의학박사 학위논문

Neuroprotective effects of an active constituent derived from *Indigofera tinctoria* extract in experimental models of Parkinson's disease.

Research Supervisor

Yoo-Hun Suh

2013 년 02 월

서울대학교 대학원

의과학과박사과정

By

Spandana Rajendra Kopalli

A thesis of the Degree of Doctor of Philosophy

Neuroprotective effects of an active constituent derived from *Indigofera tinctoria* extract in experimental models of Parkinson's disease.

Research Supervisor

Yoo-Hun Suh

February 2013

The Department of Biomedical Sciences,

Seoul National University

College of Medicine

Spandana Rajendra Kopalli

파킨슨병 모델에서 인도람 추출물 유래 활성성분의 신경보호 효과에 관한 연구

Neuroprotective effects of an active constituent derived from *Indigofera tinctoria* extract in experimental models of Parkinson's disease

지도교수 서 유 헌

이 논문을 의학박사 학위논문으로 제출함
2013 년 02 월

서울대학교 대학원
의학과 의과학전공

코팔리 스판다나 라젠드라

코팔리 스판다나 라젠드라의 의학박사 학위논문을 인준함

2013 년 02 월

위 원 장 이 광우 (인)
부위원장 서 유 헌 (인)
위 원 김 용식 (인)
위 원 김 혜선 (인)
위 원 황 은우 (인)

ABSTRACT

Introduction: As part of our drug discovery program for potential molecules in treating Parkinson's disease (PD), we have screened and isolated an active constituent from Indigenous herb *Indigofera tinctoria* Linn *(I. tinctoria*, Fabaceae) in experimental models of PD. *I. tinctoria,* is an annual herb found throughout India and other parts of Asia. The herb is widely used for several years in the traditional Indian and Chinese system of Medicine for the treatment of epilepsy, nervous disorders, bronchitis, liver ailments, sores, ulcers and hemorrhoids.

Methods: Bio-assay guided extraction, fractionation**,** separation and identification of the active constituent from *I. tinctoria* extract was done through thin layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC) and NMR techniques. To evaluate the *in vitro* protective activities the butanol sub-fraction, SF-6 was assessed using human neuroblastoma SH-SY5Y cells against α-synuclein-, 6-hydroxydopamine (6-OHDA)-, and H_2O_2- induced cytotoxicity using WST-1 cell viability assay. Further, the free radical scavenging effects of SF-6 was also evaluated using electron spin resonance (ESR) spectroscopy.

The single compound methylparaben (MP) isolated from sub-fraction SF-6 was evaluated for reactive oxygen species (ROS)

generation inhibition in 6-OHDA–induced cytotoxicity using DCF-DA in SH-SY5Y cells. MP's effect on lipid peroxidation assay was examined using the MDA analysis kits in 6-OHDA-induced mouse brain samples.

In vivo studies were evaluated against 6-OHDA-lesioned neuronal damage by stereotaxically injecting 6-OHDA unilaterally into the substantia nigra. Agents were administered intraperitoneally 30 min before and 90 min after lesion induction. Animals received a further daily injection of agents intraperitoneally for 14 days. Two weeks after the 6-OHDA injection, contralateral rotational asymmetry was observed by apomorphine challenge. Further, to investigate the behavioral deficits induced by intra-nigral administration of 6-OHDA in mouse, rotarod test was also performed in the 6-OHDA-lesioned mice. Tyrosine hydroxylase-positive (TH^+) cells in the substantia nigra of 6-OHDA-lesioned mice brain sections was performed by Immunohistochemical analysis using ABC kits.

Results: The butanol sub-fraction, SF-6 attenuated the α-synuclein-induced cytotoxicity in SH-SY5Y cells and scavenged directly hydroxyl free radicals as estimated using the ESR spectroscopy. The single compound MP isolated from butanol sub-fraction (SF-6) showed significant and potent neuroprotective effects in both *in vitro* and *in vivo* evaluations. MP at nanomolar concentrations inhibited 6-OHDA-

and H_2O_2-induced cytotoxicity and reduced the ROS generation in SH-SY5Y cells in a dose dependent fashion. Further MP attenuated the lipid peroxidation induced by 6-OHDA toxicity in mouse brain tissues. In *in vivo* studies, MP attenuated the behavioral deficits provoked by 6-OHDA injection and attenuated the contralateral rotational asymmetry in apomorphine challenged mouse in a dose dependent fashion. MP was found to be more potent when compared with known compound deprenyl. Further immunohistochemical analysis of brain tissues in MP treated subjects protected the dopaminergic neurons as evident by significantly higher number of surviving tyrosine hydroxylase-positive (TH^+) cells in the substantia nigra of 6-OHDA-lesioned mice.

Conclusions: Considering the results obtained our *in vitro* and *in vivo* findings suggest the possibility of MP to show neuroprotection in experimental models of PD via the antioxidant properties and therefore can further be developed as potential agent for treating neurodegenerative processes seen in PD.

Keywords: *Indigofera tinctoria***,** methylparaben, 6-hydroxydopamine; reactive oxygen species; Parkinson's disease, substantia nigra, SH-SY5Y cells; neurodegeneration; oxidative stress.

Student number: 2009-31325

CONTENTS

Abstract ..... **i**

Contents ..... **iv**

List of tables and figures ..... **v**

Introduction **1**

Material and Methods **5**

Results **11**

Discussion **24**

References **30**

LIST OF TABLES AND FIGURES

FIGURE	TITLE	PAGE NO
1.	Photograph of *Indigofera tinctoria* Linn.	4
2.	HPLC analysis of butanol sub-fraction, SF-6 derived from *I. tinctoria.*	15
3.	Behavioral experimental protocol	16
4.	Protective effects of SF-6 / deprenyl on α-synuclein-, H_2O_2- and 6-OHDA-induced toxicity.	17
5.	Hydroxyl radical scavenging effect of SF-6 determined by Fenton reaction system.	18
6.	Protective effects of MP on ROS generation by 6-OHDA in SH-SY5Y cells.	19
7.	MP reduces the accumulation of lipid peroxidation product, MDA in 6-OHDA-lesioned mouse.	20
8.	Protective effects of MP and/or deprenyl on apomorphine (0.5 mg/kg, s.c.)-induced rotational behavior in 6-OHDA-lesioned mice.	21
9.	Protective effects of MP and/or deprenyl on the rotarod test in 6-OHDA-injected mice.	22
10.	Effect of MP treatment on TH immunohistochemical staining in the SN in 6-OHDA-lesioned mice.	23

LIST OF ABBREVIATIONS

MP, methylparaben

PD, Parkinson's disease

HPLC, High Performance Liquid Chromatography

6-OHDA, 6-Hydroxy dopamine

ROS, Reactive oxygen species

DCF-DA, 2′, 7′-dichlorofluorescein diacetate

H_2O_2, hydrogen peroxide

MDA, malondialdehyde

TH, Tyrosine hydroxylase

ESR, Electron spin resonance

SN, Substantia nigra

DA, Dopamine

i.p., Intraperitoneal

s.c., Subcutaneous

Dep, deprenyl

INTRODUCTION

Parkinson's disease (PD) is characterized by a loss of dopaminergic neurons in the substantia nigra (SN) region of the brain (Alves da Costa, 2003; Braak and Braak, 2000; Forno, 1996; Goedert, 2001). Its characteristic feature is an increasing tremor in the resting limbs and rigidity known as dyskinesia, particularly exhibited as a shuffling gait moreover also associated with the degeneration of cognitive function and memory (Houghton and Howes, 2005).

Although the etiology of PD remains obscure, generation of reactive oxygen species (ROS) caused by oxidative stress together with relative paucity of the antioxidant defenses in the SN and nigrostriatal dopaminergic pathway is widely considered as the final cause of neuronal death (Coyle and Puttfarcken, 1993; Heh-In Im, 2006). There is an increasing amount of evidence that the neurotoxicity of 6-OHDA is mainly due to the oxidation resulting in the generation of cytotoxic free radicals which are believed to play a pivotal role in the degeneration of the nigrostriatal dopaminergic system (Hou et al., 1997). Damage induced by 6-OHDA *in vivo* and in cultured neuronal cells is similar to that induced by ROS (Naveilhan et al., 1994). Nigrostriatal 6-OHDA injections result in a Parkinsonian pattern, together with neuronal loss in the SN of the rat brain (Ungerstedt, 1968).

Several authors also reported that the urine of PD patients treated with levodopa contained an increase amount of 6-OHDA which indicates that 6-OHDA may constitute an important endogenous cause of PD pathogenesis (Hou et al., 1997; Jellinger K, 1995). The progressive neurodegeneration in PD does not halt/slow

down by the currently used drug therapies and also produce grave side-complications with long term use (Chaturvedi *et al.*, 2006). Hence the search for potential therapeutic agents continues.

Discovery of new drugs from traditional medicine is not a new phenomenon. Current researches are focusing on finding therapies, preferentially from natural products which could help in preventing/delaying the ongoing neurodegeneration in PD (Dawson and Dawson, 2002).

Many traditional herbal remedies have been recently demonstrated to possess neurotrophic and neuroprotective properties which may be useful in preventing various forms of neuronal cell loss including the nigrostriatal degeneration seen in PD (Narender et al., 2006). Research confirms that several medicinal herb-based extracts increase redox/antioxidative abilities of the body and can effectively slow the progression of PD (Todorov, 1993). As part of our drug development program for neuroprotection, we have identified a potential agent beneficial in ameliorating neurodegenerative processes in experimental models of PD.

