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ORIGINAL ARTICLE

Extract of *Styrax japonica* attenuates glutamate-induced apoptosis via regulating MAPK signaling pathway in HT22 hippocampal cells

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Abstract

Two-thirds of people with dementia suffer from Alzheimer's disease, and there is a need to develop treatments with fewer side effects. Cholinergic and glutamate-induced brain damage occurs in the early stages of Alzheimer's disease, so substances that suppress these symptoms may be potential candidates for the treatment. Ethanol extracts of 40 kinds of oriental medicine plants were examined whether they have acetylcholine esterase (Ache) inhibitory properties. We next investigated whether the ethanol extracts of six oriental medicine plants showing Ache inhibitory activity could inhibit glutamate-induced HT22 cell death. The ethanol extract of *Styrax japonica* (EESJ) was found to be relatively superior in both inhibitory activities. MTT and annexin V/PI staining assays confirmed that EESJ inhibited glutamate-induced apoptosis in the HT22 mouse hippocampal cells. EESJ also suppressed glutamate-mediated ROS production and attenuated the phosphorylation levels of MAPK members including ERK, JNK, and p38 kinases. Therefore, EESJ is a suitable candidate for developing a substance of Alzheimer's disease treatment.

Keywords Glutamate · HT22 cells · *Styrax japonica* · Apoptosis · Reactive oxygen species · Bcl-2 family · Mitogen-activated protein kinase

Abbreviations

EESJ	Ethanol extracted leaves of *Styrax japonica*
Glu	Glutamate
ROS	Reactive oxygen species
MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal regulated kinase
JNK	*c*-Jun *N*-terminal kinase
NAC	*N*-Acetylcysteine

Introduction

Alzheimer's disease, a type of dementia, is a neurodegenerative disease that deteriorates cognitive function and memory with age. The two-thirds of dementia people suffer from Alzheimer's disease (Nussbaum and Ellis 2003). According to the Korean Dementia observatory 2019 published by the Central Dementia Center, the number of dementia patients over 65 in Korea was about 750,000 in 2018, with a prevalence of 10.16%, of which 74.5% suffer from Alzheimer's disease. Despite being a well-known senile degenerative disease, the exact cause was not remaining unknown due to the complex pathological characteristics of Alzheimer's disease. Oxidative stress, Amyloid β accumulation and reduced levels of acetylcholine are believed to be the causes of Alzheimer's disease (Markesbery 1997; Laferla et al. 2007; Whitehouse et al. 1981). Especially in the early stages, cholinergic and glutamatergic synapses are considered abnormal (Selkoe 2002).

Acetylcholine (Ach) is a cholinergic neurotransmitter located at the basal forebrain in the central nervous system (CNS). The drugs for Alzheimer's disease have based on temporarily increasing the concentration of acetylcholine by suppressing acetylcholinesterase (AchE) as the cholinergic

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hypothesis (Francis et al. 1999). AchE inhibitors such as tacrine, donepezil, and rivastigmine are used as treatments for Alzheimer's disease, but they have detrimental effects like gastrointestinal injury (McGleenon et al. 1999; Schulz 2003). Therefore, finding low-risk and efficient therapeutic candidates from natural products containing a variety of compounds is also important in treatment development (Mukherjee et al. 2007).

Glutamate is the excitatory neurotransmitter which is associated with learning and memory in the central nervous system (Riedel et al. 2003). However, dysfunction of glutamatergic synapses due to early symptoms of Alzheimer's disease causes a high concentration of glutamate which results in neurotoxicity (Selkoe 2002). Glutamate-induced neuronal cell death has two typical pathways. First, excitotoxicity of glutamate makes ionotropic glutamate receptors overactive and intracellular accumulated Ca^{2+} activates cell death pathways (Choi 1988). Another pathway is the programmed nerve cell death caused by oxidative stress without going through glutamate receptors. The high concentration of extracellular glutamate inhibits the absorption of cystine from the cystine/glutamate antiporter drastically reducing the content of intracellular glutathione (GSH). Thereby reactive oxygen species (ROS) increases and causes oxidative stress which triggers cell death (Shirlee et al. 2005).

Therefore, simultaneous control of the cholinergic and glutamatergic system may be one of the important strategies for treatment Alzheimer's disease. In this study, 43 oriental medicine plant species of ethanol extracts were investigated for AchE inhibitory activity and glutamate-induced cytotoxicity inhibition. The ethanol extract of *Styrax japonica* leaves (EESJ) was found to be excellent in inhibiting AchE activity and glutamate-induced cytotoxicity, and the related signaling pathways were examined.

Materials and methods

Materials

Forty species of 80% aqueous ethanol (v/v) extracted plant samples were purchased from Jeju Biodiversity Research Institute of Jeju Technopark for acetylcholinesterase (AchE) inhibitory assay. AchE, acetylthiocholine iodide (ATCI), 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) and L-Glutamic acid potassium salt monohydrate were purchased from Sigma (St. Louis, MO, USA).

