In vivo screening of traditional medicinal plants for neuroprotective activity against Aβ42 cytotoxicity by using Drosophila models of Alzheimer’s disease

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Summary

Alzheimer’s disease (AD) is the most common neurodegenerative disorder, characterized by progressive neuronal loss with amyloid β-peptide (Aβ) plaques. Despite several drugs currently used to treat AD, their beneficial effects on AD progress remains under debate. Here, we established a rapid in vivo screening system using Drosophila AD models to assess the neuroprotective activities of medicinal plants that have been used in traditional Chinese medicine. Among 23 medicinal plants tested, the extracts from five plants, Coriandrum sativum, Nardostachys jatamansi, Polygonum multiflorum (P. multiflorum), Rehmannia glutinosa, and Sorbus commixta (S. commixta), showed protective effects against the Aβ42 neurotoxicity. We further characterized the neuroprotective activity of ethanol extracts from P. multiflorum and S. commixta. Aβ42-expressing flies that we used showed AD neurological phenotypes, such as decreased survival and motility and increased cell death and reactive oxygen species level. However, feeding these flies extracts from P. multiflorum or S. commixta showed strong suppression of such phenotypes. Similar results were observed in human cells, so that the treatment of P. multiflorum and S. commixta extracts increased the viability of Aβ-treated SH-SY5Y cells. Moreover, 2, 3, 5, 4′-tetrahydroxystilbene-2-O-β-D-glucoside, one of the main constituents of P. multiflorum, also showed similar protective activity against Aβ42 cytotoxicity in both Drosophila and human cells. Taken together, our results suggest that both P. multiflorum and S. commixta have therapeutic potential for the treatment of neurodegenerative diseases, such as AD.

Keywords: Aβ42, Alzheimer’s disease, Drosophila, Polygonum multiflorum, ROS, Sorbus commixta

Abbreviations: Aβ42, amyloid-β42; AD, Alzheimer’s disease; AO, acridine orange; DHE, dihydroethidium; EGFR, epidermal growth factor receptor; JNK, c-Jun N-terminal kinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; P. multiflorum, Polygonum multiflorum; S. commixta, Sorbus commixta.
Introduction

Alzheimer’s disease (AD) is a common neurodegenerative disease that is characterized by progressive neural loss caused by amyloid β-peptide (Aβ) plaques and cognitive deficits. During disease progression, neurotoxic forms of Aβ cause neuronal damages via various cellular abnormalities such as increased oxidative damage, impaired energy metabolism, disrupted cellular calcium homeostasis, and increased inflammatory response. Several drugs have been approved to treat AD, given the dysfunction of cholinergic and glutamatergic neurotransmission; they function as acetylcholinesterase inhibitors or N-methyl-D-aspartate (NMDA) receptor antagonists. However, the beneficial effects of these drugs during the progression of AD are still in question.

Due to its complex etiology, a combination therapy including the use of medicinal plants with effective components has been proposed as an alternative choice for treatment of AD. Chinese traditional medicine has employed a variety of herbs to treat dementia, and a number of neuroprotective agents were isolated from these herbs. For example, Polygonum multiflorum (P. multiflorum; Syn. Reynoutria multiflora) has been known as a tonic and antiaging agent in many remedies for centuries, and the use of its dried root to treat cognitive disorders including AD has been reported. Moreover, one of the main active ingredients of P. multiflorum, a monomer of stilbene (2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside), was shown to exhibit beneficial effects on some neurodegenerative diseases via its antioxidative and anti-inflammatory properties.

Drosophila has been used as a model system for human diseases as well as animal development. Several types of Drosophila AD models have been developed to reveal the molecular pathophysiology of AD. Moreover, as Drosophila AD models show various and easily-quantifiable phenotypes, such as eye degeneration, developmental defects,
shortened lifespan, locomotor defects, increased oxidative stress sensitivity, and learning and memory defects, they have been employed for finding genetic modifiers of AD-associated genes. In addition, *Drosophila* AD models have been used to search and evaluate possible candidate drugs for AD. Recently, usage of *Drosophila* models has been extended to test the efficacy of traditional medicines, and successfully played a role in the analysis of their action mechanism at the molecular level. Our laboratory study revealed that SuHeXiang Wan, a traditional prescription, is neuroprotective against the cytotoxicity of Aβ42 by using *Drosophila* AD models.

