

The Effects of *Crinum asiaticum* var. *japonicum* Baker Seeds on Neuroprotection and Antineuroinflammation in Neuronal Cell Lines

Natural Product Communications
Volume 15(10): 1–10
© The Author(s) 2020
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/1934578X20965465
journals.sagepub.com/home/npj



Hye-Sun Lim¹, Yoonju Kim¹, Yu Jin Kim^{1,2} , Eunjin Sohn¹, Joo-Hwan Kim³, and Soo-Jin Jeong¹

Abstract

Crinum asiaticum var. *japonicum* Baker is a Korean herbal medicine that is traditionally prescribed for reducing fever and inflammation. In the present study, we investigated if the ethanol extract of *C. asiaticum* seeds (ECAS) influences the hallmarks of Alzheimer's disease (AD) pathogenesis. ECAS markedly inhibited the activity of acetylcholinesterase (AChE). Concurrent treatment with hydrogen peroxide (H₂O₂) and ECAS significantly prevented the neuronal cell death by regulating phosphorylation of cyclic adenosine monophosphate response element-binding protein and p38 mitogen-activated protein kinase. ECAS revealed antineuroinflammatory effects by inhibiting nitric oxide production and suppressing inducible nitric oxide expression in lipopolysaccharide-stimulated BV-2 microglia. Furthermore, the high-performance liquid chromatographic analysis determined lycorine as a standard compound of ECAS. Our data suggest that ECAS has inhibitory effects on AD pathogenesis such as AChE activation, neuronal damage, and neuroinflammation.

Keywords

Crinum asiaticum seeds, acetylcholinesterase, Alzheimer's disease, anti-neuroinflammation, neuroprotection

Received: July 15th, 2020; Accepted: September 19th, 2020.

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is characterized by memory and cognitive impairment.¹ In the US, AD affects 15% of the population aged 65–74, and 44% of the population aged 75–84 years.² In South Korea, the occurrence of AD is also gradually increasing, affecting 1.3% of the population aged 65%–69% and 33.9% of the population over the age of 85.³ Unfortunately, there is no currently available medication for the treatment of AD. Although several acetylcholinesterase (AChE) inhibitors, such as tacrine, rivastigmine, galantamine, and donepezil, have been approved by the US Food and Drug Administration (FDA) since 1996,^{4,5} these drugs mask the symptoms of AD, but do not have therapeutic efficacies, and have severe side effects.⁶ Therefore, further research on therapeutic agents for AD is currently a major requirement.

Crinum asiaticum var. *japonicum* Baker (Amaryllidaceae) is a medicinal herbal plant found in Asian countries, including Korea, Japan, and China. In traditional oriental medicine, *C. asiaticum* has been prescribed for the treatment of rheumatoid arthritis, fever, headache, and ulcer.⁷ Several recent studies have provided evidence to support the biological activities of *C. asiaticum*, such as anti-inflammation,^{7,8} antiobesity,⁹ antiviral,¹⁰ and antioxidation.¹¹ The leaves and roots of *C. asiaticum* have been used in traditional oriental medicine; they

contain the alkaloid, lycorine, and the amino acid, tazettine.^{12–14} However, there is no report on the efficacy of *C. asiaticum* seeds.

Hence, in the present study, we investigated the effect of the ethanol extract of *C. asiaticum* seeds (ECAS) on the physiological changes of AD, such as the inhibition of AChE activity, neuroprotection, and antineuroinflammation using hippocampal and microglial cell lines. We also explored the molecular mechanisms responsible for the biological activity of ECAS on the regulation of AD pathogenesis. Additionally, a quantitative analysis of the standard compound in ECAS was achieved using high-performance liquid chromatography (HPLC).

¹Clinical Medicine Division, Korea Institute of Oriental Medicine, Daejeon, Republic of Korea

²Herbal Medicine Research Division, Korea Institute of Oriental Medicine, Daejeon, Republic of Korea

³Department of Life Science, Gachon University, Seongnam, Gyeonggi-do, Republic of Korea

Corresponding Author:

Soo-Jin Jeong, Clinical Medicine Division, Korea Institute of Oriental Medicine, Daejeon 34054, Republic of Korea.
Email: sjjeong@kiom.re.kr



Results and Discussion

AD is a serious life-threatening disease with an increasing prevalence; however, no cure is currently available, and the quality of life of patients has not yet been well studied.¹⁵ Thus, it is important to conduct research on therapeutics for AD. Currently, most of the FDA-approved AD drugs focus on a single target, AChE.¹⁶ However, since AD progression is influenced by multiple causes, such as amyloid- β (A β) aggregation,¹⁷ neuronal cell damage,¹⁸ neuroinflammation¹⁹ besides AChE activation, they should be considered as targets for AD drug development.

According to the Alzheimer's Association, many researchers believe that successful AD treatment will ultimately implicate a "cocktail" of medications aimed at multiple targets.²⁰ Of note, herbal medicines have the properties of multicomponent and multitarget therapy because they are a type of cocktail drug comprising several phytochemicals.²¹ Hence, they are attractive as potent anti-AD drug candidates. In fact, several groups suggest the use of herbal medicines for the treatment of AD.²²⁻²⁴ Choi et al reported on the protective effects of white ginseng, which attenuates neuronal damage and neuroinflammation in the A β -injected mouse hippocampus.²² Fu et al reported on the anti-AD effect of Danggui-Shaoyao-San, a traditional oriental herbal formula.²³ Leu et al reported the therapeutic potential of the traditional medicinal plants, *Polygonum multiflorum* and *Sorbus commixta*, for the treatment of neurodegenerative diseases such as AD.²⁴ Hence, in the current study, we examined the activity of the Korean herbal medicine, *C. asiaticum* seeds, for the treatment of AD using an in vitro experimental model. Our results demonstrate that ECAS has protective effects against neuronal cell damage and neuroinflammation.

A β and AChE are central biomarkers for AD.²⁵ In the brain of AD patients, extracellular plaques of aggregated A β proteins are observed and contribute to neurodegeneration. Acute exposure to A β has no toxic effect, whereas long-term exposure mediates cholinergic cellular toxicity.²⁶ In this study, the effects of ECAS on AChE and A β were measured using in vitro cell-free systems. As shown in Figure 1(A), 100 μ g/mL of ECAS dramatically inhibited AChE activity by 78.3%, whereas berberine, a positive control as an AChE inhibitor,²⁷ reduced the activity by 52.9%. By contrast, ECAS had no significant inhibitory effect on A β aggregation, whereas morin, a positive control of the A β aggregation antagonist, inhibited it by 70.0% (Figure 1(B)). To confirm further the inhibition of AChE by ECAS, dose-response activity on AChE inhibition was evaluated. ECAS increased the inhibition of AChE activity in a dose-dependent manner (Figure 1(C)).

