorbance of the mixture was determined with an ELISA reader at 700 nm. Vitamin C was treated similarly and used as a standard for the calculation of the vitamin C equivalent to determine the reducing power.

2.5 Cell culture

The cell line of PC12 (ATCC ® CRL1721) derived from rat pheochromocytoma was acquired from American Type Culture Collection (Manassas, VA, USA). PC12 cells were treated with nerve growth factor (4 ng/mL) to induce differentiation. The PC12 cells were cultivated in RPMI 1640 supplemented with 5% (v/v) FBS, 10% (v/v) HS, streptomycin (100 µg/mL) and penicillin (100 units/mL) at a temperature of 37 °C in 5% CO_2 humidified atmosphere. Changing the culture medium every other day and culturing the cells for seven days facilitated diffusion to a density of approximately 75% to 80%.

2.6 Cytotoxicity assay

The 96-well plates were inoculated with 2.5×10^5 cells/mL for one day and treated with HJE (up to 200 µg/mL) and H_2O_2 (up to 400 µM) for 20 h. Each well was treated with 0.01 mL of EZ-Cytox reagent and then cultured for four hours. The absorbance was read at 450 nm with a microplate reader (Tecan, Männedorf, Switzerland) to monitor the viability of PC12 cells. The percentage of cell survival was detected by comparing the optical density of living cells and untreated controls.

2.7 Antioxidant enzyme activities

PC12 cells (2.5×10^5 cells/mL) were cultured in plates (6-well) for one day and pre-treated with or without 25 and 50 µg/mL of HJE for 60 minutes. The cells treating with H_2O_2 (150 µM) for one day. The assay kits were utilized to analyze the activities of catalase and SOD based on the manufacturer's guidelines (Cat. 707002 and 706002 Cayman).

2.8 Intracellular ROS level

PC12 cells (2.5×10^5 cells/mL) were cultured in 6-well plates for one day and then pretreated with or without 25 and 50 µg/mL of HJE for 60 minutes. The stimulation of cells was performed by treating with H_2O_2 (150 µM) for an hour. The cells were harvested by trypsinization and suspended in DPBS solution containing 5 µM carboxy-H_2DCFDA at a density of 1×10^6 cells/mL, and incubated for 30 minutes at 37 °C, protecting the cells from light. The cells were centrifuged for five minutes at 130 × g, followed by removal of the supernatant. The cells were subsequently resuspended in pre-warmed DPBS. The procedure was repeated twice to wash cells. The cells were immediately analyzed for ROS detection using flow cytometry, and excited with a 488 nm laser and then determined at 535 nm.

2.9 Whole protein extraction and western blotting analysis

PC12 cells (2.5×10^5 cells/mL) were cultured in culture dishes (90 × 20 mm) for one day and then pretreated with or without 25 and 50 µg/mL of HJE for 60 minutes. The cells were stimulated with H_2O_2 (150 µM) for 0.5 h or 24 h. Subsequently, whole cell lysates corresponding to each treatment were prepared in RIPA buffer supplemented with phosphatase and protease suppression, and centrifuged for 15 minutes at 14,000 × g to acquire supernatant of whole protein extracts. Following protein quantification using the Bradford assay, the protein (20 mg) in each sample was analyzed on 15% or 8% of SDS-PAGE (110 V for 90 minutes). After separation, the protein was transferred to the polyvinylidene fluoride membrane (100V, 60 minutes). Subsequently, the membrane was blocked with 5% BSA for an hour, and then mixed with a variety of antibodies (AKT, HO-1, OGG1, β-actin, Sirt1, and p-AKT), followed by incubation at a temperature of 4 °C overnight. After incubation overnight, the membrane was cleaned with TBST three times (five minutes each time), and then incubated with the secondary antibody combined with relevant HRP at RT for 2 hours. Subsequently, the membranes were cleaned with TBST five times (five minutes each), followed by treatment with EZ-western Lumi Pico Alpha chemiluminescence reagent and visualized using an imaging system (Alliance version 15.11; UVITEC Cambridge, UK). The stripping buffer was employed in this study to allow imaging of multiple proteins on a single membrane. ImageJ 1.52 version (US National Institute of Health, Bethesda, MD, USA) was exploited to detect the strip densities.

2.10 Cytoplasmic and nuclear protein extraction and western blotting

PC12 cells (2.5×10^5 cells/mL) were cultivated in culture dishes (90 × 20 mm) for one day and then pretreated with or without 25 and 50 µg/mL of HJE for 60 minutes. The cells were stimulated with H_2O_2 (150 µM) for 30 minutes. In accordance with the manufacturer's guidelines, the nuclear and cytoplasmic proteins were extracted from cells under each treatment condition

using a NE-PERTM nuclear and cytoplasmic extraction reagent. Following protein quantification using the Bradford assay, 15 mg of cytoplasmic and nuclear protein was used for western blotting as described previously. Nevertheless, in this condition, the membranes were incubated with p-NF-κB/p65 or NF-κB/p65, β-actin, NRF2, or lamin b antibodies.

2.11 Statistical analysis

The SPSS program (version 22 SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. The data were expressed as mean ± SD. Statistically significant differences between groups were determined by one-way ANOVA, followed by Duncan's test. A significant difference was considered when the p-value was less than 0.05.