Indigofera tinctoria (I. tinctoria, Fabaceae*),* is an annual herb found throughout India and other parts of Asia (Fig.1). The herb is widely used for several years in the traditional Indian and Chinese system of Medicine for the treatment of epilepsy, nervous disorders, bronchitis, liver ailments, sores, ulcers and hemorrhoids (Anand et al., 1979; Anand et al., 1981; Satyavati, 1987; Todorov, 1993). Various parts of *I. tinctoria* are used to treat skin diseases, gastropathy and as brain tonics. The juice from the leaves is mixed with honey and taken orally in the treatment of liver and spleen enlargement, epilepsy and other nervous infections. The extract from

the whole plant was claimed to be good for epilepsy and neuropathy (Satyavati, 1987; Motamarri et al., 2012).

Fig. 1. *Indigofera tinctoria* Linn.

Experimental evidence based on traditional claims suggest that *I. tinctoria* possess antidiabetic (Verma et al., 2010), antibacterial, antioxidant (Prakash 2007; Bakasso et al., 2008; Renukadevi and Suhani, 2011), antiinflammatory (Oli, 2005; Tyagi et al., 2010), hepatoprotective (Anand et al., 1979; Anand et al., 1981; Singh et al., 2001; Sreepriya et al., 2001a; Sreepriya et al., 2001b, Chitra et al., 2003), antidyslipidemic (Narender et al., 2006; Singh et al., 2006; Puri et al., 2007), antiepileptic (Asuntha et al., 2010), anticancer (Han, 1994), antiproliferative (Kameswaran, 2008), anthelmintic (Balamurugan, 2009) and antinociceptive (Kumar et al., 2009)

activities. But so far its protective effects in neurodegenerative diseases like PD were untouched.

Based on the traditional claims of *I. tinctoria* for its use in epilepsy, nervous and brain disorders, in the present investigation we evaluated the neuroprotective properties of an active constituent, methylparaben (MP), isolated from *I. tinctoria* extract against selected neurotoxins using SH-SY5Y human neuroblastoma cell lines *in vitro* and 6-OHDA-induced neurotoxicity in mouse model of PD *in vivo*.

The detailed experimental study was described in the following sections

MATERIALS AND METHODS

1. Preparation of the extracts

The alcoholic extract of aerial parts of *I. tinctoria* was kindly supplied from M/s Laila Impex, R&D center, Manufacturers of Herbal Extracts, Andhra Pradesh, India. Alcoholic extract (50 g) was partitioned into hexane, ethyl acetate, n-butanol and aqueous fractions to furnish 0.63 g, 8.62 g, 10.02 g and 28.25 g respectively. The n-butanol soluble fraction (5 g) was further subjected to column chromatography over silica gel (100-200 mesh) and eluted with dianion HP 20 column chromatography, chloroform to methanol 20:1 ratio to yield 50 mg of pale yellow color sub-fraction named SF-6. Further HPLC purification and NMR data revealed that the active sub-fraction consists of a single compound methyparaben (MP, Fig. 2). The compound was solubilized with distilled water under sonication before use.

2. Cell culture and *in vitro* treatments

SH-SY5Y cells were maintained in DMEM (Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10 % FBS (Gibco BRL, Gaithersberg, MD, USA) and 0.3 % antibiotics at 37 ^{0}C in 5 % CO_2.

Initially, the four fractions (hexane, ethyl acetate, n-butanol and aqueous) were evaluated for their cytoprotective effects against 6-OHDA-induced toxicity in SH-SY5Y cells. Bio-assay guided fractionation and evaluation revealed that butanol fraction was cytoprotective. Butanol fraction was further sub-fractionated and the resulted fraction was named as SF-6. SF-6 and/or deprenyl (10 μg, Sigma, St. Louis, MO, USA) were pretreated to SH-SY5Y cells for 4 h before the treatment with various toxins [α-synuclein (2 μM, Sigma,

St. Louis, MO, USA), hydrogen peroxide (H_2O_2, 250 μM Sigma, St. Louis, MO, USA) and 6-OHDA (20 μM, Sigma, St. Louis, MO, USA)].

3. Cell viability assay

WST-1 metabolizing activity was determined according to the manufacturer's instructions (Roche, Indianapolis, IN). SH-SY5Y cells were plated in a 96 well plate at a density of $7x10^3$ cells/well. As reported previously (Park *et al.*, 2002) cells were allowed to adhere to plates for 24 h. SF-6 and/or deprenyl was introduced into the media of SH-SY5Y cells 4 h before treatment with α-synuclein (2 μM), H_2O_2 (250 μM) and 6-OHDA (20 μM). The colorimetric assay measures the metabolic activity of viable cells treated with various toxins when WST-1 (Roche, Indianapolis, IN, USA), 10 μL was added to the culture media. The culture was incubated at 37 ^{0}C in a humidified atmosphere of 95 % air and 5 % CO_2 for 1 h. The absorbance of the reaction product was measured with an ELISA reader (Bio-Rad, Germany) at a wavelength of 450 nm.

4. Measurement of hydroxyl radical activity by electron spin resonance (ESR) spectroscopy

Hydroxyl radicals ($^{\cdot}OH$) were generated using a Fenton reaction. DMPO (4.5M, 10 μL, Sigma, St. Louis, MO, USA) was added to a solution containing H_2O_2 (2.8 M, 75 μL), $FeSO_4$ (0.6M, 75 μL, Sigma, St. Louis, MO, USA) and PBS, Vitamin C (100 μM, Sigma, St. Louis, MO, USA) and/or SF-6 (0-100 μg/mL, 75 μL). The $^{\cdot}OH$ was trapped with DMPO to produce stable hydroxyl radical spin adduct (DMPO-$^{\cdot}OH$) which can be monitored by ESR

spectroscopy (JEOL ES-IPRITS/TE, Tokyo, Japan), operating as follows: microwave power, 1.00 mW; frequency 9.423 GHz; modulation amplitude 0.16 mT/100 kHz; time constant, 0.3 sec; sweep time, 2 min. The signal intensities of DMPO-·OH were represented by the relative peak height of DMPO-·OH to the intensity of the Mn (II) signal as a marker in the cavity.

5. Determination of ROS generation

Intracellular reactive oxygen species (ROS) in SH-SY5Y cells were quantified using fluorescent dye, 2′, 7′-dichlorofluorescein diacetate (DCF-DA; Sigma, St. Louis, MO, USA) as described previously (Green et al., 1982). For assay, SH-SY5Y cells were seeded in a 96 well plates and were incubated with 6-OHDA (20μM) and increasing concentrations of MP (0.01, 0.1 and 1.0 nM) and/or deprenyl for 24 h. Cells were incubated with DCF-DA at 37 ℃ for 30 min, then cells were washed three times with PBS (pH7.4) and the relative levels of fluorescence were quantified in a spectrophotofluorometer (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 485 nm and emission wavelength 538 nm. The measured fluorescence values were expressed as a percentage of the fluorescence in control cells.

6. Animals and intranigral injections of 6-OHDA

Seven-week-old male C57BL/6N mice weighing 20-25 g were housed in a specific pathogen free room automatically maintained on a 12 h light-dark cycle at 25 ^{0}C with proper humidity. Food and water was provided *ad libitum*. All experiments were carried out in accordance with the Guidelines for Animal

Experiments of Ethics and Committee of Seoul National University, Seoul, South Korea.

Desipramine (25 mg/kg, Sigma, St. Louis, MO, USA) was injected intraperitoneally (i.p) to block norepinephrine reuptake 1h before 6-OHDA injection. Animals were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and injected 10 μg of 6-OHDA (4 μg/μL containing 0.2 mg/mL L-ascorbic acid). 6-OHDA was injected unilaterally into the substantia nigra (A / P = -3.2, M / L = -1.5, D / V = -4.6, with a flat skull position (coordinates in mm, with anterior–posterior and lateral measured from bregma, and ventral from dura). Injections were made at a rate of 0.5 μL/min using a 25-gauge Hamilton syringe (Hamilton Company, USA).

MP (1, 10 and 50 μg/kg, i.p.) and deprenyl (10 mg/kg, i.p.) were administered 30 min before 6-OHDA injection. Animals received daily doses of MP or deprenyl for two weeks following the 6-OHDA injections. In addition sham (L-ascorbic acid injected) mice received MP or vehicle and 6-OHDA-injected mice received vehicle alone. The *in vivo* experimental protocol was shown in Fig. 3.

7. Rotational behavior test

Drug induced asymmetric rotational behaviors were tested using apomorphine (0.5 mg/kg, s.c., Sigma, St. Louis, MO, USA) 2 weeks after lesioning. Mice were placed in an individual plastic bowls with a diameter of 20 cm and rotational behavior was assessed using automated Rotometer (Rozas et al., 1997). Mice were acclimatized with their environment for 10 min before turns. And the number of net rotations (contralateral-ipsilateral) of animals was recorded for 60 min.

8. Rotarod performance test

Two weeks after 6-OHDA lesioning, all the animals were tested for locomotor activity (ROTA-ROD for mice 7650 by UGO Basile, Varese, Italy). After adaptation for 5 min, the mice were placed on a horizontal plastic rod rotating at a speed of 10 rpm for a maximum of 10 min. The time that each mouse was able to maintain its balance walking on the top of the rod was measured.

9. Lipid peroxidation assay

Two weeks after 6-OHDA administration, SN tissues were collected immediately after animals had been sacrificed and then frozen on dry-ice and stored at -70 °C until required. To measure malondialdehyde (MDA) levels, lipid peroxidation assay kits (Calbiochem, Laliola, CA, USA) were used as per the manufacturer's instructions. Briefly, SN tissues were homogenized in 4 volumes of ice cold 20 mM phosphate-buffer saline (pH 7.4) containing 5 nM butylated hydroxytoulene. Homogenates were centrifuged at 3000 g for 10 min at 4℃ and the supernatant (200 μL) was used for each assay. For each reaction, 650 μL of diluted R1 reagent (1:3) of methanol: N-methyl-2-phenylindole) were added and mixed with 150 μL of R2 reagent.