The pathogenesis of AD is multifaceted, and it is not enough to assess the effects on a single biomarker. Neuronal death underlies various neurodegenerative diseases, including AD as well as Parkinson's and Huntington's diseases.²⁸ Thus, we additionally investigated whether the ECAS actions are involved in the regulation of neuronal death using HT22 hippocampal cells. Because neuron cells contain high contents of

polyunsaturated fatty acids, have high oxygen consumption, and have vulnerable defense against antioxidants, they are weak to oxidative stress.²⁹ We and others reported that H₂O₂ treatment triggers oxidative stress-mediated cell death in neuron cells from the hippocampus.³⁰⁻³²

Consistently, the current study showed that cell viability was maintained at over 90% when treated with ≤ 25 μ g/mL of ECAS and only at 83.1% when treated with 50 μ g/mL in the cell counting kit (CCK) assay (Figure 2(A)). Nontoxic concentrations of extracts were used for subsequent assays. Neuronal cell damage was induced by exposing HT22 cells to H₂O₂. Cell viability was significantly reduced in H₂O₂-treated cells compared with untreated controls. Co-treatment of cells with ECAS and H₂O₂ significantly reversed the neuronal cell death induced by H₂O₂ treatment. The neuroprotective effect of ECAS peaked at 12.5 μ g/mL and was reduced in a dose-dependent manner (Figure 2(B), left). Carvedilol, a neuroprotective agent, was used as a positive control.³³ To confirm further the effect of ECAS on neuroprotection, the release of lactate dehydrogenase (LDH) was evaluated. Consistent with the results of the CCK assay, ECAS significantly prevented H₂O₂-induced LDH release (Figure 2(B), right).

Mechanisms of neuroprotection are regulated by multiple molecular signals such as cyclic adenosine monophosphate response element-binding protein (CREB) and/or mitogen-activated protein kinase (MAPK) pathways. CREB is an essential molecule in the memory system.³⁴ Many researchers have reported that inactivation of CREB contributes to memory impairment and neuroprotection in vitro or in vivo models of AD.³⁵⁻³⁷ Our data revealed that compared with the untreated control, treatment with H₂O₂ alone suppressed phosphorylation of CREB (Figure 2(C) and (D) left panel), which is crucially involved in neuronal cell survival, memory, and synaptic transmission in the brain.³⁸ p38 MAPK is another important factor involved in the regulation of neurocellular protection.^{39,40} In a recent review paper by Lee and Kim, it is of interest that p38 MAPK plays a crucial role in the orchestration of a variety of events in AD progression such as neuronal cell death, inflammation, and synaptic dysfunction, suggesting a potential of p38 MAPK signaling as a vital target for the treatment of AD.⁴¹ Furthermore, it enhanced the phosphorylation of p38 MAPK, which plays an important role in neuronal death under oxidative stress.³⁹ In accordance with previous reports, H₂O₂ treatment clearly enhanced the level of phospho-p38 MAPK (Figure 2(C) and (D) right panel). By contrast, ECAS treatment markedly suppressed the H₂O₂-mediated phosphorylation of p38 MAPK in a dose-dependent manner (Figure 2(C) and (D)). Taken together, our results suggest that the beneficial effects of ECAS on neuroprotection may be regulated by the CREB/p38 MAPK signaling pathways.

Neuroinflammation is closely related to the progression of multiple neurodegenerative diseases, including AD,^{42,43} and is characterized by microglial activation that has been observed in regions associated with A β deposition.⁴⁴ Microglial activation

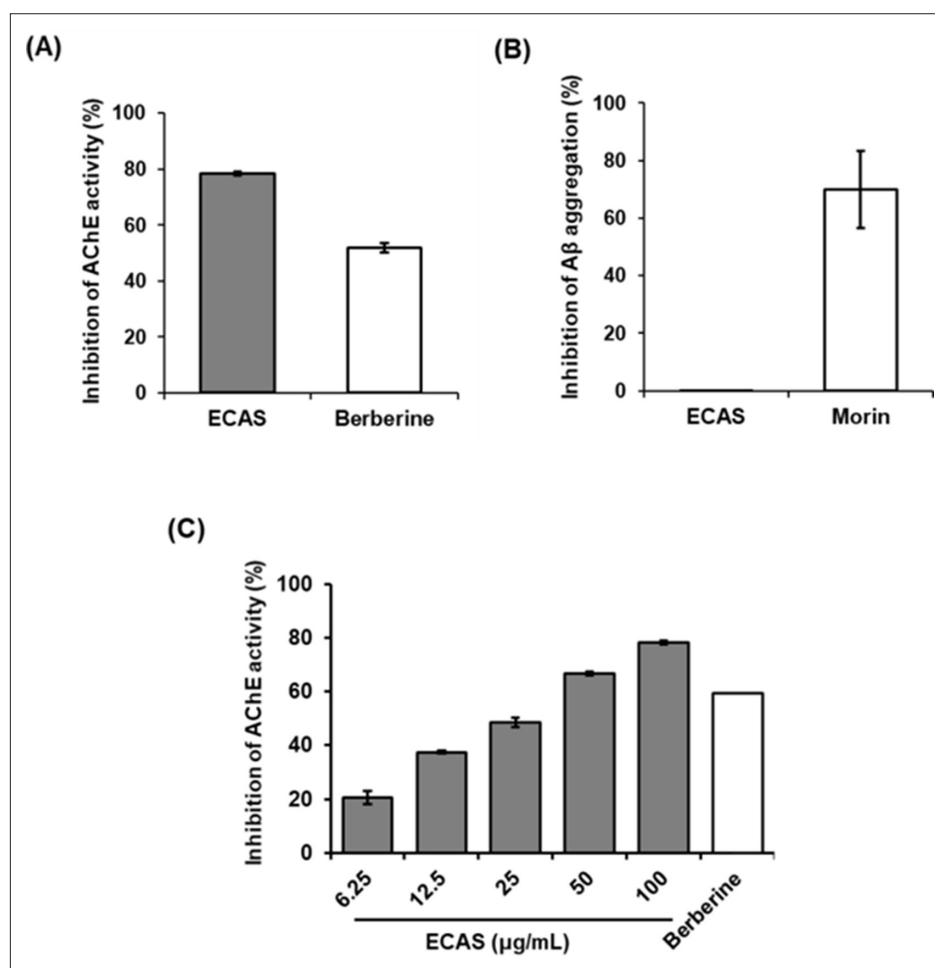


Figure 1. Inhibitory effects of ECAS on AChE activity and A β aggregation (A and C). An AChE activity assay was performed using the modified Ellman's colorimetric method (B). A β aggregation assay was performed using the SensoLyte Thioflavin T β -amyloid aggregation kit (AnaSpec, Fremont, CA, USA). (A) Effect of ECAS on AChE activity at 100 μ g/mL. Berberine was used as a positive control. (B) Effect of ECAS on A β aggregation at 100 μ g/mL. Morin was used as a positive control. (C) Effect of ECAS on AChE activity at various concentrations (6.25, 12.5, 25, 50, or 100 μ g/mL). Berberine was used as a positive control. Each value is presented as mean $\pm$ SEM. AChE, acetylcholinesterase; A β , amyloid- β ; ECAS, ethanol extract of *Crinum asiaticum* seeds.