Acetylcholinesterase inhibitory activity assay

AchE inhibitory assay was measured by Ellman method optimized for 96-well plates (Ellman et al. 1961). Briefly, 160 µL of 0.25 M potassium phosphate buffer (pH 7.4), 10 µL of 10 mg/mL sample or control (80% aqueous ethanol), and 10 µL of 1 U/mL AchE were mixed and incubated for 5 min at 37 °C. Then, 20 µL of 2.5 mM acetylthiocholine iodide (ATCI) or background (1 M phosphate buffer) was added and incubated for 60 min at 37 °C. Finally, 40 µL of 25 mM DTNB was added and measured at 410 nm absorbance. Tests were repeated at least three times.

Preparation of the ethanol extract of EESJ

Leaves of *Styrax japonica* were harvested in September 2019, washed several times, and dried in shady place. After freezing at − 70 °C for 1 h, leaves were lyophilized for 2 days and ground into small pieces. 89 g of leaf powder was mixed with 890 mL of 80% aqueous ethanol (v/v). The mixture was extracted at 60 °C in sonication for 2 h. The extract was filtered and then concentrated using rotary evaporator. Subsequently, the extract was lyophilized one more time for 2 days.

Cell culture and reagents

Mouse HT22 hippocampal cells were gifted by Dr. J-R, Lee from Korean Research Institute of Bioscience and Biotechnology (KRIBB). HT22 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (Gibco BRL), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO_2. HT22 cells were sub-cultured every 2–3 days.

Cell viability assay

HT22 cells were seeded at a density of 3×10^4 cells per well in 24-well plates. After incubating for 24 h, cells were pre-treated with various concentrations of EESJ (final 10, 20, 30, 40, or 50 µg/mL) or vehicle (80% Ethanol) for 2 h and then 0.5 M of glutamate was added to the medium for 24 h. Cells were measured by 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution. The stock solution of MTT (5 mg/mL) was added to each well at a final concentration of 0.5 mg/mL. After incubation for 3 h, the supernatant was removed, and formazan crystals were subsequently dissolved in 500 µL of dimethyl sulfoxide (DMSO). The absorbance was measured at 420 nm with a microplate reader.

Cell apoptosis assay

For detection of apoptosis cell death, HT22 cells were seeded with 2 mL of medium in 6-well plates. After incubating for 24 h, cells were pre-treated with EESJ at 10 or 50 µg/mL, and then glutamate (final concentration 4 mM) were added

and cultured for another 24 h. The supernatant and HT22 cells were gathered in 15 mL tube and centrifuged. The pellet was washed with phosphate buffer saline (PBS). Finally, cells were incubated with Annexin V-FITC and PI (FITC Annexin V apoptosis detection kit, BD Biosciences) for 15 min and detected by flow cytometry (LSRFortessa, BD).

Intracellular ROS production

To evaluate the intracellular ROS production level, HT22 cells (5×10^4) were seeded in 6-well plates. Following 24 h of incubation, they were pre-treated with or without EESJ (10 or 50 μg/mL) for 2 h, and co-treated 4 mM of glutamate for 12 h. Cells were stained with 10 μM of 2′,7′-Dichlorofluorescein diacetate (H_2DCF-DA, Sigma) in darkness for 15 min. After staining, cells were harvested and washed twice with PBS. ROS production was measured by flow cytometry (LSRFortessa, BD).

Western blot

Cells were lysed in ice-cold M-PER buffer (Thermo scientific, Bonn, Germany) consisting of protease inhibitor cocktail (Roche), PMSF (1 mM), sodium vanadate (2 mM), sodium pyrophosphate (30 mM), and sodium fluoride (100 mM). C (SDS-PAGE). After quantification of the total protein, proteins were separated by 10–12% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose membranes (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK). Membranes were blocked by 5% skim milk in TBST. Several primary antibodies were used to determine the levels of MAPKs, such as p-p38 (Thr180/Tyr182), p38, p-ERK (Thr202/Try204), ERK, p-JNK (Thr183/Tyr185), JNK (1:1000 dilution, Cell signaling technology, USA). In addition, Bax, Bcl-2 (1:500 dilution, Cell signaling technology, USA) and GAPDH (Santa Cruz Biotechnology, USA) were diluted in TBST (1:1000). Donkey anti-Mouse IgG secondary antibody (Merck Millipore, Germany) was used in TBST 1:4000 and shaken slowly for 1 h. The bands were detected using the ECL kit (Biosesang).

Statistical analysis

All data are repeated at least three times. Error bar means standard deviation (S.D.). Statistical analysis was performed by Student's *t* test and one-way ANOVA followed by Tukey's post hoc test (Graphpad Prism, version 5.0, Graphpad Software Inc, San Diego, CA, USA). $p < 0.05$ was considered to indicate significant differences.

