



*Letter*

***Magnoliae Cortex* extract protects PC12 cells from cytotoxicity induced by hydrogen peroxide or 6-hydroxydopamine through enzyme induction**

**Takahito Nishiyama, Yasuhiro Masuda, Tadashi Izawa, Tomokazu Ohnuma, Kenichiro Ogura and Akira Hiratsuka**

*Department of Drug Metabolism and Molecular Toxicology, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432-1 Horinouchi, Hachioji-shi, Tokyo 192-0392, Japan*

(Received April 10, 2019; Accepted April 17, 2019)

**ABSTRACT** — Alzheimer's and Parkinson's disease are neurodegenerative disorders of unknown cause for which there is no cure or way of preventing or slowing its progression. Various genetic and environmental factors are thought to be involved in the onset of neurodegenerative diseases. Oxidative stress, such as the generation of reactive oxygen species and lipid peroxidation, is a major factor in initiating the disease process. However, oxidative stress in cells is known to be suppressed by drug-metabolizing enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1), and antioxidant enzymes, such as catalase. Here, we used PC12 cells, which are a recognized model for neuronal cell death and neurite extension, to investigate whether *Magnoliae Cortex* derived from the *Magnoliaceae* plant family can induce these enzyme activities. Our results reveal that *Magnoliae Cortex* extract induces the activity of both NQO1 and catalase. In addition, the cytotoxic effect of hydrogen peroxide and 6-hydroxydopamine was significantly suppressed by pretreatment of the cells with *Magnoliae Cortex* extract. Based on our findings, we conclude that induction of these enzyme activities by *Magnoliae Cortex* extract leads to an enhancement of its cytoprotective effect.

**Key words:** Catalase, Hydrogen peroxide, 6-Hydroxydopamine, NAD(P)H:Quinone oxidoreductase 1, Oxidative stress, PC12 cells

**INTRODUCTION**

Oxidative stress is thought to be involved in the development of a variety of neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis and Parkinson's disease (Andersen, 2004; Lin and Beal, 2006). However, because the pathogenic mechanism of neurodegenerative diseases is not fully understood, no therapy has been established to prevent progress of the disease itself. Oxidative stress refers to a state of disruption in the cellular balance between the amount of reactive oxygen species (ROS) and the endog-

enous pool of antioxidants that results in an excessive accumulation of ROS (Sies and Cadenas, 1985). ROS are highly reactive and will readily modify proteins, lipids and nucleic acids, leading to extensive cellular damage.

Several defense mechanisms against oxidative stress are present within cells. Nonetheless, brain cells are particularly susceptible to oxidative stress because the adult human brain represents only 2% of total body weight, but accounts for 20% of oxygen consumed by the entire body. The fact that brain cells are vulnerable to oxidative stress supports the notion that ROS are involved in the development of neurodegenerative diseases.

A number of factors are known to protect cells from the effects of oxidative stress. For example, dietary antioxidants, such as vitamin E, vitamin C,  $\beta$ -carotene and flavonoids (Aruoma, 1994), attenuate the levels of ROS. Drug-metabolizing enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO 1) and glutathione *S*-transferase, and antioxidant enzymes, such as catalase, superoxide dismutase and glutathione peroxidase, are also known to suppress oxidative stress. NQO1 is thought to protect cells against oxidative stress caused by the products of dopamine metabolism, which include ROS, quinones and semiquinones (Zafar *et al.*, 2006). Catalase reduces oxidative stress in cells by converting hydrogen peroxide ( $H_2O_2$ ) into oxygen and water. These observations suggest it may be possible to prevent neurodegenerative diseases caused by oxidative stress using compounds that can induce drug-metabolizing and antioxidant enzymes (Van Muiswinkel and Kuiperij, 2005).

Potent inducers of drug-metabolizing and antioxidant enzymes have been isolated from natural products (Morimitsu *et al.*, 2002; Chen *et al.*, 2004). Most studies have focused on antioxidants derived from fruits and vegetables because these represent a ready source of such compounds for daily intake. Based on these studies, many active constituents, such as sulforaphane (Zhang *et al.*, 1992), curcumin (Balogun *et al.*, 2003), isothiocyanates (Nakamura *et al.*, 2000) and resveratrol (Chen *et al.*, 2005), were isolated and identified from natural products.