3 Results and discussion

3.1 TPC and TFC

Polyphenols exhibit antioxidant activity and decrease the RNS and ROS (Meo et al., 2016). The total flavonoid and phenolic contents in HEEs and HWE are listed in Table 1. The minimum TPC of HWE was 18.59 mg GAE/g. Increasing the concentration of ethanol in the assessed HEEs boosted the TPC from 36.04 GAE/g to 74.90 mg GAE/g. All of the TPC reported via Lee et al. exceeded 30.13 mg GAE/g (Lee et al., 2012). The TPC of 80% HEE is the highest, which may be attributed to the polarity of 80% solution of ethanol, which is close to TPC (Limmongkon et al., 2018).

Choi et al. (2018) reported that polyphenols were most abundant in 80% HEEs, followed by the stems and roots, using LC-ESI-MS/MS. This study also showed the abundance of HJ leaf polyphenols in 80% HEE. Flavonoids exhibit anti-inflammatory, antioxidant, anti-angiogenic and anti-carcinogenic activities (Shi et al., 2018; Chavez-Santiago et al., 2021; Zapata et al., 2021). As presented in Table 1, HWE contained 1.10 mg QUE/g of the lowest TFC value, which is similar to the TPC values of 149.54 mg QUE/g, which is the highest TFC found in 80% of HEE, and consistent with the TPCs. The TFC of HEEs increased from 22.31 to 149.54 mg QUE/g under increasing ethanol concentrations in the range reported here.

3.2 DPPH, ABTS, and reducing power

The scavenging activity of DPPH was described as 50% of inhibitory concentration (IC_{50}, μg/mL), which represents the concentration needed to eliminate 50% of DPPH radicals. Therefore, compounds with lower IC_{50} exhibit higher scavenging activity of DPPH. Table 1 presents the IC_{50} values of the scavenging activity of DPPH and ABTS, and reducing power of the HWE and HEEs, with 80% HEE exerting the highest (IC_{50} 249.62, μg/mL) DPPH scavenging activity, while the DPPH scavenging activity of HWE was the lowest (IC_{50} 2382.90 μg/mL).

As shown in Table 1, compared with other bioactive compounds analyzed, the total flavonoid and phenol content has a greater impact on the scavenging activity of DPPH. Furthermore, the scavenging activity of DPPH reported by Choi et al. (2018) was 316.82 μg/mL in an 80% HEE, which is slightly larger than in the present study, which may be due to different sampling locations. It also proves, to a certain extent, that the 80% HEE prepared in this study exhibits good radical scavenging ability.

The ABTS radical scavenging strategy can be applied to assess the antioxidant capacity of pure compounds and herbal or plant extracts, so as to assess the total antioxidant capacity of a variety of antioxidant solutions. Generally, the antioxidants oxidize ABTS to generate green radical cations. Antioxidants inhibit the above reaction by providing electrons and suppressing the generation of colored ABTS free radicals. Table 1 shows that among the HEEs, the 50% HEE had the highest (IC_{50} 1055.93 μg/mL) scavenging activity of ABTS, which was not markedly different from 80% of HEE ($p > 0.05$). The scavenging activity of ABTS reported by (Choi et al., 2018) was 491.86 μg/mL in an 80% ethanol extract of HJ, which was remarkably lower than in the present study, mainly because of the modification of the reference method.

The reducing power (mg Vit C/g) of HJ extracts is presented in Table 1. The HWE had the lowest (20.03 mg Vit C/g) reducing power, which is related to the lowest TPC (18.59 mg GAE/g) and TFC content (1.1 mg QUE/g) (Table 1). By contrast, the 80% HEE showed the highest reducing power (60.24 mg Vit C/g), which is likely due to its highest TPC (74.90 mg GAE/g) and TFC (149.54 mg QUE/g). The reducing power of HEEs increased from 25.89 to 60.24 mg Vit C/g with an increasing concentration of ethanol, over the range reported here.

As shown in Table 1, both antioxidant capacity and bioactive compound content of HWE are the lowest, due to poor solubility in bioactive substances and water following isomerization and inactivation at high temperatures. For HEEs, both the antioxidant capacity and bioactive compound content were enhanced with increasing ethanol concentration due to the lysis of nonpolar compounds in solvents with lower polarities. The 80% HEE showed the highest antioxidant capacity and

Table 1. TPC, TFC, and antioxidant activities of HJE.

Sample	TPC (mg GAE/g)	TFC (mg QUE/g)	DPPH scavenge IC_{50} (μg/mL)	ABTS scavenge IC_{50} (μg/mL)	Reducing power (mg VitC/g)
HWE	18.59 ± 0.26^{d}	1.10 ± 0.05^{d}	2382.90 ± 118.23^{a}	1478.85 ± 96.50^{a}	20.03 ± 0.10^{d}
30% HEE	36.04 ± 0.29^{c}	22.31 ± 0.30^{c}	2314.79 ± 205.62^{a}	1504.48 ± 88.88^{a}	25.89 ± 0.19^{c}
50% HEE	58.28 ± 0.80^{b}	116.24 ± 2.67^{b}	536.62 ± 34.47^{b}	1055.93 ± 46.02^{b}	48.13 ± 0.35^{b}
80% HEE	74.90 ± 0.27^{a}	149.54 ± 1.18^{a}	249.62 ± 30.06^{c}	1110.94 ± 69.47^{b}	60.24 ± 0.31^{a}

Each value represents the mean ± SD (n = 3). Means with different lowercase letters within the same column are significantly different ($P < 0.05$). IC_{50} represents the concentration of the HJ extract scavenging 50% of the free radicals.

bioactive compound content. Therefore, 80% HEE (referred to as HJE hereafter) was optimal based on the results of phenolic and flavonoid content, and antioxidant capacity, and was used in subsequent experiments.

