

Neuroprotective Effect of the *n*-Hexane Extracts of *Laurus nobilis* L. in Models of Parkinson's Disease

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Abstract

Free radical scavenging and antioxidants have attracted attention as a way to prevent the progression of Parkinson's disease (PD). This study was carried out to investigate the effects of *n*-hexane fraction from *Laurus nobilis* L. (Lauraceae) leaves (HFL) on dopamine (DA)-induced intracellular reactive oxygen species (ROS) production and apoptosis in human neuroblastoma SH-SY5Y cells. Compared with apomorphine (APO, IC₅₀=18.1 μM) as a positive control, the HFL IC₅₀ value for DA-induced apoptosis was 3.0 μg/ml, and two major compounds from HFL, costunolide and dehydrocostus lactone, were 7.3 μM and 3.6 μM, respectively. HFL and these major compounds significantly inhibited ROS generation in DA-induced SH-SY5Y cells. A rodent 6-hydroxydopamine (6-OHDA) model of PD was employed to investigate the potential neuroprotective effects of HFL *in vivo*. 6-OHDA was injected into the substantia nigra of young adult rats and an immunohistochemical analysis was conducted to quantitate the tyrosine hydroxylase (TH)-positive neurons. HFL significantly inhibited 6-OHDA-induced TH-positive cell loss in the substantia nigra and also reduced DA induced α-synuclein (SYN) formation in SH-SY5Y cells. These results indicate that HFL may have neuroprotective effects against DA-induced *in vitro* and *in vivo* models of PD.

Key Words: *Laurus nobilis* L, Dopamine, Parkinson's disease, Neuroprotective, α-synuclein

INTRODUCTION

Parkinson's disease (PD) is one of the most common progressive neurodegenerative disorders, affecting about 2% of the population over the age of 60 (Lo Bianco *et al.*, 2004). PD is characterized by severe motor deficits, including uncontrollable tremors, postural imbalance, slowness of movement, and rigidity. These symptoms are caused by neuronal cell death common to many neurodegenerative disorders, such as PD and Alzheimer's disease (Golde, 2009). Many studies suggest that an imbalance between cytoplasmic and vesicular dopamine (DA) may cause cell degeneration (Barzilai *et al.*, 2001), and that this imbalance may underlie the dopaminergic degeneration observed in PD. Additionally, exogenous DA has been described as a neurotoxic factor in both *in vivo* and *in vitro* studies using primary cultures and several cell lines (Gomez-Santos *et al.*, 2003). Hence, in the course of PD studies,

6-OHDA is frequently used to generate experimental models of PD for *in vivo* studies and for the induction of neuronal cell death *in vitro*. Although the etiology of idiopathic PD remains unknown, evidence points to the involvement of a broad range of factors, including oxidative stress, mitochondrial dysfunction, and environmental toxins (Mattson, 2000). However, treatment of PD remains unsatisfactory (Chen and Oebering, 2005).

Histologically, PD is distinguished by the selective loss of DA-producing neurons in the substantia nigra (Dauer and Przedborski, 2003). In addition, SYN the major component of Lewy bodies, is one of the neuropathological hallmarks of PD (Dedov *et al.*, 2001). Diffused accumulation of SYN protein occurs with aging in the substantia nigra pars compacta (Chu and Kordower, 2007) and prior to frank inclusion formation in PD patients (Chu *et al.*, 2006). These observations indicate that a simple increase in the levels of SYN expression is suf-

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ficient to cause neurodegeneration and that such an increase may underlie the pathogenesis of PD. Thus, we used SYN as a degenerative indicator of PD to screen plant extracts that inhibit SYN expression in SH-SY5Y cells. From these studies, we selected the leaves of *Laurus nobilis* as an active plant extract against PD.

Bay Laurel (*Laurus nobilis*, Lauraceae) is natively distributed in the Mediterranean Basin and has been used as a fixture in European and North American cuisines and also as a flavor in many classic French dishes. It has been reported that *Laurus nobilis* has analgesic, anti-inflammatory, anticonvulsant (Sayyah *et al.*, 2002; Sayyah *et al.*, 2003), and antioxidant activities (Conforti *et al.*, 2006). However, the neuroprotective effect of *Laurus nobilis* against PD has not been reported. In this study, the biological activity of the *n*-hexane fraction from *Laurus nobilis* (Lauraceae) leaves (HFL) and its two major compounds, costunolide and dehydrocostus lactone, were examined *in vitro* and *in vivo* to evaluate the therapeutic potential of HFL against PD.

