Korean J Physiol Pharmacol
Vol 14: 279−283, October, 2010
DOI: 10.4196/kjpp.2010.14.5.279

Bark Constituents from Mushroom-detoxified *Rhus verniciflua* Suppress Kainic Acid-induced Neuronal Cell Death in Mouse Hippocampus

Jong-Seon Byun[1], Yoon Hee Han[1,4], Sung-Jun Hong[1], Sung-Mi Hwang[1], Yong-Soo Kwon[2], Hee Jae Lee[1], Sung-Soo Kim[1], Myong-Jo Kim[3,†], and Wanjoo Chun[1,*]

[1]Department of Pharmacology, College of Medicine, [2]College of Pharmacy, [3]Division of Bio-resources Technology, Kangwon National University, Chunchon 200-701, [4]Department of Radiology, Ilsan Paik Hospital, Inje University, School of Medicine, Goyang 411-706, Korea

Urushinol, a plant allergen, has significantly restricted the medical application of *Rhus verniciflua*, although it has been reported to possess a wide variety of biological activities such as anti-inflammatory, antioxidant, and anti-cancer actions. To reduce the urushinol content while maintaining the beneficial biological activities, mushroom-mediated fermentation of *Rhus verniciflua* was carried out and this method resulted in significantly attenuated allergenicity [1]. In the present study, to examine the neuroprotective properties of mushroom-fermented stem bark of *Rhus verniciflua*, two constituents were isolated from mushroom-fermented bark and their neuroprotective properties were examined in a mouse model of kainic acid (KA)-induced excitotoxicity. KA resulted in significant apoptotic neuronal cell death in the CA3 region of mouse hippocampus. However, seven daily administrations of RVH-1 or RVH-2 prior to KA injection significantly attenuated KA-induced pyramidal neuronal cell death in the CA3 region. Furthermore, pretreatment with RVH-1 and RVH-2 also suppressed KA-induced microglial activation in the mouse hippocampus. The present study demonstrates that RVH-1 and RVH-2 isolated from *Rhus verniciflua* and detoxified using mushroom species possess neuroprotective properties against KA-induced excitotoxicity. This leads to the possibility that detoxified *Rhus verniciflua* can be a valuable asset in herbal medicine.

Key Words: Kainic acid, Neuroprotection, Stigma-4-en-3-one, Stigma-4-en-3,6-dione

INTRODUCTION

Rhus verniciflua Stokes, a deciduous tree in the anacardiaceae family and indigenous in East Asia, has been used as a traditional herbal medicine to treat diabetes mellitus and stomach diseases. In the present study, RVH-1 (stigma-4-en-3-one) and RVH-2 (stigma-4-en-3,6-dione) were isolated from *Rhus verniciflua*. RVH-1 and RVH-2 belong to the stigmasterol group, which are plant sterols, specifically phytosterols. Phytosterols have been reported to possess a variety of biological activities such as anticancer [2] and hypocholesterolemic properties [3]. Stigmasterol also has been reported to exhibit anti-osteoarthritic [4] and cytotoxic activities [5]. Although RVH-1 has been isolated from various natural plants such as *Lawsonia inermis* [6] and *Typha latifolia* [7] and RVH-2 from *Argemone mexicana* [8] and *Stephania cepharantha* [9], their biological activities have not been clearly elucidated.

Although *Rhus verniciflua* has been reported to exhibit a variety of biological activities such as antioxidant, anti-inflammatory, anti-cancer, and anti-platelet properties, its medical use was significantly neglected due to the presence of an allergenic compound, urushinol, which causes irritation, inflammation, and urushinol-induced contact dermatitis. Therefore, various approaches such as solvent extraction, far-infrared radiation, and enzyme treatment were applied to selectively remove urushinol in *Rhus verniciflua*. In the mean time, a fermentation method utilizing mushroom species was obtained and it significantly reduced the urushinol content of the stem bark of *Rhus verniciflua* [1]. To determine whether detoxified *Rhus verniciflua* possesses neuroprotective properties, the effects of isolated constituents on neuronal survival were examined in a kainic acid (KA)-induced excitotoxicity animal model.

