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Neuroprotective Activities of Some Medicinal Plants against Glutamate-induced Neurotoxicity in Primary Cultures of Rat Cortical Cells

Jin Bae Won and Choong Je Ma*

Department of Biomaterials Engineering, School of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, Republic of Korea

Abstract – Neurodegenerative diseases such as Alzheimer's disease, stroke, and Parkinson's disease, are caused by neuronal cell death. Apoptosis, oxidative stress, inflammation, excitotoxicity or ischemia are discussed to play a role of neuronal cell death. In order to find the candidate of neuroprotective agent, neuroprotective activity of some medicinal plants was investigated with in vitro assay system using glutamate-induced neurotoxicity in primary cultures of rat cortical cells. The aqueous methanolic extracts of twenty-seven medicinal plants were evaluated the protective effects against glutamate-injured excitotoxicity in rat cortical cells at the concentration of 50 μg/ml and 100 μg/ml, respectively. Among them, extracts of *Lonicera japonica, Taraxacum platycarpum, Polygonum aviculare, Gardenia jasminoides, Forsythia viridissima, Lygodium japonicum, Panax notoginseng, Akebia quinata, Anemarrhena asphodeloides and Phellodendron amurense* showed significantly neuroprotective activities against glutamate-induced neurotoxicity in primary rat cortical cells.
Keywords – Alzheimer's disease, Excitotoxicity, Glutamate, Neuroprotection, Rat cortical cell

Introduction

Glutamate has been known that played important roles in the central nervous system (CNS) such as memory, neuronal survival, neuronal differentiation, learning and behavior at the normal range of concentration (Sucher *et al*., 1996; Michaelis, 1998). However, over-expression of glutamate receptor by high concentration of glutamate resulted in neuronal cell death in the CNS, and may be responsible for neuropathological disorders such as Parkinson's disease, Alzheimer's disease, epilepsy, seizures, ischemic stroke and spinal cord trauma (Lipton and Rosenberg, 1994; Cacabelos *et al*., 1996; Lee *et al*., 1999). Thus, neuronal cell protection against neurotoxicity in the glutamate-injured neuronal cells has been one of the research targets to develop neurodegenerative diseases drugs (Rajendra *et al*., 2004). We previously applied glutamate-induced neurotoxicity in the primary rat cortical cells as an in vitro assay system to evalutate neuroprotective activity of several medicinal plants (Jeong *et al*., 2009b; Lee *et al*., 2006).

Inflammatory processes are closely related to the pathogenesis of neurodegenerative diseases such as Parkinson's diseases, Alzheimer's diseases and stroke (Hashioka *et al*., 2009). It was reported that rheumatoid arthritis patients who are administered with anti-inflammatory drug for a long period are not likely to be exposed to risk of Alzheimer's disease. Some non-steroidal anti-inflammatory drugs (NSAIDs) may be used to prevent and treat Alzheimer's diseases (Muraoka and Miura, 2009).

In this study, we have evaluated neuroprotective activity of some medicinal plants which has been used herbal medicine to treat inflammation as an anti-inflammation drug.

Experimental

Materials – All chemicals for rat cortical cell cultures and biochemical assays were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO), unless stated otherwise. Fetal bovine serum was obtained from Hyclone Co. (Logan, Utah). MK-801 and CNQX used as positive control were purchased from Research Biochemicals International (Natick, MA). Urethane and triton X-100 were purchased from Junsei Chemical Co. (Tokyo, Japan) and Yakuri Chemical Co. (Osaka, Japan), respectively. The 80% methanol extracts of each plant were used for comparison of the neuroprotective activities.

*Author for correspondence
Tel: +82-33-250-6565; E-mail: cjma@kangwon.ac.kr

Table 1. Protective effects of aqueous methanolic extracts of medicinal plants on glutamate-induced excitotoxicity in primary rat cortical cells by LDH assay