In addition to foods, such as fruits and vegetables, another source of natural antioxidants are herbal medicines that contain many active constituents. Traditional herbal medicines are used as multi-component drugs, although the efficacy and mechanism of action of individual components are often unknown. Recently, the pharmacological action of traditional herbal medicines and elucidation of their effective constituents have been studied in many laboratories. The traditional herbal medicine *Magnoliae Cortex* is known to have efficacy in diseases of the central nervous system. In this study, we investigated the effects of *Magnoliae Cortex* extract on protection against oxidative stress in PC12 cells, which are frequently used as a model system for neuronal cell death.

## MATERIALS AND METHODS

### Materials

*Magnolia Cortex* extract was kindly provided by Tsumura Co. Ltd. (Tokyo, Japan). Rat adrenal medulla PC12 cells were purchased from American Type Culture Collection (Manassas, VA, USA). 6-Hydroxydopamine (6-OHDA) was purchased from Sigma-Aldrich (St

Louis, MO, USA). CellTiter-Glo® Luminescent Cell Viability Assay kit was obtained from Promega Co. (Madison, WI, USA). Hydrogen peroxide was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Cell culture Petri dishes (collagen type I coated ware) and 24-well cell culture multidisc (collagen type I coated ware) were from AGC Techno Glass Co., Ltd. (Shizuoka, Japan). Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). All other reagents were of the highest grade commercially available.

### *Magnoliae Cortex* extract

*Magnoliae Cortex* (100 mg) was added to 2 mL of methanol, and the mixture was vigorously stirred at room temperature for 10 min and then centrifuged at  $1,000 \times g$  for 10 min. The resulting supernatant was transferred to another container and 2 mL of methanol added to the residue. The same process was repeated one more time and the resultant supernatants were combined and used as *Magnoliae Cortex* extract.

### Induction of enzyme activity

Dose- and time-dependent enzyme induction by *Magnoliae Cortex* extract were performed under the following conditions. PC12 cells were seeded at a density of  $2 \times 10^6$  cells/dish in a 100 mm diameter cell culture Petri dish and cultured at 37°C under 5%  $CO_2$  for 96 hr. After culturing, the medium was removed and the cells washed with  $1 \times$  PBS (-). Ten ml of serum-free medium containing *Magnoliae Cortex* extract (0–200  $\mu g/mL$ ) was then added. Methanol was added as a control to a final concentration of 0.2%. The cells were then cultured at 37°C under 5%  $CO_2$  conditions for a further 24 hr. Alternatively, *Magnoliae Cortex* extract was added to a final concentration of 100  $\mu g/mL$ . As a control, methanol was added to a final concentration of 0.1%. Cells were then cultured at 37°C under 5%  $CO_2$  for 12, 24 or 48 hr.

### Cytotoxicity

PC12 cells were seeded at a density of  $6.6 \times 10^4$  cells/well in a 24-well cell culture dish and cultured at 37°C and 5%  $CO_2$  for 72 hr. After culturing, the medium was removed, and 400  $\mu L$  of serum-free medium containing *Magnoliae Cortex* extract (0–300  $\mu g/mL$ ) added to each well. As a control, methanol was added to a final concentration of 0.2%. The cells were then cultured at 37°C under 5%  $CO_2$  conditions for 24 or 48 hr.

### Enzyme source

After culturing the cells for a specified period of time, the medium was removed and the cells were washed with

### *Magnoliae Cortex* extract reduces cytotoxicity in PC12 cells

1 × PBS (-). Four mL of 1 × PBS (-) was added and cells were detached from the culture dish using a cell scraper. The resulting cell suspension was centrifuged at 500 × g for 3 min. The cell pellet was resuspended in 300 µL of 50 mM Tris-HCl buffer (pH 7.4) containing 2 mM EDTA and 125 mM sucrose and then subjected to ultrasonic treatment (10 sec). The resultant homogenate was centrifuged at 15,000 × g for 15 min, and the supernatant used as the enzyme source.

#### Protein concentration

Protein concentrations were determined using a Bio-Rad Protein Assay kit according to the manufacturer's instructions.

#### Enzyme activities

NQO1 and catalase activities were determined spectrophotometrically using previously reported methods (Nishiyama *et al.*, 2008, 2010).

#### Cell viability

Cell viability was established using a CellTiter-Glo® Luminescent Cell viability Assay kit according to the manufacturer's instructions and as previously described (Nishiyama *et al.*, 2010).