Excessive release of excitatory amino acids may play an important role in the pathogenesis of neuronal injury such as ischemia, stroke, and neurodegenerative diseases [10,11]. Kainic acid (KA), a potent excitotoxin, binds to specific kainite-type receptors and causes depolarization of neurons resulting in status epilepticus and neurodegeneration [12]. The systemic or intracerebroventricular injection of KA re-

Received July 19, 2010, Revised August 25, 2010,
Accepted September 1, 2010

*Corresponding to: Wanjoo Chun, Department of Pharmacology, College of Medicine, Kangwon National University, Hyoja-2-dong, Chunchon 200-701, Korea. (Tel) 82-33-250-8853, (Fax) 82-33-250-8809, (E-mail) wchun@kangwon.ac.kr
†Co-corresponding author: Myong-Jo Kim, Division of Bio-resources Technology, Kangwon National University, Hyoja-2-dong, Chunchon 200-701, Korea. (Tel) 82-33-250-6413, (Fax) 82-33-250-6413, (E-mail) kimmjo@kangwon.ac.kr

ABBREVIATIONS: RVH-1, stigma-4-en-3-one (RVH-1); RVH-2, stigma-4-en-3,6-dione; KA, kainic acid; icv, intracerebroventricular; PDGF, platelet-derived growth factor.

sults in the death of pyramidal neurons and concurrent reactive gliosis in hippocampus [13,14].

In the present study, we examined the neuroprotective effects of RVH-1 and RVH-2, isolated from mushroom- detoxified stem bark of *Rhus verniciflua*, on hippocampal CA3 neuronal damage induced by intracerebroventricular injection of KA.

METHODS

Animals and reagents

Male ICR mice weighing 23～25 g were obtained from Folas-International, Ltd. (Seoul, Korea). All of the animal experiments were conducted in accordance with the animal care guidelines of the National Institutes of Health (NIH) and Korean Academy of Medical Sciences (KAMS). Mice were housed five per cage in a room maintained at 22±2°C with an alternating 12/12 hr light/dark cycle. Food and water were available *ad libitum*. KA was obtained from Sigma Chemical Co. (St. Louis, MO, USA). KA was prepared as a stock solution at a concentration of 1 mg/ml in sterile 0.1 M phosphate-buffered saline (PBS, pH 7.4), and aliquots were stored at −20°C until use.

Compounds isolated from detoxified Rhus verniciflua

RVH-1 (stigma-4-en-3-one) and RVH-2 (stigma-4-en-3,6-dione) were isolated and identified from mushroom- detoxified stem bark of *Rhus verniciflua* [1]. These compounds (Fig. 1) were orally administered using an oral zonde daily for 7 consecutive days at a dose of 10 mg/kg. Saline was administered to control mice. More than three mice were used for each group. KA was intracerebroventricularly administered 1 hr after the last administration of these compounds.

Intracerebroventricular (icv) injection of KA

The administration of KA (0.1 μg/5 μl) was performed according to the procedure established by Laursen and Belknap [15]. Briefly, KA was injected at the bregma point with a 50-μl Hamilton microsyringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. Mice were sacrificed 24 hr after KA administration.

Immunohistochemistry

All mice were sacrificed 24 hr after KA injection. Mice were transcardially perfused and post-fixed for 4 hr in 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose, sectioned coronally (40 μm) on a freezing microtome, and collected in cryoprotectant for storage at −20°C. For cresyl violet staining, the sections were mounted in gelatin-coated slides and allowed to air-dry overnight. The mounted sections were submerged in 0.1% cresyl violet solution for 5 min. The sections were rinsed in 70% ethanol and dehydrated in a graded series of ethanol, immersed in xylene, mounted in Permount (Fisher Scientific, NJ, USA) and cover slipped. The free-floating immunohistochemistry of the brain of the brain sections was processed as previously described [16]. Sections that were collected from cryoprotectant were washed with PBS, pre-incubated for 30 min in 0.1 M PBS with 1% bovine serum albumin and 0.2% Triton X-100, and incubated for 2 days at 4°C with the following primary antibodies: OX-6 (1 : 1,000; BD Pharmingen) or NeuN (1 : 1,000; Santa Cruz, Santa Cruz, CA, USA). After 2 days of incubation with primary antibody, the antigens were detected with 3,3-diaminobenzidine tetrahydrochloride using Vectastain Elite ABC kits (Vector, Burlingame, CA, USA). Sections were mounted, air-dried, dehydrated through graded ethanol, cleared in histoclear, and cover slipped using Permount.