3.3 Cytotoxicity

The cytotoxic activities of H_2O_2 and HJE in the PC12 cells were assessed via analysis of water-soluble tetrazolium salt (WST). Treating the cells with HJE at various concentrations (0, 25, 50, 100, and 200 μg/mL) yielded no significant cytotoxicity up to 50 μg/mL (Figure 1A). Similarly, H_2O_2 up to 100 μM had no cytotoxic activity on the PC12 cells (Figure 1B), while co-treatment including HJE (up to 50 μg/mL) and H_2O_2 (150 μM) induced cytotoxicity in PC12 cells (Figure 1C). Accordingly, concentrations of 25 and 50 μg/mL of HJE and H_2O_2 (150 μM), respectively, were selected for subsequent experiments.

3.4 Intracellular ROS level

As HJE possesses antioxidant activity in vitro, this activity of HJE in H_2O_2-induced PC12 cells was investigated. Notably, the PC12 cells represent a cell line of pheochromocytoma, which has been extensively adopted in studies investigating brain diseases. To our knowledge, the current study is the first of its kind to propose that HJE inhibits oxidative stress in PC12 cells, and thus useful in the management of neurodegenerative diseases. Previous studies have hypothesized that natural compounds and medicinal plants, for instance curcumin, ginseng, *Withania somnifera, Ginkgo biloba, Baccopa monnieri,* resveratrol, and Wolfberry can alleviate or prevent neurological diseases and ameliorate the neurological symptoms (Ratheesh et al., 2017). Although *HJ* has been expressed and used as a conventional drug in China and Korea, its potential biological benefits are limited in neurological diseases. High and uncontrolled levels of ROS have been shown to induce irreparable oxidative injury in cells and structural damage (Halliwell & Whiteman, 2004). Here, HJE was found to reduce ROS generation in PC12 cells, induced by H_2O_2 (Figure 2A-E). DCFH-DA analysis was used to screen for ROS in the PC12 cells. The DCFH-DA probe can freely penetrate the cell membrane and is oxidized to the highly fluorescent substance (DCF) after reacting with ROS. When the PC12 cells were subjected to H_2O_2 oxidative stress for 60 min, ROS levels increased by 345%, compared with the control (Figure 2E). Pre-treatment of PC12 cells with HJ extracts at 25 and 50 μg/mL remarkably prevented the accumulation of intracellular ROS, compared with the control treated with H_2O_2 ($p < 0.05$), resulting in a 40.08% and 49.6% decrease in the ROS levels, respectively. These results indicate that the antioxidant performance of HJ can reduce the oxidative stress of PC12 cells induced by H_2O_2 and protect against neuronal damage induced by oxidative stress.

3.5 Intracellular antioxidant defense system

The body can protect itself from ROS via antioxidant mechanisms. The antioxidant enzymes, including HO-1, catalase, and SOD, inactivate ROS via a variety of enzymatic reactions, thus contributing to the protection of cell (Trippier et al., 2013). SOD is an important component of the cellular antioxidant defense system, and is ubiquitous in nearly all living cells exposed to oxygen. Catalase is an important enzyme that protects cells from oxidative damage caused by ROS. HO-1 is an enzyme, which can catalyze the decomposition of heme into iron, anti-inflammatory agent carbon monoxide and antioxidant biliverdin. HO-1 protects the cells by decreasing the superoxide levels together with other ROS (Uddin et al., 2020). OGG1 is a principal protein mediating DNA demethylation induced by oxidative stress. The decrease of OGG1 contributes to resistance to DNA demethylation triggered by oxidative stress, while the excessive expression of OGG1 makes cells vulnerable to DNA demethylation caused through oxidative stress (Zhou et al., 2016).

Our results suggest that SOD and catalase levels were reduced markedly when cells were treated with only H_2O_2 (Figure 2F, 2G).

