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Amyloid β Protein (25-35) 유도 배양신경세포 독성에 대한 목단피의 억제효과

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Moutan Cortex Extract Inhibits Amyloid β Protein (25-35)-induced Neurotoxicity in Cultured Rat Cortical Neurons

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ABSTRACT : Moutan cortex, the root bark of *Paeonia suffruticosa* Andrews (Paeoniaceae), has pharmacological effects such as anti-inflammatory, antiallergic, analgesic and antioxidant activities. We investigated a methanol extract of Moutan cortex for neuroprotective effects on neurotoxicity induced by amyloid β protein (Aβ) (25-35) in cultured rat cortical neurons. Exposure of cultured cortical neurons to 10 μM Aβ (25-35) for 24 h induced neuronal apoptotic death. Moutan cortex inhibited 10 μM Aβ (25-35)-induced neuronal cell death at 30 and 50 ㎍/㎖, which was measured by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay and Hoechst 33342 staining. Moutan cortex inhibited 10 μM Aβ (25-35)-induced elevation of intracellular calcium concentration ($[Ca^{2+}]_i$), and generation of reactive oxygen species (ROS) which were measured by fluorescent dyes. Moutan cortex also inhibited glutamate release into medium induced by 10 μM Aβ (25-35), which was measured by HPLC. These results suggest that Moutan cortex prevents Aβ (25-35)-induced neuronal cell damage by interfering with the increase of $[Ca^{2+}]_i$, and then inhibiting glutamate release and ROS generation. Moutan cortex may have a therapeutic role in preventing the progression of Alzheimer's disease.

***Key Words* :** Moutan Cortex, Amyloid β protein (25-35), Neurotoxicity, Alzheimer's Disease, Neuroprotection

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized clinically by cognitive impairment and pathologically by the appearance of senile plaques and neurofibrillary tangles (Price *et al*., 1998). Amyloid β protein (Aβ), a 39- to 43-amino-acid peptide fragment derived from amyloid precursor protein, is thought to be closely related to the pathogenesis of AD as the major component of the senile plaques that characterize this disease (Ivins *et al*., 1999). In cultures, Aβ can directly induce neuronal cell death (Ueda *et al*., 1994) and can render neurons vulnerable to excitotoxicity (Koh *et al*., 1990) and oxidative insults (Goodman and Mattson, 1994). Although the precise mechanism of Aβ-induced cell death is not well understood, Aβ neurotoxicity has been speculated to be due to various factors, including oxidative stress, excessive increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and glutamate accumulation, and induction of neurotoxic cascades (Gray and Patel, 1995; Ueda *et al*., 1997; Ekinci *et al*., 2000).

Moutan cortex, the root bark of *Paeonia suffruticosa* Andrews (Paeoniaceae), has been used extensively as a traditional Chinese medicine in eastern Asian countries. Moutan cortex is used to treat a plethora of disease classes such as atherosclerosis, infection and inflammation. Various pharmacological activities of Moutan cortex include anti-hepatotoxic, anti-inflammatory, anti-allergic, anti-oxidant and analgesic effects (Shon and Nam, 2004; Tatsumi *et al*., 2004; Rho *et al*., 2005; Chun *et al*, 2007; Jiang *et al*., 2007). In the CNS, a possible neuroprotective effect of a

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traditional Chinese medical formulation containing Moutan cortex against rat brain ischemia/reperfusion injury has been reported (Le *et al*., 2007). Paeonol, a common component of Moutan cortex, reduced cerebral infarction in ischemia/reperfusion injured rats (Hsieh *et al*., 2006). Furthermore, Moutan cortex was effective to prevent oxidative stress-induced neuronal death and generation of reactive oxygen species in cultured neurons (Shimada *et al*., 2004; Rho *et al*., 2005). These reports indicate that Moutan cortex can be a potential protective agent against neurodegenerative diseases such as AD and stroke. The aim of our study was to determine whether Moutan cortex had a protective effect against Aβ (25-35)-induced neuronal damage in cultured rat cortical neurons.

