Neuroprotective activity of the methanolic extract of *Lonicera japonica* in glutamate-injured primary rat cortical cells

Jin Bae Weon[1], Hye Jin Yang[1], Bohyoung Lee[1], Bo-Ra Yun[1], Ju Hee Ahn[1], Hyeon Yong Lee[1], Choong Je Ma[1,2]

[1]Department of Biomaterials Engineering, School of Bioscience and Biotechnology, Kangwon National University, Chuncheon, [2]Research Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, Republic of Korea

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ABSTRACT

Background: We previously reported that the extracts of several Korean medicinal plants showed neuroprotective activity in glutamate-injured primary culutres of rat cortical cells. **Objective:** Among them, the effect of the methanolic extract of *Lonicera japonica* flower on the glutamate-induced neuronal cell death and its potential mechanism of action was investigated. **Results:** Treatment by the methanolic extract of *L. japonica* flower significantly protected neuronal cells against glutamate-induced excitotoxicity. It decreased the calcium influx that accompanies the glutamate induced excitotoxicity of neuronal cells, and inhibited the subsequent overproduction of nitric oxide, reactive oxygen species and peroxide to the level of control cells. In addition, it preserved cellular activity of superoxide dismutase, an antioxidative enzyme reduced by glutamate insult. **Conclusions:** According to this data, the methanolic extract of *L. japonica* flower significantly protected neuronal cells against glutamate excitotoxicity via antioxidative activity.

Key words: Antioxidant, excitotoxicity, glutamate, *Lonicera japonica*, neuroprotection

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INTRODUCTION

Glutamate is known to be involved with central excitatory neurotransmission, as seen in neuronal survival, synaptogenesis, neuronal plasticity, memory and in the brain.[1] However, high concentration of glutamate can evoke neuronal dysfunction and even damage or death.[2] Glutamate mediated neurotoxicity may be involved in several neuropathological disorders such as Alzheimer's disease, Parkinson's disease, ischemic stroke, and spinal cord trauma.[3] Thus, neuroprotection against glutamate-induced neurotoxicity has been a therapeutic strategy to treat neurodegenerative disease.[4]

During our search for neuroprotective medicinal plant extracts, we found that 80% Methanol (MeOH) extract of *Lonicera japonica* flowers had significant neuroprotective activity against glutamate induced neurotoxicity in primary cultures of rat cortical cells.[5] The flowers and buds of *L. japonica* have been well known as antiviral, anti-inflammatory, and antibacterial agents in traditional Chinese medicine, and widely used in the treatment of various diseases, including upper respiratory tract infections, fever, sores, and swelling.[6]

The present study examined the effect of *L. japonica* extract on the survival of neurons damaged by glutamate excitotoxicity, using primary cultures of rat cortical cell as an *in vitro* model of neurodegenerative disease. To elucidate the mechanism, the effects of *L. japonica* extract were tested for an increase in the Calcium [Ca^{2+}] and nitric oxide (NO) levels, cellular oxidation, mitochondrial membrane potential and antioxidative enzymes.

Address for correspondence:
Prof. Choong Je Ma, Department of Biomaterials Engineering, Division of Biotechnology and Bioengineering, Kangwon National University, Hyoja-2 Dong, Chuncheon 200-701, Republic of Korea. E-mail: cjma@kangwon.ac.kr

MATERIALS AND METHODS

Materials and reagents

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 purchased from Hyclone Co. (Logan, Utah). MK-801 used as positive control was 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. Dried flower of *Lonicera japonica* was purchased from Daejeon Oriental medicine Market, Daejeon, Korea and identified by the Dr. Young-Bae Seo, a professor of the College of Oriental Medicine, Daejeon University. Voucher specimen (CJ0001M) has been deposited in this institute.

Cell culture

Primary cultures of mixed cortical cells containing both neuronal and glial cells were prepared from 17~19-day-old fetal Sprague-Dawley rats as described previously.[7] In brief, the trypsin-dissociated cortical cells were plated on multi well culture plates (Corning, NY) coated with collagen at a density of 1×10^6 cells per well. The cortical cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum with penicillin (100 IU/ml) and streptomycin (10 µg/ml) at 37°C in a humidified atmosphere of 95% air-5% Carbon dioxide (CO_2). Cultures were allowed to mature for 17 days before being used for experiments. Our mixed cortical cultures consisted of approximately 70~75% cells immunopositive for neuron-specific enolase, and 25~30% cells immunopositive for glial fibrillary acidic protein as determined by immunocytochemical staining methods.[7] All experiments were performed with Ethical Approval of Kangwon National University.

