

Neuroprotective effect of methanol extract of *Phellodendri* Cortex against 1-methyl-4-phenylpyridinium (MPP⁺)-induced apoptosis in PC-12 cells

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Abstract

Phellodendri Cortex (PC) is a traditional herbal medicine, widely used in Korea and China. The effects of the methanol extract of *Phellodendri* Cortex (PC extract) on 1-methyl-4-phenylpyridinium (MPP⁺)-induced neuronal apoptosis in PC-12 cells have been investigated. MPP⁺-induced apoptosis in PC-12 cells was accompanied by an increased bax/bcl-2 ratio, release of cytochrome c to the cytosol and activation of caspase-3. PC extract inhibited the downregulation of bcl-2 and the upregulation of bax, as well as the release of mitochondrial cytochrome c into the cytosol. In addition, PC extract attenuated caspase-3 activation and cleavage of poly (ADP-ribose) polymerase (PARP). These results suggest that the PC extract has protective effects against MPP⁺-induced neuronal apoptosis in PC-12 cells.

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Keywords: Apoptosis; Neuroprotection; PC-12 cells; *Phellodendri* Cortex; 1-Methyl-4-phenylpyridinium

1. Introduction

Phellodendri Cortex (PC) is the dried trunk bark of *Phellodendron amurense* Ruprecht, which is widely found in Korea and China. PC is a traditional herbal medicine that has anti-inflammatory (Uchiyama et al., 1989; Mori et al., 1994; Park et al., 2007), antimicrobial (Yu et al., 2005; Wang et al., 2009), antitumor (Hoshi et al., 1976; Mitani et al., 2001), hypotensive, antiarrhythmic (Wang, 1999), antioxidant (Wang et al., 2009) activities and inhibit lipid peroxidation (Kong et al., 2001). We previously reported that the methanol extract of PC (PC extract) exerts anti-inflammatory activity via

inhibition of microglial activation in neurodegenerative brain diseases (Park et al., 2007). PC contains a number of alkaloids that are known anti-inflammatory agents including berberine, palmitine, phellodendrine and jatrorrhizine (Uchiyama et al., 1989; Mori et al., 1994, 1995). As a result, PC has long been used for the treatment of various diseases and cancer in oriental clinical fields in Korea. However, to date the neuroprotection-related pharmacological action of PC extract has not been thoroughly studied.

1-Methyl-4-phenylpyridinium (MPP⁺), the active metabolite of 1-methyl-4-phenyl-2,3,6-tetrahydropyridine (MPTP), can cause apoptosis by inhibiting complex I of the mitochondrial electron transport chain (Singer and Ramsay, 1990). It is generally accepted that low MPTP/MPP⁺ doses lead to apoptosis, whereas high MPTP/MPP⁺ doses is acutely toxic, leading to necrotic degeneration (Nicotra and Parvez, 2002). A number of genes and their proteins determine the progression of apoptosis, the bcl-2 family being among the regulatory genes; bcl-2 is anti-apoptotic, whereas bax is pro-apoptotic. Dimerization of anti-apoptotic factor with pro-apoptotic factor

Abbreviations: DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MPP⁺, 1-methyl-4-phenylpyridinium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasolium bromide; PARP, poly (ADP-ribose) polymerase; PC, *Phellodendri* Cortex; SDS, sodium dodecyl sulfate.

