Protective Effect of Aqueous Extract of *Ginseng radix* against 1-Methyl-4-phenylpyridinium-Induced Apoptosis in PC12 Cells

Ee-Hwa KIM, a Mi-Hyeon JANG, b Min-Chul SHIN, b Mal-Soon SHIN, b and Chang-Ju KIM*. b

a Department of Meridian & Acupuncture, College of Oriental Medicine, Semyung University; #21–1 Shinwol-dong, Jechon, 390–711, Korea; and b Department of Physiology, College of Medicine, Kyung Hee University; #1 Hoigi-dong, Dongdaemoon-gu, Seoul 130–701, Korea. Received June 4, 2003; accepted August 30, 2003

Ginseng radix, the root of *Panax ginseng* C. A. MEYER (Araliaceae), is one of the best-known Oriental medicinal herbs with numerous therapeutic applications. To investigate whether *Ginseng radix* possesses a protective effect against 1-methyl-4-phenylpyridinium (MPP+) -induced cytotoxicity in neuronal cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry, DNA fragmentation assay, reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, and caspase-3 enzyme assay were performed on PC12 neuronal cells. Cells treated with MPP+ exhibited various apoptotic features, while cell pretreated with *Ginseng radix* prior to MPP+ exposure showed a decrease in the occurrence of apoptotic features. These results suggest that *Ginseng radix* may exert a protective effect against MPP+-induced apoptosis in PC12 cells.

**Key words** *Ginseng radix*; Parkinson’s disease; 1-methyl-4-phenylpyridinium (MPP+); apoptosis

Parkinson’s disease is a neurodegenerative condition characterized by rigidity and akinesia. A major pathologic hallmark of Parkinson’s disease is the degeneration of nigrostriatal dopaminergic neurons. It was reported that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that can induce symptoms similar to those observed in Parkinson’s disease in mice, and MPTP induces selective loss of dopaminergic neurons in the substantia nigra of mice. The toxicity of MPTP is known to be mediated through the toxic metabolite 1-methyl-4-phenylpyridine (MPP+), and various studies have shown that MPP+ treatment leads to apoptosis.

Apoptosis, also known as programmed cell death, is a form of cell death that occurs during several pathologic situations in multicellular organisms and contributes to cell replacement, tissue remodeling, and removal of damaged cells under normal conditions. It is a complex process characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and formation of “apoptotic bodies.” In numerous studies, it has been documented that the process of apoptosis is regulated by the expression of several proteins. Of these, the p53 protein plays a proapoptotic role following stress in many types of cells and upstream activation induces cell cycle deregulation. Bax, a proapoptotic protein, is expressed abundantly and selectively during apoptosis and promotes cell death. The caspases, a class of cysteine proteases, are considered to play a central role in the apoptotic process and to trigger a cascade of proteolytic cleavage events in mammalian cells. Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executors of apoptosis, being responsible either partially or wholly for the proteolytic cleavage of various proteins.

*Ginseng radix*, the root of *Panax ginseng* C. A. MEYER (Araliaceae), is one of the best-known medicinal herbs with numerous therapeutic applications. The aqueous extract of *Ginseng radix* has been used to treat a wide variety of diseases including anemia, diabetes mellitus, insomnia, ischemia, and gastritis. It was reported that the extract of *Ginseng radix* scavenges hydroxyl radicals and protects unsaturated fatty acids from decomposition caused by iron-mediated lipid peroxidation. In addition, Jin et al. reported that *Ginseng radix* alleviates scopolamine-induced learning disability and improves spatial working memory in mice. Recently, it was also reported that ginsenosides (Rb1 and Rg1), a key family of components of *Ginseng radix*, have a protective action against MPTP-induced cell death in PC12 cells.

