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Protective Effects of *Paeonia lactiflora* and Its Active Compound, Paeoniflorin, against Neuronal Oxidative Stress in H_2O_2-Treated SH-SY5Y Cells

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SUMMARY

Background: Hydrogen peroxide (H_2O_2), a major risk factor of neuronal oxidative stress, initiates a cascade of neuronal cell death. The purpose of this study is to investigate the neuroprotective effects and mechanisms of *Paeonia lactiflora* (PL) and its active compound, paeoniflorin (PF), against oxidative stress.
Methods: Cell viability, lactate dehydrogenase (LDH) release, reactive oxygen species (ROS) production, and apoptosis-related protein expression were observed using H_2O_2-induced SH-SY5Y neuronal cells.
Results: Treatment with 300 μM H_2O_2 induced cell loss, LDH release, ROS production, and nuclei condensation as shown through Hoechst 33342 staining in SH-SY5Y cells. However, PL and PF treatments significantly diminished neuronal cell death, LDH release, ROS production, and the number of apoptotic cell body. Furthermore, our data showed that treatment with PL and PF significantly attenuated H_2O_2-induced neuronal apoptosis by down-regulating the expression of cleaved caspase-9, caspase-3, and poly (ADP-ribose) polymerase. The expression of B-cell lymphoma 2/B-cell lymphoma 2-associated X was up-regulated, and PF (10 μg/mL) showed the strongest protective activity against neuronal oxidative stress.
Conclusion: Taken together, PL shows to be a promising agent for protection against oxidative stress-related apoptotic neuronal cell death, and its effects were attributed to PF, a major compound of PL.

1. Introduction

Overproduction of reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydroxyl radical (·OH), nitric oxide (NO), and hydrogen peroxide (H_2O_2) causes lipid peroxidation and protein damage, resulting in oxidative stress.[1] Brain has a higher oxygen demand for metabolism than that of other organs, and it also has low concentrations of antioxidants and related enzymes.[2] Because unsaturated fatty acids are abundant in the brain, lipid peroxidation occurs more easily in the brain than in other organs.[3,4] Neuronal cells in the brain are constantly exposed and irreversibly damaged by oxidative stress, which may cause neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease (PD), multiple sclerosis, and Alzheimer's disease (AD).[5] Therefore, the antioxidant effects of natural plant products, such as resveratrol, green tea, and curcumin, have received much attention owing to their effects in reducing the risk of neurodegenerative disease.[6–8]

The dried roots of peony, *Paeonia lactiflora* (PL), had been widely used in Asian countries, including China, Japan, and Korea. PL has been reported to have beneficial health effects, such as anti-oxidant,[9] anti-cancer,[10] and anti-inflammatory effects.[11] Studies on component analysis have shown that PL contains monoterpene glycosides, such as paeoniflorin (PF), albiflorin, and lactiflorin.[12] Among these components, PF, a water-soluble monoterpene glucoside, is an essential active ingredient in the biological activities of PL. Previous studies showed that PF has various pharmacological effects, including anti-oxidant,[13] anti-inflammatory,[14] and neuro-protective effects.[15] In particular, PF has been reported to ameliorate cognitive impairment in mouse model.[16] However, the neuroprotective mechanisms of PL and PF against oxidative stress have not been fully understood. Therefore, we investigated the neuro-protective effects of PL and PF against oxidative stress and the underlying mechanism of these effects on apoptosis in H_2O_2-treated SH-SY5Y neuronal cells.

2. Material and methods

2.1. Preparation of sample

The PL used in this research was supplied by the Gyeongnam Oriental Anti-aging Institute (Sancheong, Korea). PL was dried by hot air and stored at -5 °C until extraction. PL was added to 20 times of its volume of purified water and heated for 3 h at 90 °C for extraction. The PL-extracted water was filtered using No.2 filter papers (Whatman, Kent, UK), and the extracted water was evaporated at 40 °C. Next, the extract was stored at 4 °C in a refrigerator. PF (purity > 98%) was supplied from Cayman Chemical Co. (Ann Arbor, MI, USA).

