Neuroprotective Effects of *Carpinus tschonoskii* MAX on 6-Hydroxydopamine-Induced Death of PC12 Cells


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**Abstract** — The present study investigated the neuroprotective effect of *Carpinus tschonoskii* MAX and its intracellular protective mechanism on 6-hydroxydopamine (6-OHDA)-induced oxidative damage in PC12 cells. We found that pretreatment of PC12 cells with *C. tschonoskii* extract significantly inhibited the cell death induced by 6-OHDA in a dose dependent manner. *C. tschonoskii* extract decreased 6-OHDA-induced apoptotic events such as chromatin condensation, DNA fragmentation, the decrease of Bcl-2/Bax ratio, caspase-3 activation and PARP cleavage. *C. tschonoskii* extract also reduced generation of 6-OHDA-induced reactive oxygen species and nitric oxide. Furthermore, *C. tschonoskii* extract up-regulated the myocyte enhancer factor 2 D (MEF2D), a critical transcription factor for neuronal survival, and Akt activity, whereas it inhibited the activity of ERK1/2 and JNK. The results suggest that *C. tschonoskii* extract decreases 6-OHDA-induced oxidative stress and could prevent PC12 cell apoptosis induced by 6-OHDA via the up-regulation of MEF2D and Akt activity, and thus may have application in developing therapeutic agents for Parkinson's disease.

**Keywords:** *Carpinus tschonoskii* MAX, 6-OHDA, PC12 cells, Apoptosis, MEF2D, Akt

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**INTRODUCTION**

Parkinson's disease (PD) is currently the most common degenerative disorder of the aging brain, after Alzheimer's disease (Bové *et al.*, 2005). The pathological hallmark of PD involves the loss of the nigrostriatal dopaminergic pathway, resulting in a marked impairment of motor control (Blum *et al.*, 2001). Although the pathogenic processes of PD remain unknown, recent findings indicate that an increase of oxidative stress may be a critical mediator of dopaminergic neuron destruction in PD (Jenner and Olanow, 1996; Przedborski and Ischiropoulos, 2005). The death of dopaminergic neurons caused by oxidative stress might produce altered activities of survival signaling factors, including myocyte enhancer factor 2 (MEF2) (Mao *et al.*, 1999), MAP Kinase pathways (Veeranna *et al.*, 2000) or the PI3K/Akt pathway (Shimo and Chiba, 2001; Greggio and Singleton, 2007). To date, however, no therapeutic drugs for PD have been identified which do not have certain adverse effects. Many studies have focused on plant components and extracts that can scavenge ROS and protect dopaminergic neurons from oxidative damage.

Recent studies showed that *Carpinus* leaves have flavonoid compounds such as myricetin, quercetin, kaempferol, apigenin and luteolin, which are known to be antioxidants (Chang and Jeon, 2004; Jeon *et al.*, 2007). In addition, cytoprotective activities of *Carpinus tschonoskii* MAX methanol extract have been reported (Zhang *et al.*, 2007). An extract of *C. tschonoskii* exhibited ROS scavenging activity which preserved V79-4 Chinese hamster lung fibroblast viability against H2O2 induced oxidative stress (Zhang *et al.*, 2007). However, a possible neuroprotective effect of *C. tschonoskii* extract, and its mechanism in a PD model, have not previously been assessed.

This study was conducted to investigate the neuroprotective effect of *C. tschonoskii* extract and its intracellular protective mechanism, using PC12 cells treated with 6-hydroxydopamine (6-OHDA) as a PD model.
MATERIALS AND METHODS

**Materials**

EtOH extract (80%) and several solvent fractions from *Carpinus tschonoskii* MAX were purchased from Jeju hi-Tech Industry Development Institute, which has professional facilities and provides extracts from plants growing on Jeju Island for research purposes in biotechnology and related industrial fields. The HPLC profile of *C. tschonoskii* EtOH extract was also obtained from Jeju hi-Tech Industry Development Institute (Fig. 1). 6-hydroxydopamine (6-OHDA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and β-actin monoclonal antibody were purchased from Sigma-Aldrich (Yongin, Kyunggi-do, Korea). MEF2D monoclonal antibody was purchased from BD Biosciences (CA, USA). ERK1/2, p-ERK1/2, p38, p-p38, SAPK/JNK, p-SAPK/JNK, Akt and p-Akt polyclonal antibodies were obtained from Cell Signaling Technology (MA, USA). HRP-conjugated goat anti-rabbit and horse anti-mouse IgGs were purchased from Vector Laboratories (MA, USA). Aprotinin, leupeptin and Nonidet P-40 were purchased from Roche Applied Science (IN, USA).

**Cell culture**

PC12, a rat pheochromocytoma cell line, was supplied by KCLB (Korea Cell Line Bank). PC12 cells were incubated in RPMI 1640 medium (HyClone, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO Inc, NY, USA) at 37°C under an atmosphere of 95% air and 5% CO₂. All experiments used cells plated at a density of 1.0×10⁵ cells/ml and were conducted 24 h after cells were seeded.

**MTT assay for cell viability**

Cell survival was evaluated by MTT reduction (Scudiero et al., 1988). PC12 cells (1.0×10⁵ cells/ml) were seeded in 24-well plates for 24 h. The PC12 cells were pretreated with 25, 50 or 100 μg/ml of 80% EtOH extract or solvent fractions from *C. tschonoskii* for 30 min prior to incubation with 250 μM 6-OHDA for 24 h. MTT was added to the cells at a final concentration of 250 μM and cultures were further incubated at 37°C with 5% CO₂ for 4 h to produce a dark blue formazan product formed by MTT reduction. Media were aspirated and the resulting formazan crystals were dissolved in DMSO (Amresco, OH, USA). The absorbance of each well was measured using a microplate reader (Amersham Pharmacia Biotech, NY, USA) at 540 nm excitatory emission wavelength. PC12 cell viability was determined as a percent of inhibition due to reduced absorbance compared to the untreated controls.

**Evaluation of intracellular reactive oxygen species (ROS)**

Intracellular ROS levels were determined using the fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Rosenkranz et al., 1992). This molecule is cleaved intracellularly by esterases to form non-fluorescent 2′,7′-dichlorofluorescin (DCFH), which is transformed to the fluorescence compound 2′,7′-dichlorofluorescein (DCF) upon oxidation by ROS. PC12 cells (1.0×10⁵ cells/ml) were seeded in 6-well plates for 24 h. The cells were pretreated with 100 μg/ml of *C. tschonoskii* 80% EtOH extract for 30 min prior to 250 μM 6-OHDA treatment for 3 h. The cells were incubated with 50 μM DCFH-DA for 20 min at 37°C under 5% CO₂ in the dark. PC12 cells were washed twice with PBS and fluorescence was monitored by a COULTER® EPICS® XL™ Flow Cytometer (Coulter, Miami, FL, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Fig. 1. LC/MS Chromatogram of flavonoids from *Carpinus tschonoskii* MAX EtOH extract by Jeju Hi-Technology Development Institute. Each peak indicates Quercetin-3-O-glucoside (a), quercitrin (b), quercetin (c), apigenin (d) and kaempferol (e).