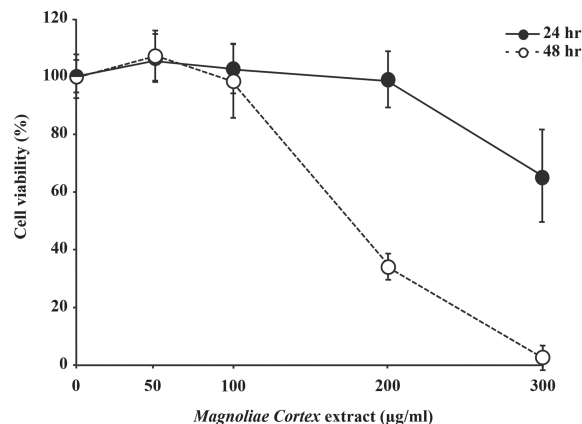
#### Cytotoxicity induction

PC12 cells were seeded at a density of  $6.6 \times 10^4$  cells/well in a 24-well cell culture dish and cultured at 37°C and 5% CO<sub>2</sub> for 72 hr. After culturing, the medium was removed, and 300 µL of serum-free medium containing *Magnoliae Cortex* extract (0-200 µg/mL) was added to each well. After culturing for 24 hr, 100 µL of serum-free medium containing H<sub>2</sub>O<sub>2</sub> (250 µM or 500 µM) or containing 6-OHDA (100 µM or 200 µM) was added to each well. After 3 hr (H<sub>2</sub>O<sub>2</sub>) or 24 hr (6-OHDA) incubation, cell viability was measured according to the methods described above.

## RESULTS

#### Cytotoxicity

Cytotoxic effects of *Magnoliae Cortex* extract on PC12 cells were determined (Fig. 1). PC12 cells were incubated with *Magnoliae Cortex* extract at various concentrations for 24 and 48 hr. No significant cytotoxicity was observed up to 200 µg/mL of extract after 24 hr treatment. However, a decrease in cell viability was observed when cultured cells were exposed to extracts at a concentration of 300 µg/mL and the survival rate was  $65.0 \pm 16.0\%$ . At 48 hr, no significant cytotoxicity was observed up to



**Fig. 1.** Cytotoxic effects of *Magnoliae Cortex* extract on PC12 cells. The PC12 cells were treated with 0-300 µg/mL of *Magnoliae Cortex* extract. Cell viabilities at 24 and 48 hr were measured using a CellTiter-Glo® Luminescent Cell Viability Assay kit as described in Materials and Methods.

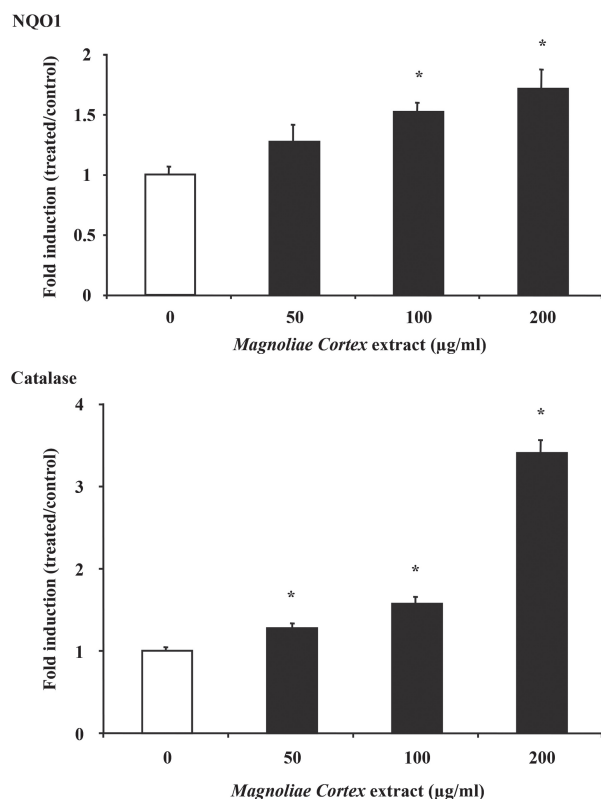
100 µg/mL of extract. However, a marked decrease in cell viability was observed after 48 hr incubation with extract at  $\geq 200$  µg/mL, and the cells were almost all killed after treatment with extract at 300 µg/mL.