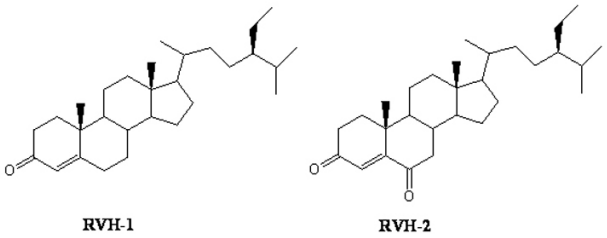

Fig. 1. Chemical structure of RVH-1 (stigma-4-ene-3-one) and RVH-2 (stigma-4-ene-3,6-dione).

In situ labeling of DNA fragmentation

The analysis of cells exhibiting DNA fragmentation, which are suggestive of apoptosis as previously described [17], was performed according to the manufacturer's instructions using terminal deoxynucleotidyl transferase with peroxide-12-UTP nick-end labeling (TUNEL) (Roche Molecular Biochemicals, Indianapolis, IN, USA). Brain sections for TUNEL staining were prepared according to the same procedure as above for immunohistochemistry. The percentage of TUNEL-positive cells was assessed by analysis of digitized images from 5 or more microscopic fields of TUNEL-stained cells from TIFF files (Adobe Photoshop).

Statistical analysis

Data were analyzed using Mann-Whitney's U test and SPSS software 12K (SPSS statistics, Chicago, IL, USA) for independent samples to compare mice treated with KA and saline, and mice treated with KA and RVH-1 or RVH-2. A value of $p < 0.05$ was accepted as statistically significant. Results are expressed as mean±SEM values.

RESULTS

Both RVH-1 and RVH-2 attenuated KA-induced neuronal cell death

Intracerebroventricular injection of KA resulted in extensive neuronal cell death in the CA3 region of mouse hippocampus, whereas neuronal cell death was negligible in vehicle-, RVH-1-, or RVH-2-treated mice (Fig. 2). Although both RVH-1 and RVH-2 attenuated KA-induced neuronal cell death, they did not completely prevent neuronal cell death at a RVH concentration of 10 mg/kg. RVH-2 appeared to be more protective than RVH-1. However, dose-dependent inhibition of KA-induced neuronal cell death was not observed (data not shown).

Both RVH-1 and RVH-2 attenuated KA-induced apoptotic neuronal cell death

Representative and quantitative analysis of apoptotic neuronal cell death was determined using TUNEL staining (Fig. 3). In accordance with the data from cresyl violet staining (Fig. 2), TUNEL-positive neurons in the CA3 region were observed abundantly in KA-injected mice. Mice pretreated with RVH-1 or RVH-2 showed attenuated neuronal cell damage (Fig. 3). There was no apparent difference between RVH-1 and RVH-2. The number of TUNEL-positive neurons was negligible in vehicle-treated mice (oral administration of saline as pretreatment and icv injection of PBS instead of KA), RVH-1-only-treated mice (oral administration of RVH-1 or RVH-2 for 7 days daily as pretreatment and icv injection of PBS instead of KA), and RVH-2-only-treated mice.

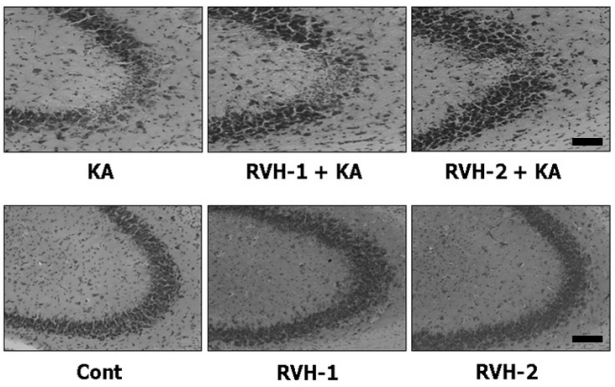

Fig. 2. Representative neuroprotective effects of RVH-1 and RVH-2 on KA-induced neuronal cell death in CA3 region of mouse hippocampus. Hippocampal cell death was examined with cresyl violet staining. Intracerebroventricular (icv) injection of KA showed marked loss of neurons in the CA3 region of hippocampus (KA). However, RVH-1 or RVH-2 treatment prior to KA injection showed attenuation of neuronal cell loss compared to KA alone. RVH-2 appeared to be more protective than RVH-1, albeit not significantly so. Neuronal cell death was not observed in vehicle (Cont)-, RVH-1-only-, or RVH-2-only-treated mice. More than three mice were used for each group. Scale bar: 100 μm.