MATERIALS AND METHODS

Materials

Propidium iodide (PI), Dulbecco's modified Eagle's medium, fetal bovine serum, penicillin, and streptomycin were purchased from Meiji Seika (Tokyo, Japan). DA, 6-OHDA, and sodium dodecylsulfate were from Sigma-Aldrich Co. (St. Louis, MO, USA). Hybond-polyvinylidene difluoride membrane was obtained from Amersham Pharmacia Biotechnology (Piscataway, NJ, USA).

Extraction and analysis of HFL

The leaves of *Laurus nobilis* were imported from Turkey by Orege Forest Agricultural & Food Products Foreign Trade, Ltd. The plant was identified by professor Youngbae Shu. Voucher specimens (NPRI-Q003) have been deposited in the Natural Products Research Institute herbarium, Seoul National University, Korea. The dried leaves of *Laurus nobilis* (40.0 kg) were extracted with methanol (3×20 L, 24 hr each) at room temperature and concentrated under vacuum at 40°C to yield a brown residue (2.4 kg). The residue was partitioned between

Table 1. IC₅₀ value represents the concentration (μM) required for 50% neuroprotective effects of the compounds against H₂O₂- or DA-induced apoptotic cell death in SH-SY5Y cells. CC₅₀ represents the concentration of compound required for 50% toxic effects

Samples	Units	IC ₅₀ mean ± S.D.		50% cytotoxic concentration (CC ₅₀) mean±S.D.
		H ₂ O ₂ induced apoptosis	Dopamine-induced apoptosis	
HFL	(μg/ml)	17.21 ± 1.2	3.02 ± 0.22	47.1
Costunolide		3.7 ± 1.1	7.3 ± 0.90	23.3 ± 0.50
Dehydrocostus lactone	(μm)	1.9 ± 0.41	3.6 ± 0.82	18.8 ± 1.1

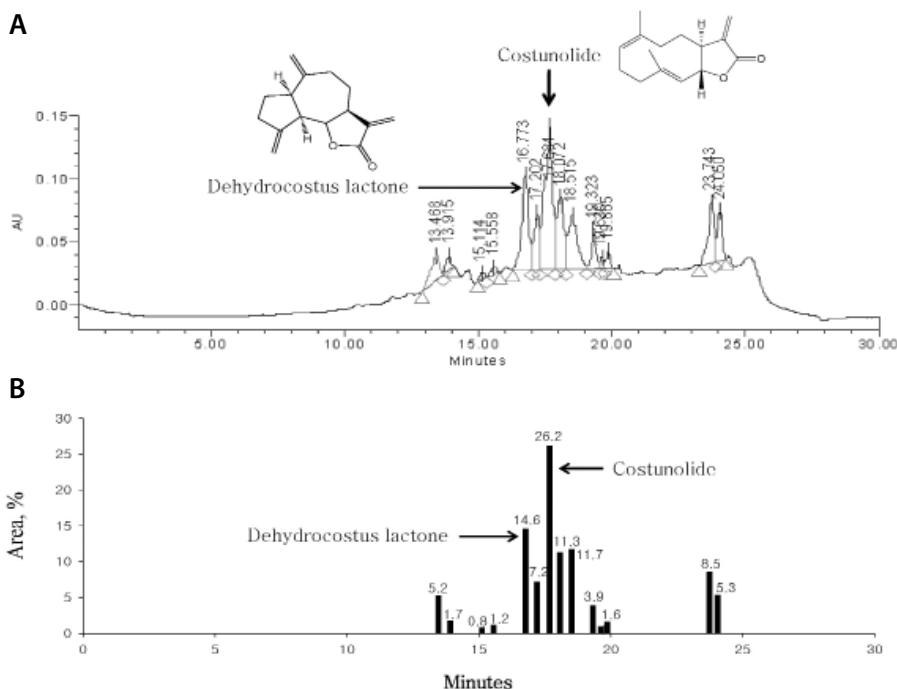


Fig. 1. (A) HPLC chromatogram of HFL. (B) The relative constituents of HFL and the major compounds, costunolide (26.2%) and dehydrocostus lactone (14.6%).

n-hexane and 10% aqueous methanol (2 L each). The re-partitioned HFL was dried under reduced pressure using a rotary evaporator. The HFL was then weighed to calculate the yield and analyzed using HPLC (RP-18, 5 μ m, 250 \times 4.6 mm). A gradient system of aqueous methanol from 90% (0 min) to 100% (30 min) was applied at a flow rate of 1 ml/min (Fig. 1).