induces the production of various biomolecules such as nitric oxide (NO) and proinflammatory factors. Among them, NO is the most potent molecule in neuroprotection⁴⁵ and regulates the release of proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in AD.⁴⁶ In our study, we utilized BV-2 microglia to examine the inhibitory effect of ECAS on neuroinflammation (Figure 3(A)). Under different ranges of nontoxic concentrations, the anti-inflammatory effects of ECAS were compared by measuring the amount of NO. As shown in Figure 3(B), ECAS significantly decreased lipopolysaccharide (LPS)-stimulated NO production. At the highest nontoxic concentrations of ECAS, NO production was inhibited by 29.4% compared with LPS alone (30.2%). The inhibitory activity of ECAS at 50 μ g/mL was similar to that of an anti-inflammatory drug ibuprofen, a positive control.⁴⁷ Inducible nitric oxide (iNOS) is a protein responsible for the production of cellular NO.⁴⁸ Western blot analyses were performed to confirm further the role

of NO in the antineuroinflammation activity of ECAS. Consistently, ECAS treatment clearly reduced the expression of iNOS at the protein level in the LPS-stimulated BV-2 microglia (Figure 3(C)). These results suggest that NO-related signaling is involved in the ECAS-induced reduction of the inflammatory response in microglia. Next, we examined the effects of ECAS on the production of proinflammatory cytokines. Microglial activation by LPS significantly increased the levels of TNF- α and IL-6 compared with untreated cells (Figure 3(D)). ECAS treatment did not have an inhibitory effect on the LPS-mediated generation of TNF- α and IL-6 in BV-2 cells. Overall, the anti-neuroinflammatory effects of ECAS are associated with NO signaling but independent of inflammatory cytokine production.

To establish quality control of ECAS, HPLC analysis was conducted. We obtained good separation chromatograms for lycorine from ECAS using mobile phases consisting of 0.02% (v/v) aqueous trifluoroacetic acid (TFA) (A) and acetonitrile

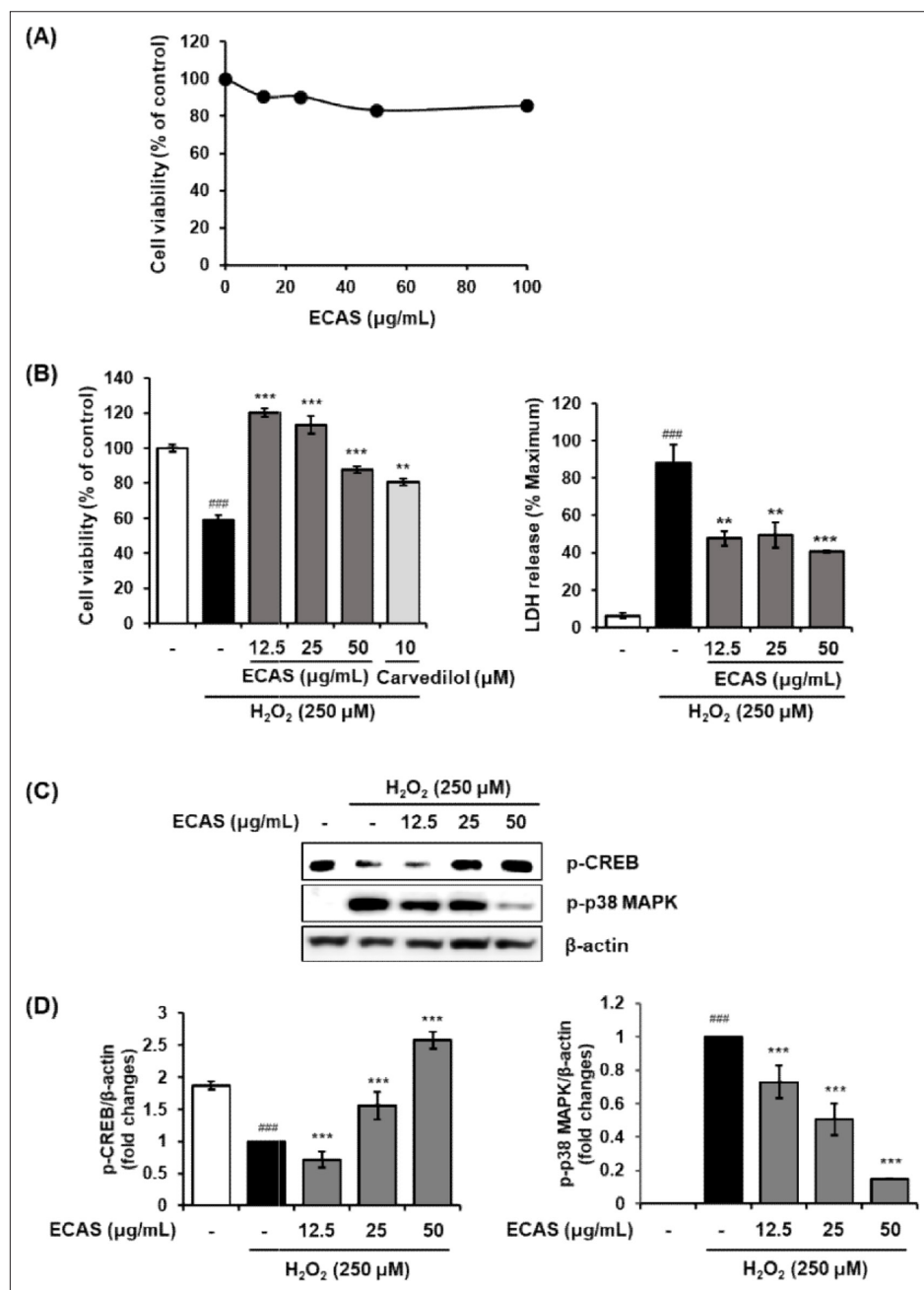


Figure 2. Neuroprotective effects of ECAS in H₂O₂-damaged HT22 cells. (A) HT22 cells were treated with various concentrations of ECAS for 24 hours. The cytotoxicity of ECAS was measured using a CCK assay. (B-D) Cells were exposed to H₂O₂ in either the absence or presence of ECAS for 6 hours. (B) Left: cell viability was determined using a CCK assay kit. Carvedilol was used as a positive control. Right: an LDH release assay was performed to evaluate the toxic effect of ECAS. The results are expressed as the mean $\pm$ SEM of 3 independent experiments. ### $P < 0.001$ versus vehicle control cells; ** $P < 0.01$ or *** $P < 0.001$ versus H₂O₂-treated cells. (C) Cell lysates were prepared and subjected to immunoblotting using anti-phospho-CREB and p38 MAPK antibodies. β -actin was used as an internal control. (D) Bar graphs represent the relative changes of phospho-levels of CREB and p38 MAPK. CCK, cell counting kit; CREB, cyclic adenosine monophosphate response element-binding protein; ECAS, ethanol extract of *Crinum asiaticum* seeds; H₂O₂, hydrogen peroxide; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase.