Each reaction was incubated at 45℃ for 60 min and centrifuged at 10,000 g for 10 min. The supernatant was measured at 586 nm. Concentrations are expressed as nM MDA/mg protein.

10. Tissue Preparation and Immunohistochemistry

After behavioral tests, mice were anesthetized with 20 to 30 μL of mixture of Zoletil (12.5 mg/kg) and Rompun (17.5 mg/kg), and then immediately cardiac perfused with PBS containing heparin. Brains were isolated and fixed in 4% paraformaldehyde solution for 24 h at 4℃.Sequential coronal sections (25 μM) of SN were prepared on a cryostat (Cryotome, Thermo electron cooperation) and were maintained in cryoprotectant solution and stored at 4℃. Immunohistochemistry was performed to stain the SN after incubation with 3% H_2O_2 in 0.05 M PBS. Tissues were then incubated overnight at room temperature in primary antibodies (Rabbit TH, 1:100; Santa Cruz Biotechnology, CA, USA). In the next step, brain tissues were incubated with biotinylated anti-rabbit IgG (Vector Laboratories Burlingame, CA, USA) for 1h at room temperature. After incubation with a Vector Elite ABC Kit (Vector Laboratories Inc., CA, USA), the antibody biotin–avidin-peroxidase complex was visualized with diaminobenzidine (DAB) substrate kit for peroxidase (Vector Laboratories Inc., CA, USA). After the reaction, brain tissues were mounted on gelatin-coated slides, dried, dehydrated and covered before being examined with a microscope.

11. Statistical Analysis

Data was expressed as mean ± S.E.M. Statistical comparisons between all groups were performed by using One-Way Analysis of Variance (ANOVA) followed by Bonferroni's test using Sigma Plot 11.1 software (San Jose, CA, USA). *p-values* less than 0.05 were considered statistically significant.

RESULTS

SF-6 attenuated α-synuclein, H_2O_2 or 6-OHDA induced cytotoxicity in SH-SY5Y cells

Preliminary bioassay guided fractionation and evaluation revealed that butanol sub-fraction, SF-6 was cytoprotective against 6-OHDA-induced toxicity in SH-SY5Y cells. SF-6 treated alone at various concentrations hardly revealed any effect on SH-SY5Y cell viability performed by WST-1 assay (Fig. 4A). Treatment of SH-SY5Y cells with α-synuclein (2 μM), H_2O_2 (250 μM) or 6-OHDA (20 μM) resulted in approximately 35 %, 30 % and 30 % reduction in cell survival with 24 h respectively, where as the samples pretreated with 1, 5 and 10 μg/mL SF-6 showed a reduction of cytotoxicity mediated by α-synuclein, H_2O_2 or 6-OHDA in a concentration dependent manner respectively.

Treatment with known compound deprenyl did not show any significant effect on attenuating α-synuclein induced toxicity, but showed considerable activity in attenuating H_2O_2 or 6-OHDA induced cell death at 10 μg/mL concentration when compared with SF-6. All the data were represented in Fig. 4B, Fig. 4C and Fig. 4D respectively.

Hydroxyl radical scavenging effects of SF-6

Hydroxyl radicals generated in Fe_2SO_4/H_2O_2 system were trapped by DMPO forming spin adduct which could be detected by ESR spectrometer. The ESR signal is inhibited by the presence of $^{\cdot}OH$ scavengers, which compete with DMPO for $^{\cdot}OH$. The height of the peak of the spectrum represented the relative amount of DMPO-$^{\cdot}OH$ adduct. After the addition of SF-6, the decrease of the amount of DMPO-$^{\cdot}OH$ adduct was shown on the ESR spectrum.

The results indicate that the scavenging activity increased with the increase in concentration of SF-6 in a dose dependent manner showing a maximum inhibition at 100 µg/mL concentration. Similar activity was found with known scavenger, Vitamin C at 100 µM concentration. All the above data were represented in Fig. 5.

MP reduced the ROS generation induced by 6-OHDA

Since the butanol sub-fraction, SF-6 showed attenuation against α-synuclein, H_2O_2 or 6-OHDA induced cytotoxicity in SH-SY5Y cells and inhibited the excessive hydroxyl radical production, the effect of the single constituent MP was evaluated for ROS generation induced by 6-OHDA in SH-SY5Y cells quantified by cell permeable dye DCF-DA, which becomes highly fluorescent when oxidized to DCF. Thus DCF-DA was loaded to cells after 6-OHDA treatment, and changes in fluorescence were measured using a spectroflurometer.

The cells treated with 20 µM 6-OHDA showed significant increase of intracellular ROS compared with the untreated cells. This increase was significantly attenuated by MP pretreatment ($p<0.05$, $p<0.05$ and $p<0.01$) respectively. The result indicates that pretreatment of SH-SY5Y cells with MP effectively prevents 6-OHDA-induced oxidative stress. On the other hand deprenyl, at 50 µM concentration also showed significant attenuation ($p<0.05$) in reducing the ROS generated by 6-OHDA induced oxidative stress. All the above data were represented in Fig. 6.

Determination of malondialdehyde (MDA) in 6-OHDA-lesioned brain samples using lipid peroxidation assay

Next, we examined the lipid peroxidation inhibiting properties of MP in 6-OHDA-induced mouse brain tissues. Two weeks after administering 6-OHDA, the lipid peroxidation product (MDA level) of lesioned SN was increased to 1.50 ± 0.17 nM/mg protein compared to those of control groups (1.01±0.10 nM/mg protein). However, MP treatment at various concentrations (1, 10 and 50 μg) reduced mean MDA levels in ipsilateral sides to 1.40 ± 0.15, 1.18 ± 0.12 and 1.09 ± 0.09 nM/mg of protein, respectively (Fig. 7). Deprenyl also showed significant reduction in MDA levels at 10 mg/kg dose. These results indicate that MP treatment inhibited 6-OHDA-induced lipid peroxidation in the lesioned substantia nigra.

Effect of MP on apomorphine–induced rotations induced by intra-nigral 6-OHDA injection

Drug-induced asymmetric rotational behaviors of mice were evaluated by recording their net rotations for 1 h. Compared to control mice that showed nearly no apomorphine-induced rotational behavior, 6-OHDA injected mice exhibited significantly higher number of net rotations. MP-treated surgical mice showed significantly lower number of net rotations compared to the surgical mice (with increasing doses of MP decreasing the number of rotations). MP at 50 μg/kg was as effective as deprenyl treated at 10 mg/kg. Nonetheless, neither MP nor deprenyl treated group were able to completely eliminate the effect of the toxin, exhibiting some rotations. Overall, these behavioral data suggested that MP treatment significantly relieved 6-OHDA-induced asymmetric rotational behavior when challenged with apomorphine. All the above data were shown in Fig. 8.

Effect of MP on rotarod performance test

In the rotarod performance test, the latency time to fall off from the rod was reduced in 6-OHDA-injected mice ($p<0.001$) compared with control mice. However, the latency time to fall off was increased ($p<0.05$, $p<0.05$ and $p<0.01$) with mice pretreated with 1, 10 and 50 µg/Kg dose of MP respectively. The results indicate that MP treatment showed a significant improvement in rotarod performance for 6-OHDA-induced motor deficits. Deprenyl also profoundly protected against the reduction in motor performance when treated at 10 mg/kg dose ($p<0.01$). All the data were represented in the Fig. 9.

MP reduces cell damage in substantia nigra caused by 6-OHDA

For TH immunostaining, representative microphotographs of the SN are shown in Fig. 10. Mice brains injected with 6-OHDA showed considerable decrease in the number of stained region compared to control group. MP treated mice brains restored the reduced TH immunoreactivity induced by 6-OHDA in the SN. Lower concentrations of MP (1 and 10 mg/kg) treatment were observed to be more effective in reducing cell damage. TH^+ cells in contralateral SN of the MP treated group showed no significant change and were similar to that of controls. Our results suggested that MP treatment reduced 6- OHDA induced dopaminergic cell loss in the SN of the mice brains.