Neurotoxicity and cell viability

Test samples were dissolved in Dimethyl sulfoxide (DMSO) (final culture concentration, 0.1%); and, preliminary studies indicated that the solvent had no effect on cell viability of control and glutamate-treated cells at the concentration used.[7] Seventeen-day-old cortical cell cultures were washed with DMEM and incubated with test samples for 2 hours. The cultured cells were then exposed to 200 µM L-glutamate. After 24 hours incubation in the presence of test samples, the cultures were assessed for the extent of neuronal damage by measuring lactate dehydrogenase (LDH) in the media.[7] Data are expressed as the percentage protection relative to vehicle-treated control cultures. Values shown are the mean ± Standard deviation (SD) of three experiments (3-4 cultures per experiment).

Measurement of intracellular calcium and nitric oxide contents

The intracellular calcium was determined by ratio fluorometry using Ca^{2+} specific dye, Fura 2-AM.[7,8] In brief, 2 hours before exposure to 200 µM glutamate, cultures grown on 48-well plates were treated with *L. japonica* extracts sample and 5 µM Fura-2 AM in phosphate-buffered saline (PBS, pH 7.2) at 37°C in a humidified atmosphere of 95% air – 5% CO_2. The change of $[Ca^{2+}]_i$ was measured 3 hours after exposure to glutamate. Fura-2 fluorescence was measured with a spectroflurometer by exciting cells at 340 and 380 nm and measuring light emission at 520 nm. The level of NO formed was determined by measuring the content of nitrite released into the medium using the method of Dawson *et al.*[9] The culture medium was reacted with Griess reagent and the absorbance was then read at 550 nm. The concentration was determined against a nitrite standard curve. Values shown are the mean ± SD of three experiments (3-4 cultures per experiment).

Measurement of cellular peroxide

The relative level of free radicals, i.e. peroxide, in cultured cells was measured with the oxidation-sensitive compound, 2',7'-dichlorofluorescein diacetate (2,7-DCF-DA) by the method of Goodman and Mattson.[10,11] Cells were loaded with DCF-DA (50 µM, 50 min-incubation) followed by three washes in Hank's Balanced Salt Solution (HBSS). DCF fluorescence was then determined after 3 hours' incubation by measuring light emitted at 530 nm of exciting cells with light at 485 nm. Values shown are the mean ± SD of three experiments (3-4 cultures per experiment).

Measurements of mitochondrial membrane potential

To measure the mitochondrial membrane potential, we used rhodamine-123, a cell-permeable cationic fluorescent probe that is selectively sequestered by viable mitochondria.[12] After exposure to glutamate, the cells were incubated for 30 minutes at 37°C with rhodamine123 (5 µM). Cells were then washed with 20 mM Hepes-Na (final pH 7.4). The fluorescence was determined at excitation and emission wavelengths of 485 and 530 nm, respectively.

Assay for the activity of antioxidant enzymes

Cells from three culture plates were pooled in 2 ml of 0.1 M phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged for 30 minutes at 3000×g at 4°C and the supernatant (cytosolic and mitochondrial fractions) collected for the measurements of antioxidative enzyme activity. The activity of superoxide dismutase was determined according to the method of McCord and Fridovich by xanthine-xanthine oxidase reaction.[13] Glutathione reductase activity was measured according to the method of Carlberg and Mannervik based on the reduction of oxidized gluthathione (GSSG) by glutathione reductase and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) .[14] Glutathione peroxidase activity was determined by quantifying the rate of oxidation of glutathione to GSSG by cumene hydroperoxide, a reaction catalyzed by glutathione peroxide.[15] Values shown are the mean ± SD of three experiments (3-4 cultures per experiment).