Results

Screening of 40 plant species for the acetylcholine esterase inhibitory activity

We examined whether 80% ethanol extracts from 40 species of native Jeju island plants had acetylcholinesterase (AchE) inhibitory activity (Fig. 1). The samples with reaction

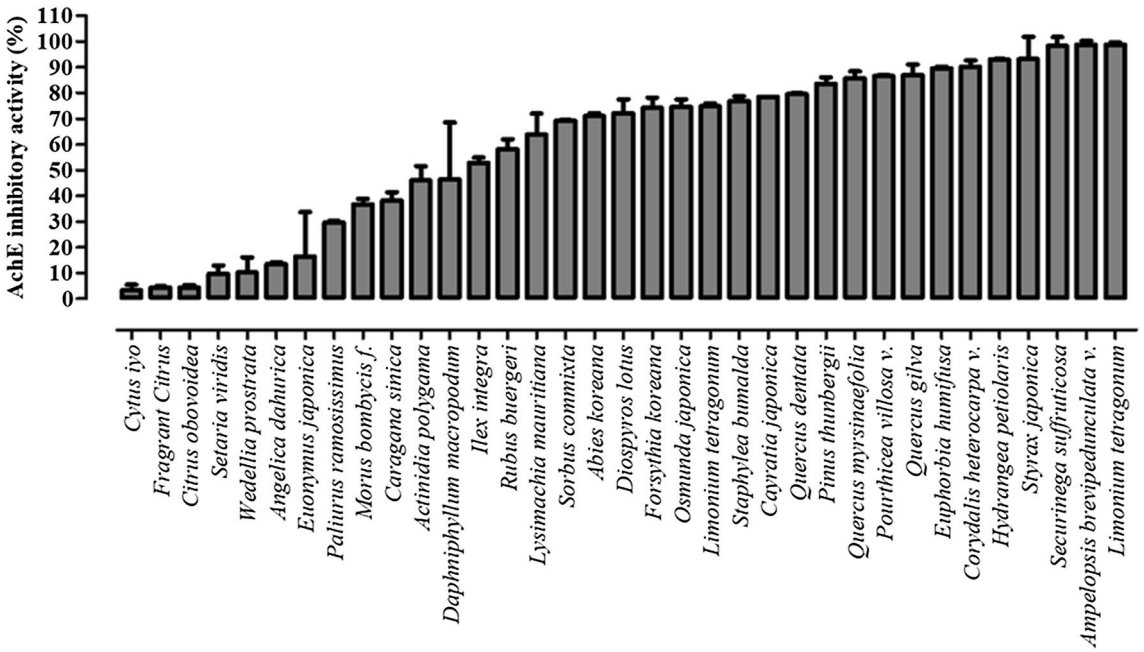


Fig. 1 HYPERLINK "sps:id::fig1||locator::gr1||mediaobject::0" Effect of treatment with extracts of forty plant species on acetylcholinesterase activities in HT22 cells. The concentration of samples was 416 μg/mL. Each bar represents the mean ± SD from two independent experiments

concentration of 10 mg/mL (final concentration 416 µg/mL) were screened, and 11 species had more than 90% AchE inhibitory activity. Among them, six species with high activity were selected (Table 1). Subsequently, we diluted the concentration of samples and measured the absorbance for calculation of IC_{50} value. As a result, the *Styrax japonica* leaves have the lowest IC_{50} value (31.25 µg/mL).

Ethanol extract of EESJ treatment reduces glutamate-induced cell death in HT22 cells

MTT assay was used to determine the effect of six plant samples on glutamate-induced cell death. First, HT22 cells were exposed to glutamate only at 1–4 mM for 24 h (Fig. 2A). HT22 cells were dead by glutamate in a dose-dependent manner and about 10% of cells survived at 4 mM glutamate (10.02 ± 1.05%). Then, we cultured the cells with or without 4 mM of glutamate and with or without six samples at a concentration of 40 µg/mL (Fig. 2B). According to the data, of the samples that showed high AchE inhibitory activity, two species inhibited cell death induced by glutamate. Between of them, we selected *S. japonica* for the next test because of its low IC_{50} value. Therefore, we treated EESJ on HT22 cells at concentrations ranging from 10 to 50 µg/mL to confirm the cytotoxic effect (Fig. 2C). When treated with EESJ alone, the cell viability was more than 80% at the highest concentration (50 µg/mL). To check out exactly, cells were pre-treated with or without EESJ at concentrations ranging from 10 to 50 µg/mL for 2 h and additionally incubated with or without 4 mM glutamate for 24 h (Fig. 2D). EESJ inhibited glutamate-induced cell death dose-dependently. Figure 2E shows the morphological changes with or without EESJ and glutamate treatment. Cells treated with glutamate alone did not maintain a healthy shape compare with cells treated with other combination treatment. For example, there were no morphological differences between cells treated with EESJ only and cells treated with EESJ and glutamate

Table 1 The IC_{50} values of six selected plant species in the AchE inhibitory activity assay.