Protection of pyramidal neurons by RVH-1 and RVH-2 was confirmed with neuronal immunostaining in the CA3 region of hippocampus

To further elucidate the neuroprotective effects of RVH-1 and RVH-2 on pyramidal neurons in the hippocampus, immunostaining of NeuN, a neuronal marker, was carried out. In accordance with the data from cresyl violet and TUNEL staining, both RVH-1 and RVH-2 attenuated KA-induced loss of pyramidal neurons in the CA3 region of mouse hippocampus (Fig. 4). Neither RVH-1 nor RVH-2 had a noticeable effect on the viability of pyramidal neurons.

Both RVH-1 and RVH-2 attenuated KA-induced microglial activation

Given the previous report that KA-induced neuronal cell death accompanies microglial activation in the hippocampus [13], the suppressive effect of RVH-1 and RVH-2 on KA-induced microglial activation was examined using immunostaining with OX-6, a microglial activation marker. The intracerebroventricular administration of KA resulted in a considerable amount of microglial activation in the hippocampus, especially in the CA3 region where neuronal cell death was most obvious (Fig. 5). However, seven daily administrations of RVH-1 and RVH-2 prior to KA markedly attenuated KA-induced microglial activation (Fig. 5).

DISCUSSION

In the present study, we demonstrated that RVH-1 and RVH-2 isolated from mushroom-detoxified stem bark of *Rhus verniciflua*, exhibited neuroprotective activity against KA-induced excitotoxic damage in the CA3 region of mouse hippocampus. RVH-1 and RVH-2 significantly attenuated KA-induced pyramidal neuronal cell death and also sup-

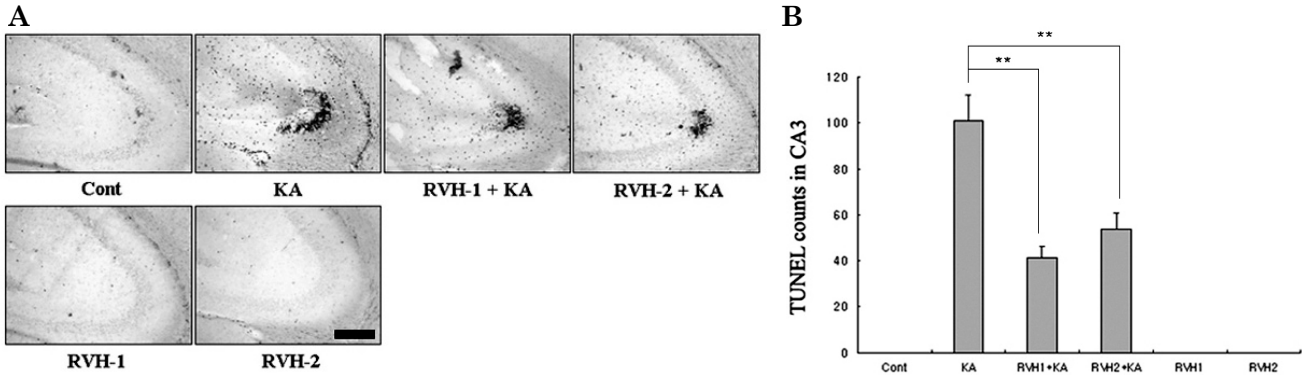

Fig. 3. Representative (A) and quantitative (B) analysis of neuronal cell death with Terminal deoxytransferase-mediated dUTP-nick end labeling (TUNEL) assay. A considerable number of TUNEL-positive neurons appeared with KA treatment within the CA3 region after 24 hr. Pre-administration of RVH-1 or RVH-2 significantly reduced the number of TUNEL-positive cells compared to the KA-only group. RVH-2 pretreatment showed fewer TUNEL-positive cells than RVH-1. A single treatment of RVH-1 or RVH-2 had no noticeable effects on cell survival. More than three mice were used for each group. Quantitative data represent three independent experiments and are expressed as mean±SEM. **$p<0.01$ indicates statistically significant difference from the KA-only treated group. Scale bar: 100 μm.