MATERIALS AND METHODS

1. Plant material and extraction

Moutan cortex was purchased from a market in Daegu, Korea and identified by Professor K.S. Song, Kyungpook National University. The voucher specimen (KNUNPC-MC1) is stored at the Natural Products Chemistry Lab., Division of Applied Biology and Chemistry, Kyungpook National University, Daegu, Korea. The dried material (1 ㎏) was refluxed in 2 L methanol twice at room temperature and the residue was filtered off using a filter paper. The filtrate was evaporated to dryness under 45℃ to obtain methanol extract (189.33 g).

2. Experimental animals

Pregnant Sprague-Dawley rats for primary neuronal culture were supplied by Daehan BioLink Co., Ltd. (Chungbuk, Korea) and housed in an environmentally controlled room at 22 ± 2℃, with a relative humidity of 55 ± 5%, a 12-h light/dark cycle, and food and water *ad libitum*. The procedures involving experimental animals complied with the animal care guidelines of the National Institutes of Health and the animal ethics committee of Chungbuk National University.

3. Induction of neurotoxicity in primary cultures of rat cerebral cortical neurons

Primary cortical neuron cultures were prepared using embryonic day 15 to 16 Sprague-Dawley rat fetuses, as described previously (Ban *et al*., 2005; Lee *et al*., 2007). Neurotoxicity experiments were performed on neurons after 3-4 days in culture. Cultured neurons were treated with 10 μM Aβ (25-35) (Bachem, Bubendorf, Switzerland) in serum-free DMEM (Sigma) at 37℃ for 24 h (unless otherwise indicated) to produce neurotoxicity. An Aβ (25-35) stock solution of 2 mM was prepared in sterile distilled water, stored at –20℃, and incubated for more than 2 days at 37 ℃ to aggregate before use. Moutan cortex was dissolved in DMSO at concentrations of 50 ㎎/㎖ and further diluted in experimental buffers. The final concentration of DMSO was ≤ 0.1%, which did not affect cell viability. For each experiment, Moutan cortex was applied 15 min prior to treatment with 10 μM Aβ (25-35). It was also present in the medium during Aβ (25-35) incubation.

4. Measurements of Aβ (25-35)–induced neuronal death and intracellular biochemical changes

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) assay and Hoechst 33342 (Molecular Probes, Eugene, OR, USA) staining were performed to measure neuronal death 24 h after exposure of cultured neurons to 10 μM Aβ (25-35), as described previously (Ban *et al*., 2005; Lee *et al*., 2007). Changes in $[Ca^{2+}]_i$ were measured with Fluo-4 AM (Molecular Probes), a calcium-sensitive fluorescent dye, using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Oberkochen, Germany) with a 488-㎚ excitation argon laser and 515-㎚ longpass emission filters (Ban *et al*., 2005; Lee *et al*., 2007). To measure glutamate secreted into the medium, cells were treated with 10 μM Aβ (25-35) in a HEPES buffer containing 8.6 mM HEPES, 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$ and 10 mM glucose at pH 7.4, and glutamate secreted over 6 h was quantified by HPLC with an electrochemical detector (MF series, BAS, IN, USA) (Ban *et al*., 2005; Lee *et al*., 2007). The microfluorescence of 2',7'-dichlorofluorescein, the fluorescent product of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes), and a laser scanning confocal microscope (MRC1024ES, Bio-Rad, Maylands, UK) with 488-㎚ excitation and 510-㎚ emission filters were used to monitor the generation of reactive oxygen species (ROS) in neurons treated with 10 μM Aβ (25-35) for 24 h (Ban *et al*., 2005).

5. Statistical analysis

Data are expressed as mean ± SEM and statistical significance was assessed by one-way analysis of variance (ANOVA) and Tukey's tests. $P < 0.05$ was considered significant.

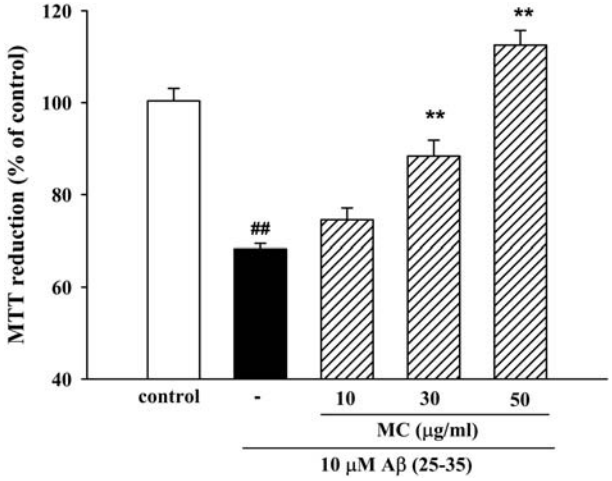

Fig. 1. Inhibitory effect of Moutan cortex (MC) on Aβ (25-35)-induced neuronal cell death in cultured cortical neurons. Neuronal cell death was measured using the MTT assay. The MTT absorbance from untreated cells was normalized to 100%. Results are expressed as mean ± S.E.M. of data obtained from 3 independent experiments. $^{\#\#}P < 0.01$ vs control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs 10 μM Aβ (25-35).