However to the best of our knowledge, no report has been published on the effect of aqueous extract of *Ginseng radix* on MPP+-induced apoptosis in PC12 cells. In the present study, the protective effect of the aqueous extract of *Ginseng radix* on apoptosis induced by MPP+ in PC12 cells was investigated. PC12 cells retain dopaminergic characteristics and have been widely used for neurobiological and neurochemical studies in Parkinson’s disease.

**MATERIALS AND METHODS**

**Drugs and Reagents** *Ginseng radix* was purchased from the Kyung-Dong marketplace (Seoul, Korea). To obtain the aqueous extract of *Ginseng radix*, 200 g of *Ginseng radix* was added to distilled water, and extraction was performed by heating at 80 °C, concentrated with a rotary evaporator, and lyophilized. The resulting powder, weighing 30 g, was dissolved in saline. H2O2 was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). The DNA fragmentation assay kit was obtained from TaKaRa (Shiga, Japan) and the caspase-3 assay kit from CLONTECH (Palo Alto, CA, U.S.A.).

**Cell Culture** Cells of the rat pheochromocytoma PC12 line were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL) at 37°C in 5% CO2, 95% O2 in a humidified cell incubator, and the medium was changed every 2 d.

**MTT Cytotoxicity Assay** Cell viability was determined using the MTT assay kit following the manufacturer’s protocol. To determine the cytotoxicity of MPP+, cells were...
treated with MPP⁺ at concentrations of 33.66 μM, 168.29 μM, 336.59 μM, 1.68 mM, and 3.37 mM for 12 h. For analysis of the protective effect of *Ginseng radix* against cell death induced by MPP⁺, cells were pretreated with *Ginseng radix* at concentrations of 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, and 5 mg/ml for 1 h before MPP⁺ was applied at a concentration of 336.59 μM for another 12 h. Cultures of the control group were left untreated. Ten microliters of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. Solubilization solution 100 μl was then added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, U.S.A.) at a test wavelength of 595 nm and a reference wavelength of 690 nm. Optical density (O.D.) was calculated as (O.D. of 595 nm and a reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.)*100.

**TUNEL Assay** For *in situ* detection of apoptotic cells, the TUNEL assay was performed using the ApoTag® peroxidase in situ apoptosis detection kit. PC12 cells were cultured on 4-chamber slides (Nalge Nunc International, Naperville, IL, U.S.A.) at a density of 2×10⁵ cells/chamber. After treatment with *Ginseng radix* and MPP⁺, the cells were washed with phosphate-buffered saline (PBS) and fixed by incubating in 4% paraformaldehyde (PFA) for 10 min at 4 °C. The fixed cells were then incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase (TdT)-catalyzed reaction for 60 min at 37 °C in a humidified atmosphere and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with antidigoxigenin antibody conjugated with peroxidase for 30 min. DNA fragments were stained using 3,3-di-aminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO, U.S.A.) as the substrate for the peroxidase.

**Flow Cytometric Analysis** For flow cytometric analysis, after treatment with *Ginseng radix* and MPP⁺, cells were collected and fixed by incubation with 75% ethanol in PBS at −20 °C for 1 h. Afterward, the cells were incubated with RNase 100 μg/ml and propidium iodide 20 μg/ml in PBS for 30 min at 37 °C and were analyzed using FACScan (Becton Dickinson, San Jose, CA, U.S.A.).

**DNA Fragmentation** For detection of apoptotic DNA cleavage, the DNA fragmentation assay was performed using the ApopLadder EX™ DNA fragmentation assay kit (TaKaRa). Cells were treated with *Ginseng radix* and MPP⁺ and then lysed with 100 μl of lysis buffer. The lysate was incubated with 10 μl of 10% SDS solution containing 10 μl of Enzyme A at 56 °C for 1 h followed by treatment with 10 μl of Enzyme B at 37 °C for 1 h. After adding 70 μl of precipitant and resuspending the resultant pellet in TE (Tris–EDTA) buffer, genomic DNA was visualized by electrophoresis in a 2% agarose gel containing ethidium bromide.