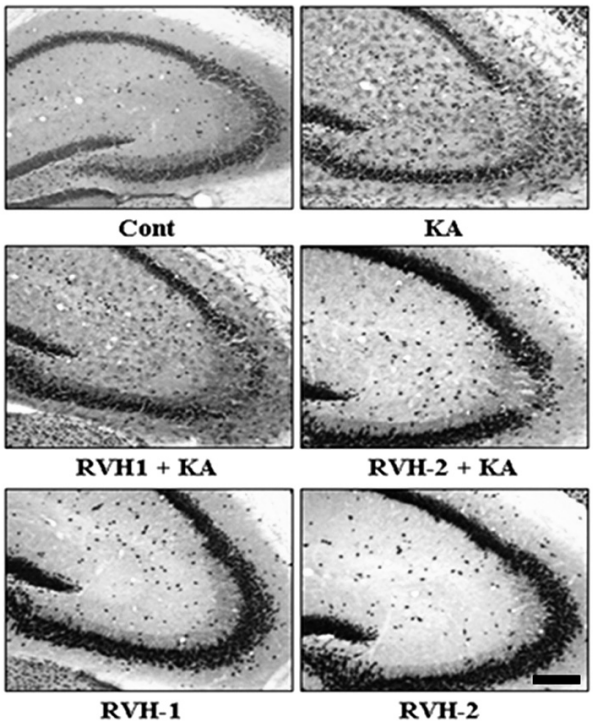

Fig. 4. Representative images of neuronal protection by RVH-1 and RVH-2 against KA-induced neuronal cell death in the CA3 region of hippocampus. In order to further elucidate the neuroprotective properties of RVH-1 and RVH-2, NeuN immunoreactivity, which specifically stains pyramidal neurons in the hippocampus, was examined. Pretreatment of RVH-1 or RVH-2 attenuated KA-induced death of pyramidal neurons in the CA3 region of hippocampus. RVH-2 showed more neuronal protection against KA-induced excitotoxicity compared to RVH-1. RVH-1 and RVH-2 showed no noticeable change in neuronal viability. More than three mice were used for each group. Scale bar: 100 μm.

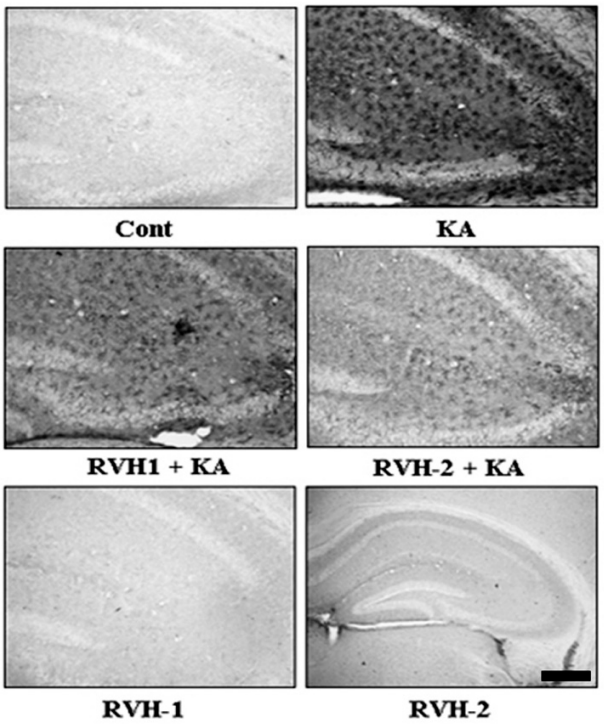

Fig. 5. Suppressive effects of RVH-1 and RVH-2 on KA-induced microglial activation. Given the previous report that KA-mediated neuronal death accompanies microglial activation, expression of OX-6, a microglial activation marker, was examined with immunohistochemical staining at 24 hr after KA or vehicle treatments. KA resulted in increased microglial activation (KA). However, KA-induced microglial activation was attenuated with RVH-1 or RVH-2 pretreatment. RVH-2 appears to be more suppressive than RVH-1. RVH-1 or RVH-2 showed negligible effects on microglial activation. Representative images were obtained from three independent experiments. More than three mice were used for each group. Scale bar: 100 μm.

pressed microglial activation in the hippocampus.