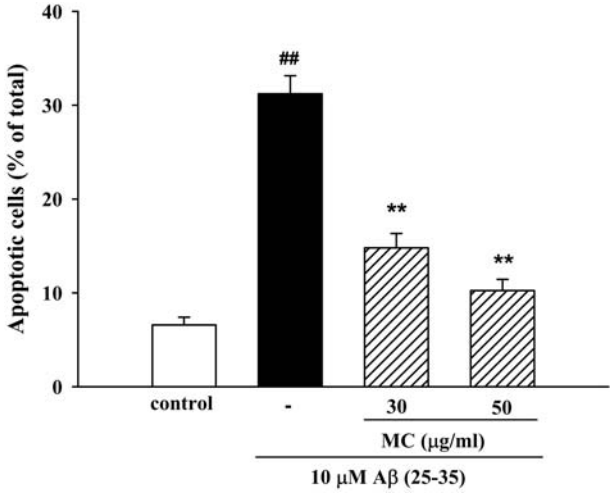

Fig. 2. Inhibitory effect of Moutan cortex (MC) on Aβ (25-35)-induced apoptosis of cultured cortical neurons. Apoptotic cells measured by Hoechst 33342 staining were counted in 5 to 6 fields per well. Results are apoptotic cells as a percentage of the total number of cells expressed as mean ± S.E.M. of data obtained from 4 independent experiments. $^{\#\#}P < 0.01$ vs control, $^{**}P < 0.01$ vs 10 μM Aβ (25-35).

RESULTS

1. Moutan cortex inhibits Aβ (25-35)–induced neuronal cell death

In previous experiments (Ban and Seong, 2005), we have demonstrated that Aβ (25-35) over the concentration range of 5-20 μM produced a concentration-dependent reduction of cell viability in cultured cortical neuron. Therefore, the concentration of 10 μM was used for the determination of Aβ (25-35)-induced neuronal cell damage in the present experiments. When cortical neurons were exposed to 10 μM Aβ (25-35) for 24 h, absorbance in the MTT assay was 68.3 ± 1.2% of that of the untreated controls (Fig. 1), indicating that Aβ (25-35) caused neuronal cell death. In cultures treated with Moutan cortex (30 or 50 ㎍/㎖), the Aβ (25-35)-induced neuronal death was significantly reduced (absorbance, 112.5 ± 3.2% of control with 50 ㎍/㎖ Moutan cortex).

Hoechst 33342 staining was used to detect condensed or fragmented DNA, which is indicative of Aβ (25-35)-induced neuronal apoptotic death. Treatment of neurons with 10 μM Aβ (25-35) induced apoptosis in 31.2 ± 1.9% of cultured cortical neurons, compared with 6.6 ± 0.8% in control cultures. The addition of Moutan cortex (50 ㎍/㎖) significantly decreased Aβ (25-35)-induced apoptotic cell death to 10.2 ± 1.2% of all neurons (Fig. 2).

2. Moutan cortex inhibits Aβ (25-35)–induced $[Ca^{2+}]_i$ elevation

Increases in $[Ca^{2+}]_i$ have been associated with Aβ-induced cell death. In our cell cultures, treatment with 10 μM Aβ (25-35) produced a slow but gradual increase in $[Ca^{2+}]_i$, with the maximum fluorescence intensity (ca. 180, compared to a basal level of 100) observed about 5 min after Aβ (25-35) application. In contrast, pretreatment with Moutan cortex (50 ㎍/㎖) significantly inhibited the increase of $[Ca^{2+}]_i$ induced by 10 μM Aβ (25-35) throughout the measurement period (Fig. 3). Moutan cortex did not affect basal $[Ca^{2+}]_i$.