(B). The photodiode array (PDA) detector wavelength ranged from 190 to 400 nm, and the ultraviolet (UV) wavelength was recorded at 240 nm for lycorine. Under these established HPLC methods, the retention time of lycorine was 8.361 minutes. We also determined the content of lycorine. The linear

relationship between the peak area (y) and concentration (x , $\mu\text{g/mL}$) of the component was expressed by the regression equation ($y = ax + b$) as shown in Table 1. The calibration curve of lycorine showed good linearity with a correlation coefficient (r^2) of 1.0000 for 7 different concentration range,

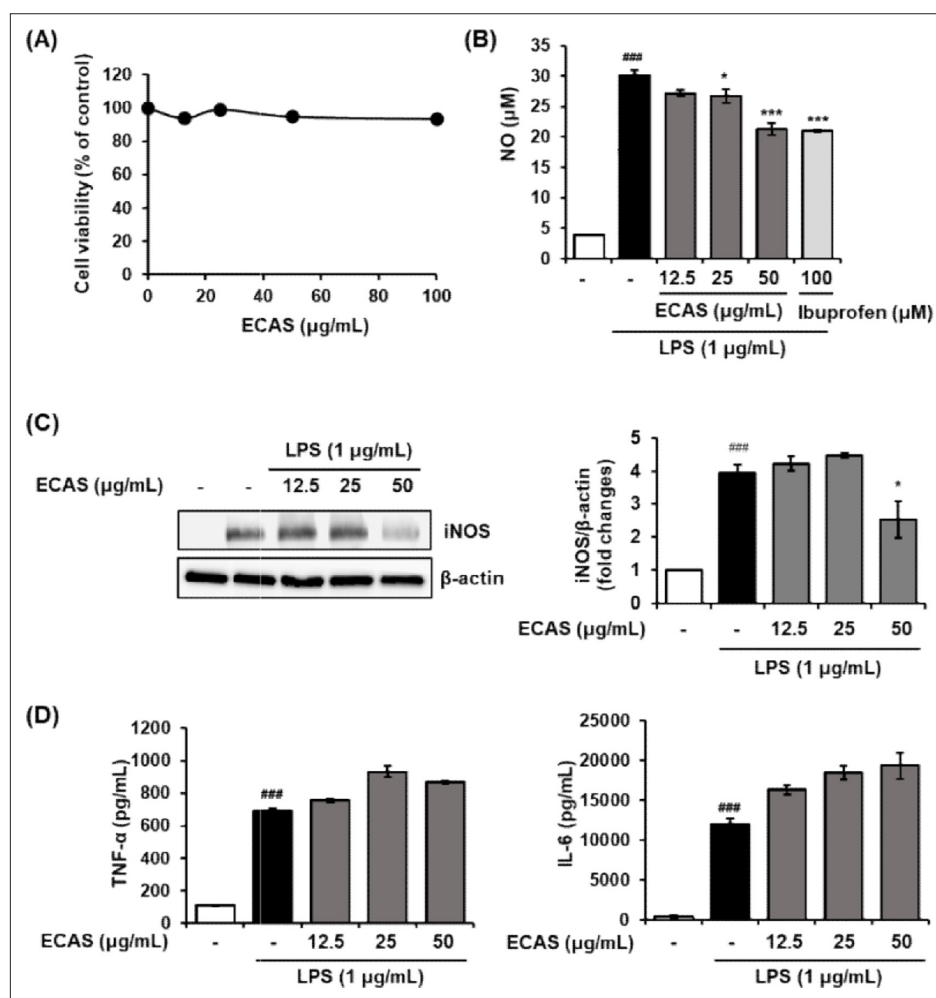


Figure 3. Inhibitory effects of ECAS on neuroinflammation in LPS-stimulated BV-2 cells. (A) BV-2 cells were treated with ECAS for 24 hours. The cytotoxicity of ECAS was measured using a CCK assay. (B) Cells were pretreated with ECAS for 2 hours and then stimulated with LPS (1 µg/mL) for an additional 22 hours. The production of NO was measured in the culture medium and assessed using Griess reagent. Ibuprofen was used as a positive control. (C and D) BV-2 cells were pretreated with various concentrations of ECAS for 2 hours and incubated with LPS (1 µg/mL) for an additional 22 hours. (C) Cell lysates were prepared and subjected to Western blotting using an anti-iNOS antibody. Bar graphs represent the relative changes in iNOS expression. (D) Cells were pretreated with ECAS for 2 hours and then stimulated with LPS (1 µg/mL) for an additional 22 hours. The production of TNF-α (A) and IL-6 (B) were measured in the culture medium and assessed using enzyme-linked immunosorbent assay kits. The results are expressed as mean ± SEM of 3 independent experiments. ^{###}*P* < 0.001 versus vehicle control cells; ^{*}*P* < 0.05 and ^{***}*P* < 0.001 versus LPS-treated cells. CCK, cell counting kit; ECAS, ethanol extract of *Crinum asiaticum* seeds; IL-6, interleukin 6; iNOS, inducible nitric oxide; LPS, lipopolysaccharide; NO, nitric oxide; TNF-α, tumor necrosis factor-alpha.

1.5625–100 µg/mL. The limit of detection (LOD) and limit of quantitation (LOQ) for the lycorine were 0.138 µg/mL and 0.420 µg/mL, respectively. The developed HPLC analytical method was applied to the quantitative analysis of lycorine in *C. asiaticum*; the amount was 4.802 mg/g. Lycorine is a pyrrolo-phenanthridine alkaloid found in many Amaryllidaceae.⁴⁹ Davey et al presented a report on the purification and analysis of lycorine from 2 *Crinum* species by HPLC.⁵⁰ Several other reports used lycorine as a standard compound for *Zephyranthes grandiflora*, *Galanthus* species, and *Hymenocallis littoralis* in HPLC analyses.^{51–53} Biologically, lycorine is known as a specific inhibitor for vitamin C synthesis.⁵⁴ Additionally, lycorine has been recorded as having antitumor, anti-inflammation, and

antioxidation properties.^{11,55,56} In our study, we observed that lycorine inhibited AChE activity by 30% (data not shown). Further investigations are required to identify the compounds responsible for the other peaks of the HPLC chromatogram and determine the bioactive compounds of ECAS.

In summary, the present study demonstrates the anti-AD properties of *C. asiaticum* seeds by inhibiting the enzymatic activity of AChE, one of the major biomarkers of AD. We also observed that *C. asiaticum* seeds have neuroprotective activity, regulating phosphorylation of CREB and p38 MAPK against damaged neuronal cells by oxidative stress. Additionally, blocking the generation of pro-inflammatory mediator NO addressed the antineuroinflammatory effects of *C. asiaticum*

Table 1. Linear Range, Regression Equation, Correlation Coefficient LOD, LOQ, and Content for Lycorine in Ethanol Extract of *Crinum asiaticum* Seeds.

Compound	Linear range ($\mu\text{g/mL}$)	Regression equation ($y = ax + b$)		Correlation coefficient (r^2)	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)	Content
		Slope (a)	Intercept (b)				
Lycorine	1.5625-100	4890	1113.5	1.0000	0.138	0.420	4.802 ± 0.025

$y = ax + b$, y means peak area and x means concentration ($\mu\text{g/mL}$).