Assay for 2,2-diphenyl-1-picrylhydrazyl radical *scavenging activity*

For the measurement of 2,2-diphenyl-1-picrylhydrazyl

(DPPH) radical scavenging activity, 100 µl of an ethanolic solution of 60 µM DPPH was mixed with 100 µl of water (H_2O) with or without the different concentrations of the tested compounds in 96-well plate. The plate was incubated in the dark room at room temperature for 30 minutes and the absorbance was recorded at 520 nm.[7,16]

Protein assay

Protein content was measured by the method of Lowry *et al.*, with bovine serum albumin (BSA) as a standard.[17]

Statistical analysis

Statistical significance was determined by one-way ANalysis Of VAriance (ANOVA) and, if significant, group means were compared by post-hoc analysis using Tukey multiple comparison of means. Values shown are the mean ± SD of three experiments (3-4 cultures per experiment).

RESULTS AND DISCUSSION

We previously reported that methanolic extract of *L. japonica* showed neuroprotective activity against glutamate-induced neurotoxicity in cultured rat cortical cells.[5] We have investigated neuroprotective activities of *L. japonica* extract, using primary cultures of rat cortical neurons injured with glutamate [Figure 1]. *L. japonica* extract protected primary cultures of rat cortical cells against glutamate-induced neurotoxicity in a dose dependent manner.

In order to reveal how *L. japonica* extract protected against glutamate-induced neurotoxicity, we have investigated the mechanisms of action using glutamate-injured primary cultures of rat cortical cells. Glutamate released to neuronal cells actiavtes several types of pre- and post-synaptic glutamate receptors such as, *N*-methyl *D*-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and/or Kainic acid (KA). Then, intracellular calcium concentration was increased by glutamate excitotoxicity and it may lead to mitochondrial dysfunction, generation of reactive oxygen species, and activation of proteases, phospholipases, and endonucleases, leading to neuronal cell death.[18] In addition, the excessive Ca^{2+} influx is followed by the activation of NO synthase (NOS) and subsequent overproduction of NO.[8] Thus, the effect of *L. japonica* extract on the increase in the content of $[Ca^{2+}]_i$ and NO induced by glutamate was determined. The $[Ca^{2+}]_i$ increased by glutamate treatment was significantly and effectively inhibited by the treatment of *L. japonica* extract at the concentration ranged from 10.0 to 100.0 µg/ml [Figure 2]. *L. japonica* extract also significantly reduced over-production of NO in cortical cells exposed to glutamate in a dose-dependent manner [Figure 3].

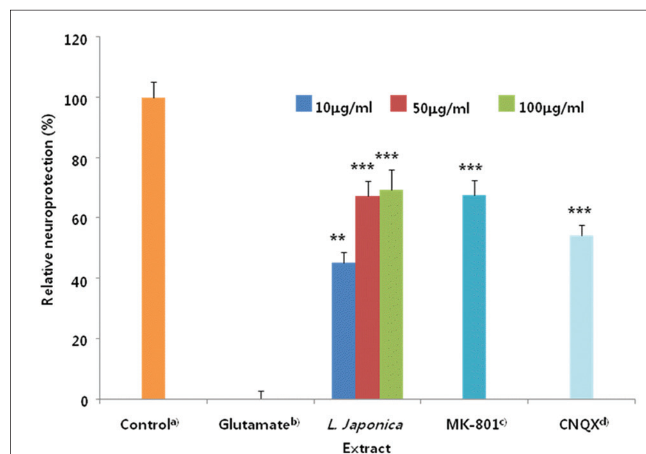

Figure 1: Rat cortical cultures were washed with Dulbecco's modified Eagle's medium and incubated with the methanolic extract of *L. japonica* for 1 hour. The cultures were then exposed to 100 mM glutamate for 24 hours. After the incubation, the cultures were assessed for the extent of neuronal damage (treatment throughout). The values shown are the mean ± Standard Deviation of three experiments (5 - 6 cultures per experiment). [a]lactate dehydrogenase (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: dizocilpine maleate, a non-competitive antagonist of the N-methyl D-aspartate (NMDA) receptor, [d]CNQX: 6-cyano-7-nitroquinoxaline-2, 3-dione, non-NMDA receptor antagonist $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs glutamate-treated cultures (Analysis of Variance [ANOVA] and Tukey)