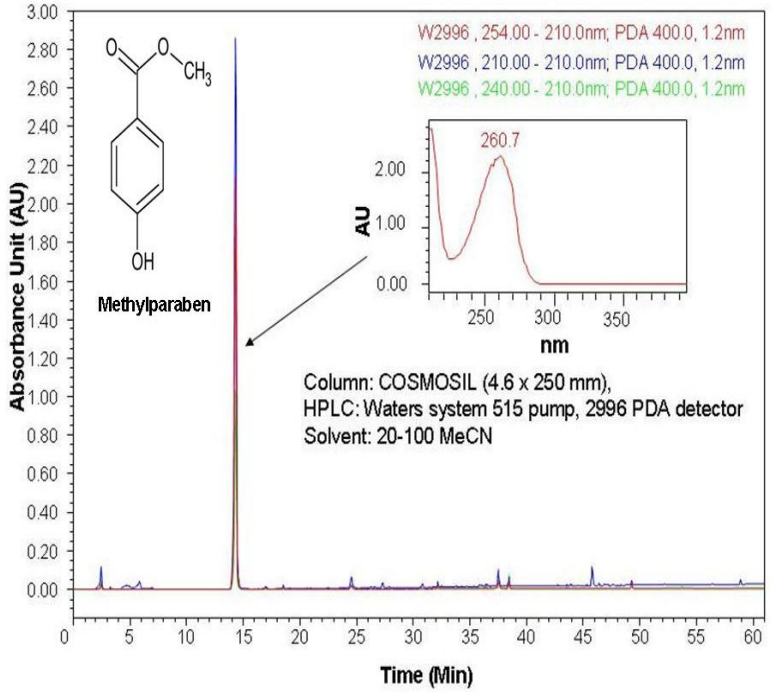

Fig. 2: HPLC analysis of butanol sub-fraction derived from *I. tinctoria.*

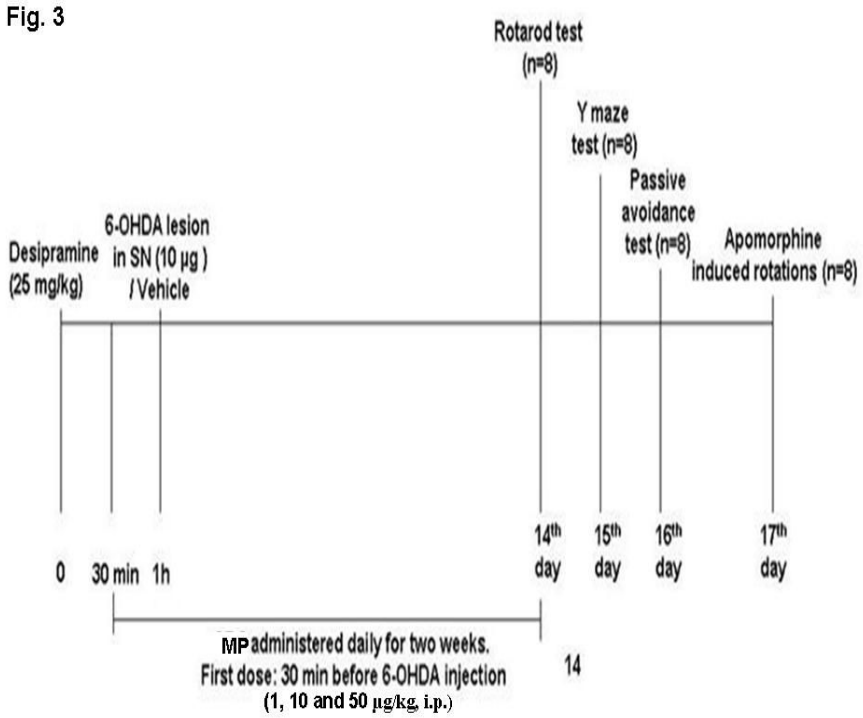

Fig. 3: Behavioral experimental protocol.

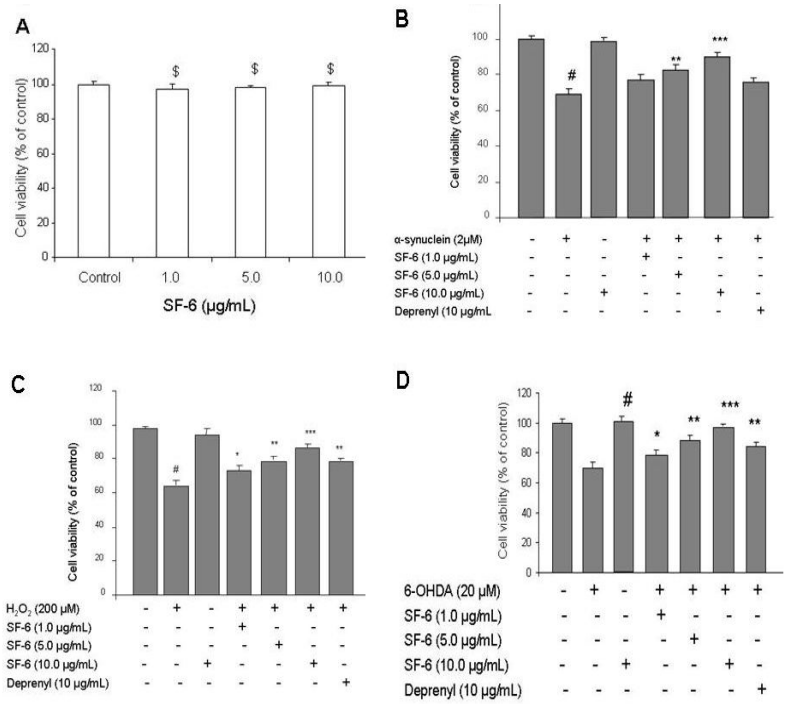

Fig. 4. Protective effects of SF-6 / deprenyl on α-synuclein-, H2O2- and 6-OHDA-induced toxicity. A: Effect of SF-6 on cell viability in SH-SY5Y cells for 24 h, B: Effect of SF-6 on exposure with α- synuclein to SH-SY5Y cells for 24 h, C: Effect of SF-6 on H_2O_2- induced toxicity in SH-SY5Y cells for 24 h and D: Effect of SF-6 on 6-OHDA-induced toxicity in SH-SY5Y cells for 24 h. Data is represented as percentage of control value ± SEM, n=6. # Significantly different from vehicle control group (# $p< 0.001$). Significantly different from toxin treated group ($^{*}p< 0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

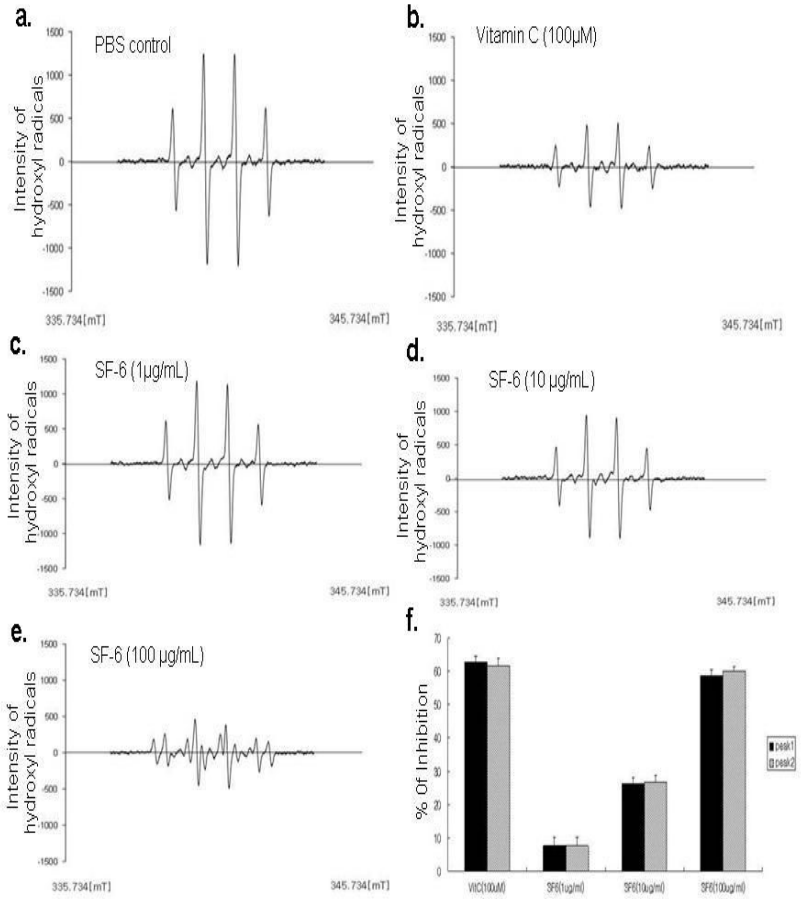

Fig. 5. Hydroxyl radical scavenging effect of SF-6 determined by ESR. Electron spin paramagnetic resonance spectra showing the effect of SF-6 on the scavenging of hydroxyl radical ($HO^{\cdot}$) and retardation of DMPO-HO adduct formation, in aqueous solution at room temperature in air. a) control, b) Vitamin C (100μM), c) SF-6 (1 μg/mL), d) SF-6 (10 μg/mL), e) SF-6 (100 μg/mL) and f) Bar diagram showing the percentage inhibition of hydroxyl radicals by SF-6 compared with known antioxidant ascorbic acid.

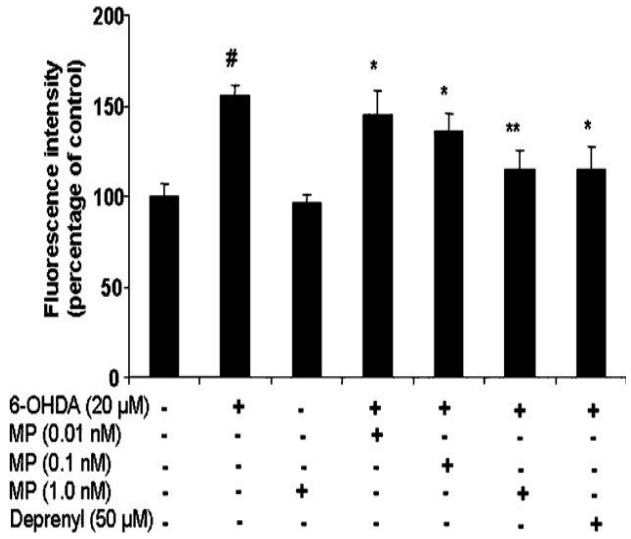

Fig. 6. Protective effects of MP on ROS generation by 6-OHDA in SH-SY5Y cells. The cells were pre-treated with MP for 4 h before stimulation with 20 μM 6-OHDA. ROS generation was measured by the fluorescence intensity of DCFH-DA after 6-OHDA stimulation for 2 h Values are given as the mean ± SEM. (n=6). $^{\#}p< 0.001$, compared with the control group, and $^{*}p < 0.05$, $^{**}p < 0.01$ and ***p<0.001, compared with the 6-OHDA-alone treated group.