LOD (limit of detection): $3.3 \times (\text{SD of the response/slope of the calibration curve})$.

LOQ (limit of quantitation): $10 \times (\text{SD of the response/slope of the calibration curve})$.

seeds in LPS-stimulated microglia. We are going to carry out in vivo assays to verify further the efficacy and molecular mechanisms of ECAS on the regulation of AD pathogenesis. Overall, our findings propose that *C. asiaticum* seeds could be a useful anti-AD drug candidate.

Materials and Methods

Plant Material and Preparation of ECAS

The seeds of *C. asiaticum* var. *japonicum* Baker were collected in Gimnyeong-ri, Gujwa-eup, Jeju-si, Jeju-do, South Korea, in September 2013, and identified by Professor Joo-Hwan Kim (Gachon University, Seongnam, South Korea). A voucher specimen has been deposited at the Herbarium in the Korea Institute of Oriental Medicine (Daejeon, South Korea). The dried seeds (30 g) were extracted 3 times with aqueous ethanol (300 mL) at room temperature for 7 days. The extracted solution was filtered using filter paper (5 μm) and concentrated using a rotary evaporator (EYELA N-1000, Rikakikai Co., Tokyo, Japan) under vacuum to obtain the ethanol extract (2.20 g). The yield of ECAS was 7.33%.

AChE Activity Assay

In vitro AChE activity was assessed according to the protocol based on Ellman's colorimetric method,⁵⁷ with modifications, using an AChE Assay Kit (Abcam, Cambridge, UK). The ECAS stock solution was dissolved in dimethyl sulfoxide at a concentration of 100 mg/mL. Assay samples were diluted with 0.1 M sodium phosphate buffer (pH 8.0). The final concentration of ECAS was 100 $\mu\text{g/mL}$. The AChE stock solution was prepared in 0.1% bovine serum albumin/water (H_2O) at 25 U/mL and dissolved in 0.1 M sodium phosphate buffer (pH 7.3, assay buffer), to a final concentration of 35.2 mU/mL, before the assay. The substrates, acetylthiocholine iodide and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), were dissolved in H_2O and assay buffer at 10 mM, and both solutions were diluted 20-fold in assay buffer to a final concentration of 0.5 mM. Briefly, 0.6 mL of H_2O and assay buffer were added in acetylthiocholine iodide and DTNB to prepare 10 mM stock solutions. For the assays, 0.25 mL of each of 10 mM acetylthiocholine iodide and DTNB were mixed in 4.75 mL of assay buffer to a final concentration of 0.5 mM and used as the reaction mixture. For the enzymatic reaction in 96-well plates, 50 μL of the sample solution and 50 μL of the reaction mixture were mixed

and preincubated for 10 minutes at room temperature. AChE solution (10 μL) was then added to the initial mixture to start the reaction, which was performed for 1 hour at room temperature. The absorbance was measured at 412 nm using an Epoch microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). The inhibition of the activity of AChE was calculated by comparing the rate of reaction of the sample with that of the blank. All experiments were performed in triplicate, and the percentage of inhibition against the AChE activity was calculated according to the following equation:

$$\text{AChE activity inhibition (\%)} = \left(1 - \frac{S - S'}{C - C'}\right) \times 100$$

S: samples (sample solution, assay buffer with DTNB, substrate, and enzyme);

S': samples (sample solution, assay buffer with DTNB and substrate, without enzyme);

C: control (0.1 M sodium phosphate buffer (pH 8.0), assay buffer with DTNB, substrate, and enzyme); and

C': control (0.1 M sodium phosphate buffer (pH 8.0), assay buffer with DTNB and substrate, without enzyme).

A β Aggregation Assay

A β (1-42) aggregation was measured using the SensoLyte Thioflavin T β -Amyloid aggregation kit (AnaSpec, Fremont, CA, USA), according to the manufacturer's instructions. The assay is based on the property of the Thioflavin T dye, the fluorescence of which increases when it is bound to aggregates of A β (1-42) peptides. Briefly, Thioflavin T was dissolved in assay buffer (50 mM Tris/150 mM sodium chloride [NaCl; pH = 7.2], 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/150 mM NaCl [pH = 7.2], 10 mM phosphate/150 mM NaCl) and used at a concentration of 100 μM . Samples were dissolved in assay buffer and used at a final concentration of 100 $\mu\text{g/mL}$. To measure the inhibition of A β (1-42) aggregation in 96-well black microplates, 5 μL of the sample and 85 μL of A β (1-42) were mixed, followed by the addition of 10 μL of Thioflavin T. Fluorescence of Thioflavin T was measured at intervals of 20 minutes for 2 hours, with an excitation wavelength (λ_{ex}) of 440 nm and an emission wavelength (λ_{em}) of 485 nm using a SpectraMax i3 Multi-Mode Detection Platform (Molecular Devices, Sunnyvale, CA, USA). This gave 7 readings for each well sample. All fluorescence readings are expressed in relative fluorescence units. Experiments were

performed in triplicate and averaged, and the percentage of inhibition of A β aggregation was calculated according to the following equation:

$$\text{A}\beta \text{ aggregation inhibition (\%)} = \left(1 - \frac{\text{fluorescence of A}\beta - \text{treated sample}}{\text{fluorescence of untreated sample}}\right) \times 100$$

Cell Lines and Culture

BV-2 and HT22 cells were maintained in Dulbecco's Modified Eagle's medium (Hyclone/Thermo, Rockford, IL, USA) supplemented with 10% fetal bovine serum (Hyclone/Thermo, Rockford, IL, USA) and penicillin/streptomycin in 5% carbon dioxide at 37 °C. BV-2 cells were pretreated with ECAS for 2 hours and treated with LPS (1 μ g/mL, Sigma-Aldrich, St. Louis, MO, USA) for an additional 22 hours. HT22 cells were co-treated with ECAS and H₂O₂ (500 μ M, Sigma-Aldrich, St. Louis, MO, USA) for 6 hours.