**RNA Isolation and RT-PCR** RNA was isolated from PC12 cells using RNAzol® (TEL-TEST, Friendswood, TX, U.S.A.) following the manufacturer's instructions. RNA 2 μg and 2 μl of random hexamers (Promega, Madison, WI, U.S.A.) were added together, and the mixture was heated at 65 °C for 10 min. AMV reverse transcriptase (Promega) 1 μl, 10 mM dNTP (Promega) 5 μl, RNasin (Promega) 1 μl, and 10×AMV RT buffer (Promega) 5 μl were then added to the mixture, and the final volume was increased to 50 μl with dimethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 °C for 1 h.

**PCR amplification** was performed in a reaction volume of 40 μl containing 1 μl of the appropriate cDNA, 1 μl of each set of primers at a concentration of 10 pm, 4 μl of 10×reaction buffer, 1 μl of 2.5 mm dNTP (iNTRON, INC., Seoul, Korea), and 2 units of Taq DNA polymerase (TaKaRa). For rat p53, the primer sequences were 5’-CTCTGTATCTTCGTCCCCTT-3’ (a 21-mer sense oligonucleotide) and 5’-AGGACGCCAAACACGAAAC-3’ (a 21-mer anti-sense oligonucleotide). For rat bax, the primer sequences were 5’- CACCACTCTGAACATCATGA-3’ (a 21-mer sense oligonucleotide) and 5’-TCAAGCCATCTTCTCCAGAT-3’ (a 21-mer anti-sense oligonucleotide). For *cyclophilin*, the internal control used in the study, the primer sequences were 5’-ACCCACCGTGTTCCTGCAG-3’ (a 20-mer sense oligonucleotide) and 5’-CATTTGCCATGGACAAGATG-3’ (a 20-mer anti-sense oligonucleotide). The expected sizes of the PCR products were 560 bp (for p53), 540 bp (for bax), and 291 bp (for *cyclophilin*).

The PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, U.S.A.) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 amplification cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s, with an additional extension step at the end of the procedure at 72 °C for 5 min. For *cyclophilin*, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular Analyst™ version 1.4.1 (Bio-Rad, Hercules, CA, U.S.A.).

**Western Blot Analysis** Cells were treated with *Ginseng radix* and MPP⁺ and collected. Cells were lysed in a lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P40, 0.1% sodium dodecyl sulfate (SDS), 1 mM PMSF, and 100 μg/ml leupeptin. Protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad). Protein of 50 μg was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane. Mouse p53 antibody (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) and mouse bax antibody (Santa Cruz Biotech) were used as primary antibodies. Horseradish peroxidase-conjugated anti-mouse antibody for p53 and bax (Amersham Pharmacia Biotech GmbH, Freiburg, Germany) were used as secondary antibodies. Band detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech GmbH).

**Caspase Enzyme Activity Assay** Caspase enzyme activity was measured using the ApoAlert® caspase-3 assay kit according to the manufacturer's protocol. In brief, after treatment with *Ginseng radix* and MPP⁺, cells were lysed with 50 μl of chilled cell lysis buffer. 2×reaction buffer (containing DTT) 50 μl and 5 μl of the appropriate conjugated substrate at a concentration of 1 mM were added to each lysate. The mixture was incubated in a water bath at 37 °C for 1 h, and the absorbance was measured with a microtiter plate reader at a test wavelength of 405 nm.
Statistical Analyses  Results are expressed as mean±standard error of the mean (S.E.M.). Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan’s post-hoc test. Differences were considered statistically significant at p<0.05.