Cell culture

Numerous studies have used the human neuroblastoma SH-SY5Y cell line as a model cell system for studying the mechanisms involved in DA-mediated neurotoxin for PD (Kobayashi *et al.*, 2008). Human neuroblastoma SH-SY5Y cells (ATCC No. CRL-2266) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified 5% CO₂ atmosphere at 37°C.

Evaluation of apoptosis

The fluorescent DNA label PI was used to evaluate apoptosis by differentiating between dead cells and living cells (Zhang and Zhao, 2003). For the experiments, SH-SY5Y cells were plated at a density of 3 \times 10⁵ cells/well in 6-well plates and cultured for 24 hrs. After the cells were pretreated with HFL (0.2, 1, and 5 μ g/ml, in 0.5% dimethyl sulfoxide) for 24 hrs, the medium was replaced with fresh medium containing 600 μ M of DA for another 24 hrs. The cells were harvested after a brief wash with PBS, suspended in 400 μ l PBS, and incubated with PI (40 μ g/ml) for 30 min in the dark. The amount of PI incorporated was analyzed by flow cytometry (Coulter EPICS XL, Ramsey, Minnesota, USA).

Detection of intracellular reactive oxygen species (ROS) content

The intracellular ROS level was detected using the oxidation-sensitive fluorescence probe DCFH-DA (LeBel *et al.*, 1992). Briefly, cells were seeded into 6-well plates (3 \times 10⁵ cells/well) and treated with HFL or its two major compounds and 600 μ M of DA for 4 hrs. DCFH-DA was added in the dark at 10 μ M final concentration 30 minutes before the end of incubation. Then, cells were collected, washed in 10 mM sodium phosphate and pH 7.2 buffer containing 150 mM NaCl (PBS), and finally resuspended in 500 μ l PBS for the fluorimetric analysis using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., CA, USA). Excitation and emission wavelengths were 485 nm and 530 nm, respectively; both excitation and emission slits were set at 10 nm.

Western blot analysis

SH-SY5Y cells were seeded at a density of 3 \times 10⁵ cells/well in 100-mm dishes and cultured for 48 hrs. The cells were then incubated with 0.2, 1, and 5 μ g/ml of HFL and 600 μ M of DA for 15 hrs. To extract proteins, the cells were washed twice with PBS and then lysed in PRO-PREP protein extraction solution (Intron Biotechnology, Seongnam, Korea). Equal amounts of cell lysates were loaded onto each lane (10 μ g/lane), separated by 12% SDS-PAGE with tris-glycine running buffer, and transferred to polyvinylidene difluoride membranes by electrotransfer for 1 hr at 265 mA. The membranes were incubated overnight at 4°C with the anti-human primary antibody (1:1,000) for SYN (Abcam, Cambridge, UK). Following 1 hr of incubation at room temperature with secondary antibody (1:5,000) of anti-mouse HRP-conjugated IgG (Chem-

con, Temecula, CA, USA), they were analyzed using image analyzer LAS1000 (Fuji, Japan). The blots were then stripped and re-probed with anti-human β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected as described above. The blots were performed at least three times to confirm data reproducibility.

Animals

Male Sprague-Dawley rats, weighing 220-230 g, were purchased from Samtako (Bio Korea, Osan-shi, Korea). The animals were housed at room temperature for 1 week prior to the experiments in a humidity-controlled environment with 12 h light/dark cycles and unlimited access to food and water. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Seoul National University.

6-OHDA lesions

Rats weighed approximately 250 g at the beginning of the experiment and were anesthetized with ketamine (80 mg/kg, i.p.; Ketaset[®]; Fort Dodge, IA, USA) followed by xylazine (10 mg/kg, i.p.; Rompun[®], Bayer, UK) and placed in a stereotaxic frame (Dae-Jong Instrument Industry Co., Seoul, Korea). They were then injected with 20 μ g of 6-OHDA into the left substantia nigra 4.8 mm posterior, 1.8 mm lateral, and 7.8 mm ventral to the bregma and dura (Paxinos and Watson, 1998) at 1 μ l/min using a Hamilton 10- μ l syringe with a 26-gauge needle. Saline was injected instead of 6-OHDA into the sham control group. Following injection, the needle was left in place to allow complete diffusion of the medium, and wounds were clipped. After lesion, the animals were returned to their cages for 1 week.