Cell Viability Test

The cytotoxic effects of ECAS against either HT22 or BV-2 cells were evaluated using the CCK-8 assay. BV-2 and HT22 cells were plated on 96-well microplates at a density of 3×10^4 /well and 5×10^3 /well, respectively. Cells were treated with various concentrations of ECAS for 24 hours. CCK-8 solution (Dojindo, Kumamoto, Japan) was added, and the cells were incubated for 4 hours. The absorbance was read at 450 nm on an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Mean OD in ECAS-treated cells}}{\text{Mean OD in untreated cells}} \times 100$$

LDH Assay

To determine the cytotoxic effect of ECAS, LDH release from H₂O₂-damaged HT22 cells was measured using the CytoTox 96 Nonradioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA). Cells were lysed to induce maximal LDH release, and supernatants were collected to measure experimental LDH release. Cell lysates or supernatants were reacted with a substrate mixture at room temperature for 30 minutes in the dark. After adding stop solution, the absorbance at 490 nm was measured on an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The cytotoxicity of ECAS was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{Experimental LDH release}}{\text{Maximum LDH release}} \times 100$$

Western Blotting

HT22 and BV-2 cells were treated with various concentrations of ECAS. Cell lysates were prepared using CellLytic MT Cell Lysis Reagent (Sigma-Aldrich, St. Louis, MO, USA) containing

a protease inhibitor cocktail (GenDEPOT, Barker, TX, USA). The protein concentration was determined using Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA). Equal amounts of cell extract were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4%-20% Mini-PROTEANTGX Precast gels and transferred to polyvinylidene fluoride membranes. The membranes were incubated with a blocking solution (5% skim milk in Tris-buffered saline containing Tween 20 [TBST]), followed by overnight incubation at 4 °C with the appropriate primary antibodies: phospho-CREB, phospho-p38 MAPK, iNOS (Cell Signaling Technology, Beverly, MA, USA), and β -actin (Santa Cruz Biotechnology, Dallas, TX, USA). The membranes were washed 3 times with TBST and then incubated with a horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA, USA) for 1 hour at room temperature. The membranes were again washed 3 times with TBST and then developed using an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL, USA). The images were captured using an ImageQuant LAS 4000 mini Luminescent Image Analyzer (GE Health Care Bio-Sciences, Piscataway, NJ, USA).

NO Assay

BV-2 cells were pretreated with various concentrations of ECAS for 2 hours and stimulated with LPS (1 μ g/mL) for an additional 22 hours. The NO synthesis was analyzed by determining the accumulation of nitrite (NO₂⁻) in the culture supernatant using the Griess Reagent System (Promega, Madison, WI, USA). Equal volumes of supernatant and sulfanilamide solution were mixed and incubated for 10 minutes at room temperature and then added to naphthylethylenediamine dihydrochloride solution for an additional 5 minutes. The absorbance was measured at 540 nm using an Epoch Microplate Spectrophotometer. The nitrite concentration was determined from a standard curve generated with sodium nitrite (NaNO₂).

Measurement of TNF- α and IL-6 Production

BV-2 cells were treated with various concentrations of ECAS for 2 hours and stimulated with LPS for 22 hours. An enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems; Minneapolis, MN, USA) was used for the measurement of TNF- α and IL-6 according to the manufacturers' protocols. Briefly, the supernatant of the cell culture was collected and added to each well of the ELISA plates with the appropriate antibodies. Optical density was measured at 450 nm, and the concentrations of TNF- α and IL-6 were calculated by creating a standard curve.

Chemicals and Reagents

Lycorine, purchased from Biopurify Phytochemicals Ltd. (Chengdu, China), had a purity of $\geq 98.0\%$ by HPLC analysis. HPLC-grade solvents, acetonitrile, and water were purchased

from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA), and analytical grade reagent TFA was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Chromatographic Conditions

The quantitative analysis was performed using a Waters Alliance e2695 HPLC system (Waters Corp., Milford, MA, USA) equipped with a pump, degasser, column oven, auto sample injector, and PDA detector (Waters Corp., #2998). The data were acquired and processed using Empower software (version 3; Waters Corp). The chromatographic separation for the standard component was performed at room temperature using a Luna C₁₈ analytical column (250 × 4.6 mm, 5 μm, Phenomenex, Torrance, CA, USA). The gradient elution system with 2 mobile phase consisting of 0.02% (v/v) aqueous TFA (A) and acetonitrile (B) was as follows: 0%-20% B for 0-10 minutes, 20%-100% B for 10-11 minutes, and 100% B for 11-18 minutes. The flow rate of 1.0 mL/min and the volume injection of 10 μL were constant in all cases. The UV wavelength for detecting lycorine was 240 nm.

Preparation of Standard and Sample Solutions

Lycorine, weighed accurately, was dissolved in methanol at 1.0 mg/mL and stored below 4 °C. This stock solution was diluted with methanol to yield a series of standard solutions with different concentrations for quantitative analysis.

The dried seeds of *C. asiaticum* (30 g) were extracted 3 times with aqueous ethanol (300 mL) at room temperature for 7 days. The extracted solution was filtered through a filter paper (5 μm) and concentrated using a rotary evaporator (EYELA N-1000, Rikakikai Co., Tokyo, Japan) under vacuum to give the ethanol extract (2.20 g). The yield of ECAS was 7.33%. The ECAS was weighed accurately and dissolved in methanol at a concentration of 10 mg/mL. The sample solution was filtered through a syringe filter (0.45 μm) before HPLC analysis.

Calibration Curve, LOD, and LOQ

The calibration curve of the component was obtained by assessment of the peak areas from the standard solutions at 7 different concentrations. The tested concentration range was 1.5625-100 μg/mL for lycorine. The LOD and LOQ for the component were calculated using the slope of the calibration curve and the SD of the intercept as follows:

LOD = 3.3 × (SD of the response/slope of the calibration curve); and

LOQ = 10 × (SD of the response/slope of the calibration curve).

Statistical Analysis

The data are expressed as the mean ± SEM. Data were analyzed using a one-way analysis of variance and Dunnett's multiple comparisons test. *P* < 0.05 was considered significant.

Acknowledgments

We thank the Herbal Medicine Research Division, Korea Institute of Oriental Medicine (Dr Jin Sook Kim) and the Korean Seed Association for the kind donation of plant material.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the Korea Institute of Oriental Medicine under grant (KSN1515293 and KSN2013240).

ORCID ID

Yu Jin Kim  <https://orcid.org/0000-0001-5152-9439>

References

1. Rakonczay Z, Horváth Z, Juhász A, Kálmán J. Peripheral cholinergic disturbances in Alzheimer's disease. *Chem Biol Interact.* 2005;157-158:233-238. doi:10.1016/j.cbi.2005.10.034
2. Hebert LE, Weuve J, Scherr PA, Evans DA. Alzheimer disease in the United States (2010-2050) estimated using the 2010 census. *Neurology.* 2013;80(19):1778-1783. doi:10.1212/WNL.0b013e31828726f5
3. Kim YJ, Han JW, So YS, Seo JY, Kim KY, Kim KW. Prevalence and trends of dementia in Korea: a systematic review and meta-analysis. *J Korean Med Sci.* 2014;29(7):903-912. doi:10.3346/jkms.2014.29.7.903
4. Birks J, Harvey RJ. Donepezil for dementia due to Alzheimer's disease. *Cochrane Database Syst Rev.* 2006;25(1):CD001190.
5. Birks JS, Grimley Evans J. Rivastigmine for Alzheimer's disease. *Cochrane Database Syst Rev.* 2015;10(4):CD001191.
6. Alldredge BK, Corelli RL, Ernst ME, et al. *Applied Therapeutics: The Clinical Use of Drugs*, 10th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins; 2013:2385.
7. Kim YH, Kim KH, Han CS, et al. Anti-inflammatory activity of *Crinum asiaticum* Linne var. *japonicum* extract and its application as a cosmeceutical ingredient. *J Cosmet Sci.* 2008;59(5):419-430.
8. Samud AM, Asmawi MZ, Sharma JN, Yusof AP. Anti-inflammatory activity of *Crinum asiaticum* plant and its effect on bradykinin-induced contractions on isolated uterus. *Immunopharmacology.* 1999;43(2-3):311-316. doi:10.1016/S0162-3109(99)00132-0