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2.2. Instruments and chemicals

Dulbecco's modified eagle medium (DMEM) and penicillin/streptomycin were obtained from Welgene (Daegu, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (MO, USA). H_2O_2 was supplied from Junsei Chemical Co. (Tokyo, Japan). Bis-acrylamide 30% solution and radioimmunoprecipitation assay (RIPA) buffer were obtained from Elpis Biotech (Dajeon, Korea). Pre-stained protein size markers were obtained from GenDEPOT Inc. (Katy, TX, USA).

2.3. Cell culture

SH-SY5Y cells were grown in DMEM medium containing with 10% fetal bovine serum (Welgene), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere with 5% CO_2 incubator. The cells were sub-cultured using 0.05% trypsin-EDTA solution in phosphate buffered saline (PBS) when the cells were reached an 80% confluence. To assess the effect of PL and PF on SH-SY5Y cells, they were divided into 8 groups; 'normal' group represents non-treated group, 'control' group represents H_2O_2 (300 μM)-treated control group, 'PL10', 'PL50', and 'PL100' group represents PL (10, 50, or 100 μg/mL)-treated group stimulated with H_2O_2 (300 μM), and 'PF1', 'PF5', and 'PF10' group represents PF (1, 5, or 10 μg/mL)-treated group stimulated with H_2O_2 (300 μM).

2.4. MTT assay

After reaching approximately 80% confluence, the SH-SY5Y cells were seeded at a density of 3×10^4 cells/well into a 96-well plate and incubated for 24 h. Various concentrations of PL (10, 50, or 100 μg/mL) or PF (1, 5, or 10 μg/mL) utilized to incubate the cells for 4 h. Next, the cells were stimulated with H_2O_2 300 μM for 24 h. Afterward, MTT solution was added to the cells in a 96-well plate, which was then incubated 4 h at 37 °C. After incubation, the medium containing MTT was removed. The insoluble formazan crystals were dissolved in 200 μL of dimethyl sulfoxide (Sigma Chemical Co.) and absorbance was read at 540 nm using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA).[17]

2.5. Measurement of intracellular ROS levels

The ROS scavenging effect of PL and PF was determined using dichloro-dihydro-fluorescein diacetate (DCFH-DA; Sigma Chemical Co.).[18] 80 μM DCFH-DA were added into the each well and incubated the cells for 30 min at 37 °C. Fluorescence was determined by a FLUOstar OPTIMA (BMG Labtech., Ortenberg, Germany) at excitation and emission absorbances of 480 nm and 535 nm, respectively.

2.6. Lactate dehydrogenase (LDH) release assay

The LDH release assay was detected by an LDH Kit (Takara Bio Inc., Shiga, Japan) following the manufacture's protocol. 100 μL of cell supernatant and 100 μL of reaction mixture were added to the plate and were incubated for 30 min at room temperature. Absorbances were detected at 490 nm using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA).[19]

2.7. Hoechst 33342 staining

For the observation of apoptosis nuclei, SH-SY5Y cells were plated onto 8 chamber slides (1×10^5 cells/well). Cells were bathed with PBS (pH 7.4) and fixed with 4% paraformaldehyde for 10 min. Fixed cells were bathed with PBS and then stained with Hoechst 33342 (Life Technologies, Grand Island, NY, USA) for 20 min. Nuclear DNA staining was viewed under an Olympus BX50 fluorescence microscope (Tokyo, Japan).[20]

2.8. Western blotting

SH-SY5Y cells were scraped and rinsed with PBS. The cells were then centrifuged and subsequently lysed using RIPA buffer supplemented with 1 x protease inhibitor cocktail (Sigma Chemical Co.) according to the manufacturer's instructions. Equal amounts of protein lysates were analyzed by electrophoresis in a precast 10–13% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride membranes (Millipore, MA, USA). Next, the membrane was incubated with 5% skimmed milk (dissolved in PBS-Tween® 20) for 60 min. The membranes incubated with a primary antibody [Cleaved caspase-9, cleaved caspase-3, poly (ADP-ribose) polymerase (PARP; 1:1000, Cell Signaling, MA, USA); B-cell lymphoma 2-associated X (BAX), B-cell lymphoma 2 (BCL-2; 1:200, Santa Cruz, CA, USA); β-actin (1:1000, Cell Signaling)] for 12 h at 4 °C. The membranes were washed and incubated with secondary antibodies. Lastly, the immunostaining was visualized using a chemiluminescent, and the results were normalized to β-actin expression.