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is probably a critical interaction (Zhou and Zhu, 2000). Apoptosis induced by oxidative stress is associated with the release of cytochrome c, activation of caspases and cleavage of poly (ADP-ribose) polymerase (PARP) (Nicholson et al., 1995; Slater et al., 1995; Tewari et al., 1995; O'Brien et al., 2000). Furthermore, PC-12 cells from the rat pheochromocytoma line (Greene and Tischler, 1976) serve as a useful model system for studying the molecular mechanisms responsible for neuronal survival under MPP⁺-induced oxidative stress (Kim et al., 2003; Chen et al., 2006; Guan et al., 2006; Wu et al., 2007). No specific or effective therapeutic agents to restrict neuronal damage and neurological dysfunction without undesirable side-effects are currently available. Thus, there is a need to develop new protective agents that can prevent the progression of neuronal apoptosis. Recently, several preclinical and clinical studies reported that several oriental herbal plants or prescriptions have protective actions against apoptosis, and are potential therapeutic agents. In the present study, MPP⁺ was used to induce apoptosis in PC-12 neuronal cells, and the protective effect of PC extract against MPP⁺-induced damages was investigated to provide a new insight to elucidate the mechanism by which it prevented apoptosis.

2. Materials and methods

2.1. Materials

Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT), 1-methyl-4-phenylpyridinium (MPP⁺), dimethyl sulfoxide (DMSO) and β -actin were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum (FBS) and RPMI-1640 were purchased from HyClone (Logan, UT, USA). Horse serum, penicillin and streptomycin were obtained from Gibco BRL (Div. of Invitrogen, Gaithersburg, MD, USA). Affinity-purified goat anti-mouse bcl-2, bax and caspase-3 p17 antibodies, anti-rabbit and anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Affinity-purified rabbit anti-mouse cytochrome c antibody was obtained from BD Pharmingen (BD Biosciences, San Diego, CA, USA), and rabbit anti-mouse PARP antibody was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Nitrocellulose membrane and ECL Western detection reagent was purchased from Amersham Bioscience (Piscataway, NJ, USA).

2.2. Preparation of methanol extract of PC

Dried PC was purchased from an oriental drug store in Yeongchun Province (Kyungpook, Republic of Korea), and its identity was verified by Dr. Y.-K. Park, College of Oriental Medicine, Dongguk University (DUCOM). A voucher specimen (PC-M0401) has been deposited in the Herbarium of DUCOM.

The PC was air-dried, after which 500 g samples were cut into small pieces and extracted 3 times with 2L of 80% methanol in a reflux condenser for 24 h. The PC extract was filtered through Whatman No. 1 filter paper, concentrated using a rotary vacuum evaporator, and lyophilized (11.16 g,

a yield of 10%). Finally, the lyophilized PC extract was dissolved in DMSO (the concentration of DMSO in the final culture medium was $\leq 0.01\%$, which had no effect on the cell viability).

2.3. Cell culture and treatment

PC-12 cells obtained from the American Type Culture Collection (ATCC) were cultured with RPMI-1640 supplemented with 10% horse serum, 5% heat-inactivated FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin, after which they were maintained in a humidified incubator in air with 5% CO₂. In all experiments, the cells were pre-incubated with different concentrations of PC extract for 2 h, after which MPP⁺ was added to the medium for an additional 24 h.

2.4. Cell viability assay

To evaluate the cytotoxic effect of the PC extract against PC-12 cells, the cell viability was determined using an MTT assay. Briefly, PC-12 cells (2.5×10^4 cells/well) were treated with different concentrations of PC extract in 96-well culture plates, after which they were cultured for 24 h at 37 °C in an air with 5% CO₂ incubator. MTT (0.5 mg/ml) was added to each well, after which the cells were cultured for an additional 4 h at 37 °C under 5% CO₂. Next, the MTT was removed, and the resulting dark blue crystals were dissolved with 100 μ l DMSO. The absorbance at 550 nm was read using an automated microplate reader (Tecan GENios, ReTiSoft Inc., Ontario, Canada).