RESULTS

Effect of Ginseng radix against MPP⁺-Induced Cytotoxicity  As shown in Fig. 1, the viability of cells incubated with MPP⁺ at concentrations of 33.66 μM, 168.29 μM, 336.59 μM, 1.68 mM, and 3.37 mM for 12 h was 99.79±3.60%, 72.99±2.39%, 60.51±0.76%, 56.24±0.83%, and 17.71±0.30% of the control value, respectively. A trend of decreasing viability with increasing MPP⁺ concentration was observed. The viability of cells exposed to MPP⁺ 336.59 μM for 12 h was 56.24±0.83% of the control value, while cells pretreated for 1 h with Ginseng radix 0.01 mg/ml, 0.1 mg/ml, and 1 mg/ml before exposure to MPP⁺ increased in a statistically significant fashion to 69.01±3.08%, 72.32±3.36%, and 82.25±2.15% of the control value, respectively. Results of the MTT assay showed a significant decrease in the viability of MPP⁺-treated cells, while Ginseng radix was shown to exert a protective effect against MPP⁺-induced cytotoxicity.

Morphological Changes Induced by Ginseng radix and MPP⁺  To confirm the induction of apoptosis by MPP⁺ and the preventive effect of Ginseng radix in PC12 cells, cells treated with MPP⁺ and Ginseng radix were analyzed using the TUNEL assay. As shown in Fig. 2, TUNEL-positive cells stained dark brown under the light microscope, and nuclear condensation was observed in cultures treated with MPP⁺ 336.59 μM, while the appearance of cells pretreated with Ginseng radix 1 mg/ml prior to MPP⁺ exposure was similar to that of control cells.

Fig. 1. MPP⁺-Induced Cytotoxicity and Protective Effect of Ginseng radix
Above: Cytotoxic effect of MPP⁺. a, Control group; b, MPP⁺ 33.66 μM-treated group; c, MPP⁺ 168.29 μM-treated group; d, MPP⁺ 336.59 μM-treated group; e, MPP⁺ 1.68 mM-treated group; f, MPP⁺ 3.37 mM-treated group. Below: Effect of Ginseng radix on cell viability. A, Control group; B, MPP⁺ 336.59 μM-treated group; C, Ginseng radix 0.01 mg/ml-pretreated and MPP⁺ 336.59 μM-treated group; D, Ginseng radix 0.1 mg/ml-pretreated and MPP⁺ 336.59 μM-treated group; E, Ginseng radix 1 mg/ml-pretreated and MPP⁺ 336.59 μM-treated group. Results are expressed as mean±standard error of the mean (S.E.M.). *p<0.05 compared with the control. #p<0.05 compared with the MPP⁺-treated group.

Fig. 2. PC12 Cells Stained in the TUNEL Assay
Black arrows indicate where condensed and margined chromatin were labeled. Cells were pretreated with Ginseng radix at a concentration of 1 mg/ml for 1 h and then with MPP⁺ 336.59 μM for 12 h. Scale bars represent 100 μm.

Fig. 3. Results of Flow Cytometric Analysis
The fraction of cells in the sub-G1 phase was increased in the MPP⁺-treated cultures but reduced in the Ginseng radix-pretreated cultures to a level comparable to that seen in the control. A, Control group; B, MPP⁺ 336.59 μM-treated group; C, Ginseng radix 1 mg/ml-pretreated and MPP⁺ 336.59 μM-treated group.
Cell Cycle Distribution Changes  Flow cytometric analysis was also used to analyze the protective effect of Ginseng radix against MPP⁺-induced cell death. The fraction of cells in the sub-G₁ phase increased from 6.71% (value from the control group) to 26.62% following treatment with MPP⁺ 336.59 μM for 12 h, and this figure was reduced with pretreatment with Ginseng radix at a concentration of 1 mg/ml for 1 h to the respective value of 15.57% (Fig. 3).

Characterization of Apoptosis via Examination of DNA Fragmentation  To ascertain the protective effect of Ginseng radix against MPP⁺-induced cell death, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was analyzed. As shown in Fig. 4, treatment with MPP⁺ at a concentration of 336.59 μM for 12 h resulted in the formation of definite fragments that could be seen upon electrophoresis as a characteristic ladder pattern; pretreatment with Ginseng radix 1 mg/ml for 1 h resulted in a significantly decreased intensity of MPP⁺-induced DNA laddering.