Scientific name	Parts used	IC_{50} (µg/mL)
Ampelopsis brevipedunculata	Leaf	100.28 ± 16.36
Corydalis heterocarpa	Whole plant	122.47 ± 9.27
Hydrangea petiolaris	Leaf	37.04 ± 7.61
Limonium tetragonum	Whole plant	115.72 ± 1.94
Securinega suffruticosa	Leaf	190.11 ± 0.79
Styrax japonica	Leaf	30.94 ± 18.02

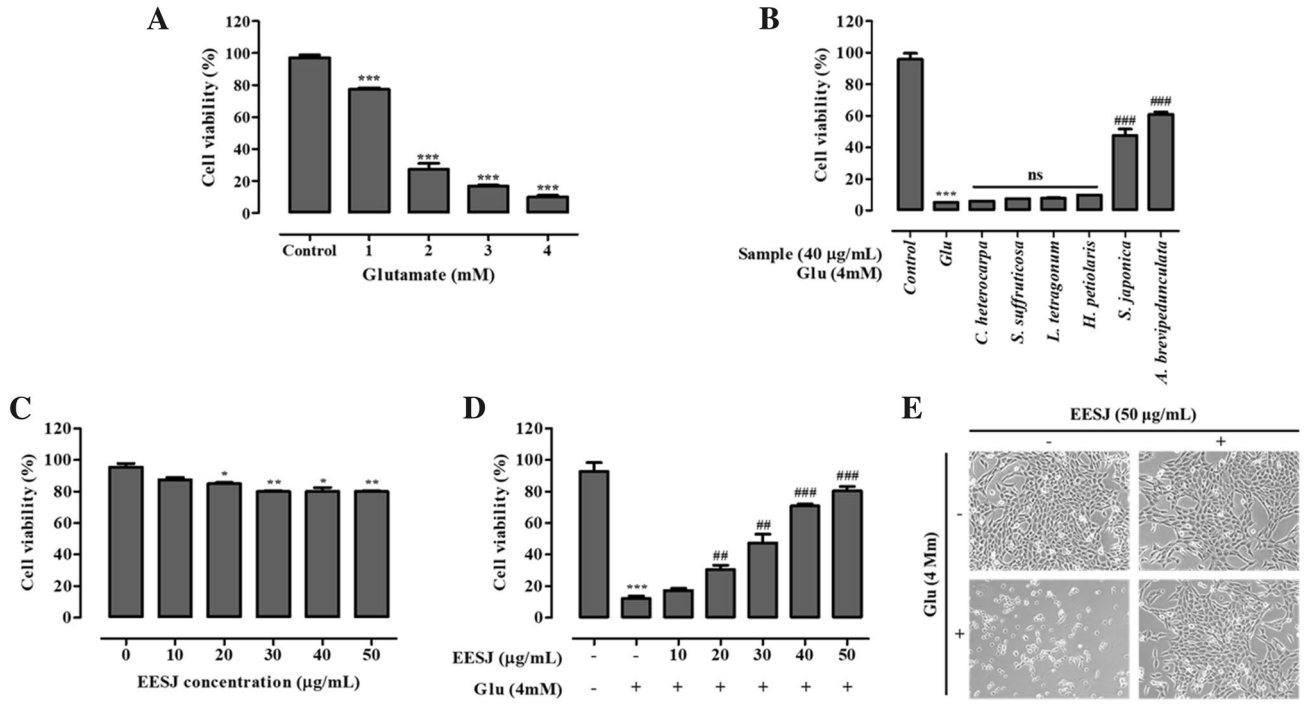


Fig. 2 Ethanol extracted *Styrax japonica* (EESJ) inhibited glutamate-induced cell death in HT22 neuronal cells. MTT assay was performed. **A** Glutamate induces cell death in HT22 cells in concentration-dependent manner. **B** Two samples showed inhibition of glutamate-induced cell death. **C** Different concentrations of EESJ were treated on HT22 cells to confirm cytotoxic effects. **D** EESJ inhibited glutamate-induced cell death in concentration-dependent manner. **E** The morphological changes of which cells treated with or without EESJ (50 µg/mL) and glutamate (4 mM) were observed (×100). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus treatment 4 mM glutamate alone. Each bar represents the mean ± SD from three independent experiments

together. Based on these data, it can be expected that EESJ inhibits glutamate-induced cytotoxicity.

EESJ treatment inhibited cell apoptosis induced by glutamate

In HT22 cells, both necrosis and apoptosis are known to occur due to oxidative stress, and apoptosis is induced at a relatively late time (Fukui et al. 2009). Annexin V/propidium iodide double staining was used to investigate how EESJ affects glutamate-induced apoptosis (Fig. 3). Glutamate treatment with a high concentration (4 mM) for 24 h increased mainly the proportion of Q_2 [FITC-annexin V(+)/propidium iodide(+); late apoptotic cell death] and Q_4 [FITC-annexin V(+)/propidium iodide(−); early apoptotic cell death] regions, reaching a total of 81.13 ± 7.78%. On the other hand, the apoptotic cell death was clearly reduced in cells treated with glutamate and EESJ together. In cells pre-treated with EESJ concentrations of 10 μg/mL, the proportion of Q_2 and Q_4 regions were reduced to