Drug application

Rats in each PD model were randomly split into four groups: the sham group, 6-OHDA lesion group, apomorphine (APO) has been known that it has protective effects against 6-OHDA-induced nigrostriatal damage in rat (Yuan *et al.*, 2006). So, APO was used as a positive control group and the HFL treatment group. The rats in the sham group were injected intraperitoneally (i.p.) with vehicle. The treatment groups were i.p. injected one time with 0.8 mg/kg, 4 mg/kg, or 20 mg/kg of HFL and 5 mg/kg of APO at 1 h after 6-OHDA surgery.

Immunohistochemistry

For immunohistochemistry, brains were fixed in 3.5% neutral formalin for 24 hours, then immersed in a phosphate-buffered 30% sucrose solution for storage at 4°C for at least 24 hrs prior to sectioning. Brains were sectioned into 10- μ m sections using a cryotome (Leica Microsystems, Nussloch, Germany). A monoclonal anti-tyrosine hydroxylase (TH) antibody (Chemicon International, Temecula, CA, USA) and the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) were used for immunohistochemical detection of TH protein. The secondary antibody (biotinylated anti-mouse IgG, Vector Laboratories, Burlingame, CA, USA) was incubated at room temperature for 1 h, followed by ABC solution for an additional 1 h. The stained slides were mounted using cover slips and mounting fluid. TH immunoreactivity in the rat substantia nigra regions were visualized by optical microscopy (Olympus, Tokyo, Japan).

Image capture and analysis

Images of substantia nigra neurons displaying robust TH-positive signals were captured using an optical microscope (Olympus). Neuron counting in the area of TH-positive neurons in the substantia nigra was determined by imaging using a computerized image analyzer (Image J software, version 1.34 s, National Institutes of Health, Maryland, USA). Initially in all treatment groups, TH-positive neurons were counted in the contralateral and ipsilateral hemispheres of the brain at the substantia nigra (4.80 mm from bregma), with reference to a standard rat brain atlas (Paxinos and Watson, 1998). Images were subsequently captured at $\times 100$ magnification for quantitative neuron counting using Image J software. For each animal, four to five adjacent sections were counted, and

these were pooled to give a mean neuron count for each individual animal. These counts were further combined to give a total mean cell count for the contralateral and ipsilateral hemispheres in each treatment group. In all cases, the lesion size was then expressed as a mean percentage loss compared with the contralateral side.

Statistical analysis

All data are presented as mean \pm S.D. Statistical significance was determined by *t*-test (Sigma Plot 10.0 software, SPSS, Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