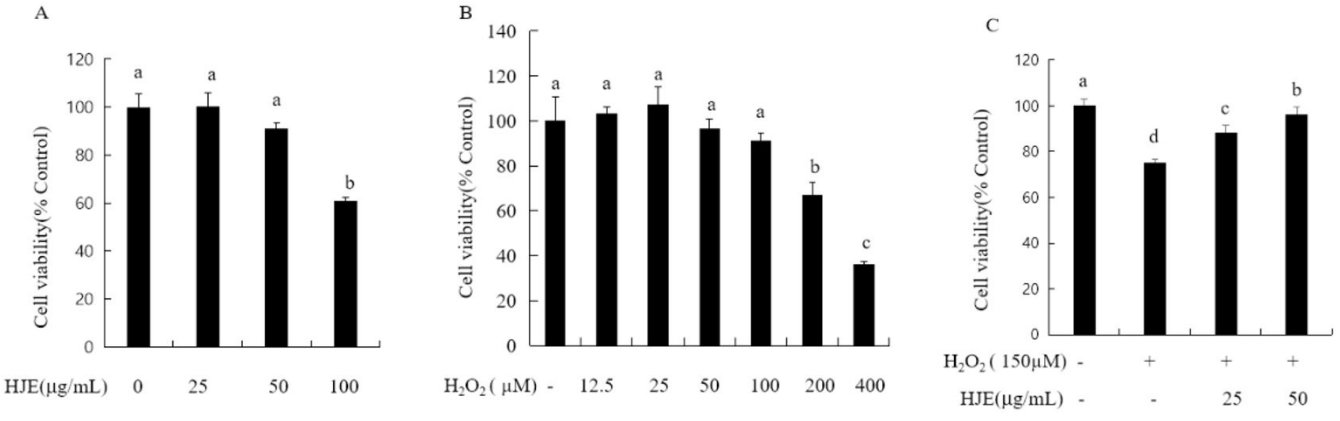

Figure 1. The viability of PC12 cells treated with *Humulus japonicus* extract (HJE). PC12 cells were incubated with HJE alone at the indicated concentrations (A), hydrogen peroxide at indicated concentrations (B), and HJE at 0, 25, and 50 μg/mL and treated with 150 μM hydrogen peroxide (C) for 24 h before cell viability studies were performed. The results are presented as the mean ± standard deviation (SD) (n = 3). Different small case letters indicate significance at $p < 0.05$ by one-way ANOVA analysis followed by Duncan's test.

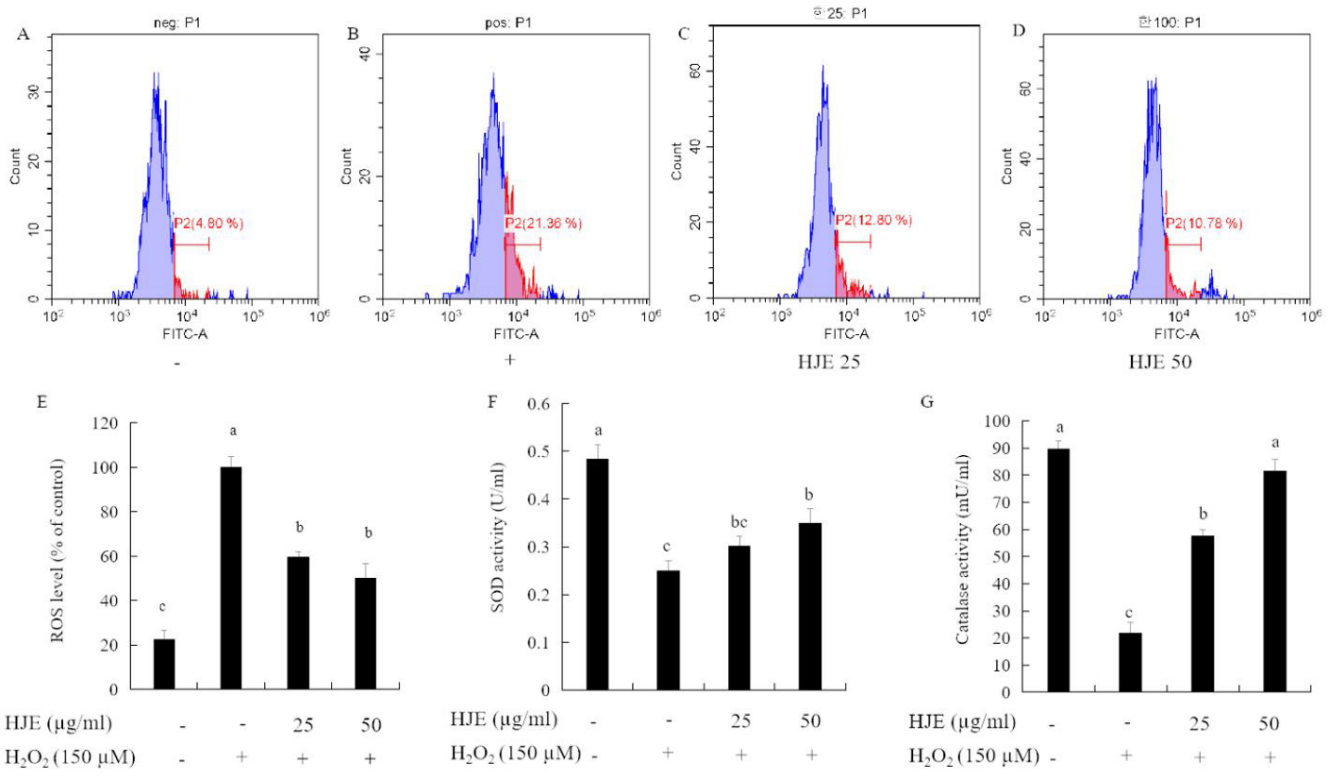

Figure 2. Effect of HJE on intracellular ROS levels (A-E), SOD (F), and catalase (G) activity in H_2O_2-stimulated PC12 cells. Cells were pretreated with HJE at indicated concentrations for 1 h and subsequently stimulated with 150 μM of H_2O_2 for 24 h. Each bar represents the mean ± SD. Different small case letters indicate significant at $p < 0.05$ by one-way ANOVA analysis followed by Duncan's test.