2.9. Statistical analysis

All data are provided as mean ± standard deviation (SD). Statistical analysis evaluated using the program IBM SPSS version 23 by Duncan's multiple range test (IBM Corporation, NY, USA). Significance was set at $p < 0.05$.

3. Results

3.1. The protective effects of PL and PF on H_2O_2-induced cell death in SH-SY5Y cells

Cell viability in the H_2O_2-induced control group was decreased to 38.77% compared to that of the normal group (100%) (Figure 1). However, treatment with PL and PF expressively increased cell

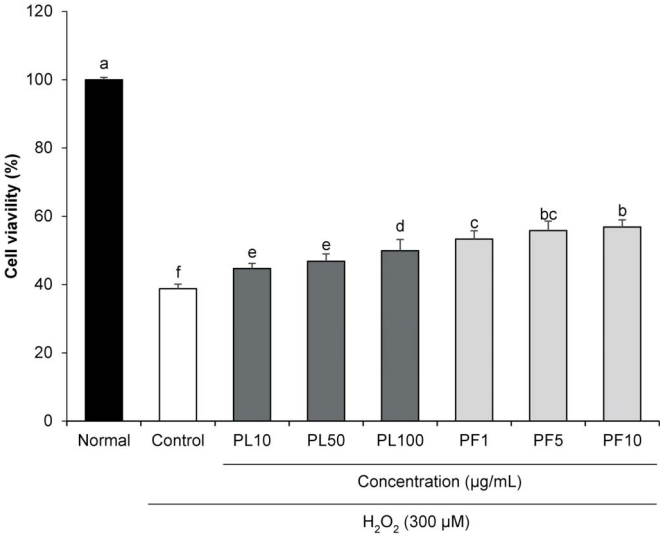

Figure 1. The protective effects of PL and PF on H_2O_2-induced cell death in SH-SY5Y cells. All the data are provided as the mean ± SD. $^{a\sim f}$ Means categorized with different letters for each experimental variable were expressed statistically significance ($p < 0.05$). PL: *Paeonia lactiflora*; PF: Paeoniflorin.

viability. Especially PL (100 μg/mL) and PF (10 μg/mL) showed 49.90% and 56.87% of viability, respectively.

3.2. The inhibitory effects of PL and PF on H_2O_2-induced intracellular ROS levels in SH-SY5Y cells

Our result shows that ROS levels in H_2O_2-treated SH-SY5Y cells significantly increased in a time-dependent manner (Figure 2). Compared to normal group (83.76%), ROS production in H_2O_2 (300 μM)-treated group was increased (100%). However, PL (100 μg/mL) and PF (10 μg/mL) significantly inhibited the excessive ROS production by decreasing it to 90.82% and 87.04%, respectively.