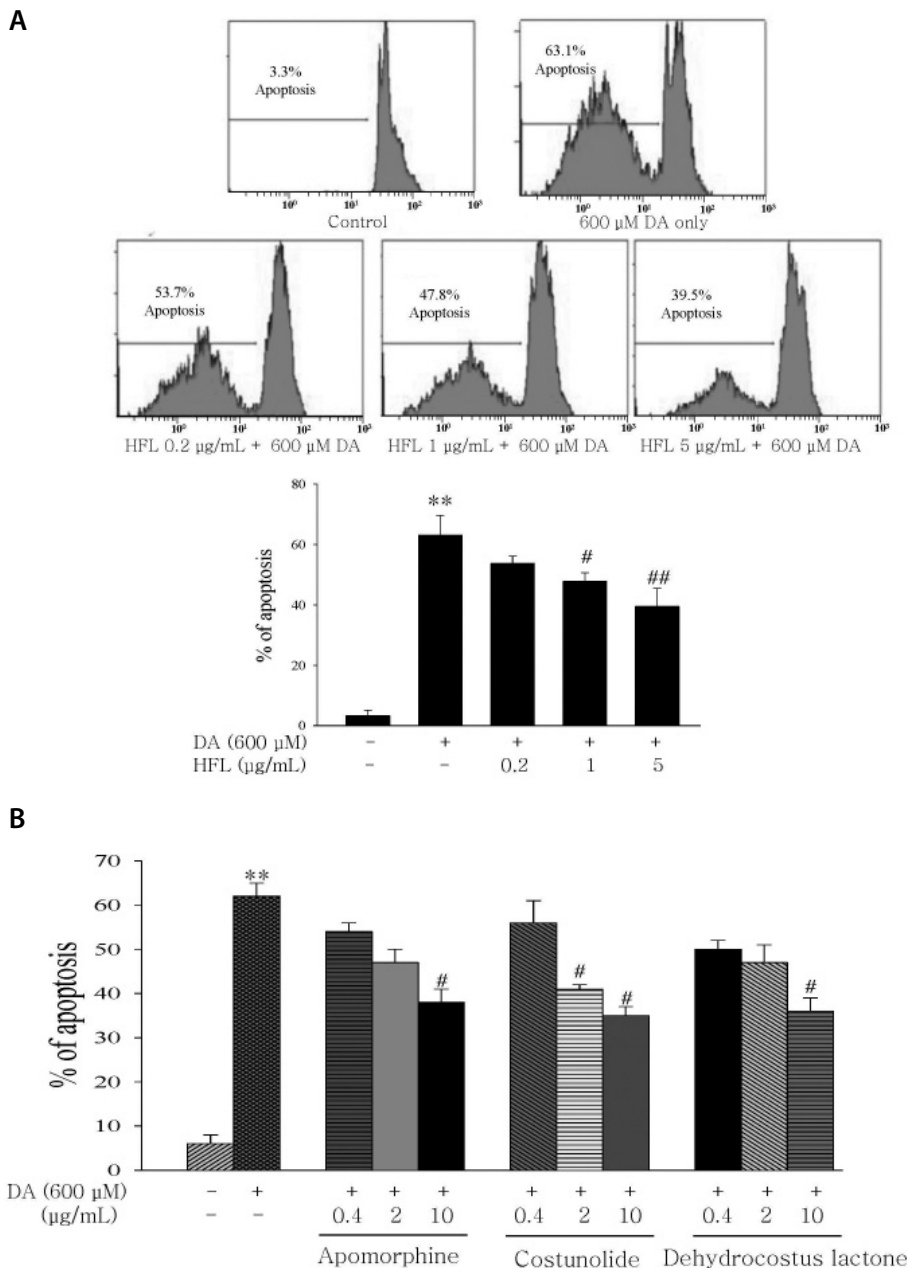


Fig. 2. Neuroprotective effects of HFL and its major compounds against DA-induced cell death. SH-SY5Y cells were pre-incubated with three concentrations of HFL (0.2, 1, 5 μ g/ml) or with the two major compounds of HFL (0.4, 2, 10 μ M) for 24 hrs and then exposed to 600 μ M DA for additional 24 hrs. Cellular apoptosis was determined by flow cytometry. (A) Histograms representing the distribution of apoptotic cells relative to total cells. (B) Plots calculated from the histograms distribution (mean \pm SD, n=3). ** $p < 0.01$ vs. normal control, # $p < 0.05$ vs. DA-treated cells, ## $p < 0.01$ vs. DA-treated cells.