2.5. Morphological analysis

To observe nuclear changes that occurred during apoptosis, the chromatin specific dye, Hoechst 33342, was used. Briefly, PC-12 cells were harvested and fixed with 4% paraformaldehyde for 20 min at room temperature, after which they were washed 3 times with 0.02 M PBS and exposed to 10 μ g/ml Hoechst 33342 for 10 min at room temperature in the dark. The samples were observed under a fluorescence microscope (Olympus Imaging America Inc., Center Valley, PA 18034-0610, USA) at 200 \times magnification.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The mRNA levels of bcl-2 and bax were assessed by RT-PCR. Briefly, PC-12 cells (1×10^5 cells/ml) were treated with different concentrations of PC extract for 2 h, after which they were stimulated with 70 μ M MPP⁺ for 24 h. Total RNA was prepared by adding TRIzol reagent according to the manufacturer's protocols (Gibco BRL). The total RNA was stored at -70 °C until use. Two μ g of total RNA from each sample were reverse transcribed at 42 °C for 1 h in a reaction mixture containing 5 \times RT-buffer (Promega Corp., Madison, WI 53711, USA), 0.5 mM of each dNTP, 3 mM MgCl₂, RNase inhibitor and Improm-IITM reverse transcriptase (2 U).

The PCR reaction mixture comprised of the following: 2 μ l cDNA, 4 μ M 5' and 3' primers, a 10 \times buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100, 25 mM MgCl₂; Takara Bio Ltd., Shiga, Japan), 250 μ M of dNTP and 1 U of Tag polymerase. PCR was conducted by subjecting the reaction mixture to the following incubation conditions: 95 °C for 5 min, followed by 27 cycles of 95 °C for 1 min, 61 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min for bcl-2; 95 °C for 5 min, followed by 27 cycles of 95 °C for 1 min, 65 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 10 min for bax; 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The RT-PCR products were then electrophoresed on a 1% (w/v) agarose gel, stained with ethidium bromide and visualized by UV light.

The PCR primers used for this study were as follows: 5'-CAC CCC TGG CAT CTT CTC CT-3' (sense), 5'-GTT GAC GCT CCC CAC ACA CA-3' (antisense) for bcl-2 (Accession No.; 1111, 349 bp); 5'-GCA GGG AGG ATG GCT GGG GAG-3' (sense), 5'-TCC AGA CAA GCA GCC GCT CAC G-3' (antisense) for bax (Accession No.; 1111, 352 bp); and 5'-CTC GTG GAG TCT ACT GGT GT-3' (sense), 5'-GTC ATC ATA CTT GGC AGG TT-3' (antisense) for GAPDH (Accession No.; 1111, 420 bp) as an internal control for PCR.

2.7. Western blot analysis

PC-12 cells (1×10^5 cells/ml) were pretreated with different concentrations of PC extract for 2 h, after which they were stimulated with 70 μ M MPP⁺ for 24 h. The cells were lysed with 0.1 ml of 50 mM Tris–HCl (pH 7.2) containing 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl and 1% NP-40, after which they were centrifuged at 12,000 rpm for 10 min. The supernatant was collected and used for further analyses.

To analyze the cytochrome c release, the cytosolic fractions were prepared by differential centrifugation using the method described by Sugawara et al. (2002). The protein concentration was determined using the Bradford staining method, after which equal amounts of protein (20 μ g) were electrophoresed on 10–15% density SDS-acrylamide gels. Following electrophoresis, the proteins were transferred from the gel to a nitrocellulose membrane using an electric transfer system. Non-specific binding was blocked with 5% skim milk in TBST buffer (5 mM Tris–HCl, pH 7.6, 136 mM NaCl, 0.1% Tween-20) for 1 h. The blots were incubated with antibodies against bcl-2 (1:500), bax (1:1000), cytochrome c (1:250), PARP (1:1000), caspase-3 p17 (1:200) and actin (1:5000) overnight at 4 °C, after which they were washed 3 times with 1 \times TBST. The blots were incubated for 1 h at room temperature with a 1:5000 dilution of horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG. They were washed 3 times with 1 \times TBST, after which they were developed using the ECL Western detection reagents.

2.8. Statistical analysis

Data are presented as the mean \pm standard deviation (S.D.). Statistical analysis was conducted using one-way ANOVA followed by a post-hoc LSD test. $P < 0.05$ was considered to indicate statistical significance. The SPSS software package (SPSS program, version 13.0) was used for all statistical tests.