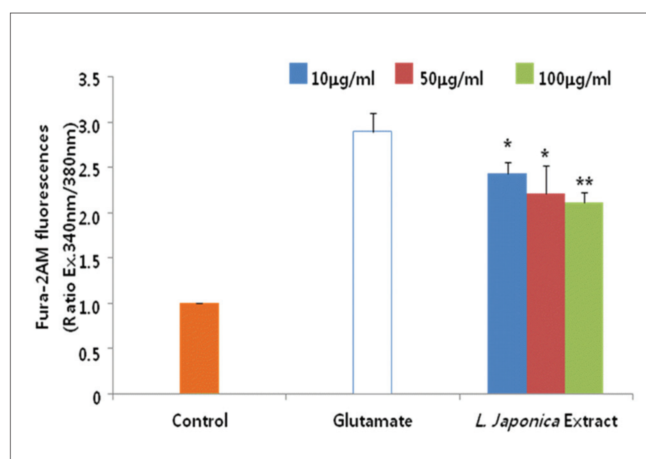

Figure 2: Cultures were treated with the methanolic extract of *L. japonica* and 5 mM Fura-2 AM 1 hour before exposure to 100 µM glutamate. The change of $[Ca^{2+}]_i$ was measured 3 hour after glutamate exposure. The values shown are means ± Standard deviation of three experiments (3-4 cultures per experiment). $^{*}P<0.05$, $^{**}P<0.01$ vs. Glutamate-injured cells (Analysis of Variance [ANOVA] and Tukey)

It is one of the characteristic features of glutamate-induced neurotoxicity that mitochondrial membrane potential is decreased.[19] The excessive Ca^{2+} accumulation in mitochondria triggers mitochondrial damage.[20] Thus, we

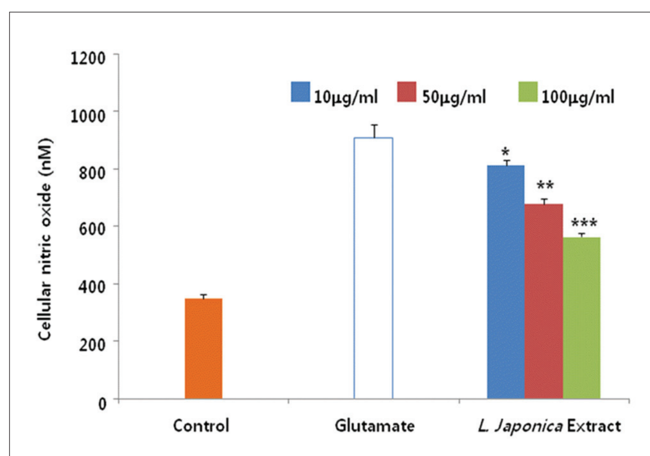

Figure 3: Cultures were pretreated with the methanolic extract of *L. japonica* 1 hr before glutamate exposure. The change of cellular nitric oxide was determined using the griess reagent. The values shown are means ± Standard deviation of three experiments (3-4 cultures per experiment). Glutamate-injured value differs significantly from the control at a level of $P<0.001$. ** $P<0.01$, *** $P<0.001$ vs. Glutamate-injured cells (Analysis of Variance [ANOVA] and Tukey)

investigated effect of *L. japonica* extract on mitochondrial membrane potential. *L. japonica* extract treatment significantly restored mitochondrial membrane potential up to 90% of control cells [Figure 4]. This result might be partially due to reduced oxidative stress and inhibition of Ca^{2+} influx.

Glutamate-induced toxicity is known to be mediated by oxidative stress which involves reactive oxygen species such as hydroxyl radicals and superoxide anions.[21] We measured the effect of *L. japonica* extract on the content of cellular peroxide using the specific fluorescent dye, 2,7-DCF-DA. When cultured cortical cells were treated with glutamate, the cellular peroxide content was increased up to 3 hours after treatment. The increased cellular peroxides induced by glutamate were effectively reduced by the treatment with *L. japonica* extract [Figure 5]. Glutamate-induced oxidative stress is also known to deplete intracellular Glutathione (GSH), and reduce the activities of antioxidative enzymes