In the present study, *Drosophila* AD models were used to screen 23 medicinal plants from Chinese traditional medicine to identify those that possess protective activity against the Aβ42 neurotoxicity. The study revealed that the extracts of five plants showed neuroprotective activities in Aβ42-expressing flies, as seen by their increased survival rates and recovered eye development. These neuroprotective plants include *P. multiflorum* and *Sorbus commixta* (*S. commixta*), the extracts of which suppressed the Aβ42 neurotoxicity in both *Drosophila* and mammalian cells. Our results suggest that these plants maybe sources of AD treating reagents.
**Materials and Methods**

**Fly stocks and maintenance** w^{118}, embryonic lethal abnormal vision-GAL4 (pan-neurons, elav-GAL4), glass multimer reporter-GAL4 (eye, GMR-GAL4), sevenless-GAL4 (photoreceptor cells, sev-GAL4), UAS-epidermal growth factor receptor (EGFR), and 33770 (a Bloomington Drosophila Stock Center version of UAS-Aβ42) were obtained from Bloomington Drosophila Stock Center (Bloomington, USA). UAS-Aβ42, UAS-hemipterous\textsuperscript{Ca} (the constitutively active form of Drosophila JNKK, hep\textsuperscript{Ca}), and UAS-TDP-43 were gifts from Dr. M. Konsolaki (Rutgers University, USA), Dr. K. Mastsumoto (Nagoya University, Japan), and Dr. N. Bonini (University of Pennsylvania, USA), respectively. All flies were maintained on standard medium (cornmeal-bean powder/yeast/sugar) at 25°C with a 12 h light-dark cycle incubator.

**Preparation of plant extracts** All plants including *P. multiflorum* Thunb. root (*Polygonaceae*) (voucher number: CYWDU-KP0003) and *S. commixta* Hedl. stem (*Rosaceae*) (voucher number: CYWDU-KP0001) were purchased from Dong Yang herb Pharm. Co. (Seoul, Korea). Each voucher specimen was identified by Dr. Byung-Soo Koo, Department of Oriental Medicine, Dongguk University and deposited in the Medicinal Herb Garden of Dongguk University for Reference Purposes at December 11, 2014. All plants (100 g each) were grinded and extracted twice in 1 L of 30% ethanol at 100°C by using a reflux condenser for 3 h. The extracts were filtered with a 50 μm filter, and concentrated by using a lyophilizer at -60°C. The dried materials were mixed with fly food at indicated concentrations or stored at -80°C until further use. The donepezil- or KSOP1009-containing fly food were made as previously described.\textsuperscript{24)}

**Treatment of 2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-d-glucoside** To evaluate the neuroprotective effect of 2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-d-glucoside against Aβ42
cytotoxicity in vivo and in vitro, 2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-D-glucoside (Sigma-Aldrich) was dissolved in distilled water at concentration of 10 mg/mL for a stock solution. Then, the stock solution was diluted to the indicated concentration to test the effects on survival rate of Drosophila or viability of SH-SY5Y cells.

Survival and pupariation assays Embryos of indicated genotype brought up in appropriate media were collected on grape juice-agar plates. These embryos were transferred to the fly media with 30% ethanol or indicated concentration of plant ethanol extract, and raised at 25°C in upright standard plastic shell vials. Forty hatched larvae were transferred to each of new vials with same media. The numbers of pupae and enclosed adult flies were counted.