#### Enzyme induction

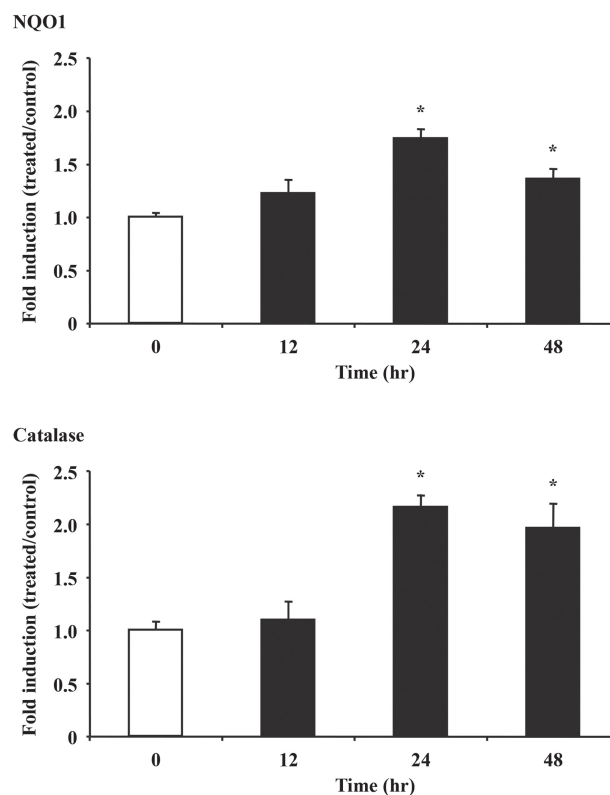
The concentration-dependent effects of *Magnoliae Cortex* extract on NQO 1 and catalase activities were determined (Figs. 2 and 3). NQO 1 and catalase activities were significantly increased after treatment with *Magnoliae Cortex* extract in a concentration-dependent manner. After treatment at the highest concentration of extract (200 µg/mL), the activities of NQO 1 and catalase were  $1.71 \pm 0.16$ -fold and  $3.38 \pm 0.17$ -fold greater than that of the control, respectively. In addition, the time-dependent effects of *Magnoliae Cortex* extract on NQO 1 and catalase activities were measured. Both NQO 1 and catalase activity increased until 24 hr after treatment with *Magnoliae Cortex* extract, and thereafter showed a slight decrease. The induction rate of NQO 1 and catalase activity after 24 hr treatment was  $1.74 \pm 0.09$ -fold and  $2.16 \pm 0.12$ -fold greater than that observed for the control, respectively.

#### Protective effects

PC12 cells either treated or untreated with *Magnoliae Cortex* extract were incubated with H<sub>2</sub>O<sub>2</sub>, which is a ROS, and with 6-OHDA, which is a neurotoxic substance. The cell survival rate was then measured (Fig. 4). Cell viability of the untreated PC12 cells decreased significant-



**Fig. 2.** Dose-dependent effects of *Magnoliae Cortex* extract treatment on NQO1 and catalase activities in PC12 cells. PC12 cells were treated with 0-200 µg/mL *Magnoliae Cortex* extract for 24 hr. After incubation, NQO1 and catalase activities were determined as described in Materials and Methods. \*, significantly different from control at  $P < 0.05$  (Student's *t*-test).



**Fig. 3.** Time-dependent effects of *Magnoliae Cortex* extract treatment on NQO1 and catalase activities in PC12 cells. The PC12 cells were treated with 100 µg/mL *Magnoliae Cortex* extract for 0-48 hr. After incubation, NQO1 and catalase activities were determined as described in Materials and Methods. \*, significantly different from 0 hr at  $P < 0.05$  (Student's *t*-test).