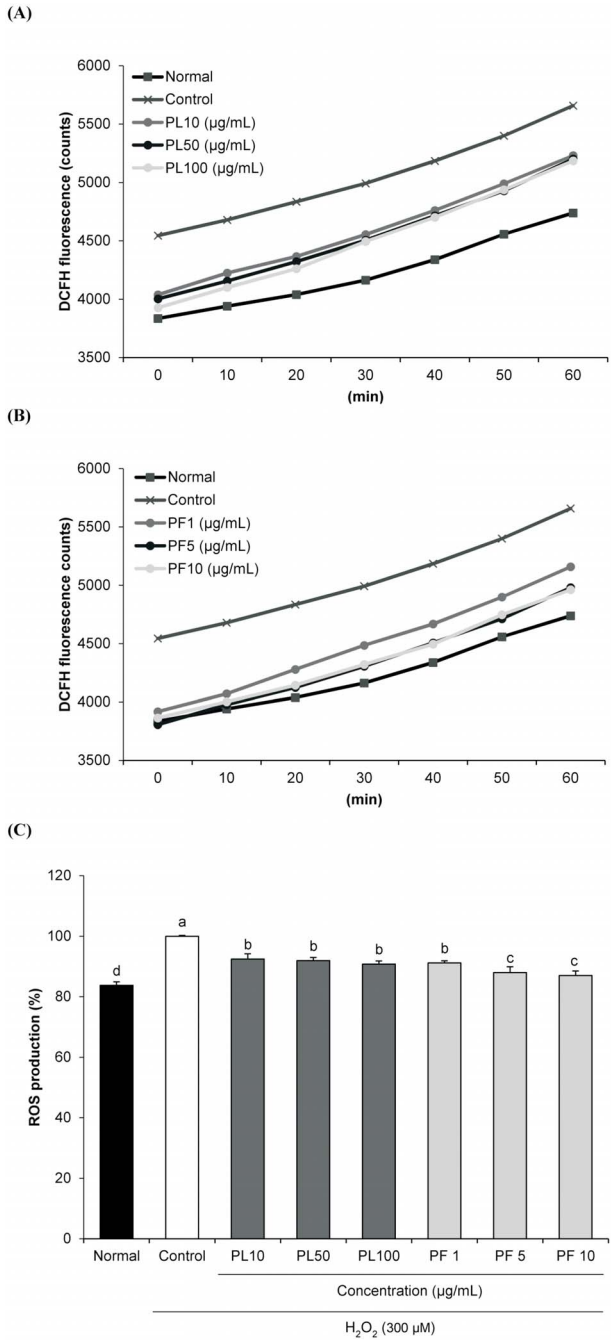

Figure 2. The inhibitory effects of PL and PF on H_2O_2-induced intracellular ROS levels in SH-SY5Y cells. (A) Fluorescence changes of the DCFH-DA in response to ROS in SH-SY5Y cells treated with PL. (B) Fluorescence changes of the DCFH-DA in response to ROS in SH-SY5Y cells treated with PF. (C) Scavenging activity of PL and PF on ROS generation treated with H_2O_2. All the data are provided as the mean ± SD. $^{a\sim d}$ Means categorized with different letters for each experimental variable were expressed statistically significance ($p < 0.05$). PL: *Paeonia lactiflora*; PF: Paeoniflorin.

3.3. The protective effects of PL and PF on H_2O_2-induced LDH release in SH-SY5Y cells

H_2O_2 treatment significantly increased LDH activity to 100%, compared with normal group (68.84%) (Figure 3). However, LDH release was decreased to 96.82% and 93.37% in the cells treated with 100 μg/mL PL and 10 μg/mL PF, respectively. Our results revealed that PL and PF have protective effects against neuronal damage induced by H_2O_2.

3.4. The anti-apoptotic activities of PL and PF against H_2O_2 by staining with Hoechst 33342 in SH-SY5Y cells

To elucidate the neuroprotective effects of PL and PF against H_2O_2-induced apoptosis, we carried out Hoechst 33342 staining. A significant increase in the number of shrunken nucleus and condensed chromatin stained with vital dye Hoechst 33342 was observed in the control group treated with H_2O_2 (300 μM) (Figure 4). On the contrary, the SH-SY5Y cells treated with PL (100 μg/mL) and PF (10 μg/mL) revealed inhibition of H_2O_2-induced nucleus condensation and apoptotic cell body.

3.5. The effects of PL and PF on the H_2O_2-induced protein expression of cleaved capase-9, -3, cleaved PARP, and BCL-2/BAX ratio in SH-SY5Y cells

To examine the protective effects of PL and PF on neuronal oxidative stress-induced apoptosis-related pathway, the caspase activation and PARP regulation were analyzed by Western blotting in H_2O_2-induced SH-SY5Y cells. Treatment with 300 μM of H_2O_2 enhanced the levels of cleaved caspase-9, and -3, and cleaved PARP. However, treatment of SH-SY5Y cells with PL and PF significantly diminished the expression levels of these proteins (Figure 5A, 5B, and 5C). In addition, in the cells treated with 10 μg/mL PF group, the expression of cleaved PARP protein, which is the final stage of apoptosis-related protein activated by the cleaved caspase-3, was nearly 2 times lower than that in the control group.