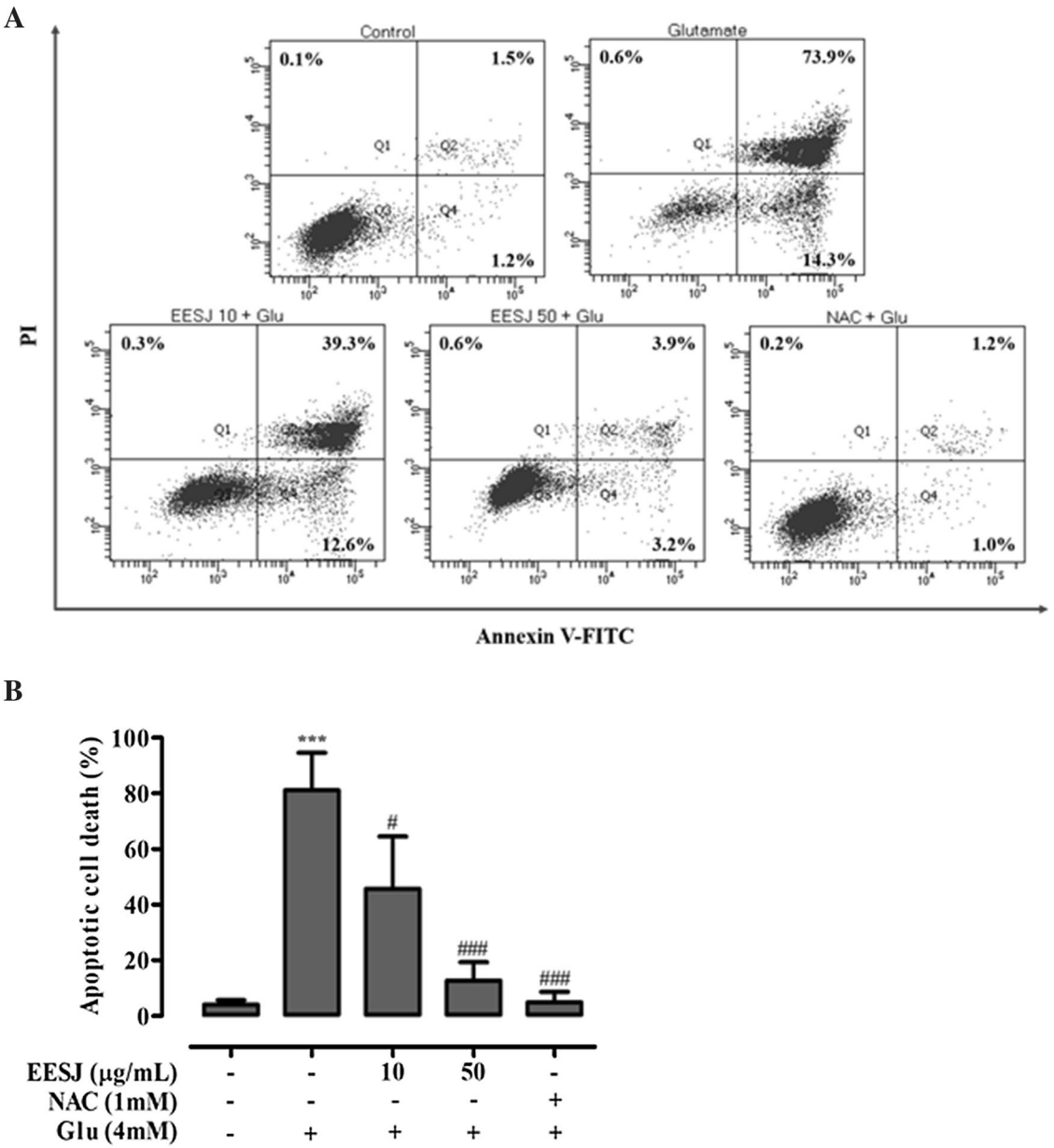


Fig. 3 EESJ reduced apoptosis induced by glutamate. After pre-treated with two concentration (10 and 50 μg/mL) of EESJ for 2 h, HT22 cells were co-treated with glutamate for 24 h. **A** For detection, cells stained with Annexin V and PI, and then measured by flow cytometry. **B** The histogram represented the percentage of apoptotic cells. $^{***}p < 0.001$ versus control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ versus treatment 4 mM glutamate alone. Each bar represents the mean ± SD from three independent experiments

45.70 ± 10.84%, and the proportion was 12.77 ± 3.77% in cells with 50 µg/mL EESJ. N-acetylcysteine (NAC), positive control, also reduced glutamate-induced apoptotic cell death (4.97 ± 2.11%). Taken together, these results suggest that the high concentration of glutamate induces HT22 cell death via early and late apoptosis and EESJ effectively, concentration-dependently, inhibits glutamate-induced apoptosis.

EESJ treatment reverses the expression levels of Bax and Bcl-2, apoptotic regulators

We observed the expression level of Bcl-2 family members related to glutamate-mediated cell death to confirm whether EESJ changes intracellular signal through western blot. Glutamate treatment switches the ratio of pro-apoptotic protein Bax to anti-apoptotic protein Bcl-2 (Jin et al. 2014; Yang et al. 2012). Figure 4A shows, in glutamate only treated cells for 12 h, Bax increased to 1.5-fold compared to control cells but Bcl-2 decreased to 0.7-fold. However, EESJ treatment changed the expression levels to 0.7- and 1.3-fold, respectively. These changes in Bax/Bcl-2 ratio are represented in Fig. 4B. These results suggest that EESJ may affect intracellular signals.