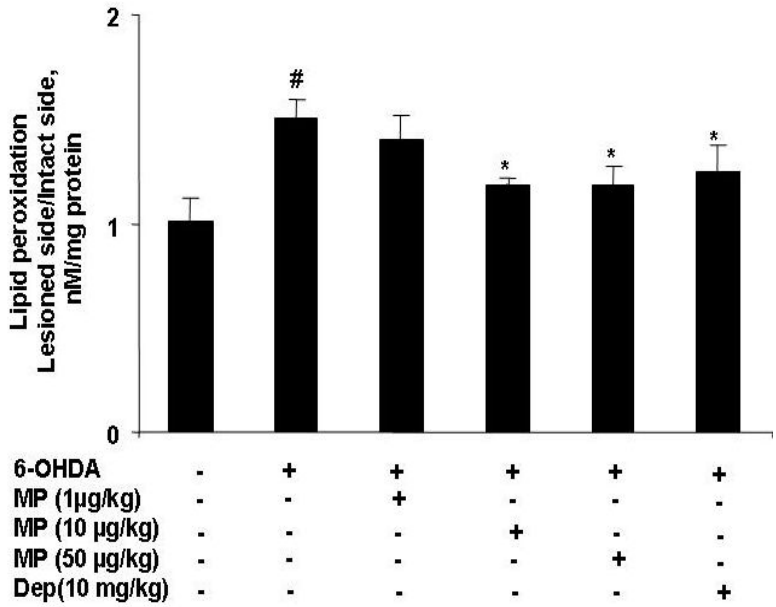

Fig. 7. MP reduces the accumulation of lipid peroxidation product, MDA in 6-OHDA-lesioned mouse. Notice the return of MDA levels to normal following MP treatment at 10 μg/kg dose. Data are represented as mean ± SEM. (n=4). $^{\#}p< 0.05$, compared with the control group, and $^{*}p < 0.05$, compared with the 6-OHDA-alone treated group respectively.

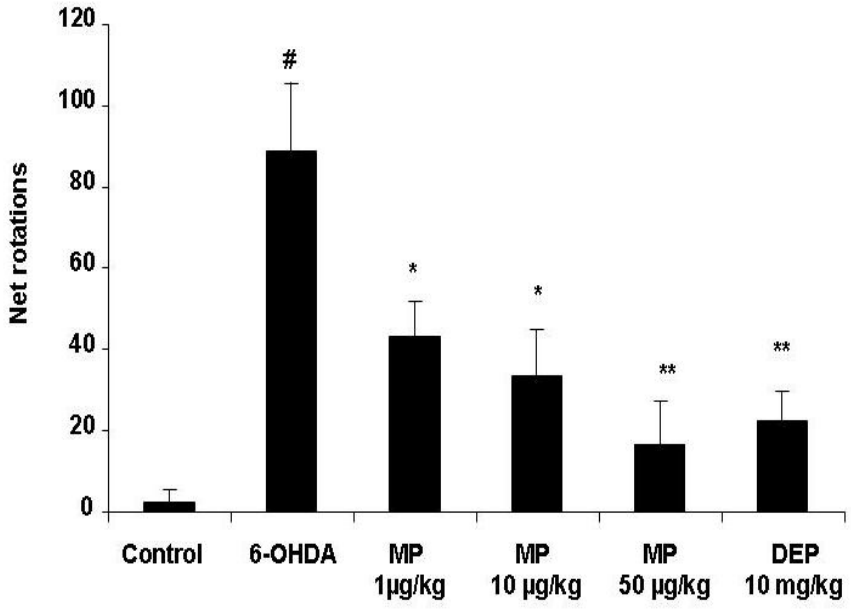

Fig. 8. Protective effects of MP and/or deprenyl on apomorphine (0.5 mg/kg, s.c.)-induced rotational behavior in 6-OHDA-lesioned mice. Two weeks after 6-OHDA injection, the number of net rotation contralateral to the lesion side was increased. However, MP treatment (1, 10 and 50 μg/kg) and deprenyl (10 mg/kg) restored the increase of number of net rotation. All data are represented as means ± S.E.M. (n=6). $^{\#}p<0.001$, significantly different from control group. $^{*}p<0.05$ and $^{**}p<0.01$ compared with 6-OHDA only treated group.

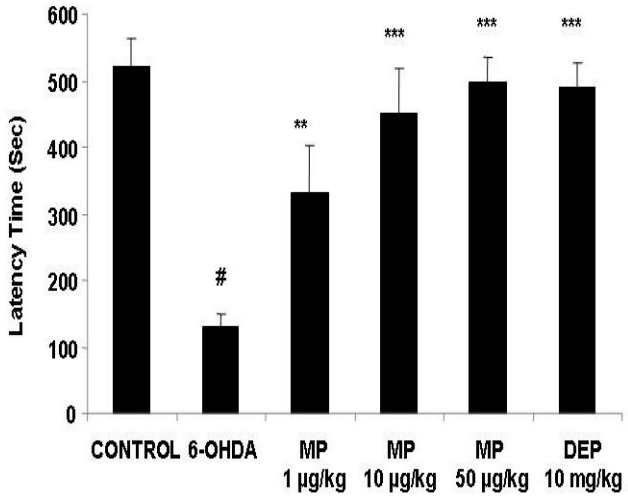

Fig. 9. **Protective effects of MP and/or deprenyl on the rotarod test in 6-OHDA-injected mice.** Two weeks after 6-OHDA injection, latency time to fall off was recorded maximally for 600 s. Data were expressed as the mean ± S.E.M. (n=6). $^{\#}p<0.001$, significantly different from control group. $^{**}p<0.01$ and $^{***}p<0.001$ compared with 6-OHDA only treated group.

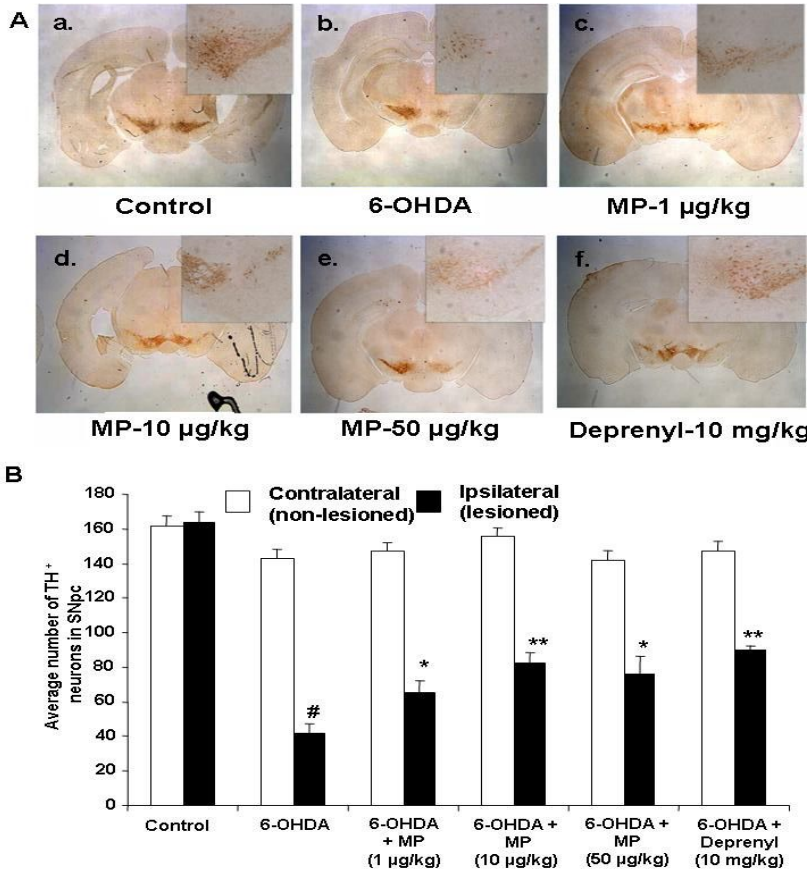

Fig. 10. Effect of MP treatment on TH immunohistochemical staining in the SN in 6-OHDA-lesioned mice. A: Control group (a), 6-OHDA treated group with loss of cell bodies in SN(b), MP treated (1, 10 and 50 μg/kg) group (c, d, e) and deprenyl (10 mg/kg) treated group (f) appeared to increase the number of neurons in the SN. B: Quantification of TH immunopositive neurons in the SN. All data are represented as means ± SEM (n=3), $^{\#}p<0.001$, compared with control group. $^{*}p<0.05$, $^{**}p<0.01$ compared to 6-OHDA treated group.

DISCUSSION

The experimental studies discussed report that the butanol sub-fraction SF-6 isolated from alcoholic extract from the aerial parts of *I. tinctoria* can protect against α-synuclein-, $H_2O_2^-$ or 6-OHDA-induced cytotoxicity in human neuroblastoma SH-SY5Y cells *in vitro*. SF-6 also scavenged hydroxyl radical estimated using ESR spectrometry. Further bioassay guided separation and characterization lead to a single compound MP which also suppressed the 6-OHDA-induced ROS generation thereby reducing the oxidative damage in SH-SY5Y cells. Further MP attenuated the behavioral and cognitive impairments in 6-OHDA-induced mouse model of PD and inhibited the lipid peroxidation in brain tissues of SN damaged by 6-OHDA induction in mice.

Mounting evidence suggests that the human neuroblastoma SH-SY5Y cells possess many characteristics of dopaminergic neurons such as dopamine transporter and are widely used as an *in vitro* PD model (von Coelln *et al.*, 2001). 6-OHDA which causes dopaminergic neuronal cell death *in vivo* and *in vitro* is also elaborately used neurotoxin to study the mechanisms related to PD (Ding *et al.*, 2004). PD is characterized by a degenerative loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). It has been known that α-synuclein, one of the major causative agents of PD, is involved in the neuronal cytotoxicity. Several reports suggest that the pathology of PD is characterized by the accumulation and aggregation of the presynaptic protein α-synuclein in the cytosol as Lewy bodies (LB's) and in neuronal processes as Lewy neurites (LN's) or Spheroids (Pandey *et al.*, 2008). A variety of evidence

implicates the aggregation of alpha-synuclein as a key step in the etiology of PD. Inhibiting the alpha synuclein aggregation, there by its toxicity to the neurons might be helpful in preventing the neurodegeneration in PD (Pandey *et al.*, 2008; Zhu *et al.*, 2004). *In vitro* experiments have demonstrated that α-synuclein induces toxic effects to SH-SY5Y cells (Pandey *et al.*, 2008; Park *et al.*, 2002). Supporting the earlier reports, our results demonstrated that α-synuclein exerted cytotoxicity to SH-SY5Y cells *in vitro* and pretreatment with SF-6 ameliorated the toxicity induced by α-synuclein in SH-SY5Y cells.