9. Jeong YJ, Sohn E-H, Jung Y-H, et al. Anti-obesity effect of *Crinum asiaticum* var. *japonicum* Baker extract in high-fat diet-induced and monogenic obese mice. *Biomed Pharmacother*. 2016;82:35-43. doi:10.1016/j.biopha.2016.04.067
10. Min BS, Kim YH, Tomiyama M, et al. Inhibitory effects of Korean plants on HIV-1 activities. *Phytother Res*. 2001;15(6):481-486. doi:10.1002/ptr.751
11. Ilavenil S, Kaleeswaran B, Sumitha P, Tamilvendan D, Ravikumar S. Protection of human erythrocyte using *Crinum asiaticum* extract and lycorine from oxidative damage induced by 2-amidinopropane. *Saudi J Biol Sci*. 2011;18(2):181-187. doi:10.1016/j.sjbs.2010.11.001
12. Chen C-K, Lin F-H, Tseng L-H, Jiang C-L, Lee S-S. Comprehensive study of alkaloids from *Crinum asiaticum* var. *sinicum* assisted by HPLC-DAD-SPE-NMR. *J Nat Prod*. 2011;74(3):411-419. doi:10.1021/np100819n
13. Kogure N, Katsuta N, Kitajima M, Takayama H. Two new alkaloids from *Crinum asiaticum* var. *sinicum*. *Chem Pharm Bull*. 2011;59(12):1545-1548. doi:10.1248/cpb.59.1545
14. Zhang X, Huang H, Liang X, et al. Analysis of amaryllidaceae alkaloids from *Crinum* by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2009;23(18):2903-2916. doi:10.1002/rcm.4205
15. Zucchella C, Bartolo M, Bernini S, Picascia M, Sinforiani E. Quality of life in Alzheimer disease: a comparison of patients' and caregivers' points of view. *Alzheimer Dis Assoc Disord*. 2015;29(1):50-54. doi:10.1097/WAD.0000000000000050
16. Sayer R, Law E, Connelly PJ, Breen KC. Association of a salivary acetylcholinesterase with Alzheimer's disease and response to cholinesterase inhibitors. *Clin Biochem*. 2004;37(2):98-104. doi:10.1016/j.clinbiochem.2003.10.007
17. Cohen SIA, Cukalevski R, Michaels TCT, et al. Distinct thermodynamic signatures of oligomer generation in the aggregation of the amyloid- β peptide. *Nat Chem*. 2018;10(5):523-531. doi:10.1038/s41557-018-0023-x
18. Cho HW, Jung SY, Lee GH, Cho JH, Choi IY. Neuroprotective effect of *Citrus unshiu* immature peel and nobiletin inhibiting hydrogen peroxide-induced oxidative stress in HT22 murine hippocampal neuronal cells. *Pharmacogn Mag*. 2015;11(Suppl 2):284-289. doi:10.4103/0973-1296.166047
19. Fan Z, Dani M, Femminella GD, et al. Parametric mapping using spectral analysis for 11C-PBR28 PET reveals neuroinflammation in mild cognitive impairment subjects. *Eur J Nucl Med Mol Imaging*. 2018;45(8):1432-1441. doi:10.1007/s00259-018-3984-5
20. Sanabria-Castro A, Alvarado-Echeverría I, Monge-Bonilla C. Molecular pathogenesis of Alzheimer's disease: an update. *Ann Neurosci*. 2017;24(1):46-54. doi:10.1159/000464422
21. Jeong S-J, Yoo S-R, Seo C-S, Shin H-K. Traditional Korean herbal formula Samsocum attenuates adipogenesis by regulating the phosphorylation of ERK1/2 in 3T3-L1 cells. *Evid Based Complement Alternat Med*. 2015;2015(10):893934-11. doi:10.1155/2015/893934
22. Choi JG, Kim N, Huh E, et al. White ginseng protects mouse hippocampal cells against amyloid-beta oligomer toxicity. *Phytother Res*. 2017;31(3):497-506. doi:10.1002/ptr.5776
23. Fu X, Wang Q, Wang Z, Kuang H, Jiang P. Danggui-Shaoyao-San: new hope for Alzheimer's disease. *Aging Dis*. 2016;7(4):502-513. doi:10.14336/AD.2015.1220
24. Liu QF, Lee JH, Kim Y-M, et al. *In vivo* screening of traditional medicinal plants for neuroprotective activity against A β 42 cytotoxicity by using *Drosophila* models of Alzheimer's disease. *Biol Pharm Bull*. 2015;38(12):1891-1901. doi:10.1248/bpb.b15-00459
25. Reale M, D'Angelo C, Costantini E, et al. Expression profiling of cytokine, cholinergic markers, and amyloid- β deposition in the APPSWE/PS1dE9 mouse model of Alzheimer's disease pathology. *J Alzheimers Dis*. 2018;62(1):467-476. doi:10.3233/JAD-170999
26. Kar S, Slowikowski SP, Westaway D, Mount HT. Interactions between beta-amyloid and central cholinergic neurons: implications for Alzheimer's disease. *J Psychiatry Neurosci*. 2004;29(6):427-441.
27. Kaufmann D, Kaur Dogra A, Tahrani A, Herrmann F, Wink M. Extracts from traditional Chinese medicinal plants inhibit acetylcholinesterase, a known Alzheimer's disease target. *Molecules*. 2016;21(9):1161. doi:10.3390/molecules21091161
28. Mattson MP. Apoptosis in neurodegenerative disorders. *Nat Rev Mol Cell Biol*. 2000;1(2):120-130. doi:10.1038/35040009
29. Rego AC, Oliveira CR. Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. *Neurochem Res*. 2003;28(10):1563-1574. doi:10.1023/A:1025682611389
30. Byun EB, Park WY, Kim WS, Song HY, Sung NY, Byun EH. Neuroprotective effect of *Capsicum annuum* var. *abbreviatum* against hydrogen peroxide-induced oxidative stress in HT22 hippocampus cells. *Biosci Biotechnol Biochem*. 2018;82(12):2149-2157.
31. Zhao Z-Y, Luan P, Huang S-X, et al. Edaravone protects HT22 neurons from H₂O₂-induced apoptosis by inhibiting the MAPK signaling pathway. *CNS Neurosci Ther*. 2013;19(3):163-169. doi:10.1111/cns.12044
32. Kim YJ, Lim H-S, Kim B-Y, Seo C-S, Jeong S-J. Quantitative analysis and biological efficacies regarding the neuroprotective and antineuroinflammatory actions of the herbal formula Jodeung-san in HT22 hippocampal cells and BV-2 microglia. *Evid Based Complement Alternat Med*. 2017;2017(4):1-10. doi:10.1155/2017/6360836
33. Ouyang Y, Chen Z, Tan M, et al. Carvedilol, a third-generation β -blocker prevents oxidative stress-induced neuronal death and activates Nrf2/ARE pathway in HT22 cells. *Biochem Biophys Res Commun*. 2013;441(4):917-922. doi:10.1016/j.bbrc.2013.10.160
34. Kandel ER. The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. *Mol Brain*. 2012;5(1):14. doi:10.1186/1756-6606-5-14
35. YH K, Kim SY, Lee SY, Jang CG. 6,7,4'-Trihydroxyisoflavone, a major metabolite of daidzein, improves learning and memory via the cholinergic system and the p-CREB/BDNF signaling pathway in mice. *Eur J Pharmacol*. 2018;826:140-147.