3. Results

3.1. Effect of PC extract on MPP⁺-induced cytotoxicity

To exclude the possibility that reductions in the viability of the cells occurred due to direct toxicity of the PC extract against the cells, we evaluated cell viability in response to different concentrations of PC extract using an MTT assay. PC extract-induced cell toxicity was negligible at concentrations of 10 and 30 μ g/ml in PC-12 cells (Fig. 1A).

The viability of cells exposed to 70 μ M MPP⁺ for 24 h was $80.2 \pm 2.2\%$ that of the control value, while cells that were pretreated with for 2 h with PC extract at a concentration of 10 and 30 μ g/ml prior to exposure to MPP⁺ increased significantly to $89.6 \pm 3.0\%$ and $92.9 \pm 2.6\%$ of that of the control

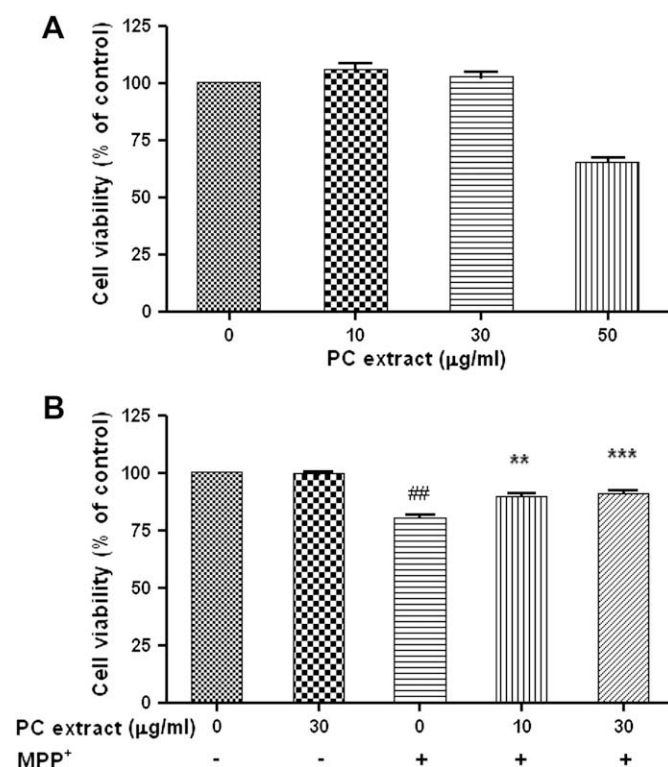


Fig. 1. Cytotoxic effect and protective effect of PC extract in PC-12 cells. Cells were treated with different concentrations of PC for 24 h, after which the cell viability was measured by MTT assay (A). PC-12 cells were pretreated for 2 h with 10 or 30 μ g/ml PC extract prior to exposure to 70 μ M MPP⁺ for 24 h. Treatment with PC extract attenuated the MPP⁺-induced reductions in the viability of PC-12 cells (B). Three independent experiments were performed, and the data shown are reported as the mean percentage of treated cells when compared to untreated control cells \pm S.D. ## $P < 0.01$ vs. control; ** $P < 0.01$, *** $P < 0.001$ vs. MPP⁺.

value, respectively (Fig. 1B). Taken together, these results indicate that the viability of MPP⁺-treated cells decreased significantly, but that the PC extract exerted a protective effect against the MPP⁺-induced cytotoxicity.

3.2. Effect of PC extract on the morphology of cell nuclei

To determine if the cell protection conferred by PC extract occurred due to the inhibition of apoptosis, PC-12 cells were treated with MPP⁺ and the indicated concentrations of PC extract. The cells were stained with Hoechst 33342, which revealed that the nuclei of the control cells were round to oval, with a uniform pattern of blue fluorescence (Fig. 2A). Following treatment with MPP⁺ at a concentration of 70 μ M for 24 h, the cell nuclei that contained chromatin became brighter and shrunk (Fig. 2B). Finally, the nuclei decreased in size, indicating apoptosis. In contrast, cells that were pretreated with PC extract appeared remarkably preserved and the alterations observed in the MPP⁺ treated cells were significantly attenuated (Fig. 2C and D).