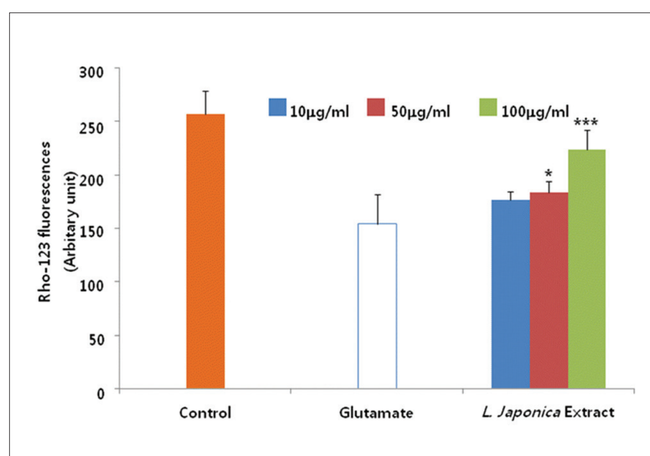

Figure 4: Cultures were pretreated with the methanolic extract of *L. japonica* 1 hour before glutamate exposure. The membrane potential of mitochondria was determined using the fluorescent dye rhodamine 123 (Rho-123). The values shown are means ± Standard deviation of three experiments (3-4 cultures per experiment). Glutamate-injured value differs significantly from the control at a level of $P<0.001$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. Glutamate-injured cells (Analysis of Variance [ANOVA] and Tukey)

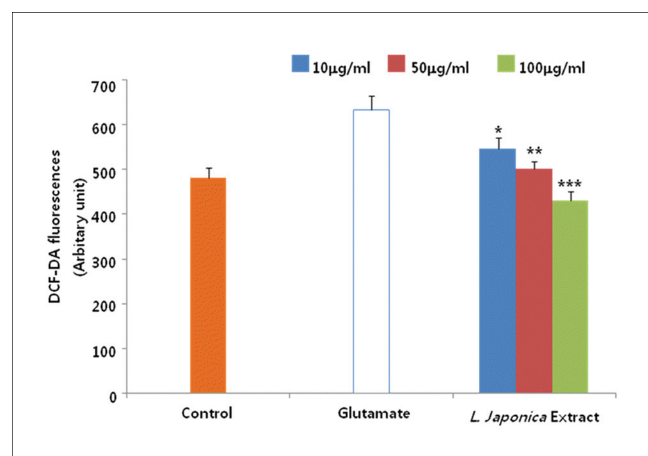

Figure 5: Cultures were pretreated with the methanolic extract of *L. japonica* 1 hr before glutamate exposure. The relative content of intracellular peroxide was determined using the fluorescent dye 2',7'-dichlorofluorescein diacetate (2,7-DCF-DA). The values shown are means ± Standard deviation of three experiments (3-4 cultures per experiment). Glutamate-injured value differs significantly from the control at a level of $P<0.001$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. Glutamate-injured cells (Analysis of Variance [ANOVA] and Tukey)

Table 1: The effect of the methanolic extract of *L. japonica* on antioxidative enzymes such as superoxide dismutase, glutathione reductase and glutathione peroxidase in primary cultures of glutamate-injured rat cortical cells

	Concentration (µg/ml)	Superoxide dismutase (U/mg protein)	Glutathione reductase (mU/mg protein)	Glutathione peroxidase (mU/mg protein)
Control		25.2 ± 1.8	30.6 ± 3.8	35.8 ± 3.9
Glutamate		12.3 ± 3.2	20.1 ± 2.3	19.3 ± 1.8
L. japonica Extract	10	19.2 ± 2.1	21.2 ± 1.1	28.3 ± 4.1
	50	19.8 ± 2.3	24.8 ± 2.3	29.9 ± 1.9
	100	21.2 ± 0.3	28.8 ± 2.6	32.2 ± 1.2

such as superoxide dismutase, glutathione reductase and glutathione peroxidase.[22-24] We investigated the effect of *L. japonica* extract on the activities of antioxidant enzymes in glutamate-injured rat cortical cells. Treatment with *L. japonica* extract significantly preserved the activities of superoxide dismutase, glutathione peroxidase and glutathione reductase to the control level in primary cultures of rat cortical cells injured with glutamate [Table 1]. From these results, it could be suggested that *L. japonica* extract reduced the formation of reactive oxygen species (ROS) in cells by enhancing the antioxidative defense system.[25] Also, *L. japonica* extract showed free radical scavenging activity (data not shown).

At present, the cellular and molecular mechanisms that underlie the action of *L. japonica* extract are not fully understood. However, our results show that *L. japonica* extract significantly protected primary cultured neuronal cells against glutamate-induced oxidative stress via antioxidative activities. Therefore, we conclude that *L. japonica* extract might offer useful therapeutic choices in the treatment of neurodegenerative disorders caused by excitotoxicity.

ACKNOWLEDGEMENTS

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