Nonetheless, cells pretreated with HJE (25 and 50 μg/mL) prior to H_2O_2 treatment showed increases in SOD and catalase levels compared with cells treated with H_2O_2 alone (Figure 2F, 2G). Further,cells treated with H_2O_2 alone markedly reduced the OGG1 and HO-1 expression in comparison with untreated cells (Figure 3A, 3B). Similarly, in contrast to cells treated with H_2O_2 alone, cells pretreated with HJE (25 and 50 μg/mL) showed an obvious increase in the expression of OGG1 and HO-1 expression prior to H_2O_2 treatment (Figure 3A, 3B). On the whole, these results suggest that HJE protects PC12 cells from oxidative stress, principally by increasing the antioxidant defense and decreasing the generation of ROS. The trend of HJE suppressing the antioxidant defense system in the PC12 cells is in accordance with the results suggesting that HJE decreases the intracellular ROS generation.

3.6 Sirtuin 1 (SIRT 1) and Protein Kinase B (AKT)

Sirtuins is a conserved evolutionarily enzyme that employs NAD+ as the co-substrate. Seven kinds of proteins exist in the family of human sirtuin (SIRT1-7), of which SIRT1 is the most characteristic and conserved. In the brain, SIRT1 has been reported to prevent neurodegeneration by deacetylating several transcription factors involved in stress resistance and neuronal protection (Donmez & Outeiro, 2013). The effect of HJE on SIRT1 expression in stimulated PC12 cells was also investigated. The findings suggested that cells treated with H_2O_2 alone evidently up-regulated the SIRT1 expression. However, when cells were pretreated with 25 or 50 μg/mL HJE before stimulation with H_2O_2, the expression of SIRT1 continued to increase significantly in a dose-dependent manner (Figure 3C). In this study, HJE increased the expression of SIRT1 in PC12 cells treated with H_2O_2, which alleviates intracellular ROS and may promote cell survival and/or delay cell senescence, similar to the lifespan-extending effects of caloric restriction (Bordone et al., 2007). A possible mechanism for the promotion of cell survival by HJE includes its effect on SIRT1 expression. For instance, resveratrol, a polyphenol found in grapes and red wine, targets SIRT1 and has a beneficial influence on life span (Baur et al., 2006).

Both the Akt and PI3K-Akt signaling pathways represent signal transduction pathways, which facilitate growth and survival in response to extracellular signals. The major proteins include Akt and PI3K (phosphatidylinositol 3-kinase) (Wang et al., 2019). The influence of HJE on AKT phosphorylation in stimulated PC12 cells was also investigated. The results showed that cells treated with H_2O_2 alone showed enhanced phosphorylated AKT expression (Figure 3D). However, the expression of phosphorylated AKT was reversed in a dose-dependent manner when cells were pretreated with HJE (25 or 50 μg/mL), prior to H_2O_2 stimulation (Figure 3D). The results showed that HJE strongly suppressed the AKT activation in PC12 cells stimulated by H_2O_2, thereby promoting the survival and growth of neuronal cells.