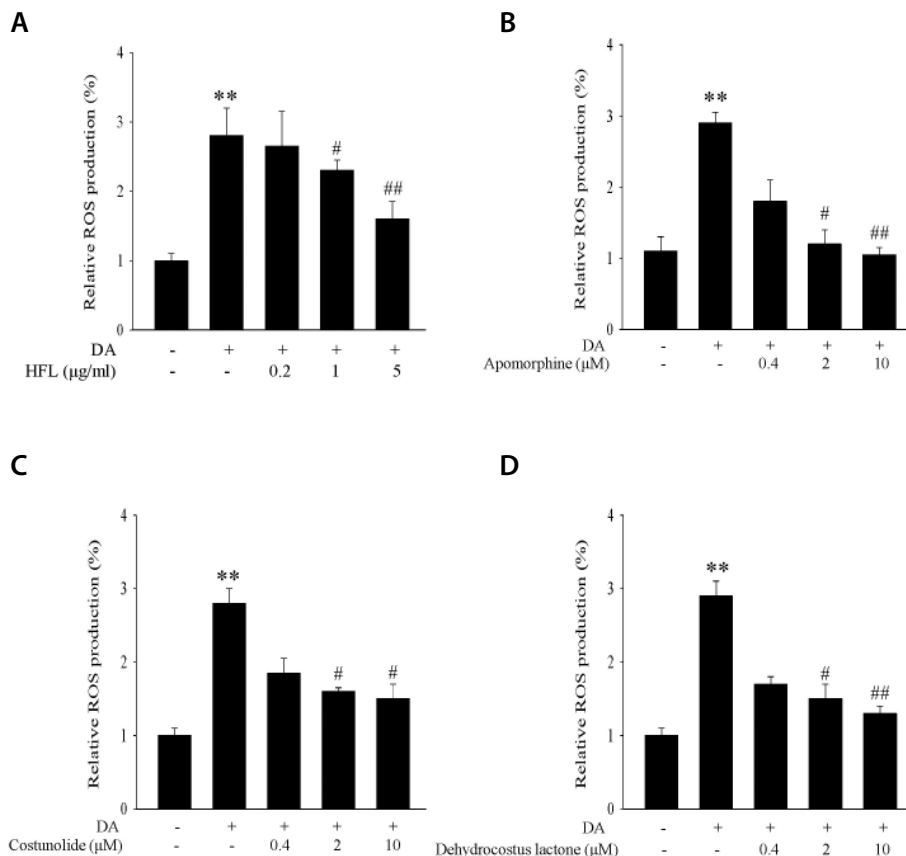


Fig. 3. Effect of HFL against DA-induced ROS production. SH-SY5Y cells were exposed to different concentrations of HFL or the two major compounds with 600 μ M of DA for 4 hrs. The intracellular ROS level was evaluated by incubation with the fluorescent probe DCF-DA (10 μ M) at 37°C for 30 min. Data are mean \pm S.E.M. of a ratio of fluorescence intensity of the treated cells to that of the controls (** p <0.01, compared with control cells, # p <0.05, ## p <0.01 compared with DA-treated cells).

RESULTS

HPLC analysis of HFL

We used HPLC to identify the compounds in HFL. HFL is composed of multiple compounds, with each of the 12 identified compounds comprising more than 1% of the total mass. Among these 12 compounds, costunolide (26.2 \pm 0.2%) and dehydrocostus lactone (14.6 \pm 0.1%) together constitute 40.8% of HFL (Fig. 1).

Anti-apoptotic effects of HFL, costunolide, and dehydrocostus lactone in SH-SY5Y cells

The effects of HFL on DA-induced cell death were verified by flow cytometry using PI staining. Fig. 2A shows that cell death was observed in only 3.3 \pm 1.8% of the control cells. Cell death was increased to 63.1 \pm 6.5% in the presence of DA, but in cells pre-treated with 0.2, 1, and 5 μ g/ml of HFL, the apoptotic ratio was reduced to 53.7 \pm 2.5%, 47.8 \pm 2.9%, and 39.5 \pm 4.9%, respectively. These results indicated that HFL significantly attenuated DA-induced cell death in SH-SY5Y cells. The two major compounds of HFL, costunolide and dehydrocostus lactone, along with the positive control, apomorphine, were also tested using the same methods. The ratio of apoptotic cell death is presented in Fig. 2B, which shows that costunolide and dehydrocostus lactone also exhibited neuroprotective effects in a dose-dependent manner.

Effects of HFL, costunolide, and dehydrocostus lactone on intracellular ROS levels

We investigated whether HFL, costunolide, and dehydrocostus lactone affected intracellular ROS levels in SH-SY5Y cells using the fluorescent probe DCFH-DA. When incubated with HFL, costunolide, dehydrocostus lactone, and DCFH-DA, an early increase in the ROS level was detected compared with control cells. However, at later time points, HFL, costunolide, and dehydrocostus lactone significantly inhibited ROS production in the range of 0.2-5 μ g/ml (Fig. 3A), and 0.4-10 μ M (Fig. 3B-D), respectively.

Identification of overexpressed SYN

Expression of SYN was analyzed by Western blot analysis, and β -actin expression levels confirmed equal loading of cell lysate per lane (Fig. 4A). SYN levels in the cytosol were increased 4-fold in SH-SY5Y cells with DA treatment. However, the addition of HFL significantly inhibited SYN expression in a dose-dependent manner (Fig. 4B).