Plant Name (Used Part)	Yield (%)	Neuroprotection (%)	
		50 μg/ml	100 μg/ml
Control		100.0 ± 2.1	
Glutamate-injured[a),b)]		0.0 ± 3.8	
Lonicera japonica (Flower)	21.2	59.8 ± 1.8***	67.3 ± 5.1***
Taraxacum platycarpum (Herb)	18.9	46.6 ± 3.5**	54.4 ± 3.9***
Polygonum aviculare (Herb)	15.4	47.4 ± 2.4**	48.8 ± 2.4**
Dianthus chinensis (Aerial Part)	20.0	–	11.2 ± 2.4
Lindera strichnifolia (Tuber)	19.2	9.5 ± 1.4	12.1 ± 1.6
Dioscorea tokora (Rhizoma)	12.3	5.5 ± 3.9	–
Gardenia jasminoides (Fruit)	38.8	35.3 ± 4.5*	46.5 ± 3.9**
Forsythia viridissima (Fruit)	12.9	44.1 ± 4.1**	42.1 ± 5.2**
Alpinia oxyphylla (Fruit)	11.1	11.1 ± 1.9	9.5 ± 0.5
Cornus officinalis (Fruit)	9.8	24.5 ± 3.6	31.2 ± 3.9*
Curcuma longa (Tuber)	23.3	20.8 ± 2.1	23.2 ± 1.2
Lygodium japonicum (Spore)	3.2	54.3 ± 4.5***	60.3 ± 5.1***
Panax notoginseng (Root)	15.2	48.9 ± 2.1**	50.5 ± 2.1***
Rheum palmatum (Root)	12.1	–	–
Akebia quinata (Stem)	11.9	38.0 ± 1.4*	40.2 ± 2.4*
Plantago asiatica (Seed)	9.2	12.4 ± 3.5	22.1 ± 1.1
Juncus effusus (Stem)	12.7	16.2 ± 2.7	13.2 ± 2.1
Gentiana scabra (Root)	9.1	14.2 ± 2.7	11.8 ± 0.9
Bupleurum falcatum (Root)	18.2	11.6 ± 1.1	10.4 ± 3.2
Alisma orientale (Root)	12.1	–	11.2 ± 4.1
Scutellaria baicalensis (Root)	12.7	–	9.2 ± 2.1
Rehmannia glutinosa (Root)	8.3	21.3 ± 3.7	22.2 ±1.4
Dioscorea batatas (Rhizome)	7.5	18.8 ± 2.8	21.2 ± 2.5
Paeonia suffruticosa (Root)	11.4	21.3 ± 2.9	25.3 ± 3.1*
Cinnamomum cassia (Cortex)	10.2	18.2 ± 1.0	20.6 ± 1.4
Anemarrhena asphodeloides (Rhizome)	15.3	34.8 ± 3.8*	42.5 ± 3.4**
Phellodendron amurense (Cortex)	17.3	33.1 ± 4.1*	43.7 ± 3.6**
MK-801[c)]		67.6 ± 4.9***	
CNQX[d)]		54.2 ± 3.6***	

[a)] LDH released from control and glutamate-treated cultures was 115.8 ± 3.8 and 212.8 ± 5.9 mU/mL, respectively. Cell viability was calculated as 100 × (LDH released from glutamate-treated − LDH released from glutamate + test compound) / (LDH released from glutamate-treated − LDH released from control). [b)] Glutamate-treated value differs significantly from the untreated. Control at a level of $p < 0.001$. [c)] MK-801 (10 μM) : dizocilpine maleate, a non-competitive antagonist of the NMDA receptor. [d)] CNQX (10 μM) : 6-cyano-7-nitroquinoxaline-2,3-dione, non-NMDA receptor antagonist. The values are expressed as means ± SD of triplicate experiments. Results differ significantly from the glutamate-treated. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Plant material – The list of medicinal plants is given in Table 1. Using parts of medicinal plants were purchased from Daejeon Oriental medicine Market, Daejeon, Korea and identified by the Dr. Young-Bae Seo, professor of the College of Oriental Medicine, Daejeon University. Voucher specimen has been deposited in the Herbarium of College.

Preparation of sample – The fifty gram of each air-dried plant material was extracted with 500 ml of 80% methanol (MeOH) in an ultrasonic apparatus which, upon removal of the solvent *in vacuo*, yielded aqueous methanolic extracts. All methanolic extracts were dissolved in DMSO (final culture concentration of DMSO, 0.1%) and samples were used after appropriate dilution. Preliminary studies indicated that the solvent had no effect on cell viability at the concentration used (data not shown).