3. Moutan cortex inhibits Aβ (25-35)–induced elevation of glutamate release

We next quantified the glutamate released into the extracellular medium after treatment with 10 μM Aβ (25-35) for 6 h. As shown in Fig. 4, 10 μM Aβ (25-35) elevated the basal glutamate level from 0.39 ± 0.02 μM in control neurons to 0.78 ± 0.10 μM. Moutan cortex (50 ㎍/㎖) significantly blocked the Aβ (25-35)-induced elevation of glutamate release, resulting in maximal values of 0.40 ± 0.05 μM.

4. Moutan cortex inhibits Aβ (25-35)–induced ROS generation

To clarify the involvement of oxidative stress in Aβ

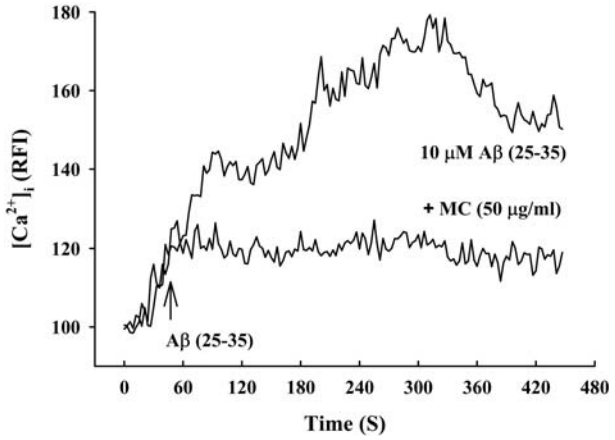

Fig. 3. Inhibitory effect of Moutan cortex (MC) on Aβ (25-35)-induced $[Ca^{2+}]_i$ elevation in cultured cortical neurons. $[Ca^{2+}]_i$ was monitored using Fluo-4 AM dye and a confocal laser scanning microscope. All images were processed to analyze changes in $[Ca^{2+}]_i$ at the single cell level. Results are expressed as the relative fluorescence intensity (RFI). Each trace shows a single cell that is representative of at least 3 independent experiments.

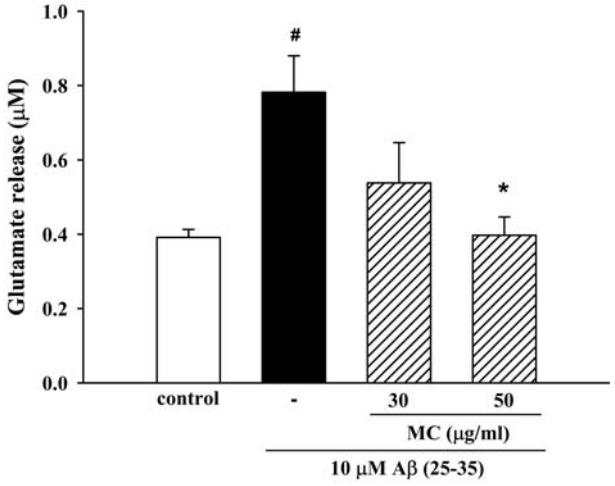

Fig. 4. Inhibitory effect of Moutan cortex (MC) on Aβ (25-35)-induced glutamate release in cultured cortical neurons. The amount of glutamate released over 6 h was measured by HPLC with an electrochemical detector. Results are expressed as mean ± S.E.M. of data obtained from 3 independent experiments. $^{\#}P < 0.05$ vs control; $^{*}P < 0.05$ vs 10 μM Aβ (25-35).

neurotoxicity, we measured the accumulation of ROS after the exposure of the cells to Aβ (25-35) for 24 h. In H_2DCF-DA-loaded cerebral cortical neurons, 10 μM Aβ (25-35) increased the fluorescence intensity, indicating that ROS were generated. In neurons treated with 10 μM Aβ (25-35), the relative fluorescence increased approximately 3-fold to 121.7 ± 7.1 compared with the value in control neurons (24.5 ± 1.9). The Aβ (25-35)-induced increase in ROS