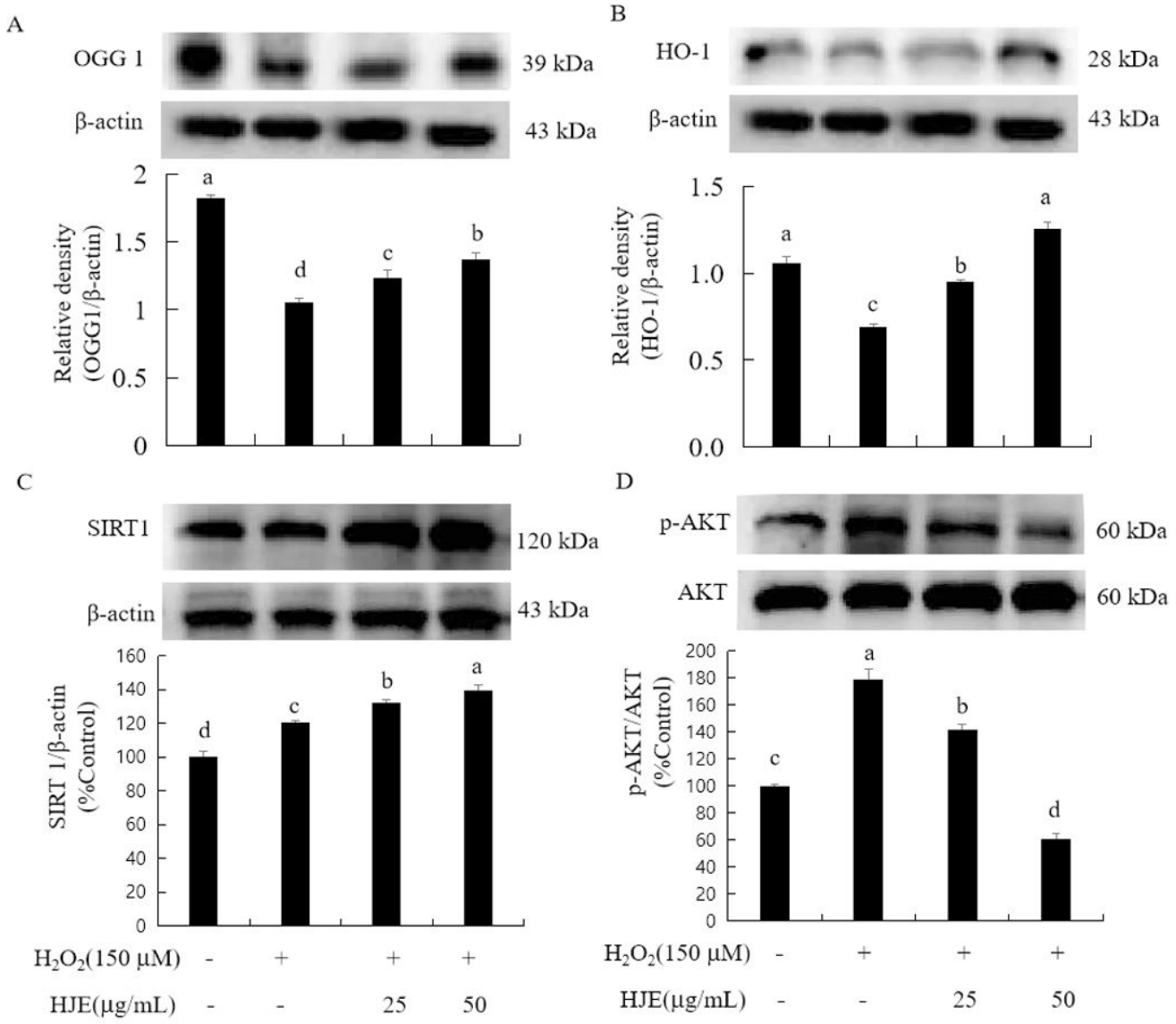

Figure 3. Effects of HJE on OGG1 (A), HO-1(B), SIRT 1(C), and AKT(D) expression in PC12 cells treated with hydrogen peroxide. Cells were pretreated with HJE at indicated concentrations for 1 h and subsequently stimulated with 150 μM of H_2O_2 for 24 h. Each bar represents the mean ± SD. Different small case letters indicate significance at $p < 0.05$ by one-way ANOVA analysis followed by Duncan's test.

3.7 Mitogen-Activated Protein Kinase (MAPK) signal pathway

Accumulating evidence has shown that the sustained activation of MAPK signaling exerts a principal effect in regulating the generation of ROS, the expression of pro-apoptotic protein, and cell proliferation (Dewanjee et al., 2018; Peti & Page, 2013). The signaling pathway of MAPK is a member of the highly conserved threonine kinase/protein serine family, comprising JNK, p38, and ERK subfamilies. ERK1/2 mediates the activation of multiple pathways, which regulate cell proliferation, translation, transcription, survival, and growth, for example MAPK, AKT (protein kinase B)-ERK1/2, and protein kinase C (PKC)-ERK1/2. JNK and P38 are activated specifically by stress and regulate the apoptosis in a variety of cells and tissues (Hotamisligil & Davis, 2016). As a result, the JNK, p38, and ERK phosphorylation levels in the PC12 cells were assessed. As shown in Figure 4, treatment with H_2O_2 for 30 minutes promotes the conversion of P38, JNK and ERK to p-P38, p-JNK, and p-ERK, respectively. Nevertheless, the PC12 cells pretreated with HJE revealed a remarkable decrease in the levels of p-P38, p-JNK, and p-ERK. The findings of this study confirmed that HJE strongly inhibited the signaling pathway of MAPK in PC12 cells stimulated by H_2O_2, which can decrease ROS production and ameliorate oxidative stress, thus promoting cell proliferation and viability.

3.8 Nuclear Factor Erythroid 2–Related Factor 2 (NRF2)

ARE/Nrf-2/Keap1 is the principal protective pathway of exogenous and endogenous ROS (Kim et al., 2008). The interaction between bioactive molecules and Keap1 results in the destruction