In vitro neuroprotective activity – Female Sprague-Dawley rats (20 - 23 °C; 12 h light cycle from 09:00 to 21:00; food, Agribrand Purinar Korea, and water ad libitum) were provided by the Laboratory Animal Center, Kangwon National University. All experiments were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of the Kangwon National University. Primary cultures of mixed cortical cells containing both neuronal and glial cells were prepared from late fetal SD rats (17 - 19-days gestation in utero) as described previously (Jeong *et al*., 2009b). All samples were dissolved in DMSO (final culture concentration, 0.1%). Preliminary studies indicated that the solvent had no effect on cell viability at the concentration used (data not shown). Two known glutamate receptor antagonists, MK-801 (dizocilpine maleate, a non-competitive antagonist of the NMDA receptor) and CNQX, were used as positive controls for the assessment of neuroprotective activity (Kim *et al*., 1999; Kim *et al*., 2003). Cortical cell cultures were washed with DMEM and incubated with the test samples for 1 h. The cultures were then exposed to 100 μM glutamate. After 24 h incubation, the cultures were assessed for the extent of neuronal damage. Neuronal viability and integrity were quantified by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and/or LDH (lactate dehydrogenase) assay described in our previous report (Kim *et al*., 1999).

Statistical Analysis – Data were evaluated for statistical significance by "ANOVA" test using a computerized statistical package (control vs 50 μg/ml and control vs 100 μg/ml, respectively). We performed all the statistics on the raw data prior to transformation to percentage against control.

Results and Discussion

Inflammatory processes are proposed to be associated with the etiology of a wide range of neurodegenerative diseases including of Alzheimer's disease. It was reported that non-steroidal anti-inflammation drugs (NSAIDs) such as aspirin, acetyl salicylate and ibuprofen protect against Alzheimer's disease. Because aspirin rapidly metabolize to salicylic acid with hydrolysis in vivo, salicylic acid may play a contributable role to prevent Alzheimer's disease (Muraoka and Miura, 2009). Also, lansoprazole (LPZ) and omeprazole (OPZ), which are used to treat gastric ulcer, exerted anti-inflammatory effects and diminished neurotoxicity in human neuroblastoma SH-SY5Y cell line (Hashioka *et al*., 2009). LPZ and OPZ are act as proton pump inhibitors that inhibit the H^+/K-ATPase located in parietal cells of gastric mucosa (Isomoto *et al*., 2007). These results suggest that various anti-inflammatory reagents including NSAIDs or proton pump inhibitors may be effective therapeutic target in the treatment of Alzheimer's disease and other neurodegenerative disorders. Thus, we investigated neuronal cell protective activity of the aqueous methanolic extracts of some medicinal plant, which have been used to treat anti-inflammatory diseases for a long time as folk medicine, in the primary cultures of rat cortical cells against glutamate excitotoxicity as a in vitro assay system. We previously applied this assay system to evaluate neuroprotective activity of other natural product extracts or compounds isolated from them.

We evaluated neuroprotective activity of the aqueous methanolic extracts of twenty-seven medicinal plants in the primary cultures of rat cortical cells. Among them, ten plant extracts including *Lonicera japonica, Taraxacum platycarpum, Polygonum aviculare, Gardenia jasminoides, Forsythia viridissima, Lygodium japonicum, Panax notoginseng, Akebia quinata, Anemarrhena asphodeloides* and *Phellodendron amurense* exerted significant neuroprotective activity (over 40% protection) (Table 1). An MTT assay showed the same trend as the LDH assay (Table 2). Of the ten medicinal plant extracts, aqueous methanolic extracts of *L. japonica* showed the most potent neuroprotective activity against glutamate-induced neurotoxicity. The potency of *L. japonica* extract is similar to that of MK-801 and superior to that of CNQX which were used as positive controls, respectively (100% and 125%). Also, *L. japonicum* showed potent neuroprotective activity. The potency of this extract is about 90% of that of MK-801 and 110% of that of CNQX. The flowers and buds of *L. japonica* has been well known as an antiviral, anti-inflammatory, and antibacterial agent in traditional medicine and widely used in the treatment of various diseases, including upper respiratory tract infections, fever, sores, and swelling. Various compounds such as alkaloids, cerebrosides, flavonoids, iridoids, polyphenols, and triterpenoid saponins have been isolated from this plant (Lin *et al*., 2008). It was reported that rutin isolated from *L. japonica* inhibited oxidative stress-induced myocardial damage in vitro model and in vivo model which might be useful in treatment of myocardial infarction (Jeong *et al*., 2009a). Information on bioactivity of *Lygodium japonicum* was very little. The aqueous ethanol extract of *Lygodium japonicum* exhibited in vitro testosterone 5α-reductase inhibitory activity and in vivo anti-androgenic activity using growth of flank organ in

Table 2. Protective effects of aqueous methanolic extracts of medicinal plants on glutamate-induced excitotoxicity in primary rat cortical cells by MTT assay