Effects of Ginseng radix on MPP⁺-Induced Changes in mRNA Expression of P53 and Bax  RT-PCR analysis of the mRNA levels of p53 and bax was performed to provide an estimate of the relative level of expression of these genes. In the present study, the mRNA level of p53 in the control cells was set at 1.00. The level of p53 mRNA following treatment with MPP⁺ 336.59 μM markedly increased to 4.19±0.34, but decreased to 2.89±0.12 and 1.82±0.04 in cells pretreated with Ginseng radix at concentrations of 0.1 mg/ml and 1 mg/ml, respectively. The level of bax mRNA following treatment with MPP⁺ 336.59 μM markedly increased to 6.82±0.51, but decreased to 3.22±0.16 and 2.04±0.06 in cells pretreated with Ginseng radix at concentrations of 0.1 mg/ml and 1 mg/ml, respectively (Fig. 5).

Western Blot Analysis of P53 and Bax  In cells in the MPP⁺ 336.59 μM-treated groups, increased level of p53 protein (53 kDa) and bax protein (21 kDa) was detected. When pretreated with Ginseng radix, however, the level of expression of p53 and bax decreased (Fig. 6).

Caspase-3 Enzyme Activity Analysis  Caspase-3 enzyme activity was measured using DEVD-peptide-nitroanilide (pNA). After 12 h of exposure to MPP⁺ at a concentration of 336.59 μM, the amount of DEVD-pNA cleaved during 6 h was significantly increased from 4.57±0.44 pmol (the control value) to 10.59±2.64 pmol, and this number decreased to 8.41±1.23 pmol and 6.42±1.32 pmol with Ginseng radix pretreatment for 1 h at concentrations of 0.1 mg/ml and 1 mg/ml, respectively. The present results demonstrate an increase in caspase-3 enzyme activity in MPP⁺-treated cells and a dampening in this increment in cells pretreated with Ginseng radix (Fig. 7).

DISCUSSION

Parkinson’s disease is a common neurodegenerative disorder. Increasing evidence from epidemiologic studies has implicated a variety of environmental factors in the selective...
dopaminergic cell loss in the substantia nigra. Epidemiologic analyses of the causes of Parkinson’s disease have identified several neurotoxicants. Of these, MPP⁺ is converted from MPTP by the monoamine oxidase B in the inner mitochondrial membrane. At the level of the central nervous system, this process takes place mainly in the glial cells. MPP⁺ is thus selectively taken up by dopaminergic neurons via the dopamine transporter of the membrane. Various studies reported that MPP⁺ induces apoptosis in several cell types such as PC12,18 cerebellar granule cells,19 and SHSY-5Y neuroblastoma cells.20

The pharmacologic effects of *Ginseng radix* in the central nervous, cardiovascular, endocrine, and immune systems have been demonstrated.12–14 In addition, *Ginseng radix* has been used as a medicinal herb for the treatment of various neurodegenerative disorders such as ischemia21 and Alzheimer’s disease.22 Saponins are key components of the pharmacologic application of *Ginseng radix*. Saponins can be classified into three major groups according to their chemical structures: protopanaxadiol (ginsenoside Rb₁, Rb₂, Rc, and Rd), protopanaxatriol (ginsenoside Re, Rf, Rg₁, and Rg₂), and oleandric acid saponins.20 Of these, Rb₁ and Rg₁ have a protective effect against MPTP-induced cell death in PC12 cells.17

In the present study, MTT assay confirmed that MPP⁺ exerts cytotoxicity in a dose-dependent manner and that administration of *Ginseng radix* has a protective effect against the cytotoxic action of MPP⁺ (Fig. 1). From flow cytometric analysis of DNA contents, an increase in the fraction of cells in the sub-G₁ phase was observed in the MPP⁺-treated group. This observation is similar to the results reported by Wang and Zhu23 in their study involving cultured PC12 neuronal cells. This increased sub-G₁ phase fraction was reduced by pretreatment with *Ginseng radix* to a level comparable to that seen in the control group (Fig. 3).