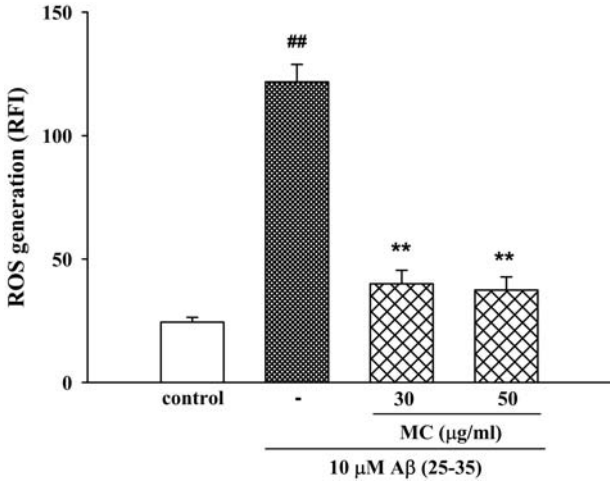

Fig. 5. Inhibitory effect of Moutan cortex (MC) on Aβ (25-35)-induced ROS generation in cultured cortical neurons. ROS was monitored using H_2DCF-DA dye and a confocal laser scanning microscope. Results are expressed as mean ± S.E.M. of RFI obtained from 3 independent experiments. $^{\#\#}P < 0.01$ vs control; $^{**}P < 0.01$ vs 10 μM Aβ (25-35).

generation was significantly inhibited by Moutan cortex (30 and 50 ㎍/㎖) (Fig. 5).

DISCUSSION

As an active partial fragment of Aβ, Aβ (25-35) forms a β-sheet structure and induces neuronal cell death, neuritic atrophy, synaptic loss, and memory impairment, although it is not found in the AD brain (Pike *et al*., 1995; Grace *et al*., 2002; Tohda *et al*., 2004). The present study demonstrated that Aβ (25-35) causes $[Ca^{2+}]_i$ increase, glutamate release, ROS generation, and neuronal cell death in cultured cortical neurons, all of which were blocked by treatment with Moutan cortex. In our previous reports, Aβ (25-35)-induced $[Ca^{2+}]_i$ increase, glutamate release, ROS generation, and neuronal apoptotic death were blocked by treatment with MK-801, an N-methyl-$_D$-aspartate (NMDA) antagonist; verapamil, an L-type Ca^{2+} channel blocker; and N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase (NOS) inhibitor (Ban and Seong, 2005; Lee *et al*., 2005). These results supporting the involvement of NMDA glutamate receptor activation, increased Ca^{2+} influx, and generation of ROS in Aβ (25-35)-induced neurotoxicity in cultured neurons are consistent with the results of other studies (Harkany *et al*., 1999; Ekinci *et al*., 2000). Regardless of the relative contribution of these events to Aβ (25-35)-induced neurotoxicity, the primary event following

Aβ (25-35) treatment of cultured neurons has been suggested to be Ca^{2+} influx, apparently via L-type voltage-dependent Ca^{2+} channels (L-VDCC), because blockade of this channel or Ca^{2+} chelation prevents other consequences (Ueda *et al*., 1997; Mattson and Chan, 2003). Furthermore, Aβ (25-35)-induced elevation of $[Ca^{2+}]_i$ and neurotoxicity were inhibited by MK-801, suggesting that Ca^{2+} influx through NMDA receptor-coupled L-VDCC plays a critical role in the neurotoxicity (Harkany *et al*., 1999; Ban and Seong, 2005; Lee *et al*., 2005). In the present study, Aβ (25-35) elicited gradual and significant $[Ca^{2+}]_i$ increase, which was blocked by Moutan cortex. Moutan cortex also significantly inhibited the Aβ (25-35)-induced glutamate elevation. These findings indicate that the sustained inhibition on $[Ca^{2+}]_i$ elevation by Moutan cortex resulted in the decrease of the Aβ (25-35)-induced glutamate release.