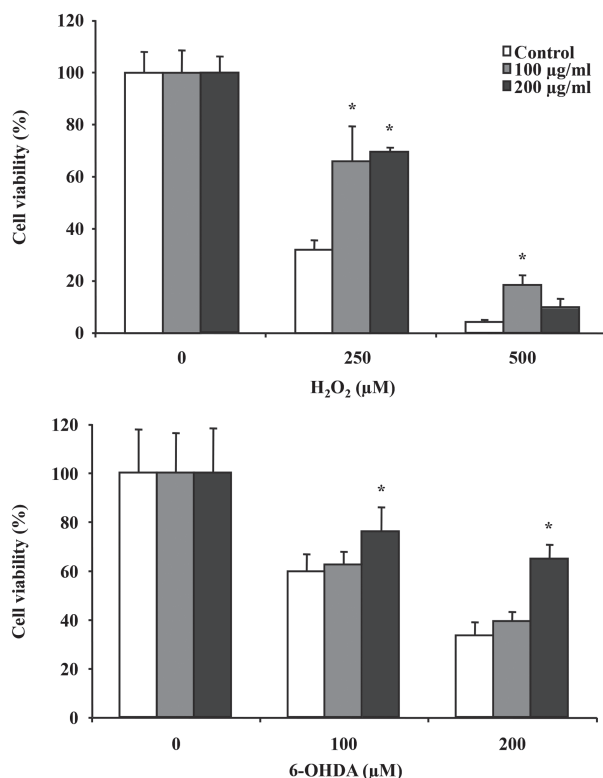
ly after exposure to  $H_2O_2$  (250 µM or 500 µM). However, pretreating the PC12 cells with *Magnoliae Cortex* extract drastically reduced cytotoxicity brought about by exposure to  $H_2O_2$ . Cells pretreated with 100 µg/mL of *Magnoliae Cortex* extract and then exposed to 250 µM or 500 µM  $H_2O_2$  displayed cell viability that was 2.1- and 4.4-fold greater, respectively, than that of untreated cells. Moreover, cells pretreated with 200 µg/mL of *Magnoliae Cortex* extract and then exposed to 250 µM or 500 µM  $H_2O_2$  displayed cell viability 2.2- and 2.3-fold greater, respectively, than the untreated group. The possible protective effect of *Magnoliae Cortex* extract against 6-OHDA-induced cell death was assessed using PC12 cells. The cells were pretreated with increasing concentrations of *Magnoliae Cortex* extract (0, 100 and 200 µg/mL) before the addition of 6-OHDA (100 or 200 µM). Cells

pretreated with 200 µg/mL of *Magnoliae Cortex* extract and then exposed to 100 µM or 200 µM 6-OHDA gave cell viabilities that were 1.3- and 1.9-fold greater, respectively, than the untreated group.

## DISCUSSION

Oxidative stress is thought to be involved in the onset of neurodegenerative diseases. Here, we focused on drug-metabolizing and antioxidant enzymes as a potential factor to reduce oxidative stress. Specifically, PC12 cells were treated with *Magnoliae Cortex* extract, which is known to have efficacy in diseases of the central nervous system, at non-cytotoxic concentrations. We investigated the induction effect of *Magnoliae Cortex* extract on NQO 1 and catalase activity in PC12 cells and also exam-

*Magnoliae Cortex* extract reduces cytotoxicity in PC12 cells



**Fig. 4.** Protection of PC12 cells against the cytotoxicity of H<sub>2</sub>O<sub>2</sub> and 6-OHDA by pretreatment with *Magnoliae Cortex* extract. PC12 cells were pretreated with 0-200 μg/mL *Magnoliae Cortex* extract for 24 hr, followed by incubation with 0, 250 and 500 μM H<sub>2</sub>O<sub>2</sub> or 0, 100 and 200 μM 6-OHDA for a further 3 hr (H<sub>2</sub>O<sub>2</sub>) or 24 hr (6-OHDA). After incubation, cell viabilities were determined using a CellTiter-Glo® Luminescent Cell Viability Assay kit. \*, significantly different from each control at P < 0.05 (Student's t-test).

ined the cytoprotective effect of the extracts against H<sub>2</sub>O<sub>2</sub> and 6-OHDA induced toxicity.

Hydrogen peroxide is a ROS, which is converted to the highly reactive hydroxyl radical via the Fenton reaction. The hydroxyl radical causes damage to cell function by modifying functional molecules such as proteins, lipids or DNA. Pretreatment of the cells with *Magnoliae Cortex* extract significantly inhibited the cytotoxic effect of H<sub>2</sub>O<sub>2</sub> in a concentration-dependent manner as compared with untreated PC12 cells. However, no such inhibition in the cytotoxic effect of H<sub>2</sub>O<sub>2</sub> was observed upon simultaneous treatment of the cells with *Magnoliae Cortex* extract and H<sub>2</sub>O<sub>2</sub> (results not shown). These observations suggest the drug-metabolizing and antioxidant enzymes

induced by pretreatment of the cells with *Magnoliae Cortex* extract plays an important role in protecting against oxidative stress.