It was well documented that H_2O_2 can induce cytotoxicity and apoptosis in many different cell types and this effect can be blocked by the addition of antioxidants (Gao et al., 2008; Gupta, 2004; Jang and Surh, 2001; Zhang et al., 2007). H_2O_2 has been extensively used as an inducer of oxidative stress *in vitro* model (Satoh et al., 1997). The exposure of cultured cells to H_2O_2 results in an imbalance in energy metabolism and the deleterious effects of hydroxyl and peroxyl radicals on membrane lipids and proteins. Our present studies confirmed that treating cells with H_2O_2 resulted in a dose-dependent viability loss in SH-SY5Y cells. However, pretreatment with different concentrations of SF-6, greatly decreased the cell viability loss. These results indicated that SF-6 did significantly protect SH-SY5Y cells from H_2O_2-induced cytotoxicity.

The selective loss of dopaminergic neurons in the substantia nigra appears to be the direct cause of neurodegeneration in cases of PD. 6-OHDA, which is commonly used for the induction of PD in experimental animals, is believed to cause dopaminergic cell death via a free radical mechanism. Our results indicate that 6-OHDA-

induced oxidative stress and that its blockage resulted in attenuated cell death. These results indicate that ROS play a primary role in the induction of neuronal damage by 6-OHDA. In the present study, SF-6 effectively reduced ROS generation leading to the attenuation of 6-OHDA-induced cell death in SH-SY5Y cells.

Increased oxidative stress, depletion of endogenous antioxidants and decreased activities of free radical scavenging enzymes have been reported in brains of patients with Parkinson's disease (Ambani et al., 1975; Dexter et al., 1989; Kish et al., 1985). Accordingly, free radicals and oxidative stress are thought to be important contributors to the pathogenesis of the disease (Olanow, 1992). In our study, SF-6 suppressed hydroxyl radicals in a dose-dependent fashion. The hydroxyl radical scavenging activity of SF-6 was found to be similar or greater when compared to that of known anti-oxidant, L-ascorbic acid.

Preliminary results from the butanol sub-fraction suggests that SF-6 posses significant effect in attenuating cytotoxicity in SH-SY5Y cells and is also a strong hydroxyl radical scavenger. The single compound methylparaben, isolated from this fraction is one of the most commonly used forms of paraben as preservatives in cosmetics and pharmaceuticals. The usage of MP is also found in several foods and other consumable products (Daniel, 1986; Smolinske, 1992). MP has been reported to obtain naturally in several plants species such as blueberries, cloudberry, yellow passion fruit and in products such as white wine, botrytised wine and bourbon vanilla (TNO, 2000).

Earlier reports revealed that MP possess antioxidant, antibacterial and antifungal activities and has promising potential in preventing microbial contamination in pharmaceutical and cosmetic

industries for over 50 years (McDonnell et al., 1988; Bao-Liang, 1989; Soni et al., 2002).

Reports also indicated that MP is capable of entering the brain and may definitely show pharmacological effects within the CNS (Jones, 1956). Since MP showed inhibitory effect against 6-OHDA-induced ROS generation in SH-SY5Y cells, we extended our studies *in vivo* using 6-OHDA-induced mouse model of PD. Animals with unilateral 6-OHDA lesions exhibit asymmetrical rotational behavior in response to dopamine agonists, such as apomorphine (Iancu et al. , 2005). Dopaminergic receptor supersensitivity caused by the loss of nigrostriatal TH terminals might be the cause of apomorphine-induced rotations (Carman et al., 1991; Fisher et al., 1991). The findings from our study indicated that MP treatment ameliorated the apomorphine-induced asymmetric rotations suggesting that MP rescued the dopaminergic neurons that are associated with a subsequent reduction of receptor super sensitivity.

Behavioral tests in PD models can be used to detect therapeutic effects and/or to characterize the extent of lesions in the brain. Since toxin-induced lesions are often variable, these tests can also serve to detect animals with a high degree of cell loss. Rotarod test was used for the assessment of neurological deficits in rodents, usually following pharmacological treatments, genetic manipulations, or brain injuries (Rogers et al., 2001). However, this test has also been used as a drugfree test for unilaterally 6-OHDA-lesioned animals to assess for akinetic symptoms and may also be used in detecting loss of tyrosine hydroxylase-immunoreactive cells in the SN (Rozas, 1997; Whishaw IQ, 2003). Our results were in agreement with previous works that the time spent on the rotating rod was lower

in 6-OHDA-induced mouse and MP treatment significantly improved the latency time.

Previous reports indicated that 6-OHDA exerts its neurodegenerative process via oxidative stress, which causes generation of ROS followed by brain membrane lipid peroxidation (Storch et al., 2000). These reports are in agreement with our present result that the level of lipid peroxidation product, MDA (an index of oxidative stress), was increased in the lesioned SN in 6-OHDA-induced mouse brain tissues. In addition, MP improved 6-OHDA-induced neurotoxicity through protecting dopaminergic neurons in the SN in the brain tissues. Histological examination of the mice brain samples indicated that MP effectively improved dopaminergic cell survivability as evidenced by increased TH^+ neurons against 6-OHDA in the SN. Thus, we propose that MP maintains nigrostriatal pathway function by enhancing the survival of dopaminergic neurons in 6-OHDA-lesioned mice.

The present results indicated that butanol sub-fraction SF-6 from *I. tinctoria*, and its isolated compound MP possesses cytoprotective actions and possess strong hydroxyl radical scavenging, ROS and lipid peroxidation inhibiting effects *in vitro* in SH-SY5Y cells. Mounting evidence suggests that antioxidants may act directly by scavenging free radicals or indirectly by increasing endogenous cellular antioxidant defenses such as activation of nuclear factor erythroid 2 (Nrf2). The potential for Nrf2-mediated transcription to protect from neurodegeneration resulting from oxidative stress mechanisms has been extensively studied (Haddad, 2002; Kelsey et al., 2010; Chen et al., 2012; Zhang et al., 2012). Further, redox regulation of neuronal survival at the signal-

transduction level influences several redox sensitive apoptotic effectors such as caspases, Bcl-2 and cytochrome *c* and their functions can be significantly affected by cellular ROS (Kowaltowski et al., 2000; Zhao et al., 2003; Matsuzawa and Ichijo et al., 2005). Reports also revealed that 6-OHDA neurotoxicity in PD models may be influenced by decreasing neuronal Nrf2 activation thereby producing oxidative insult in PD (Hara et al., 2006; Jakel et al., 2007; Tobón-Velasco et al., 2012). Since MP attenuated the 6-OHDA-induced oxidative damage its beneficial actions as a neuroprotective agent in PD may be by scavenging free radicals and/or directed towards regulation of oxidative stress mechanisms by activating Nrf2-antioxidant response element and other signaling pathways. Further research on MP in regulating endogenous oxidative mechanisms may provide valuable insights into its potential neuroprotective actions in PD.

In conclusion, this study reports for the first time that *I. tinctoria* extract contains MP and protects against 6-OHDA-induced neuronal damage both *in vitro* and *in vivo* models of PD. The marked neuroprotection exhibited by MP might be via its antioxidant defense mechanisms. The tremendous characters of MP such as low cost, long history of safe use in food/pharmaceuticals, practically non-toxic in animals and crossing of blood brain barrier provides us with immense scope to re-visit this compound as a potential therapeutic agent for neurodegenerative diseases including PD. Further our results substantiate the traditional claims of *I. tinctoria* for its use in epilepsy and brain diseases.

REFERENCES

Alves da Costa C (2003). Recent advances on alpha-synuclein cell biology: functions and dysfunctions. *Curr Mol Med,* 3(1): 17-24.

Ambani LM, Van Woert MH, Murphy S (1975). Brain peroxidase and catalase in Parkinson disease. *Arch Neurol,* 32(2): 114-118.

Anand KK, Chand D, Ghatak BJ (1979). Protective effect of alcoholic extract of Indigofera tinctoria Linn. in experimental liver injury. *Indian J Exp Biol,* 17(7): 685-687.

Anand KK, Chand D, Ray Ghatak BJ, Arya RK (1981). Histological evidence of protection by Indigofera tinctoria Linn. against carbontetrachloride induced hepatotoxicity--an experimental study. *Indian J Exp Biol,* 19(3): 298-300.

Asuntha G, Prasannaraju Y, Prasad KVSRG. 2010. Effect of ethanol extract of *Indigofera tinctoria* Linn (Fabaceae) on lithium / pilocarpine-induced s*tatus epilepticus* and oxidative stress in wistar rats". Tropical Journal of Pharmaceutical Research, 9(2):149-156.

Bakasso S, Lamien-Meda A, Lamien CE, Kiendrebeogo M, Millogo J, Ouedraogo AG, Nacoulma OG. 2008. Polyphenol contents and antioxidant activities of five *Indigofera species* (Fabaceae) from Burkina Faso. Pakistan Journal of Biological Sciences, 11(11):1429-35.

Balamurugan G SS (2009). Preliminary Phytovhrmicsl Screening and Anthelmintic Activity of Indigofera Tinctoria Lin. . *Internal Jurnal Drug Development and Researc,* 1(1): 157-160.