3.3. Effects of PC extract on the mRNA levels of bcl-2 and bax

RT-PCR was conducted to evaluate the mRNA expression of bcl-2 and bax in PC-12 cells. The mRNA expression of bcl-2 decreased, while that of bax was increased in response to treatment with 70 μ M MPP⁺. Conversely, the mRNA expression of bcl-2 increased, while that of bax decreased in response to pretreatment with PC extract at concentrations of

10 μ g/ml and 30 μ g/ml, respectively (Fig. 3A). Furthermore, a dose-dependent decrease in the bax to bcl-2 mRNA ratio was observed in response to pretreatment with PC extract (Fig. 3B).

3.4. Effects of PC extract on the protein expression of bcl-2 and bax

The bax to bcl-2 expression ratio can be used to determine whether a cell has undergone apoptosis. In MPP⁺-treated cells, the expression of bcl-2 protein was downregulated, whereas the expression of bax protein was upregulated (Fig. 4A), which resulted in a high bax to bcl-2 ratio. However, pretreatment with 10 and 30 μ g/ml PC extract attenuated the change in bax and bcl-2 that was induced by MPP⁺, resulting in a decrease in the bax to bcl-2 ratio (Fig. 4B).

3.5. Effects of PC extract on cytochrome c release, caspase-3 activity and PARP cleavage

To determine whether PC extract inhibits the level of cytochrome c released from the mitochondria into the cytosol, MPP⁺-treated cells was analyzed by Western blot. The release of cytochrome c from the mitochondria into the cytosol increased in response to treatment with 70 μ M MPP⁺ for 24 h (Fig. 5A); however, this increase was inhibited by pretreatment with PC extract at 10 and 30 μ g/ml. The effect of PC extract on the MPP⁺-induced upregulation of active caspase-3 is shown in Fig. 5B. Active caspase-3 expression increased dramatically following 24 h of treatment with MPP⁺ alone.