6-OHDA is a neurotoxin commonly used to selectively destroy dopaminergic neurons and noradrenergic neurons and is widely used to generate experimental models of Parkinson's disease. The molecular mechanism of neurotoxicity mediated by 6-OHDA in PC12 cells is thought to be due to H<sub>2</sub>O<sub>2</sub> and *p*-quinone generated during the metabolism of this compound (Saito *et al.*, 2007). Pretreatment of the cells with *Magnoliae Cortex* extract significantly suppressed the cytotoxic effect of 6-OHDA as compared with the untreated PC12 cells. Pretreatment of PC12 cells with *Magnoliae Cortex* extract induced NQO 1 and catalase activity, resulting in enhanced detoxification of H<sub>2</sub>O<sub>2</sub> and *p*-quinone. These results suggest that the induction of drug-metabolizing and antioxidant enzymes by the *Magnoliae Cortex* extract leads to an enhanced cell protective effect.

*Magnolia* can elicit various biological effects such as a muscle relaxant (Watanabe *et al.*, 1983), inducing sedation (Yang *et al.*, 2007), lowering anxiety (Kuribara *et al.*, 1998) and in the treatment of gastric ulcers (Li *et al.*, 2005). The efficacy of herbal medicines is exerted by synergistic effects of many active constituents found in herbal extracts. It is possible that *Magnoliae Cortex* induced enhancement of NQO1 and catalase activity may also be involved in the biological effects mentioned above. For example, gastric ulcers are associated with cytotoxicity caused by increased ROS and of gastric juice secretion caused by vasoconstriction due to catecholamines and subsequent increased blood flow due to vasorelaxation (Das *et al.*, 1997). It is thought that the anti-gastric ulcer effect of *Magnolia* is based on β-eudesmol, which inhibits gastric acid secretion by blocking the H<sub>2</sub> receptor (Nogami *et al.*, 1986; Kimura *et al.*, 1991). As demonstrated in this study, enhancement in the detoxification of ROS by induction of drug-metabolizing and antioxidant enzymes may also contribute to the anti-stress ulcer action of *Magnoliae Cortex* extract.

Studies to identify active constituents of traditional herbal medicines are being conducted in many laboratories. It is essential to identify the constituents of an extract that has enzyme-inducing ability in order to clarify the nature and mechanism of action of herbal medicines. Studies on the isolation and identification of active constituents involved in enzyme induction by *Magnoliae Cortex* extract are currently underway in our laboratory.