36. Ettcheto M, Abad S, Petrov D, et al. Early Preclinical changes in hippocampal CREB-binding protein expression in a mouse model of familial Alzheimer's Disease. *Mol Neurobiol.* 2018;55(6):4885-4895. doi:10.1007/s12035-017-0690-4
37. Guo H, Cheng Y, Wang C, et al. FPPM, a PDE4 inhibitor, reverses learning and memory deficits in APP/PS1 transgenic mice via cAMP/PKA/CREB signaling and anti-inflammatory effects. *Neuropharmacology.* 2017;116:260-269. doi:10.1016/j.neuropharm.2017.01.004
38. Mi Y, Qi G, Fan R, et al. EGCG ameliorates high-fat- and high-fructose-induced cognitive defects by regulating the IRS/AKT and ERK/CREB/BDNF signaling pathways in the CNS. *FASEB J.* 2017;31(11):4998-5011. doi:10.1096/fj.2017.00400RR
39. Amin FU, Shah SA, Badshah H, Khan M, Kim MO. Anthocyanins encapsulated by PLGA@PEG nanoparticles potentially improved its free radical scavenging capabilities via p38/JNK pathway against A β_{1-42} -induced oxidative stress. *J Nanobiotechnology.* 2017;15(1):12. doi:10.1186/s12951-016-0227-4
40. Shen J-N, Xu L-X, Shan L, Zhang W-D, Li H-L, Wang R. Neuroprotection of (+)-2-(1-hydroxyl-4-oxocyclohexyl) ethyl caffeate against hydrogen peroxide and lipopolysaccharide induced injury via modulating arachidonic acid network and p38-MAPK signaling. *Curr Alzheimer Res.* 2015;12(9):892-902. doi:10.2174/156720501209151019111244
41. Lee JK, Kim N-J. Recent advances in the inhibition of p38 MAPK as a potential strategy for the treatment of alzheimer's disease. *Molecules.* 2017;22(8):1287. doi:10.3390/molecules22081287
42. McGeer PL, Itagaki S, Boyes BE, McGeer EG. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology.* 1988;38(8):1285-1291. doi:10.1212/WNL.38.8.1285
43. Rogers J, Webster S, Lue LF, et al. Inflammation and Alzheimer's disease pathogenesis. *Neurobiol Aging.* 1996;17(5):681-686. doi:10.1016/0197-4580(96)00115-7
44. Edison P, Archer HA, Gerhard A, et al. Microglia, amyloid, and cognition in Alzheimer's disease: An [11C](R)PK11195-PET and [11C]PIB-PET study. *Neurobiol Dis.* 2008;32(3):412-419. doi:10.1016/j.nbd.2008.08.001
45. Pansiot J, Loron G, Olivier P, et al. Neuroprotective effect of inhaled nitric oxide on excitotoxic-induced brain damage in neonatal rat. *PLoS One.* 2010;5(6):e10916. doi:10.1371/journal.pone.0010916
46. Tai Y, Qiu Y, Bao Z. Magnesium lithospermate B suppresses lipopolysaccharide-induced neuroinflammation in BV2 microglial cells and attenuates neurodegeneration in lipopolysaccharide-injected mice. *J Mol Neurosci.* 2018;64(1):80-92. doi:10.1007/s12031-017-1007-9
47. Carty ML, Wixey JA, Reinebrant HE, Gobe G, Colditz PB, Buller KM. Ibuprofen inhibits neuroinflammation and attenuates white matter damage following hypoxia-ischemia in the immature rodent brain. *Brain Res.* 2011;1402:9-19. doi:10.1016/j.brainres.2011.06.001
48. McAdam E, Haboubi HN, Forrester G, et al. Inducible nitric oxide synthase (iNOS) and nitric oxide (NO) are important mediators of reflux-induced cell signalling in esophageal cells. *Carcinogenesis.* 2012;33(11):2035-2043. doi:10.1093/carcin/bgs241
49. Ghosal S, S. Saini K, Razdan S. *Crinum* alkaloids: their chemistry and biology. *Phytochemistry.* 1985;24(10):2141-2156. doi:10.1016/S0031-9422(00)83001-0
50. Davey MW, Persiau G, De Bruyn A, Van Damme J, Bauw G, Van Montagu M. Purification of the alkaloid lycorine and simultaneous analysis of ascorbic acid and lycorine by micellar electrokinetic capillary chromatography. *Anal Biochem.* 1998;257(1):80-88. doi:10.1006/abio.1997.2544
51. Katoch D, Kumar S, Kumar N, Singh B. Simultaneous quantification of Amaryllidaceae alkaloids from *Zephyranthes grandiflora* by UPLC-DAD/ESI-MS/MS. *J Pharm Biomed Anal.* 2012;71:187-192. doi:10.1016/j.jpba.2012.08.001
52. Kaya GI, Polat DC, Sarikaya B, Onur MA, Somer NU. Quantitative determination of lycorine and galanthamine in *Galanthus trojanus* and *G. Cilicicus* by HPLC-DAD. *Nat Prod Commun.* 2014;9(8):1157-1158. doi:10.1177/1934578X1400900824
53. Subramaniam S, Sundarasekar J, Sahgal G, Murugaiyah V. Comparative analysis of lycorine in wild plant and callus culture samples of *Hymenocallis littoralis* by HPLC-UV method. *Sci World J.* 2014;2014(24):1-6. doi:10.1155/2014/408306
54. De Gara L, Tommasi F. Further researches upon the inhibiting action of lycorine on ascorbic acid biosynthesis. *Boll Soc Ital Biol Sper.* 1990;66(10):9530-9560.
55. Min BS, Gao JJ, Nakamura N, Kim YH, Hattori M. Cytotoxic alkaloids and a flavan from the bulbs of *Crinum asiaticum* var. *japonicum*. *Chem Pharm Bull.* 2001;49(9):1217-1219. doi:10.1248/cpb.49.1217
56. Kang J, Zhang Y, Cao X, et al. Lycorine inhibits lipopolysaccharide-induced iNOS and COX-2 up-regulation in RAW264.7 cells through suppressing P38 and STATs activation and increases the survival rate of mice after LPS challenge. *Int Immunopharmacol.* 2012;12(1):249-256. doi:10.1016/j.intimp.2011.11.018
57. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol.* 1961;7(2):88-95. doi:10.1016/0006-2952(61)90145-9