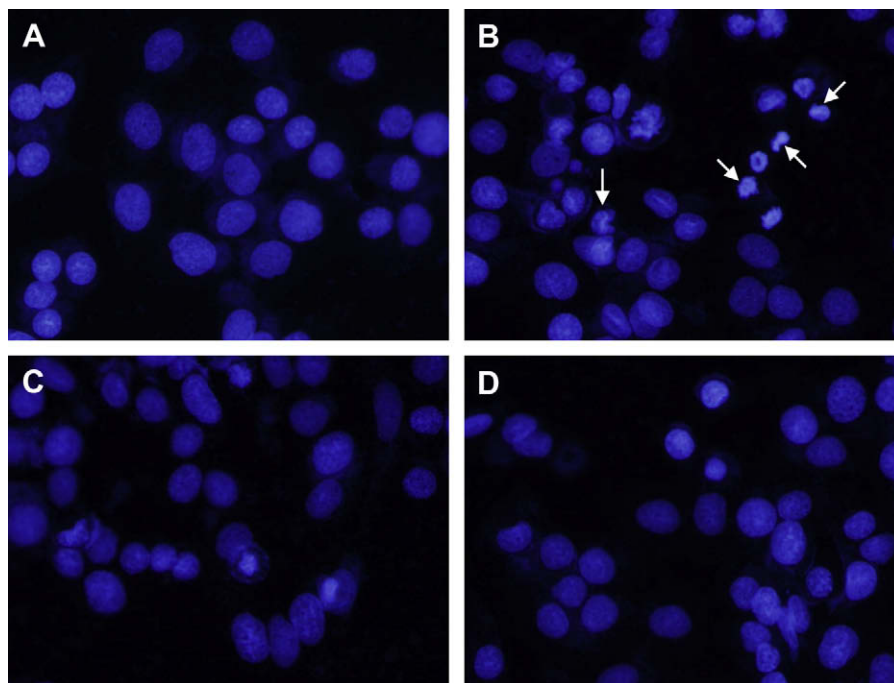


Fig. 2. Morphological analysis of nuclear chromatin in PC-12 cells as determined by Hoechst 33342 staining. The cells were treated with MPP⁺ in the absence or presence of PC extract. (A), control conditions; (B), after exposure to 70 μ M MPP⁺ for 24 h; (C) and (D), cells displayed condensed chromatin and apoptotic nuclei. PC-12 cells were pretreated with different concentrations of PC extract for 2 h prior to exposure to 70 μ M MPP⁺ for another 24 h. Photographs were taken using a fluorescence microscope at 200 \times magnification. Representative data shown were selected from 3 independent experiments. The arrows indicate apoptotic cells.

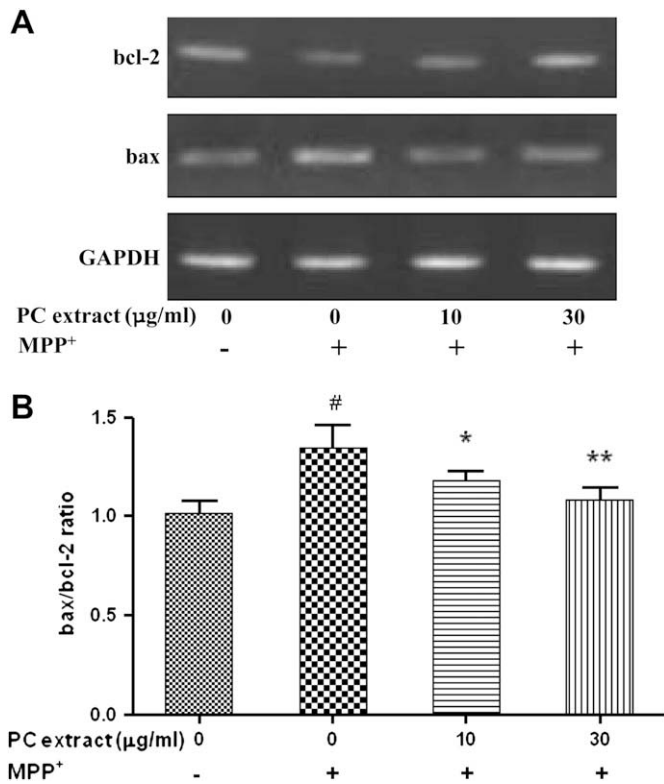


Fig. 3. Effect of PC extract on the mRNA expression of bcl-2 and bax induced by MPP⁺ treatment of PC-12 cells. The cells were treated with MPP⁺ in the absence or presence of PC extract. RT-PCR was performed to determine the bax and bcl-2 mRNA levels (A). The bax to bcl-2 ratio was determined by densitometric analysis (B). GAPDH transcripts are shown as an internal reference for amplification of cDNA. [#]*P* < 0.05 vs. control; ^{*}*P* < 0.05, ^{**}*P* < 0.01 vs. MPP⁺.

However, pretreatment with the PC extract attenuated this MPP⁺-induced caspase-3 activity in a dose-dependent manner. We also verified the MPP⁺ induced apoptotic cell death by examining the cleavage of PARP, which is a 116-kDa nuclear protein that is cleaved to an 89-kDa fragment by activated caspase-3. Treatment with 70 µM MPP⁺ caused a marked increase in the cleavage of PARP, which was attenuated by treatment with 30 µg/ml PC extract (Fig. 5B).