Inhibitory effects of HFL against the loss of dopaminergic neurons in rat models of PD

The loss of dopaminergic neurons in the substantia nigra was examined by TH immunohistochemistry following 6-OHDA lesion (Fig. 5A). The infusion of 6-OHDA caused a rapid and consistent loss of TH immunoreactivity in the substantia nigra. TH-positive neurons were reduced to 29.3 \pm 7.2% in the lesioned substantia nigra compared with the intact substantia

nigra. HFL considerably reduced the TH-positive neurons in the substantia nigra to $31.5 \pm 9.1\%$, $67.7 \pm 7.6\%$, and $70.6 \pm 5.5\%$ in the groups at 0.8, 4, and 20 mg/kg, respectively. Treatment with APO at 5.0 mg/kg resulted in loss of $32.8 \pm 5.7\%$ of

TH-positive neurons (Fig. 5B).

DISCUSSION

In the present study, *in vitro* and *in vivo* models of PD were used to examine the neuroprotective properties of HFL. The two major compounds of HFL, costunolide and dehydrocostus lactone, have been reported to inhibit ethanol absorption (Yoshikawa *et al.*, 2000), inflammatory cytokine production, and lymphocyte proliferation (Koch *et al.*, 2001). However, no earlier reports have examined the neuroprotective effects of *Laurus nobilis* with regard to the two sesquiterpenes or HFL. When SH-SY5Y cells are treated with DA, cellular apoptosis occurs (Von Coelln *et al.*, 2001), and in this study, treatment with HFL reduced DA-induced cell death in a dose-dependent manner. Although the precise mechanism underlying DA-induced neurotoxicity remains unknown, it has been reported that DA directly inhibits mitochondrial respiratory complexes I and IV, leading to depletion of ATP (Tirmenstein *et al.*, 2005). After being selectively taken up via the DA transporter present in dopaminergic neurons, DA is thought to be auto-oxidized, resulting in the generation of ROS. Previous studies also have shown that antioxidants prevent the loss of dopaminergic neurons caused by DA (Zbarsky *et al.*, 2005; Guo *et al.*, 2007; Khan *et al.*, 2010). These results suggest that HFL could have a critical role as an antioxidant in DA-induced neuronal cells. We demonstrated here that HFL reduces both DA- and H₂O₂-induced ROS production in SH-SY5Y cells, indicating that HFL effectively inhibits cell death caused by ROS.

In PD, SYN present in Lewy bodies is a key molecule involved in the pathogenesis of PD. Under normal physiological conditions, SYN adopts a soluble, unfolded structure. However, in the pathological state, it aggregates and forms fibrillar deposits within Lewy bodies (Desplats *et al.*, 2009). The expression of at least one of the mutant forms of SYN in mice

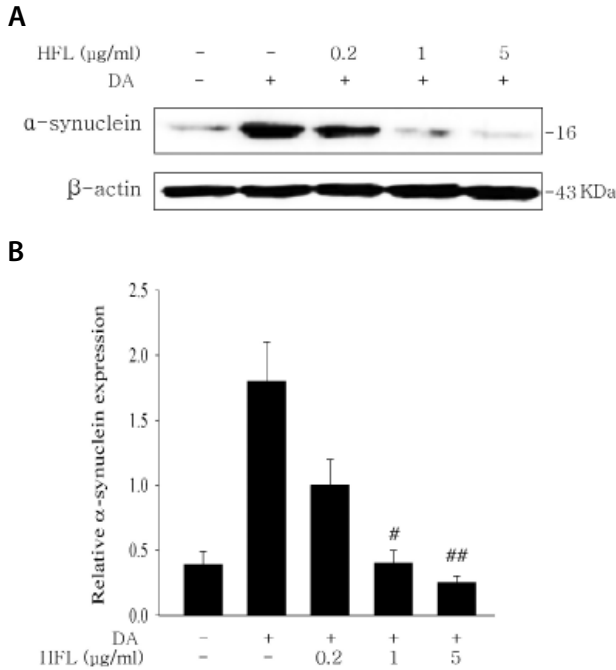


Fig. 4. Western blot analysis of SYN protein on DA-induced SH-SY5Y cells. (A) Cells were treated with 600 µM of DA and HFL (0.2, 1, 5 µg/ml) for 15 hrs and were assayed by Western blot analysis (10 µg protein per lane) using an antibody against SYN. (B) Mean amount of SYN measured from three independent experiments was normalized to β-actin. #*p*<0.05, ##*p*<0.01 vs. DA-treated cells.