Isolation and quantitative analysis of 2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-D-glucoside In order to obtain a marker compound from extract of P. multiflorum root, the extract was subjected to reversed-phase medium pressure liquid chromatography (MPLC) fractionation using a gradient mixture of MeOH and H2O (10:90 to 100:0) to give 25 sub-fractions. Of the sub-fractions, the eighth sub-fraction was applied to HPLC separation (MeOH : H2O = 35:65, 2 mL/min, Luna C18-ODS 10 ×250 mm), which yielded compound 1 (3.0 mg, tR 19.3). The structure of compound 1 was confirmed as 2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-D-glucoside, one of chemical constituents isolated from the radix of P. multiflorum26, by comparing the measured ¹H and ¹³C NMR data of (1) with the published values.26) The purity of (1) was determined to be over 95%. The amount of (1) in this extract was determined using HPLC. HPLC system (Gilson) consisted of a quaternary pump (321 pump), and UV detector (172 Diode Array Detector). The wavelength was set at 210 nm for quantification. Quantitative analysis was performed by using an Inno C18 column (4.6 × 250 mm, 5 μm) and a gradient mixture of 0.1% (v/v) formic acid in acetonitrile (A) and 0.1% (v/v) formic in H2O (B) with a flow rate of 1.0 mL/min. The mobile phase
consisting of (A) and (B) was delivered at a flow rate of 1.0 mL/min by the following programmed gradient elution: 10% (A) isocratic for 5 min, 10-40% (A) in 40 min, 40-100% (A) in 0.5 min, 100% (A) isocratic for 5 min, 100%-10% (A) in 0.5 min, 10% (A) isocratic for 5 min as post-run for reconditioning.

**Isolation and quantitative analysis of lyoniresinol 3a-O-β-D-xylopyranoside** To obtain a marker compound from ethanol extract of *S. commixta* Hedl. stem, the extract was subjected to MPLC fractionation. Of the 35 sub-fractions, the fifth sub-fraction was separated by HPLC (acetonitrile : H2O = 15:85, 2 ml/min, Luna C18-ODS, 10 × 250 mm), which yielded compound 1 (4.0 mg, *t*R 22.5). The structure of compound (1), one of the chemical constituents isolated from the stem of *S. commixta*, was confirmed as lyoniresinol 3a-O-β-D-xylopyranoside by comparing the measured ¹H and ¹³C NMR data of (1) with the published values.²⁷)

**Climbing ability assay** Locomotive activity of each experimental group was tested with 50 male flies. Ten male flies were collected in each of climbing assay vial, and incubated for 1 h at 25°C for adaptation. To measure the climbing ability, the flies were gently tapped down to the bottom of the test vial. The number of flies that reached to the top of the vial within 8 s, was recorded. Ten trials were performed for each group. The recorded climbing ability for 10 repeated tests was compared with that of the control flies. All climbing assay experiments were performed at 25°C.

**Acridine orange staining** The brains of *Drosophila* larvae (stage L3) were dissected in phosphate-buffered saline (PBS, Sigma-Aldrich). Then, they were incubated for 5 min in 1.6 × 10⁻⁶ M acridine orange solution (Sigma-Aldrich), and briefly washed 3 times with PBS. The samples were examined under Axiophot2 fluorescence microscope (Carl Zeiss).

**Cell culture and viability test** SH-SY5Y cells were obtained from the American Type Culture Collection (Rockville) and cultured in Dulbecco’s Modified Eagle’s Medium.
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(DMEM) containing 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% antibiotics (Hyclone Laboratories Inc). SH-SY5Y Cells were grown at 37°C in an incubator with 5% CO₂. To investigate the effects of *P. multiflorum* and *S. commixta* extracts on cell viability, cells were pre-treated with *P. multiflorum* or *S. commixta* extract for 30 min, and then treated with 25 μM Aβ25-35 (Sigma-Aldrich) for a further 24 h. Then, the cells were incubated with 2 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) at 37°C in a CO₂ incubator for 3 h. MTT medium was carefully aspirated, and the formazan dye was eluted using DMSO. The plate was shaken, and the absorbance was read using a spectrophotometer (Versamax) at a wavelength of 580 nm. All of the experiments were repeated at least three times.

**Antioxidant activity assay** The free radical scavenging activity of *P. multiflorum* and *S. commixta* extracts were measured by using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. 100 μL of DPPH solution (0.2 mg/mL in ethanol) was added to 100 μL of *P. multiflorum* or *S. commixta* extract solution with different concentrations (0.1 - 100 μg/mL). After 30 min of incubation at 25°C, the absorbance of the solution was measured at 517 nm. The activity of each extract concentration was determined by comparing its absorbance with that of a blank solution (reagents without *P. multiflorum* and *S. commixta* extracts). The capability to scavenge the DPPH radical was measured by using the following formula: [DPPH scavenging effect (%) = (Xcontrol - Xextract/Xcontrol) × 100]. Xcontrol and Xextract are the absorbance of the control and the solution with *P. multiflorum* or *S. commixta* extract, respectively.