4. Discussion

MPP⁺ induces apoptosis in several cell types such as PC-12, cerebellar granule cells and SH-SY5Y neuroblastoma cells (Halvorsen et al., 2002; Sheng et al., 2002; Gonzalez-Polo et al., 2003). Regarding the possible protective effect of PC extract pretreatment, our results indicate that the viability of MPP⁺-treated cells decreased significantly, but that the PC extract exerted a protective effect against the MPP⁺-induced cytotoxicity. Also we observed a significant increase in the number of apoptotic PC-12 cells subjected to MPP⁺. The cell nuclei were decreased in size that contained chromatin became brighter and shrunk, indicating apoptosis. In contrast, cells that were pretreated with PC extract significantly attenuated the alterations observed in the MPP⁺ treated cells.

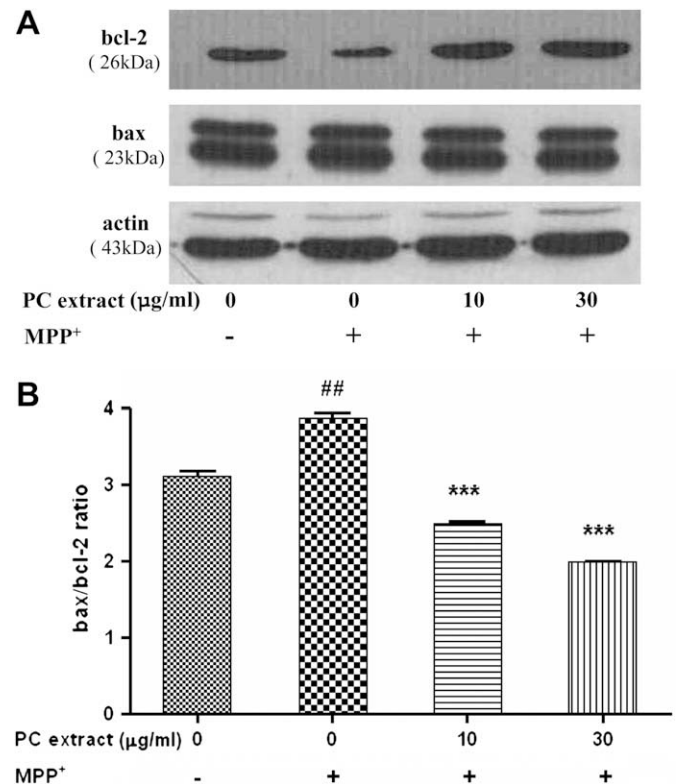


Fig. 4. Effect of PC extract on the protein expression of bcl-2 and bax induced by MPP⁺ treatment of PC-12 cells. Western blot analysis was performed to determine the bax and bcl-2 protein levels (A). The bax to bcl-2 ratio was determined using a densitometer and then plotted (B). ^{##}*P* < 0.01 vs. control; ^{***}*P* < 0.001, ^{***}*P* < 0.001 vs. MPP⁺.

It is now clear that the mitochondria lie at the centre of the cell death regulation process. A number of genes and their proteins can influence or determine the progression of apoptosis along the mitochondrial pathway (Tenneti et al., 1998; Zhou and Tang, 2002; Grammatopoulos et al., 2004). The bcl-2 family has the ability to regulate apoptosis in the mitochondrial apoptotic pathway (Oltvai et al., 1993). Bax and bcl-2, which are the two primary members of the bcl-2 family, affect the permeability of the mitochondrial membrane. Specifically, bax is a pore-forming cytoplasmic protein that translocates to the outer mitochondrial membrane. After translocation, bax influences the permeability of the membrane and induces the release of cytochrome c from the inter-membrane space of the mitochondria into the cytosol, which subsequently leads to apoptosis (Kostic et al., 1991; Yang et al., 1998). Bcl-2, which has anti-apoptotic properties, is associated with the outer mitochondrial membrane. Bcl-2 stabilizes membrane permeability, thereby preserving mitochondrial integrity, suppressing the release of cytochrome c and inhibiting apoptosis (Jang and Surh, 2004). Cell survival during the early phases of the apoptotic cascade depends primarily on the balance between the pro- and anti-apoptotic proteins of the bcl-2 family. Therefore, the bax/bcl-2 ratio may be a better predictor of apoptotic fate than the absolute concentrations of either bax or bcl-2 (Oltvai et al., 1993). The bax/bcl-2 ratio inhibits the release of cytochrome c and the activation of caspase-3 (Gross et al., 1999); therefore, any shift