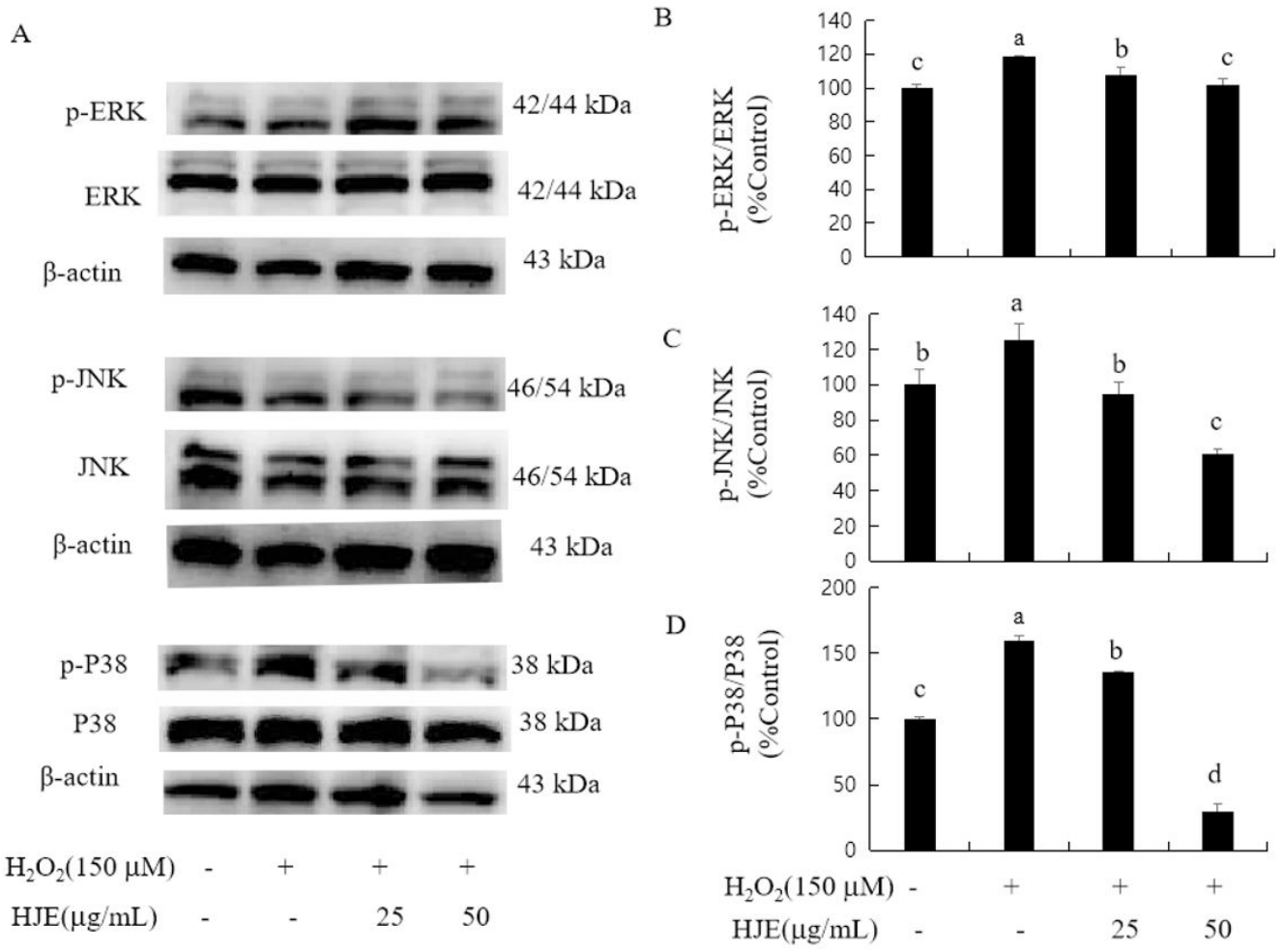

Figure 4. HJE regulates the activation of mitogen-activated protein kinase (MAPK) signal pathways in PC12 cells treated with hydrogen peroxide. Cells were pretreated with HJE at indicated concentrations for 1 h and subsequently stimulated with 150 μM of H_2O_2 for 24 h. A. Representative western blot images. B, C, D. Densitometric analysis of protein levels in (A). Quantitative results were normalized against the levels of β-actin. The results are expressed as mean ± SD (n = 3). Different small case letters indicate significance at $p < 0.05$ by one-way ANOVA analysis followed by Duncan's test.

of Nrf2/Keap1 complex, facilitating the nuclear translocation of Nrf2 and complexation with uridine- and adenylate-rich elements to trigger the expression of antioxidant proteins, for instance, HO-1 (Bhakkiyalakshmi et al., 2016). The cytosolic and nuclear activation of NRF2 in the PC12 cells was also explored. The findings suggested that both the levels of nuclear NRF2 and cytosolic NRF2 were remarkably up-regulated in cells treated with H_2O_2 only (Fig.5A, 5B). Nonetheless, cells pretreated with HJE (25 and 50 μg/mL) prior to H_2O_2 treatment revealed a remarkable upregulation in the levels of cytosolic NRF2, in comparison with cells treated with H_2O_2 alone. While a slight decrease in NRF2 was found when treated with HJE 25 to 50 μg/mL (Figure 5A), the nuclear NRF2 expression was evidently down-regulated in the cells pretreated via HJE (25 and 50 μg/mL) before treatment with H_2O_2, which was dose-dependent in comparison with the cells treated via H_2O_2 alone (Figure 5B). Nevertheless, the cytoplasmic NRF2 activation, translocation to nucleus, and the combination of the antioxidant response elements in promoter regions of several cell protective genes is also affected by many factors, which require in-depth investigation in the future.

3.9 Nuclear Factor-κB (NF-κB) pathways

NF-κB is a type of protein complex, which controls the cell survival, the generation of cytokines, and DNA transcription. Further, NF-κB exhibits various functions in nervous system, including memory, learning, and plasticity (Mattson & Meffert, 2006). NF-κB also is the major transcription factor, which regulates the inducible and basal expression of many antioxidant genes and regulates the ROS (Morgan & Liu, 2011). Our results revealed that both cytosolic and nuclear phosphorylation of NF-κB increased significantly when cells were treated with H_2O_2 alone (Figure 5C, 5D). Nonetheless, the cells pretreated with HJE (25 and 50 μg/mL) before treatment with H_2O_2 showed a significant dose dependence, in contrast to H_2O_2-treated cells