**Conflict of interest----** The authors declare that there is no conflict of interest.



## REFERENCES

- Andersen, J.K. (2004): Oxidative stress in neurodegeneration: cause or consequence? *Nat. Med.*, **10**, S18-S25.
- Aruoma, O.I. (1994): Nutrition and health aspects of free radicals and antioxidants. *Food Chem. Toxicol.*, **32**, 671-683.
- Balogun, E., Hoque, M., Gong, P., Killeen, E., Green, C.J., Foresti, R., Alam, J. and Motterlini, R. (2003): Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem. J.*, **371**, 887-895.
- Chen, C., Pung, D., Leong, V., Hebban, V., Shen, G., Nair, S., Li, W. and Kong, A.N. (2004): Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: effect of chemical structure and stress signals. *Free Radic. Biol. Med.*, **37**, 1578-1590.
- Chen, C.Y., Jang, J.H., Li, M.H. and Surh, Y.J. (2005): Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. *Biochem. Biophys. Res. Commun.*, **331**, 993-1000.
- Das, D., Bandyopadhyay, D., Bhattacharjee, M. and Banerjee, R.K. (1997): Hydroxyl radical is the major causative factor in stress-induced gastric ulceration. *Free Radic. Biol. Med.*, **23**, 8-18.
- Kimura, M., Nojima, H., Muroi, M. and Kimura, I. (1991): Mechanism of the blocking action of  $\beta$ -eudesmol on the nicotinic acetylcholine receptor channel in mouse skeletal muscles. *Neuropharmacology*, **30**, 835-841.
- Kuribara, H., Stavinoha, W.B. and Maruyama, Y. (1998): Behavioural pharmacological characteristics of honokiol, an anxiolytic agent present in extracts of Magnolia bark, evaluated by an elevated plus-maze test in mice. *J. Pharm. Pharmacol.*, **50**, 819-826.
- Li, Y., Xu, C., Zhang, Q., Liu, J.Y. and Tan, R.X. (2005): In vitro anti-Helicobacter pylori action of 30 Chinese herbal medicines used to treat ulcer diseases. *J. Ethnopharmacol.*, **98**, 329-333.
- Lin, M.T. and Beal, M.F. (2006): Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, **443**, 787-795.
- Morimitsu, Y., Nakagawa, Y., Hayashi, K., Fujii, H., Kumagai, T., Nakamura, Y., Osawa, T., Horio, F., Itoh, K., Iida, K., Yamamoto, M. and Uchida, K. (2002): A sulforaphane analogue that potently activates the Nrf2-dependent detoxification pathway. *J. Biol. Chem.*, **277**, 3456-3463.
- Nakamura, Y., Morimitsu, Y., Uzu, T., Ohigashi, H., Murakami, A., Naito, Y., Nakagawa, Y., Osawa, T. and Uchida, K. (2000): A glutathione S-transferase inducer from papaya: rapid screening, identification and structure-activity relationship of isothiocyanates. *Cancer Lett.*, **157**, 193-200.
- Nishiyama, T., Ohnuma, T., Inoue, Y., Kishi, T., Ogura, K. and Hiratsuka, A. (2008): UDP-glucuronosyltransferases 1A6 and 1A10 catalyze reduced menadione glucuronidation. *Biochem. Biophys. Res. Commun.*, **371**, 247-250.
- Nishiyama, T., Izawa, T., Usami, M., Ohnuma, T., Ogura, K. and Hiratsuka, A. (2010): Cooperation of NAD(P)H:quinone oxidoreductase 1 and UDP-glucuronosyltransferases reduces menadione cytotoxicity in HEK293 cells. *Biochem. Biophys. Res. Commun.*, **394**, 459-463.
- Nogami, M., Moriura, T., Kubo, M. and Tani, T. (1986): Studies on the origin, processing and quality of crude drugs. II. Pharmacological evaluation of the Chinese crude drug "zhu" in experimental stomach ulcer. (2). Inhibitory effect of extract of *Atractylodes lancea* on gastric secretion. *Chem. Pharm. Bull. (Tokyo)*, **34**, 3854-3860.
- Saito, Y., Nishio, K., Ogawa, Y., Kinumi, T., Yoshida, Y., Masuo, Y. and Niki, E. (2007): Molecular mechanisms of 6-hydroxydopamine-induced cytotoxicity in PC12 cells: involvement of hydrogen peroxide-dependent and -independent action. *Free Radic. Biol. Med.*, **42**, 675-685.
- Sies, H. and Cadenas, E. (1985): Oxidative stress: damage to intact cells and organs. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **311**, 617-631.
- Van Muiswinkel, F.L. and Kuiperij, H.B. (2005): The Nrf2-ARE Signalling pathway: promising drug target to combat oxidative stress in neurodegenerative disorders. *Curr. Drug Targets CNS Neurol. Disord.*, **4**, 267-281.
- Watanabe, K., Watanabe, H., Goto, Y., Yamaguchi, M., Yamamoto, N. and Hagino, K. (1983): Pharmacological Properties of Magnolol and Honokiol Extracted from *Magnolia officinalis*: Central Depressant Effects. *Planta Med.*, **49**, 103-108.
- Yang, Q.S., Xu, P.X., Li, Y.H., Jiang, S., Zhang, X. and Xue, M. (2007): Effects of theanine and houpu extract in 7-day chick social separation-stress procedure. *Zhongguo Zhongyao Zazhi*, **32**, 2040-2043.
- Zafar, K.S., Inayat-Hussain, S.H., Siegel, D., Bao, A., Shieh, B. and Ross, D. (2006): Overexpression of NQO1 protects human SK-N-MC neuroblastoma cells against dopamine-induced cell death. *Toxicol. Lett.*, **166**, 261-267.
- Zhang, Y., Talalay, P., Cho, C.G. and Posner, G.H. (1992): A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc. Natl. Acad. Sci. USA*, **89**, 2399-2403.