Plant Name (Used Part)	Neuroprotection (%)	
	50 μg/ml	100 μg/ml
Control	100.0 ± 4.6	
Glutamate-injured[a)]	0.0 ± 2.1	
Lonicera japonica (Flower)	47.2 ± 2.4***	53.5 ± 3.7***
Taraxacum platycarpum (Herb)	42.1 ± 3.6**	48.9 ± 4.8***
Polygonum aviculare (Herb)	45.1 ± 1.1**	50.1 ± 2.4**
Dianthus chinensis (Aerial Part)	12.4 ± 2.1	10.9 ± 1.3
Lindera strichnifolia (Tuber)	–	10.1 ± 2.6
Dioscorea tokora (Rhizome)	–	–
Gardenia jasminoides (Fruit)	28.9 ± 2.8*	39.1 ± 2.8**
Forsythia viridissima (Fruit)	41.8 ± 2.1**	36.9 ± 4.4**
Alpinia oxyphylla (Fruit)	10.8 ± 1.9	12.6 ± 1.5
Cornus officinalis (Fruit)	21.5 ± 2.1	28.2 ± 1.1*
Curcuma longa (Tuber)	24.8 ± 1.8	31.7 ± 2.2*
Lygodium japonicum (Spore)	56.8 ± 2.8***	61.2 ± 2.3***
Panax notoginseng (Root)	43.1 ± 3.5**	46.5 ± 1.7***
Rheum palmatum (Root)	5.9 ± 1.2	7.3 ± 2.1
Akebia quinata (Stem)	29.9 ± 2.4*	35.2 ± 3.4*
Plantago asiatica (Seed)	10.4 ± 4.5	17.7 ± 1.1
Juncus effusus (Stem)	15.2 ± 2.7	18.1 ± 4.2
Gentiana scabra (Root)	16.9 ± 1.5	14.3 ± 1.9
Bupleurum falcatum (Root)	11.6 ± 1.1	10.4 ± 3.2
Alisma orientale (Root)	6.3 ± 0.4	10.2 ± 2.7
Scutellaria baicalensis (Root)	15.3 ± 2.7	18.9 ± 3.2
Rehmannia glutinosa (Root)	24.5 ± 6.8	20.1 ±5.6
Dioscorea batatas (Rhizome)	16.8 ± 4.8	19.2 ± 5.5
Paeonia suffruticosa (Root)	17.3 ± 3.3	28.1 ± 4.2*
Cinnamomum cassia (Cortex)	14.9 ± 1.8	22.7 ± 3.4
Anemarrhena asphodeloides (Rhizome)	32.7 ± 2.2*	38.1 ± 2.8**
Phellodendron amurense (Cortex)	31.9 ± 3.9*	37.7 ± 4.6**
MK-801[b)]	70.8 ± 2.8***	
CNQX[c)]	59.2 ± 5.6***	

[a)] Cell viability was calculated as 100 × (MTT released from glutamate-treated – MTT released from glutamate + test compound) / (MTT released from glutamate-treated – MTT released from control). Glutamate-treated value differs significantly from the untreated. Control at a level of $p < 0.001$. [b)] MK-801 (10 μM) : dizocilpine maleate, a non-competitive antagonist of the NMDA receptor. [c)] CNQX (10 μM) : 6-cyano-7-nitroquinoxaline-2,3-dione, non-NMDA receptor antagonist. The values are expressed as means ± SD of triplicate experiments. Results differ significantly from the glutamate-treated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

castrated Syrian hamsters and hair re-growth after shaving in testosterone-treated C57Black/6CrSlc mice (Matsuda *et al*., 2002). Its main active principles were lipophilic compounds such as oleic acid, linolenic acid and palmitic acid (Ye *et al*., 2007). It was not reported that these potent active two plants showed neuroprotective activity or cognitive enhancing activity to treat Alzheimer's diseases, yet. Therefore, we conclude that *Lonicera japonica* and *Lygodium japonicum* might offer useful therapeutic choices in the treatment of neurodegenerative disorders caused by excitotoxicity. Furthermore, we will attempt to elucidate the neuroprotective mechanisms of *L. japonica* which showed the most potent neuroprotective activity against glutamate-induced toxicity and isolate active principles as single compounds. Also, we will attempt to isolate neuroprotectvie compounds from nine other plant extracts to identify active principles inhibiting neurotoxicity by glutamate.

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