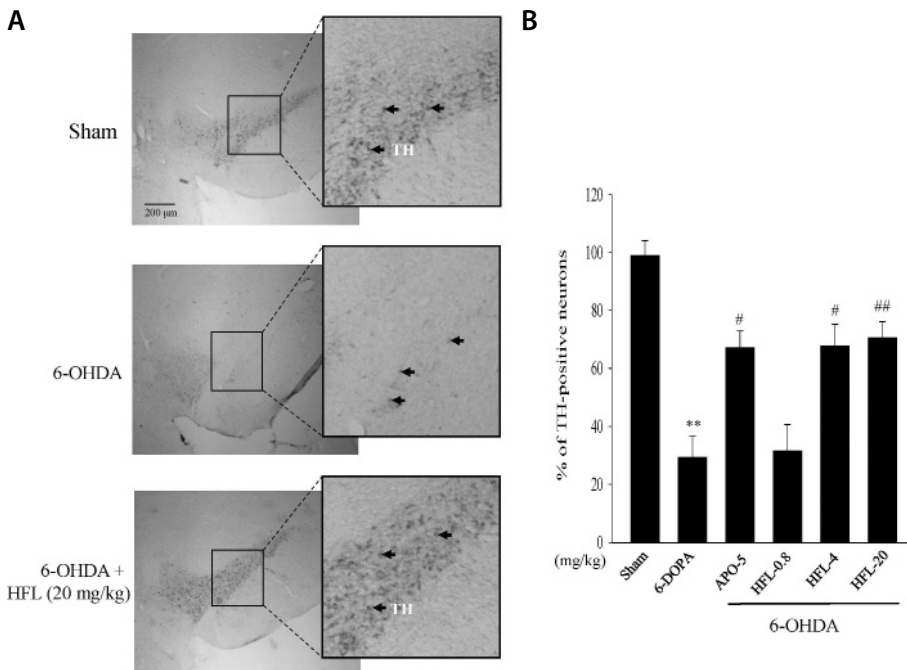


Fig. 5. Neuroprotective effects of HFL treatment against 6-OHDA-induced neurotoxicity in rats. (A) Photomicrograph of TH-immunostained neurons in the right hemisphere of 6-OHDA-lesioned rats. Sham, only 6-OHDA (600 µM), APO (5 mg/kg), HFL (0.8 mg/kg), HFL (4 mg/kg), HFL (20 mg/kg) were treated. HFL attenuated the neuronal cell death in the substantia nigra of rats. (B) Quantification of TH-positive neurons in the substantia nigra. ***p*<0.01 compared with sham group, #*p*<0.05, ##*p*<0.01 compared with the 6-OHDA-lesioned group. Mean counting value of the five slices in each animal represents TH-positive neuron number of the brain in rats.

causes locomotor dysfunction (Waxman and Giasson, 2010). These findings suggest that SYN has an important role in the pathogenesis of PD. It has been reported that intracellular DA is a stress agent for the promotion of SYN expression (Gomez-Isla *et al.*, 2003). We also found that DA increases the expression of SYN in SH-SY5Y cells treated with 600 μ M of DA for 15 hrs and that HFL significantly inhibited this DA-induced increase in SYN in a concentration-dependent manner. These results suggest that HFL may improve parkinsonism through the inhibition SYN formation.

The neuroprotective effects of HFL were further examined in a 6-OHDA-induced rat PD model. Reductions in the levels of TH immunoreactivity were observed in the substantia nigra of rats at 7 days after 6-OHDA lesion, consistent with previous reports (Watanabe *et al.*, 2004). TH is the rate-limiting enzyme in DA synthesis and is used as a marker for dopaminergic neurons in the substantia nigra (Masliah *et al.*, 2000). The loss of dopaminergic neurons in the substantia nigra was examined by TH immunohistochemistry following 6-OHDA lesion. The infusion of 6-OHDA caused a significant loss of TH-positive neurons in the lesioned substantia nigra. HFL considerably reduced the loss of TH-positive neurons and was more potent than the well-known DA agonist APO (Hughes *et al.*, 1993). These results indicate that HFL inhibits dopaminergic neuronal cell death in rat PD models.

In conclusion, we found that HFL significant reduced DA-induced cell apoptosis, ROS generation, and the over-expression of SYN in human neuroblastoma SH-SY5Y cells. In addition, HFL inhibited the 6-OHDA-induced loss of TH-positive neurons in the substantia nigra of a rat model of PD in a dose-dependent manner. These findings indicate that HFL has the potential to be used to reduce or prevent the progress of PD.

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