RVH-1 and RVH-2, which belong to the stigmasterol family, were previously isolated from various natural plants [6]. Although RVH-1 and RVH-2 have been reported to inhibit platelet-derived growth factor (PDGF)-driven proliferation of hepatic stellate cells [18], biological activity of these compounds has not been extensively studied. In the meantime, it has been reported that stigmasterol exhibits anti-inflammatory effects in a primary culture model of osteoarthritis chondrocytes by inhibiting the production of proinflammatory mediators [4]. The authors suggested that the anti-inflammatory properties of stigmasterol might be mediated through the inhibition of the NF-kB pathway. Given the previous report that activation of the NF-kB pathway might contribute to neuronal death in a KA-induced excitotoxicity model [19], it is plausible that RVH-1 and RVH-2 might exert a protective effect through inhibition of NF-kB signaling in a KA-induced excitotoxicity model.

Excitotoxicity may be involved in a variety of pathological conditions such as stroke, traumatic brain injury, and spinal cord injury [20-22]. Furthermore, accumulating evidence suggests that excitotoxicity contributes to the pathogenesis of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease [23,24]. In accordance with a previous study [13], we found that icv administration of KA resulted in the death of neurons mainly in the CA3 region of mouse hippocampus and daily pretreatment of RVH-1 and RVH-2 for 7 day significantly reduced KA-induced death of pyramidal neurons in mouse hippocampus. A single pretreatment with these compounds showed no noticeable inhibition of KA-induced neuronal death (data not shown), suggesting that a single treatment with these compounds is not sufficient to be effective. Although these compounds exerted protective effects against KA-induced excitotoxicity, it is not certain whether the neuroprotection by RVH-1 and RVH-2 is through the suppression of the NF-kB pathway. Therefore, further studies are necessary to determine the mechanism by which these compounds protect KA-induced neuronal cell death.

KA-induced excitotoxicity also accompanies activation of microglia in the hippocampus [13]. Microglia, the major immunocompetent cells in the CNS, are believed to play an important role in inflammatory processes in the brain [25]. Concomitant microglial activation during excitotoxicity might contribute to secondary damage by producing neurotoxic factors such as proinflammatory cytokines and nitric oxide [25,26]. It has been reported that a pathogenic

amount of nitric oxide contributes to neuronal damage in many disease conditions including neurodegenerative diseases [27-29]. In the present study, RVH-1 and RVH-2 significantly attenuated KA-induced microglial activation in the mouse hippocampus. However, it is not clear whether these compounds directly suppressed the microglia or whether the microglial suppression is secondary to the neuroprotection of these compounds.

In conclusion, the present study demonstrates that RVH-1 and RVH-2 possess neuroprotective properties in an animal model of KA-induced excitotoxicity. These compounds might be potentially valuable in the treatment of brain pathologies associated with excitotoxic neuronal damage such as epilepsy, stroke, and traumatic brain injury. However, further studies are necessary to clearly understand the mechanism by which these compounds protect neurons against excitotoxicity. The present study suggests the possibility that detoxification of *Rhus verniciflua* utilizing mushrooms could be a valuable approach to yield neuroprotective compounds from *Rhus verniciflua*.

ACKNOWLEDGEMENTS

This study was supported by Rural Development Administration of Bio-Green 21 project (PJ007090).