A variety of events occur downstream of neuronal Ca^{2+} overloading, including cytosolic ROS generation due to the influx of Ca^{2+} (Pereira *et al*., 2000). Many reports have demonstrated the role of ROS formation in Aβ-induced neurotoxicity (Yatin *et al*., 1999; Parks *et al*. 2001). Although researchs show that Ca^{2+} signals activate enzymes associated with ROS generation, ROS can also facilitate $[Ca^{2+}]_i$ increases by damaging $[Ca^{2+}]_i$ regulatory mechanisms and by activating Ca^{2+} release from intracellular Ca^{2+} stores (Butterfield *et al*., 2007). We found that Moutan cortex inhibited an Aβ (25-35)-induced increase in ROS generation, but we have not determined whether Moutan cortex suppresses ROS generation via inhibition of a $[Ca^{2+}]_i$ increase or vice versa. It can be suggested that Moutan cortex might prevent Ca^{2+} entry through VDCC- and/or NMDA-receptor-coupled channels to inhibit glutamate release and ROS generation, and then Aβ (25-35)-induced neuronal death, although the mechanism by which Moutan cortex blocks the channels is not clear. A variety of compounds such as paeonoside, paeoniflorin, paeonolide and paeonol have been identified and determined in Moutan cortex (Chen *et al*., 2006). Paeonol inhibited NMDA receptor-coupled Ca^{2+} influx to protect oxygen-glucose deprivation-induced neuronal death in cultures and protected against myocardial injury due to its VDCC blocking effect (Zhang *et al*., 2003; Wu *et al*., 2008). Paeoniflorin also inhibited VDCC in neuronal cell lines to produce neuronal or neuroendocrine function. The inhibition by Moutan cortex on Aβ (25-35)-induced $[Ca^{2+}]_i$ increase might be due to these compounds, which could stabilize membranes in a manner that blocks Ca^{2+} influx via VDCC. Further, recent pharmacological studies revealed that Moutan cortex can inhibit the production of ROS and some paeonol glycosides exhibited radical scavenging effects (Yoshikawa *et al*., 1992; Matsuda *et al*., 2001; Rho *et al*., 2005), suggesting that inhibition of Aβ (25-35)-induced neuronal death by Moutan cortex might be due to its ROS scavenging activity. Further study to elucidate the precise mechanism should be performed.

Many researchers have demonstrated that Aβ triggers apoptotic degeneration in *in vitro* neuronal experiments (Yan *et al*., 1999; Ekinci *et al*., 2000). In the present work, cultured cortical neurons exposed to Aβ (25-35) for 24 h showed increased chromatin condensation, a typical feature of apoptotic cell death, which was reduced by Moutan cortex. Aβ-induced apoptosis has been reported to be associated with COX-2 upregulation, and COX has been suggested to be an important source of ROS in the pathologic brain (Chan, 2001; Jang and Surh, 2005). Therefore, the protective effect of Moutan cortex on the Aβ-induced apoptosis of cultured neurons might result from the inhibition of COX and inflammatory cytokine production by Moutan cortex and its active compounds such as paeonol and paeoniflorin (Chun *et al*., 2007; Wu and Gu, 2007). The molecular mechanism for the prevention of neuronal apoptosis by Moutan cortex should be further clarified.

Since ROS and inflammation are part of the complex series of pathophysiological events that contribute to neurodegenerative diseases such as AD and stroke (Pitchumoni and Doraiswamy, 1998; Hoehn *et al*., 2005), free radical scavengers and anti-inflammatory agents have attracted considerable attention as potential neuroprotective agents. Moutan cortex and its common active compound, paeonol, have been reported to reduce rat brain ischemia/reperfusion injury (Hsieh *et al*., 2006; Le *et al*., 2007) and to prevent oxidative stress-induced neuronal death and generation of reactive oxygen species in cultured neuron (Shimada *et al*., 2004; Rho *et al*., 2005). Furthermore, anti-inflammatory effect of Moutan cortex attributable to the inhibition on COX and cytokine expression has been confirmed (Chun *et al*., 2007; Wu and Gu, 2007), suggesting that Moutan cortex has good potential for prevention of neurodegenerative diseases. Aβ is believed to play a central role in the pathophysiology of AD (Ueda *et al*., 1994; Yatin *et al*.,

1999; Butterfield *et al*., 2007). Moutan cortex blocked Aβ (25-35)-induced neuronal cell damage in the present study. Although there has been no evidence up until now to show that Moutan cortex antagonizes Aβ (25-35)-induced neurotoxicity, the present study demonstrates a novel pharmacological activity of Moutan cortex in neurons. In conclusion, the protection against Aβ (25-35)-induced neurotoxicity by Moutan cortex provides as a promising therapeutic approach to control the progression of neurodegeneration in brain of AD. Forthcoming studies will be attempted to clarify the *in vivo* effect of Moutan cortex.

ACKNOWLEDGEMENTS

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