It is known that apoptosis involves the activation of endonucleases and that this activation results in the cleavage of genomic DNA into well-defined fragments, which appear as a characteristic ladder pattern upon agarose gel electrophoresis.24 To provide evidence supporting the involvement of apoptosis in MPP⁺-induced cytotoxicity and the protective action of *Ginseng radix* against MPP⁺-induced apoptosis, the DNA fragmentation assay was performed. The MPP⁺-treated group showed the distinctive ladder pattern characteristic of apoptosis, similar to the results observed by González-Polo et al.19 in cultured rat cerebellar granule cells and those presented by Chun et al.25 in their study involving a substantia nigra-derived dopaminergic cell line. On the other hand, the cells pretreated with *Ginseng radix* showed noticeable decrease in the intensity of MPP⁺-induced DNA laddering (Fig. 4). DNA strand breaks are known to occur during apoptosis, and it is known that nicks in the DNA molecules can be detected in the TUNEL assay.26 In this study, TUNEL-positive cells were detected in the MPP⁺-treated group, while the occurrence of such cells was decreased in cultures pretreated with *Ginseng radix* (Fig. 2).

MPP⁺ has been reported to induce neuronal apoptosis,16–20 and understanding the molecular events triggering apoptosis is an important step toward the development of effective treatment strategies for such neurologic disorders. In various studies, reactive oxygen species (ROS) have been implicated in Parkinson’s disease.27,28 Cytotoxicity induced by MPP⁺ exposure is mediated by oxidative stress.19,25,28 Recently, González-Polo et al.19 reported that free radicals play an active role in the early events of MPP⁺-induced apoptosis. Numerous studies have shown that *Ginseng radix* has potent antioxidative property29 and ginsenoside Rg₁ attenuates dopamine-induced apoptosis by suppressing oxidative stress in PC12 cells.25 In addition, it was also reported that ROS generated by MPP⁺ lead to the activation of p53, bax,23 and caspase-3.19

It is known that p53 plays a crucial role in the regulation of cell cycle and apoptosis. Duan et al.30 reported that p53 inhibitors preserve MPTP-induced cell death in dopamine neuron MN9D cells. The results of the present study demonstrated increased p53 expression in MPP⁺-treated PC12 cells, and cells pretreated with *Ginseng radix* showed a decrease in this increment (Fig. 6). Bax is a member of the bcl-2 family that acts as a promoter of cell death.31,32 MPTP administered *in vivo* to mice induces an increase in the amount of bax in the substantia nigra.32 It was reported that MPP⁺ induces bax expression in dopaminergic cell neuronal cell line MN9D.33 The present results demonstrated an increase of bax expression in MPP⁺-treated PC12 cells, and cells pretreated with *Ginseng radix* showed a dampening in this increment (Fig. 6).

Caspases, a family of cysteine proteases, are an integral part of the apoptotic pathway.11 Recent reports have indicated that caspases play a role in neuronal cell death during development as well as after neuronal injury.34 Bilsland et al.35 also reported that MPP⁺-induced apoptosis involves the caspase-3 pathway. The present results demonstrated an in-
crease in caspase-3 enzyme activity in MPP⁺-treated PC12 cells, and cells pretreated with Ginseng radix showed a dampening in this increment (Fig. 7).

At present, the cellular and molecular mechanisms that underlie the action of Ginseng radix are not fully understood. Results presented in this study demonstrate that Ginseng radix exerts a significant neuroprotective effect against MPP⁺-induced apoptosis in the PC12 cells. It is possible that Ginseng radix, by attenuating MPP⁺-induced apoptosis, may offer a valuable means of therapy in the treatment of Parkinson’s disease.

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REFERENCES