EESJ treatment suppresses glutamate-induced intracellular ROS production in HT22 cells

The high concentration of extracellular glutamate increases the intracellular ROS products which cause cell death (Shirlee et al. 2005). The cells were cultured for 12 h after glutamate treatment and dyed in 10 µM H_2DCF-DA and measured with flow cytometry. In Glutamate-only treated cells, ROS products increased by 4.58 ± 0.41-fold compared to control cells, while those treated with 10 µg/mL and 50 µg/mL of EESJ decreased by 2.66 ± 0.34 and 2.64 ± 0.5-fold, respectively (Fig. 5). Cells treated with N-acetylcysteine (NAC), positive control, were 0.56 ± 0.06-fold of ROS product compared to control cells. From these

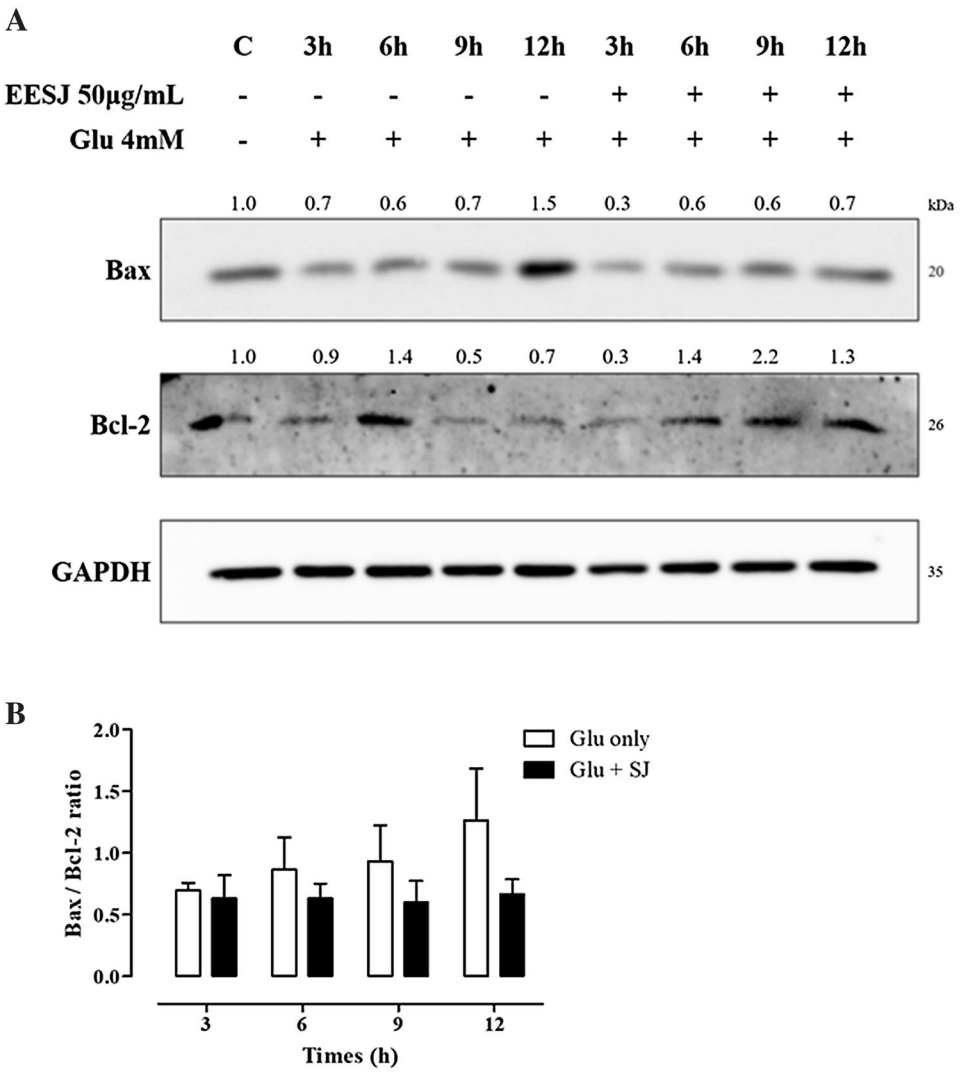


Fig. 4 Effects of EESJ on glutamate-induced apoptotic regulators in HT22 cells. HT22 cells treated with or without 50 µg/mL of EESJ for 2 h and then co-treated with 4 mM glutamate for the indicated times. Protein expression levels were determined by western blot assay using indicated antibodies. The relative intensity of band was analyzed by using Image J software