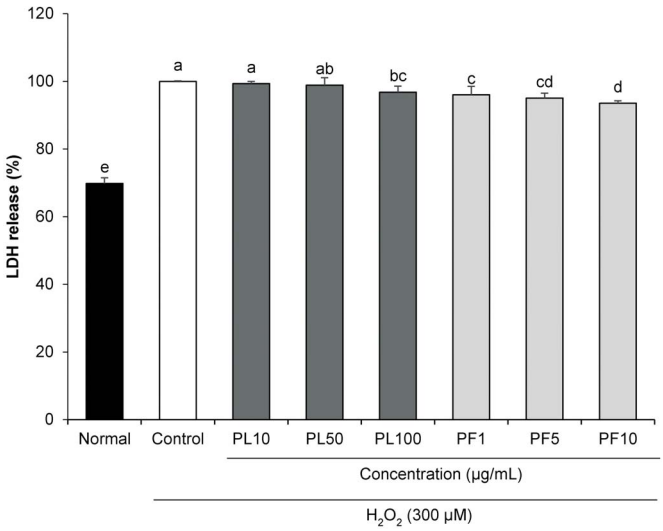

Figure 3. The protective effects of PL and PF on H_2O_2-induced LDH release in SH-SY5Y cells. All the data are provided as the mean ± SD. $^{a\sim e}$ Means categorized with different letters for each experimental variable were expressed statistically significance ($p < 0.05$). PL: *Paeonia lactiflora*; PF: Paeoniflorin.

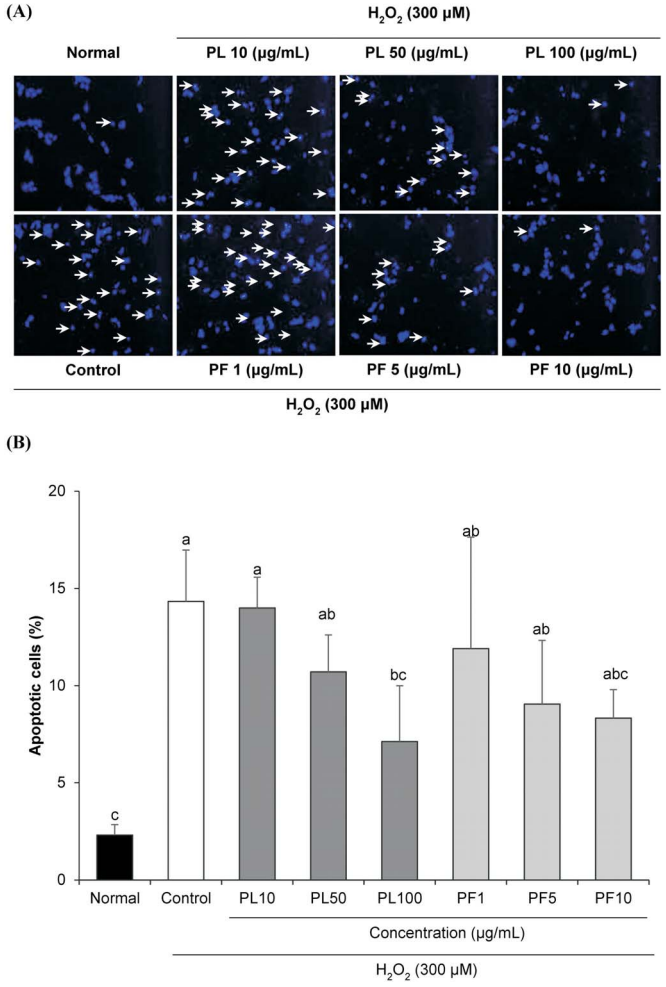

Figure 4. The anti-apoptotic activities of PL and PF against H_2O_2 by staining with Hoechst 33342 in SH-SY5Y cells. (A) Fluorescence images of SH-SY5Y cells stained with Hoechst 33342. Arrows illustrated apoptotic bodies. (B) Qualitative analysis of the apoptotic cells using a fluorescence microscope under UV illumination. All the data are provided as the mean ± SD. $^{a\sim c}$ Means categorized with different letters for each experimental variable were expressed statistically significance ($p < 0.05$). PL: *Paeonia lactiflora*; PF: Paeoniflorin.

To investigate the effects of PL and PF on upstream caspase activation, we measured the levels of BCL-2/BAX proteins expression. Treatment with 300 μM H_2O_2 increased the expression levels of the BAX and decreased the expression levels of BCL-2. However, treatment of SH-SY5Y cells with PL and PF significantly enhanced BCL-2 protein expression levels and attenuated BAX (Figure 5D). These results showed that PL and PF inhibited caspase-3, -9, and PARP cleavage by up-regulating the expression of BCL-2/BAX.