**Immunohistochemistry** The eye imaginal discs of *Drosophila* larvae (stage L3) were fixed in 4% paraformaldehyde, and then washed briefly with PBST (PBS + 0.1% Triton X-100). The tissues were blocked with 2% normal goat serum and 2% bovine serum albumin in PBST for 1 h at 25°C. The eye imaginal discs were incubated with mouse anti-Aβ42
(1:200 in PBST, DE2B4, Santa Cruz) antibodies. The samples were observed under a Zeiss LSM 710 confocal microscope (Carl Zeiss).

**Western blot analysis** Western blot analysis was performed according to the standard protocol. Anti-Aβ42 [1:2,000 in Tris-buffered saline with Tween 20 (TBST), 6E10, Covance] or anti-Actin (1:2,000 in TBST, Developmental Studies Hybridoma Bank) antibodies were used as primary antibodies, and horseradish peroxidase-conjugated anti-mouse IgG antibody (1:2,000 in TBST, Cell Signaling) was used as a secondary antibody. Immunoreactive Aβ42 and actin were visualized with enhanced chemiluminescence and LAS-4000 mini (GE healthcare).

**Reactive oxygen species measurement** To measure reactive oxygen species (ROS) level, dihydroethidium (DHE, Invitrogen Molecular Probes) has been used. The eye imaginal discs of *Drosophila* larvae (stage L3) were dissected in Schneider’s medium at 25°C. The discs were incubated with the 30 μM DHE dye dissolved in 1 mL of Schneider’s medium for 5 min in dark multi-well glass, and then washed with Schneider’s medium. The samples were examined under an Axiophot2 fluorescence microscope (Carl Zeiss).
Results

Screening of medicinal plants with neuroprotective activities against neurotoxicity of Aβ42

To identify novel medicinal plants with neuroprotective activities against neurotoxicity of Aβ42, 23 candidates were examined by using Drosophila AD models overexpressing Aβ42 (Table 1). All neurons or developing eyes of the Drosophila models expressed human Aβ42, which resulted in several AD-like phenotypes, including reduced survival rate during development and malformation of the eye. We first quantitatively examined whether the survival rate of a pan-neuronally overexpressing Aβ42 model fly (elav>Aβ42) increased or not after treating the extract of each candidate plant. The extracts of five plants demonstrated increments in the survival rate of the Drosophila AD model (Fig. 1A). These plants include Coriandrum sativum L. (C. sativum), Nardostachys jatamansi (D. Don) DC. (N. jatamansi), Polygonum multiflorum Thunb. (P. multiflorum), Rehmannia glutinosa (Gaertn.) DC. (R. glutinosa), and Sorbus commixta Hedl. (S. commixta). We also evaluate the neuroprotective activity of 2, 3, 5, 4’-tetrahydroxystilbene-2-O-β-D-glucoside, which is the main chemical constituent of P. multiflorum root, against Aβ42 cytotoxicity. Interestingly, intake of 2, 3, 5, 4’-tetrahydroxystilbene-2-O-β-D-glucoside (THSG) also significantly increased the survival rate of the Aβ42-expressing flies (Fig. 1B).

For the second round of screening, we investigated the effects of the selected plant extracts on the reduction of eye size in eye-specific Aβ42-expressing flies (GMR>33770). The results showed that extracts from the five plants improved the decreased eye size induced by Aβ42 expression (Fig. 2). To evaluate our in vivo screening system using Drosophila, we further studied the effect of P. multiflorum, which showed the strong suppressive effect in our initial screening and was associated with AD in a previous study. Additionally, the
neuroprotective effect of *S. commixta* extract, which has not been associated with the treatment of AD, also examined in *Drosophila* and human cell AD models.