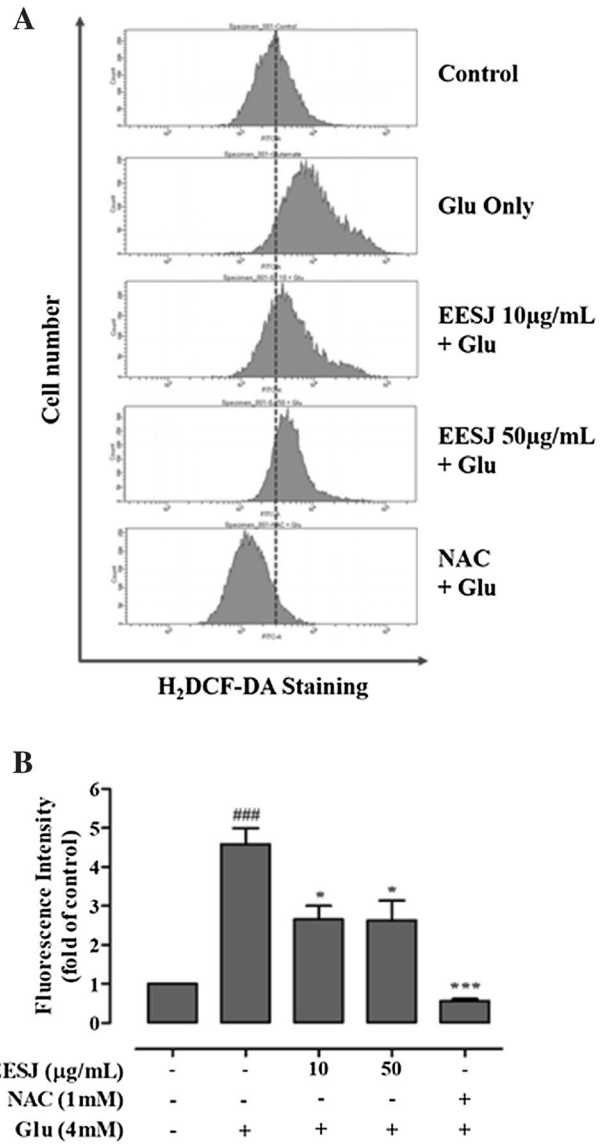


Fig. 5 Intracellular ROS production induced by glutamate in HT22 cells was measured using flow cytometry. **A** The histograms express intracellular ROS production. **B** The graph expresses quantitative analysis. $^{###}p < 0.001$ versus control; $^{*}p < 0.05$, $^{***}p < 0.001$ versus treatment 4 mM glutamate alone. Each bar represents the mean ± SD from three independent experiments

results, it was confirmed that EESJ effectively inhibits ROS production, resulting in nerve cell protection.

EESJ treatment attenuates glutamate-induced neuronal cell death via regulating MAPK signaling pathway

We investigated the expression levels of phosphorylated MAPK members closely related to oxidative stress induced by glutamate through western blot analysis. According to the reported study, while the ERK pathway is fundamentally activated by mitogenic stimuli, JNK and p38 pathways are mostly activated by cellular stressors including oxidative stress (Zhu et al. 2001). As shown in Fig. 6, glutamate upregulated expression levels of phospho-ERK 1/2 mainly from 6 to 9 h but treatment with 50 μg/mL EESJ suppressed the phosphorylated levels at the same time. In addition, EESJ inhibited glutamate-induced phosphorylation of JNK and p38 from 6 to 12 h compared to cells treated glutamate alone. These data represented that neuroprotective effects of EESJ may result from attenuation against activated MAPK pathway induced by glutamate.

Discussion

In this study, EESJ was screened for its high acetylcholine esterase (AchE) inhibitory ability. Subsequent experiments have also demonstrated that glutamate-induced oxidative stress is inhibited. Given that studies have shown that AchE inhibitors such as tacrine and galantamine, which are used as treatments for Alzheimer's disease, are reported to prevent oxidative stress, this is considered a significant result. Tacrine inhibited hydrogen peroxide (H_2O_2)-mediated apoptosis by regulating the expression of apoptosis-contributed genes in rat PC12 cells, and galantamine inhibited amyloid-beta-induced oxidative stress in cortical neurons (Wang et al. 2002; Melo et al. 2009).

Our research using HT22 hippocampal neuronal cells evaluated glutamate-induced cell death and neurotoxicity as MTT assay and confirmed that EESJ contributed to cell death suppression. In addition, when measuring Annexin V/PI-stained cells with flow cytometry, it was confirmed that the type of cell death caused by glutamate was apoptosis and inhibited by EESJ.

Western blot revealed that EESJ affects apoptotic cell signaling through changes in the expression levels of Bax and Bcl-2, the apoptosis modulators. During oxidative stress, the mitochondrial outer membrane becomes permeabilized, releasing cytochrome c and other death-promoting proteins (Adams 2003). This situation stimulates apoptosis, which is regulated by Bcl-2 family. In our result, EESJ decreased the Bax/Bcl-2 ratio increased by glutamate. Therefore, EESJ influences intracellular apoptotic cell signaling pathway by regulating the Bcl-2 family members.

We confirmed that EESJ attenuated glutamate-induced intracellular ROS production in HT22 cells using flow cytometry analysis. The accumulation of ROS production plays an important role in most programmed cell deaths (Shirlee et al. 2005; Esteve et al. 1999). According to Tan, ROS generation after 6 h increases exponentially, which may be the result of EESJ's neuroprotection effect by suppressing accumulation.