4. Discussion

PL has been known for its various effects, in particular, PL has been reported to ameliorate neuronal cell apoptosis and neurodegeneration induced by oxidative stress.[21] PF has been identified as a main active compound in PL. PF constitutes approximately 7% of components found in the root of PL.[22,23] Among the seven monoterpenes from PL extracts (albiflorin, benzoylpaeoniflorin, paeoniflorin, paeonilactone-B, paeonilactone-C, paeoniflorigenone, and oxypaeoniflorin), PF showed effective anti-inflammatory and neuroprotective properties.[24,25] However, the underlying mechanism involved in the protective effect of PL and PF, a major bioactive compound of PL, against oxidative stress-induced neuronal cell death has not been fully studied.

Firstly, we investigated the effect of PL and PF on cell viability, LDH release, and ROS production in SH-SY5Y neuronal cells treated with H_2O_2. Treatment with H_2O_2 significantly decreased the cell viability, compared with normal group. However, PL and PF significantly increased the cell viability. DCFH-DA is deacetylated to non-fluorescent DCFH by intracellular esterase, and this DCFH can be oxidized by H_2O_2.[26] Our results showed that PL and PF, especially PF, significantly decreased H_2O_2-induced ROS production. Also, when cell plasma membrane is damaged, LDH, a soluble cytosolic enzyme, is released into culture medium. Our results indicated that PL and PF significantly decreased LDH release, compared with H_2O_2-treated control group.

We explored whether PL and PF exert neuro-protective activities against H_2O_2-induced neuronal cell apoptosis, by using Hoechst 33342 staining to observe DNA condensation and morphological changes caused by H_2O_2. In our results, PL- and PF-treated cells showed reductions in H_2O_2-induced nucleus shrunken and nuclei condensation. According to a previous study, PL protects PC12 cells from H_2O_2-induced apoptosis and corticosterone-induced neurotoxicity mediated by its antioxidant action.[27] Additionally, PF attenuates amyloid $\beta_{1\text{-}42}$-induced chemotaxis and inflammation of microglia by inhibiting the nuclear factor kappa-light-chain-enhancer of activated B cells and vascular endothelial growth factor receptor Flt-1 signaling pathways.[28] Considering our current findings, the antioxidant ability of the PL may be strongly related with their active compound, PF.

Many reports showed that PARP activated by caspase-3 plays an important role in apoptosis.[29,30] According to our result, compared with H_2O_2 treatment only, treatments with PL and PF significantly diminished PARP cleavage by suppressing the activation of caspase-3 and -9. Especially, treatment with PF, compared with any concentration of PL, mostly suppressed the expression of the apoptosis-related proteins, cleaved caspase-9 and -3. Therefore, PF exerted an anti-apoptotic effect against H_2O_2-induced neuronal cell death through inactivation of the apoptosis-related signaling pathway.

The BCL-2 family composed of the anti-apoptotic members BCL-2 and BCL-X_L, as well as the pro-apoptotic members BAX and BID. Previous study has reported that BCL-2 blocks the apoptotic pathway by decreasing oxidative damage from ROS and increasing the activity of antioxidants, leading to protection of mitochondrial membrane from oxidative damage.[31] On the contrary, BAX promotes apoptosis by perturbing the permeability of the mitochondrial outer membrane and facilitating cytochrome c release.[32] In our current study, treatments with PL and PF up-regulated the expression of the anti-apoptotic protein BCL-2 and down-regulated that of the pro-apoptotic protein BAX. Treatment with PF particularly up-regulated the ratio of BCL-2/BAX protein expression. This evidence indicates that PL and PF blocked the H_2O_2-mediated neuronal cell apoptotic pathway, and PF contributed to these anti-apoptotic effects by regulating the protein expression of cleaved PARP, cleaved caspase-3, -9, and BCL-2/BAX.

5. Conclusion

PL and PF, an active compound of PL, increased cell viability and decreased ROS production. In addition, LDH release and nuclei condensation by H_2O_2 were reduced by treatments with PL and PF. Our data clearly showed that treatments with PL and PF, especially PF, exhibited a potential anti-apoptotic effect against H_2O_2 by down-regulating the protein expression of cleaved capase-3 and -9, cleaved