First, we confirmed the identification of the plants used in this study by isolating marker compounds of *P. multiflorum* and *S. commixta* extracts. 2, 3, 5, 4'-tetrahydroxystilbene-2-**O**-β-**D**-glucoside, a marker for the roots of *P. multiflorum*, and lyoniresinol 3a-**O**-β-**D**-xylopyranoside, which is one of the chemical constituents isolated from the stem of *S. commixta*, were identified by reversed-phase MPLC fractionation and HPLC separation (Fig. 3), followed by an NMR analysis (Figs. S1 and S2).26,27) The content of 2, 3, 5, 4'-tetrahydroxystilbene-2-**O**-β-**D**-glucoside and lyoniresinol 3a-**O**-β-**D**-xylopyranoside in each extract used was determined to be 0.75% and 0.28%, respectively.

*P. multiflorum* and *S. commixta* extracts ameliorated the neurological phenotypes of *Drosophila* AD models

To evaluate the toxicity of *P. multiflorum* and *S. commixta* extracts on *Drosophila*, we examined the survival and the pupariation rates during *Drosophila* development of wild-type flies (*w^1118*). As shown in Figs. 4A and B, the survival rates of flies fed with *P. multiflorum* or *S. commixta* extract did not show any difference from that of the control fly. However, the *P. multiflorum* extract intake, but not *S. commixta*, slightly retarded the pupariation rates (Figs. 4C and D). This was similar to previous reports29,30, which showed that liver injury occurred following the ingestion of *P. multiflorum*. This suggested that *P. multiflorum* extract exerts a modest toxic effect in the tested dosage. Yet, both *P. multiflorum* and *S. commixta* extracts significantly increased the pupariation rates and the motor activity of *Aβ42*-expressing flies (*elav>*Aβ42) (Figs. 4E-H).

*P. multiflorum* and *S. commixta* extracts intake reduced Aβ42-induced cell death
We further investigated the neuroprotective effects of *P. multiflorum* and *S. commixta* at the cell level. As previously reported, expression of Aβ42 in the neurons (elav> Aβ42) induced a high level of cell death in the larval brain, while no prominent cell death was detected in the brains of the controls (w^{1118}) (Figs. 5A and B). The Aβ42-induced cell death was strongly suppressed by the intake of *P. multiflorum* or *S. commixta* extract (Figs. 5A and B). The neuroprotective effects of *P. multiflorum* and *S. commixta* extracts were also confirmed by MTT assay in the mammalian cell AD model that used an Aβ-treated SH-SY5Y cells. Both *P. multiflorum* and *S. commixta* extracts had no harmful effect on the viability of the SH-SY5Y cells under the Aβ-untreated normal condition (Figs. 5C and D). Consistent with the results obtained in the *Drosophila* model, the treatment of *P. multiflorum* or *S. commixta* extract increased the viability of Aβ-treated cells in a dose-dependent manner (Figs. 5C and D). Moreover, 2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-D-glucoside (THSG) also showed the protective effect in the Aβ-treated cells (Fig. 5C).

*P. multiflorum* and *S. commixta* extracts intake decreased ROS levels in Aβ42-expressing flies

Previous studies have shown that *P. multiflorum* and *S. commixta* have a strong antioxidant effect and decreases ROS level, which is an important pathogenic pathway of AD. Accordingly, we examined whether *P. multiflorum* or *S. commixta* intake alters the increased ROS levels induced by Aβ42 expression, in the eye imaginal discs of *Drosophila*. The ROS levels in the Aβ42-expressing eye imaginal discs were significantly reduced by *P. multiflorum* or *S. commixta* intake (Figs. 6A and B). We also investigated the antioxidant activity of *P. multiflorum* and *S. commixta* extracts by measuring its DPPH radical

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scavenging capacity. Both *P. multiflorum* and *S. commixta* extracts showed strong scavenging activity, with IC$_{50}$ values of 11.87 μg/mL and 23.4 μg/mL, respectively (Fig. 6C).

Additionally, we investigated whether the neuroprotective effects of *P. multiflorum* and *S. commixta* extracts are specific for Aβ42 toxicity or if the effects are the results from the broad neuroprotective role against ROS. To address this question, the protective effects of *P. multiflorum* and *S. commixta* extracts against the cytotoxicity of TDP-43, which is the major disease protein in both frontotemporal lobar degeneration and amyotrophic lateral sclerosis,$^{35}$ were examined. Interestingly, as shown in Fig. 6D, *P. multiflorum* and *S. commixta* extracts reduced the rough eye phenotype-induced by human TDP-43 expression. This result suggests that the neuroprotective effects of *P. multiflorum* and *S. commixta* extracts are not only limited to the Aβ42 toxicity.