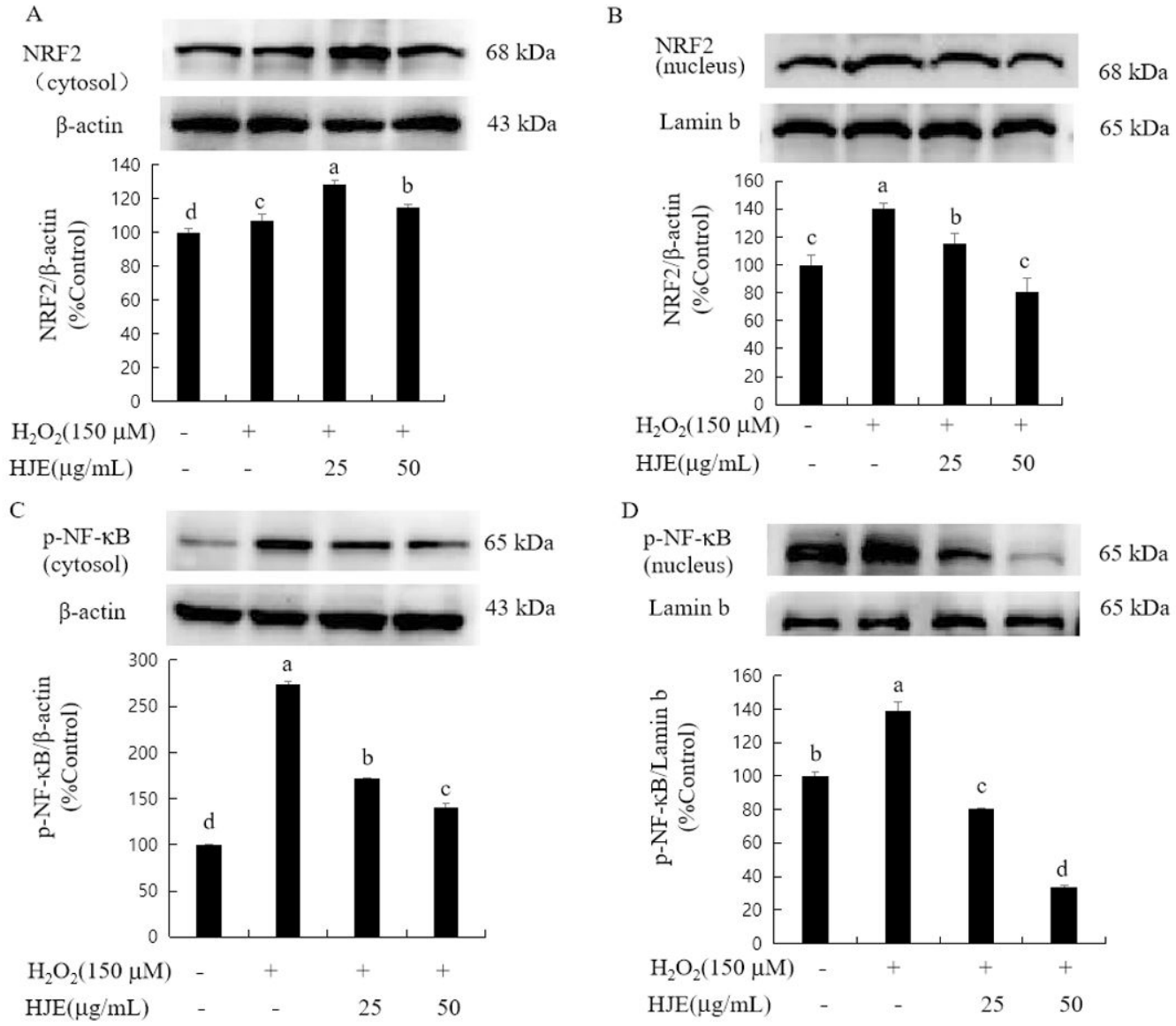

Figure 5. HJE regulates NRF2 signal pathways (A), inhibits the nuclear translocation of NRF2 (B), NF-κB signal pathways (C), and inhibits the nuclear translocation of NF-κB (D) in PC12 cells treated with hydrogen peroxide. Cells were pretreated with HJE at indicated concentrations for 1 h and subsequently stimulated with 150 μM of H_2O_2 for 0.5 h. The protein expression levels of cell signaling kinases and activated NRF2 and NF-κB in nuclear extract were investigated by western blot and the band densities were analyzed using ImageJ analysis software, with respect to β-actin and Lamin b. The results are presented as the mean ± SD (n = 3). Different small case letters indicate significant at $p < 0.05$ by one-way ANOVA analysis followed by Duncan's test.

(Figure 5C, D). Our results suggest that HJE downregulated the NF-κB phosphorylation in a concentration-dependent mode, thereby preventing oxidative stress. However, oxidative stress can cause inflammation, which increases ROS in cells, resulting in chronic inflammation (Hussain et al., 2016). As a result, oxidative stress prevention should be considered as a principal target for treating neuroinflammatory diseases.

Besides, MAPKs can activate the pathway of NF-κB (Kim & Choi, 2015), indicating that the reduction mediated by HJE in the NF-κB phosphorylation level may be downstream of MAPKs, which requires further investigation.

4 Conclusions

In conclusion, the optimal extraction method of HJ identified in the present study is 80% HEE, which provides a simple and effective method to obtain extracts with the highest TPC, TFC, and antioxidant properties. The TPC and TFC content was proportional to the antioxidant capacity. Additionally, 80% HJE exhibits antioxidant activity in PC12 cells, mediated via regulation of the MAPK and/or AKT-NRF2 and NF-κB signaling pathways, resulting in the down-regulation of antioxidant enzymes, for instance, catalase, SOD, OGG1 and HO-1 in the PC12 cells, thereby reducing the oxidative stress-induced ROS in the cell.

In addition, HJE promoted neuronal cell survival by increasing the expression of SIRT1. The present study provides evidence supporting the potential benefits of HJE in preventing oxidative stress in the PC12 cells, and hence represents a promising therapeutic agent or functional food additive for preventing and treating neurodegenerative diseases related to oxidative stress. Nevertheless, further animal and human studies are needed to completely measure the potential of the present medicinal plant for the improvement of human health.

Conflict of interest

The authors have no conflicts of interest to declare.

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