Bao-Liang, S., Hai-Ying, L., Dun-Ren, P., 1989. In-vitro spermicidal

activity of parabens against human spermatozoa. Contraception 39 (3), 331–336.

Braak H, Braak E (2000). Pathoanatomy of Parkinson's disease. *J Neurol,* 247 Suppl 2: II3-10.

Carman LS, Gage FH, Shults CW (1991). Partial lesion of the substantia nigra: relation between extent of lesion and rotational behavior. *Brain Res,* 553(2): 275-283.

Chaturvedi RK, Shukla S, Seth K, Chauhan S*, et al.* (2006). Neuroprotective and neurorescue effect of black tea extract in 6-hydroxydopamine-lesioned rat model of Parkinson's disease. *Neurobiol Dis,* 22(2): 421-434.

Chen JH, Ou HP, Lin CY, Lin FJ, Wu CR, Chang SW, Tsai CW. Carnosic acid prevents 6-Hydroxydopamine-induced cell death in SH-SY5Y cells via mediation of glutathione synthesis. Chem Res Toxicol. 2012; 25:1893-901.

Chitra M, Muthusudha N, Sasikala R. 2003. Bioefficiency of *Indigogera tinctoria* Linn. on isoniazid induced hepatotoxicity in albinorats. Ancient Science of Life, 23(2) : 79-89.

Coyle JT, Puttfarcken P (1993). Oxidative stress, glutamate, and neurodegenerative disorders. *Science,* 262(5134): 689-695.

Daniel, J.W., 1986. Metabolic aspects of antioxidants and preservatives. Xenobiotica 16 (10–11), 1073–1078.

Dawson TM, Dawson VL (2002). Neuroprotective and neurorestorative strategies for Parkinson's disease. *Nat Neurosci,* 5 Suppl: 1058-1061.

Dexter DT, Carter CJ, Wells FR, Javoy-Agid F*, et al.* (1989). Basal lipid peroxidation in substantia nigra is increased in

Parkinson's disease. *J Neurochem,* 52(2): 381-389.

Ding YM, Jaumotte JD, Signore AP, Zigmond MJ (2004). Effects of 6-hydroxydopamine on primary cultures of substantia nigra: specific damage to dopamine neurons and the impact of glial cell line-derived neurotrophic factor. *J Neurochem,* 89(3): 776-787.

Fisher LJ, Young SJ, Tepper JM, Groves PM*, et al.* (1991). Electrophysiological characteristics of cells within mesencephalon suspension grafts. *Neuroscience,* 40(1): 109-122.

Forno LS (1996). Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol,* 55(3): 259-272.

Gao M, Zhang WC, Liu QS, Hu JJ*, et al.* (2008). Pinocembrin prevents glutamate-induced apoptosis in SH-SY5Y neuronal cells via decrease of bax/bcl-2 ratio. *Eur J Pharmacol,* 591(1-3): 73-79.

Goedert M (2001). Parkinson's disease and other alpha-synucleinopathies. *Clin Chem Lab Med,* 39(4): 308-312.

Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem. 1982;126:131–138.

Gupta RC (2004). Brain regional heterogeneity and toxicological mechanisms of organophosphates and carbamates. *Toxicol Mech Methods,* 14(3): 103-143.

Haddad JJ. Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. Cell Signal. 2002; 14: 879–897.

Han R. 1994. Highlight on the studies of anticancer drugs derived

from plants in China. Stem Cells, 12(1):53-63.

Hara H, Ohta M, Adachi T. Apomorphine protects against 6-hydroxydopamine-induced neuronal cell death through activation of the Nrf2-ARE pathway. J Neurosci Res. 2006; 84:860–866.

Heh-In Im EN, Eun-sun Lee, Yu-jin Hwang, and Yong Sik Kim., (2006). Baicalein Protects 6-OHDA-induced Neuronal Damage by Suppressing Oxidative Stress. *Korean J Physiol Pharmacol,* 10: 309-315.

Hou JG, Cohen G, Mytilineou C (1997). Basic fibroblast growth factor stimulation of glial cells protects dopamine neurons from 6-hydroxydopamine toxicity: involvement of the glutathione system. *J Neurochem,* 69(1): 76-83.

Houghton PJ, Howes MJ (2005). Natural products and derivatives affecting neurotransmission relevant to Alzheimer's and Parkinson's disease. *Neurosignals,* 14(1-2): 6-22.

Iancu R, Mohapel P, Brundin P, Paul G (2005). Behavioral characterization of a unilateral 6-OHDA-lesion model of Parkinson's disease in mice. *Behav Brain Res,* 162(1): 1-10.

Jakel RJ, Townsend JA, Kraft AD, Johnson JA. Nrf2-mediated protection against 6-hydroxydopamine. Brain Research. 2007; 1144: 192–201, 2007.

Jang JH, Surh YJ (2001). Protective effects of resveratrol on hydrogen peroxide-induced apoptosis in rat pheochromocytoma (PC12) cells. *Mutat Res,* 496(1-2): 181-190.

Jellinger K LL, Kienzl E, Herlinger E, Youdim MB (1995). Chemical evidence for 6-hydroxydopamine to be an endogenous toxic

factor in the pathogenesis of Parkinson's disease. *J Neural Transm Suppl,* 45: 297-314.

Jones DT, J.L. Morrison, A.P. Richardson. p-Hydroxybenzoic acid esters as preservatives. III. The physiological disposition of p-hydroxybenzoic acid and its esters. Journal of American Pharmaceutical Association, Science Edition. 1956;45:265–73.

Kameswaran R. RR (2008). Protective effect of flavinoidal fraction of Indigofera tinctoria Benzo (α) pyrene induced lung carcinogenicity in Swiss Albino mouse. *Int. J. Cancer Res,* 4(3): 71–80.

Kelsey NA, Wilkins HM, Linseman DA. Nutraceutical antioxidants as novel neuroprotective agents. Molecules. 2010; *15:* 7792-7814.

Kish SJ, Morito C, Hornykiewicz O (1985). Glutathione peroxidase activity in Parkinson's disease brain. *Neurosci Lett,* 58(3): 343-346.

Kowaltowski AJ, Vercesi AE, Fiskum G. Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine nucleotides. Cell Death Differ 2000;7:903-10.

Kumar SA, Gandhimathi R, Amudha P (2009). Study on the Anti-Seizure Activity of Methanolic Extracts of Indigofera Tinctoria (L.). . *Pharmacologyonline,* 1: 1341-1351.

Matsuzawa A, Ichijo H. Stress-responsive protein kinases in redox-regulated apoptosis signaling. Antioxid Redox Signal. 2005; 7: 472-81.

McDonnell TJ, Chang SW, Westcott JY, Voelkel NF. Role of oxidants, eicosanoids, and neutrophils in amphotericin B lung injury in

rats. J Appl Physiol. 1988 Nov;65(5):2195-206.

Motamarri SN, Karthikeyan M, Rajasekar S, Gopal V. 2012. *Indigofera tinctoria Linn* - A phytopharmacological review. International Journal of Research in Pharmaceutical and Biomedical Sciences, 3(1), 164-169.

Narender T, Khaliq T, Puri A, Chander R (2006). Antidyslipidemic activity of furano-flavonoids isolated from Indigofera tinctoria. *Bioorg Med Chem Lett,* 16(13): 3411-3414.

Naveilhan P, Berger F, Haddad K, Barbot N*, et al.* (1994). Induction of glioma cell death by 1,25(OH)2 vitamin D3: towards an endocrine therapy of brain tumorsm. *J Neurosci Res,* 37(2): 271-277.

Olanow CW (1992). An introduction to the free radical hypothesis in Parkinson's disease. *Ann Neurol,* 32 Suppl: S2-9.

Oli RG ML, Swarna FB, Manikandan P, Khosa RL (2005). Evaluation of anti-inflammatory potential of Indigofera tinctoria extract in rats. *Indian Journal of Natural Products,* 21, : 12-15.

Pandey N, Strider J, Nolan WC, Yan SX*, et al.* (2008). Curcumin inhibits aggregation of alpha-synuclein. *Acta Neuropathol,* 115(4): 479-489.

Park CH, Choi SH, Koo JW, Seo JH*, et al.* (2002). Novel cognitive improving and neuroprotective activities of Polygala tenuifolia Willdenow extract, BT-11. *J Neurosci Res,* 70(3): 484-492.

Prakash D, Suri S, Upadhyay G, Singh BN. 2007. Total phenol, antioxidant and free radical scavenging activities of some

medicinal plants. International journal of Food Science and Nutrition, 58(1):18-28.

Puri A, Tanvir K, Rajendran SM, Geetika B, Ramesh C, Tadigoppula N. 2007. Antidyslipidemic activity of *Indigofera tinctoria.* Journal of Herbal Phamacotherapy, 7(1):59-64.

Renukadevi KP and Suhani Sultana S. 2011. Determination of Anti bacterial, Antioxidant and Cytotoxicity effect of Indigofera tinctoria on Lung cancer cell line NCI-h69. International Journal of Pharmacology, 7(3):356-362.

Rogers KL, Grice ID, Griffiths LR. Modulation of in vitro platelet 5-HT release by species of Erythrina and Cymbopogon. Life Sci. 2001;69:1817-29.

Rozas G, Guerra MJ, Labandeira-Garcia JL (1997). An automated rotarod method for quantitative drug-free evaluation of overall motor deficits in rat models of parkinsonism. *Brain Res Brain Res Protoc,* 2(1): 75-84.

Satoh T, Enokido Y, Aoshima H, Uchiyama Y*, et al.* (1997). Changes in mitochondrial membrane potential during oxidative stress-induced apoptosis in PC12 cells. *J Neurosci Res,* 50(3): 413-420.