REFERENCES

1. **Choi HS.** Biological Detoxification of Lacpuer Tree (*Rhus verniciflua* Stokes) Stem Bark by Mushroom Species. *Food Science and Biotechnology.* 2007;16:935-942.
2. **Awad AB, Fink CS.** Phytosterols as anticancer dietary components: evidence and mechanism of action. *J Nutr.* 2000;130: 2127-2130.
3. **Martins SL, Silva HF, Novaes MR, Ito MK.** Therapeutic effects of phytosterols and phytostanols in cholesterolemia. *Arch Latinoam Nutr.* 2004;54:257-263.
4. **Gabay O, Sanchez C, Salvat C, Chevy F, Breton M, Nourissat G, Wolf C, Jacques C, Berenbaum F.** Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. *Osteoarthritis Cartilage.* 2010;18:106-116.
5. **Chung IM.** Cytotoxic Chemical Constituents from the Mushroom of Pholiota adiposa. *Food Science and Biotechnology.* 2005;14:255-258.
6. **Gupta S.** Isolation and characterization of a dihydroxysterol from *LAWSONIA INERMIS. Natural Product Letters.* 1994;4: 195-201.
7. **Greca MD.** Stigmasterols from *Typha latifolia. J Nat Prod.* 1990;53:1430.
8. **Chang YC.** Cytotoxic Benzophenanthridine and Benzylisiquinoline Alkaloids from *Argemone mexicana. Z Naturforsch.* 2003;58c:521-526.
9. **Itokawa.** Several oxidized sterols isolated from callus tissue of *Stephania cepharantha. Chem Pharm Bull.* 1973;21:1386-1387.
10. **Choi DW, Rothman SM.** The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annu Rev Neurosci.* 1990; 13:171-182.
11. **Doble A.** The role of excitotoxicity in neurodegenerative disease: implications for therapy. *Pharmacol Ther.* 1999;81:163-221.
12. **Izquierdo LA, Barros DM, Ardenghi PG, Pereira P, Rodrigues C, Choi H, Medina JH, Izquierdo I.** Different hippocampal molecular requirements for short- and long-term retrieval of one-trial avoidance learning. *Behav Brain Res.* 2000;111:93-98.
13. **Byun JS, Lee SH, Jeon SH, Kwon YS, Lee HJ, Kim SS, Kim YM, Kim MJ, Chun W.** Kainic Acid-induced Neuronal Death is Attenuated by Aminoguanidine but Aggravated by L-NAME in Mouse Hippocampus. *Korean J Physiol Pharmacol.* 2009;13: 265-271.
14. **Giusti P, Lipartiti M, Franceschini D, Schiavo N, Floreani M, Manev H.** Neuroprotection by melatonin from kainate-induced excitotoxicity in rats. *FASEB J.* 1996;10:891-896.
15. **Laursen SE, Belknap JK.** Intracerebroventricular injections in mice. Some methodological refinements. *J Pharmacol Methods.* 1986;16:355-357.
16. **Baker H, Farbman AI.** Olfactory afferent regulation of the dopamine phenotype in the fetal rat olfactory system. *Neuroscience.* 1993;52:115-134.
17. **Henshall DC, Bonislawski DP, Skradski SL, Araki T, Lan JQ, Schindler CK, Meller R, Simon RP.** Formation of the Apaf-1/ cytochrome c complex precedes activation of caspase-9 during seizure-induced neuronal death. *Cell Death Differ.* 2001;8: 1169-1181.
18. **Badria FA, Dawidar AA, Houssen WE, Shier WT.** In vitro study of flavonoids, fatty acids, and steroids on proliferation of rat hepatic stellate cells. *Z Naturforsch C.* 2005;60:139-142.
19. **Nakai M, Qin ZH, Chen JF, Wang Y, Chase TN.** Kainic acid-induced apoptosis in rat striatum is associated with nuclear factor-kappaB activation. *J Neurochem.* 2000;74:647-658.
20. **Choi DW.** Glutamate neurotoxicity and diseases of the nervous system. *Neuron.* 1988;1:623-634.
21. **Lee JM, Zipfel GJ, Choi DW.** The changing landscape of ischaemic brain injury mechanisms. *Nature.* 1999;399:A7-14.
22. **McNamara JO.** Emerging insights into the genesis of epilepsy. *Nature.* 1999;399:A15-22.
23. **Coyle JT, Puttfarcken P.** Oxidative stress, glutamate, and neurodegenerative disorders. *Science.* 1993;262:689-695.
24. **Salinska E, Danysz W, Lazarewicz JW.** The role of excitotoxicity in neurodegeneration. *Folia Neuropathol.* 2005;43:322-339.
25. **Suzumura A, Takeuchi H, Zhang G, Kuno R, Mizuno T.** Roles of glia-derived cytokines on neuronal degeneration and regeneration. *Ann N Y Acad Sci.* 2006;1088:219-229.
26. **Milatovic D, Gupta RC, Dettbarn WD.** Involvement of nitric oxide in kainic acid-induced excitotoxicity in rat brain. *Brain Res.* 2002;957:330-337.
27. **Dehmer T, Lindenau J, Haid S, Dichgans J, Schulz JB.** Deficincye of inducible nitric oxide synthase protects against MPTP toxicity in vivo. *J Neurochem.* 2000;74:2213-2216.
28. **Kim YM, Bombeck CA, Billiar TR.** Nitric oxide as a bifunctional regulator of apoptosis. *Circ Res.* 1999;84:253-256.
29. **Sugimoto K, Iadecola C.** Effects of aminoguanidine on cerebral ischemia in mice: comparison between mice with and without inducible nitric oxide synthase gene. *Neurosci Lett.* 2002;331: 25-28.