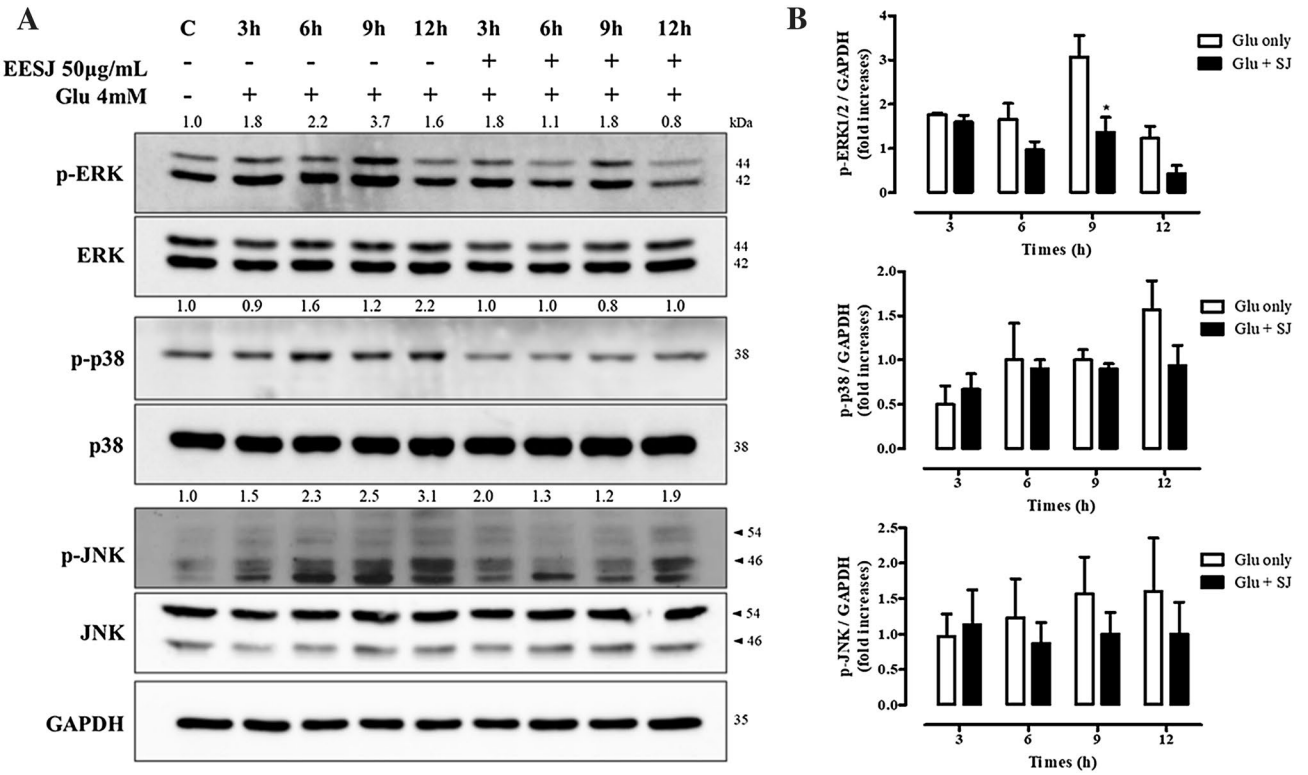


Fig. 6 Effects of EESJ on glutamate-induced phosphorylation of mitogen-activated protein kinases (MAPKs) in HT22 cells. HT22 cells treated with or without 50 μg/mL of EESJ for 2 h and then co-treated with 4 mM glutamate for the indicated time. Protein expression levels were determined by western blot assay using indicated antibodies. The relative intensity of band was analyzed using Image J software. $^{*}p < 0.05$ versus treatment 4 mM glutamate alone

Mitogen-activated protein kinase (MAPK) is a major constituent of pathways controlling cell differentiation, cell proliferation, and cell death (Pearson et al. 2001; Yang et al. 2014). ERK pathway influences not only cell growth and differentiation, but also damaging role in oxidative neuronal injury (Chu et al. 2004. In addition, JNK and p38 kinases are known to regulate cell differentiation and death (Harper and Lograsso 2001). In this study, the high concentration of glutamate (4 mM) promoted the phosphorylation of MAPK members including ERK, JNK, and p38 kinases. However, after the treatment of EESJ, the phosphorylation levels of MAPKs are decreased.

In earlier reported, the seeds or leaves of Styrax species such as *Styrax japonica* are a rich in benzofurans, benzofuran esters, benzofuran glycosides, and sapogenins (Moon et al. 2005). These benzofurans derivatives and sapogenins have been shown have neuroprotection effects (Wakabayashi et al. 2016; Montanari et al. 2019; Ye et al. 2014). No research has yet been published on whether the leaves of *S. japonica* contain these substances, but judging from the results of this study, it is believed that the leaves of the *S. japonica*, which have a neuroprotective effect, contain similar substances.

In conclusion, our results showed that EESJ reduced glutamate-induced ROS and inhibited cell apoptosis through changes in the expression of apoptosis regulator Bax and Bcl-2. It was also confirmed that EESJ had the protection against glutamate-induced neuronal cell death in HT22 cells, by inhibiting phosphorylation of MAPKs such as ERK, JNK, and p38 kinase related to oxidative stress. Therefore, the leaves of *Styrax japonica* have potential as a source of potential therapeutic candidates for neurodegenerative diseases such as Alzheimer's disease.

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Data availability Data are available upon resonable requests.

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