Satyavati GV, Gupta, A., Tandon, N., (1987). Medicinal Plants of India. *Indian Council for Medical Research, New Delhi, India*: 138.

Singh B, Chandan BK, Sharma N, Bhardwaj V, Satti NK, Gupta VN, Gupta BD, Suri KA, Suri OP. (2006). Isolation, structure elucidation and in vivo hepatoprotective potential of trans-tetracos-15-enoic acid from Indigofera tinctoria Linn.

Phytotherapy Research, 20(10): 831-9.

Singh B. SAK, Chandan B.K., Bhardwaj V., Gupta V.N., Suri O.P., Honda S.S (2001). Hepato protective activity of indigtone-a bioactive fraction from Indigofera Tinctoria linn. *Phytotherapy research,* 15: 294–297.

Smolinske SC. Parabens. In: Handbook of Food, Drug, and Cosmetic Excipients. . Boca Raton, FL: CRC Press; 1992. p. 251–8.

Soni MG, Taylor SL, Greenberg NA, Burdock GA (2002). Evaluation of the health aspects of methyl paraben: a review of the published literature. Food Chem Toxicol 40: 1335–1373.

Sreepriya M, Devaki T, Balakrishna K, Apparanantham T. 2001a. Effect of *Indigofera tinctoria* Linn on liver antioxidant defense system during D-galactosamine/endotoxin-induced acute hepatitis in rodents. Indian Journal of Experimental Biology, 39 (2):181-4.

Sreepriya M, Devaki T, Nayeem M. 2001b. Protective effects of *Indigofera tinctoria* L. against D-Galactosamine and carbon tetrachloride challenge on 'in situ' perfused rat liver. Indian Journal of Physiology and Pharmacology, 45(4):428-34.

Storch A, Burkhardt K, Ludolph AC, Schwarz J. Protective effects of riluzole on dopamine neurons: involvement of oxidative stress and cellular energy metabolism. J Neurochem. 2000;75:2259-69.

TNO, 2000. Volatile Compounds in Food. Database Software. Boelens Aroma Chemical Information Service, The Netherlands.

Tobón-Velasco JC, Vázquez-Victorio G, Macías-Silva M, Cuevas E, Ali SF, Maldonado PD, González-Trujano ME, Cuadrado A,

Pedraza-Chaverrí J, Santamaría A. (2012). *S*-allyl cysteine protects against 6-hydroxydopamine-induced neurotoxicity in the rat striatum: Involvement of Nrf2 transcription factor activation and modulation of signaling kinase cascades. Free Radic Biol Med.;53:1024-40.

Todorov IN, Zaikov, G.E., Degterev, I.A., 1993 (1993). Bioactive Compounds: Biotransformation of Biological Action, Mechanism of Antistress and Anabolic Action of Eleutherococcus. *Nova Science Publishers, Inc., Commack, NY*: 26-27.

Tyagi PK, Rai VK, Pahria AK, Kumar SS, Singh Y, Sharma M, Goval M. 2010. Preliminary phytochemical screening and evaluation of antiinflammatory activity of ethanolic extract of leaves of *Indigofera tinctoria* Linn. Journal of Current Pharmaceutical Research, 3(1):47- 50.

Ungerstedt U (1968). 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. *Eur J Pharmacol,* 5(1): 107-110.

Verma S.M SKB, Verma Amit ., (2010). Antidiabetic Activity of Leaves of Indigofera tinctoria Linn (Fabaceae). *International Journal of Toxicological and Pharmacological Research,* 1(2): 42-43.

von Coelln R, Kugler S, Bahr M, Weller M*, et al.* (2001). Rescue from death but not from functional impairment: caspase inhibition protects dopaminergic cells against 6-hydroxydopamine-induced apoptosis but not against the loss of their terminals. *J Neurochem,* 77(1): 263-273.

Whishaw IQ LK, Whishaw PA, Gorny B, Metz GA. Distinct forelimb and hind limb stepping impairments in unilateral dopamine-

depleted rats: use of the rotorod as a method for the qualitative analysis of skilled walking. J Neurosci Meth. 2003;126:13-23.

Zhang L, Yu H, Sun Y, Lin X, *et al.* (2007). Protective effects of salidroside on hydrogen peroxide-induced apoptosis in SH-SY5Y human neuroblastoma cells. *Eur J Pharmacol,* 564(1-3): 18-25.

Zhang Z, Cui W, Li G, Yuan S, Xu D, Hoi MP, Lin Z, Dou J, Han Y, Lee SM. Baicalein protects against 6-OHDA-induced neurotoxicity through activation of Keap1/Nrf2/HO-1 and involving PKCα and PI3K/AKT signaling pathways. J Agric Food Chem. 2012; 60:8171-82.

Zhao Y, Wang ZB, Xu JX. Effect of cytochrome c on the generation and elimination of O2*- and H2O2 in mitochondria. J Biol Chem 2003; 278:2356-60.

Zhu M, Rajamani S, Kaylor J, Han S, *et al.* (2004). The flavonoid baicalein inhibits fibrillation of alpha-synuclein and disaggregates existing fibrils. *J Biol Chem,* 279(26): 26846-26857.

ACKNOWLEDGEMENTS

Abstract in Korean

서론: 파킨슨 병(PD) 치료에 도움이 되는 물질을 발굴하기 위하여, 인도 및 여러 아시아 지역에서 자생하는 다년생식물인 인디고페라 틴토리아에서 추출한 활성성분들을 이용하여 PD 실험모델에서 그 효능을 연구하였다. 인디고페라 틴토리아는 수 세기 동안 인도 및 중국과 같은 동양권에서 간질, 신경계 질환, 기관지 질환, 간질환, 궤양, 치질 등 각종 질환에서 전통의약으로 사용되어 왔다.

방법: 활성성분물질의 분리 정제를 위하여, 박막크로마토그래피 (TLC), 고성능 액체크로마토그래피 (HPLC), 핵자기공명분광기 (NMR) 등의 기법들을 사용하였다. 또한 부탄올 분획층 (SF-6) 의 효능 평가를 위해서, α-synuclein, 6-hydroxydopamine (6-OHDA), 그리고 과산화수소 (H_2O_2)가 유도한 SH-SY5Y 세포의 독성에 대하여 SF-6의 신경 보호 효과를 세포 생존률 검사 (WST-1) 의 방법을 이용하여 확인하였다. 또한 SF-6의 자유라디칼 소거능을 확인하기 위하여 전자 스핀 공명 (ESR) 기법을 이용하였다.

SF-6로부터 분리한 단일화합물인 methylparaben (MP) 이 SH-SY5Y 세포에서 6-OHDA이 유도한 활성산소종을 억제하는 것을 DCF-DA 방법을 통하여 확인하였다. 또한 마우스 뇌 조직에서 6-OHDA에 의해 유도된 지질 과산화물을 Malondialdehyde (MDA) 키트를 이용하여 MP가 감소시키는 효능을 확인하였다.

동물 실험에서는 6-OHDA을 지정된 장소인, 뇌흑질 (SN)에 정위 방식 (stereotaxic)으로 주사하여 만들었으며, 이때 신경세포의 손상을 얼마나 억제하는지에 대하여 연구 하였다. 6-OHDA로 파킨슨병 동물모델을 만든 후 2주 뒤에

아포모르핀(apomorphine)에 의한 회전운동을 관찰하였고, 행동학적으로 운동기능 및 균형감각의 저하 기능을 평가하기 위하여 rotarod test를 시행하였다. 생물학적 변화를 확인하기 위하여, Tyrosine hydroxylase-positive (TH^+) cells 염색을 ABC kit를 사용하여 면역조직염색법(IHC)을 이용하여 확인하였다.

결론: SF-6 (부탄올 분획층)는 α-synuclein이 유도한 SH-SY5Y 세포의 독성을 약하게 하였고, 자유라디칼의 하나인 hydroxyl free radicals을 소거하는 것을 전자스핀공명 (ESR) 방법을 통하여 확인하였다. SF-6로부터 분리된 단일 화합물인 MP는 *in vivo* 모델과 *in vitro* 모델에서 동일하게 신경보호 효능을 가지는 것을 확인하였다. MP는 나노몰 농도에서 6-OHDA과 과산화수소 (H_2O_2)가 유도하는 세포 독성 및 활성산소종을 SH-SY5Y에서 농도의존적으로 저해하는 것을 확인하였다. 또한, 마우스 뇌조직에 6-OHDA를 주입했을 때 발생하는 지질과산화물의 축적을 MP가 억제하는 것을 확인하였다. 행동학적 실험에서 MP가 6-OHDA에 의한 운동기능 손상 및 아포모르핀에 의한 회전운동을 농도의존적으로 억제하는 것을 확인하였다. 뇌 조직을 적출하여 면역조직염색법 (IHC) 기법으로 Tyrosine hydroxylase-positive (TH^+) cells 염색을 확인한 결과, 동일하게 6-OHDA으로 독성을 유발한 쥐에서 MP를 처리한 군이 처리하지 않은 군에 비해서 Tyrosine hydroxylase-positive (TH^+) cells의 수가 유의하게 많은 것을 확인할 수 있었다.

고찰: *in vivo* 모델과 *in vitro* 모델에서 얻은 결과들을 통해 MP가 PD 실험모델에서 항산화 효능을 가짐으로 신경보호 효능을 하는 것을 알 수 있었다. 이러한 결과들을 조합하여 볼 때, MP가 PD와 같은 퇴행성 뇌 질환의 치료제로 개발

가능성이 있다는 것을 확인 하였다.

Keywords: *Indigofera tinctoria*, methylparaben, 6-hydroxydopamine; reactive oxygen species; Parkinson's disease, substantia nigra, SH-SY5Y cells; neurodegeneration; oxidative stress.

Student number: 2009-31325