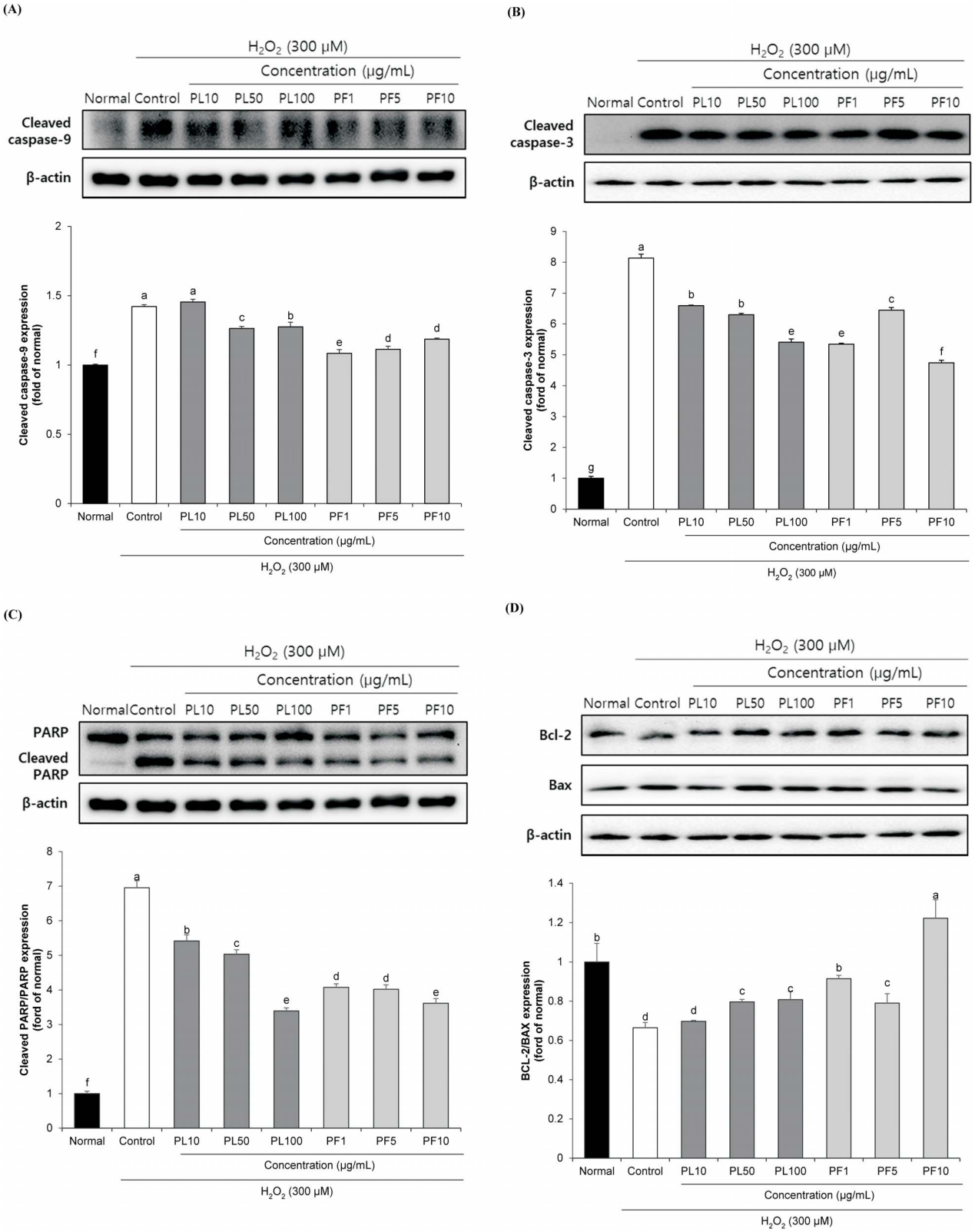

Figure 5. The effects of PL and PF on the H_2O_2-induced protein expression of cleaved capase-9 (A), -3 (B), cleaved PARP (C), and BCL-2/BAX ratio (D) in SH-SY5Y cells. All the data are provided as the mean ± SD. $^{a\sim g}$ Means categorized with different letters for each experimental variable were expressed statistically significance ($p < 0.05$). PL: *Paeonia lactiflora*; PF: Paeoniflorin.

PARP and BAX, as well as up-regulating BCL-2 protein levels. In addition, PL exerted a modulating effect against oxidative stress-related apoptotic neuronal cell death, and this effect was attributed to PF, a major compound of PL. This evidence strongly showed that PF is a preventive and therapeutic agent for oxidative stress-induced neuronal apoptosis.

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Declarations of interest

None.

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