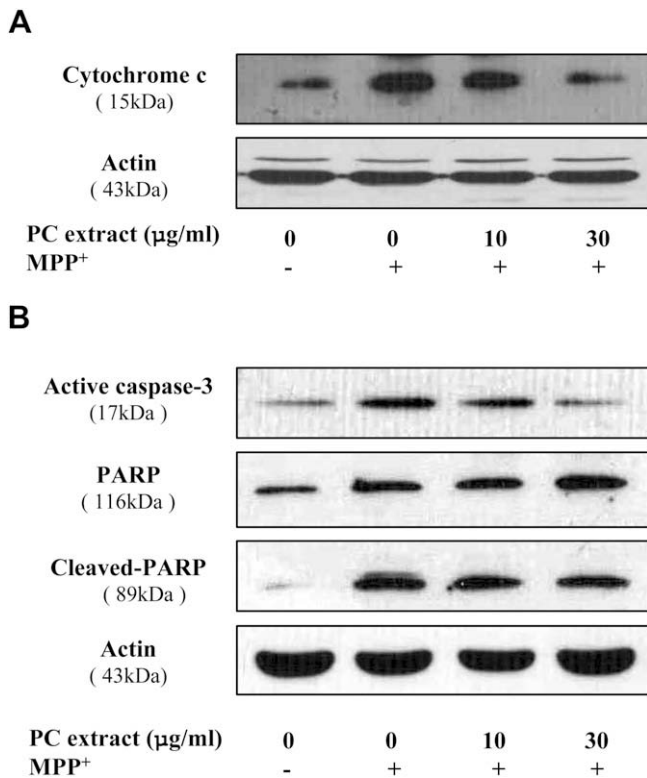


Fig. 5. Effect of PC extract on the release of cytochrome c from the mitochondria into the cytosol and on the caspase-3 activity and PARP cleavage induced by MPP⁺ treatment in PC-12 cells. (A) Cytosolic proteins were prepared from cells that were or were not pretreated with PC extract for 2 h prior to incubation in the presence of 70 µM MPP⁺ for 24 h. Western blot analysis was used to evaluate the release of cytochrome c from the mitochondria into the cytosol while the PC extract treatment blocked the release of cytochrome c from the mitochondria into the cytosol. (B) Attenuation of MPP⁺-induced caspase-3 activity and PARP cleavage by PC extract as determined by Western blot analysis.

in the balance of pro- and anti-apoptotic proteins will affect cell death. As a result, bcl-2 family members are intimately involved in cell death processes caused by MPP⁺ (Cadet et al., 2000).

In the present study, PC extract was found to ameliorate the MPP⁺-induced increase in the bax/bcl-2 ratio in PC-12 cells and that may be, at least in part, mediated by regulation of bax and bcl-2 expression in an anti-apoptotic mechanism. Taken together, the PC extract inhibits MPP⁺-induced apoptosis by suppressing the release of cytochrome c from the mitochondria to the cytosol, which, in turn, blocks the MPP⁺ enhanced activation of caspase-3 and the cleavage of PARP.

In summary, treatment with PC extract attenuated the PC-12 cell apoptosis that was induced by MPP⁺ and inhibited the release of cytochrome c into the cytosol. Indeed, PC extract was found to exert anti-apoptotic properties that led to protection against MPP⁺-induced cytotoxicity. These findings provide a further pharmacological basis for the therapeutic efficacy of PC extract during the treatment of progressive neurodegenerative diseases. However, further studies evaluating the neuroprotective mechanisms of PC extract are necessary. Future studies should be conducted to identify the active components of this extract.

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