Aβ42 accumulation or JNK and EGFR signal transduction pathways were not altered by *P. multiflorum* and *S. commixta* extracts intake

As Aβ42 accumulation is an important process of AD, we investigated whether the intake of *P. multiflorum* and *S. commixta* extracts affects Aβ42 level in the adult *Drosophila* heads by immunohistochemical and western blot analysis.$^{36}$ As shown in Figs. 6E-G, we found no prominent difference in Aβ42 accumulation between the control and the flies fed *P. multiflorum* or *S. commixta*.

Previous reports have implicated the hyperactivation of JNK and EGFR signal transduction pathways in the cytotoxicity of Aβ42.$^{24,25,37,38}$ Therefore, we investigated the effects of *P. multiflorum* and *S. commixta* extracts intake on JNK or EGFR activity. As shown in Figs. 6H and I, the intake of *P. multiflorum* and *S. commixta* extracts did not affect the constitutively active form of hep (an activator of JNK)- or EGFR-induced eye.
malformation. These results suggested that the neuroprotective effects of *P. multiflorum* and *S. commixta* are not related with the alteration of JNK or EGFR signal transduction pathways.
Discussion

Although a growing number of patients suffer from AD due to population aging, the efficacy of currently commercially available drugs of AD, known to regulate cholinergic or glutamatergic neurotransmission, is doubtful. Therefore, it is necessary to develop new drugs based on different approaches. Especially, given the complex characteristics of AD pathology, oriental medicinal plant extracts that contain various beneficial components would provide a good source for novel drugs for AD.

To find beneficial medicinal plants in Chinese traditional medicine for AD, based on their suppressive efficacies against Aβ42 toxicity, here we screened the extracts of 23 medicinal plants using in vivo and in vitro models, and identified five neuroprotective medicinal plants. Our study suggests that the screening of traditional medicinal plants is an effective and valuable searching strategy for finding novel AD drugs.

In the present study, we employed an in vivo screening system by using Drosophila AD models which have been used for a number of genetic studies. As a disease model organism for drug screening, Drosophila has several advantages, which make it a suitable model organism for drug screening. Well-conserved disease-associated genes and the ease of genetic manipulation enable Drosophila to be an excellent model system for various diseases including AD. Compared to in vitro screening platforms that are commonly adopted by the traditional drug discovery process, the use of an in vivo model like Drosophila would greatly increase the success rate of drug discovery by reflecting the in vivo conditions such as absorption, distribution, metabolism, excretion, and toxicity. In addition, the time needed to express the disease-like phenotypes in Drosophila models is much shorter than that in mammalian models, which makes it possible to evaluate drug efficacy in a reasonable amount of time. Drosophila also requires less space and a lower budget than mammalian models.
Based on these advantages, we successfully screened and characterized medicinal plants with neuroprotective activity against Aβ42 cytotoxicity. Five neuroprotective plants were identified, out of 23 medicinal plants that have been used in Chinese traditional medicine. Several phenotypes of AD model flies confirmed the beneficial effects of *P. multiflorum* and *S. commixta*, identified plants in this study, against Aβ42 neurotoxicity. An experiment with cultured human cells also confirmed the neuroprotective activities of *P. multiflorum* and *S. commixta* extracts found in the *Drosophila* models. Therefore, our results suggested that *Drosophila* models are useful *in vivo* screening systems for drug discovery.

Among the identified plants, *P. multiflorum*, *R. glutinosa*, and *C. sativum* were associated with AD. The antioxidant properties of *P. multiflorum* water extract suppressed cognitive deficits induced by Aβ accumulation in mice. We also found that *P. multiflorum* ethanol extract exerts neuroprotective effect and has antioxidant properties in both *Drosophila* and human cell AD models; this suggested that both water and ethanol extracts of *P. multiflorum* contains beneficial components for neuronal health, and that the neuroprotective mechanism of *P. multiflorum* extract may be conserved from fly to human.

As oxidative damage is an important pathological pathway of AD, antioxidant property of *P. multiflorum* would be responsible for its protective activity. In addition, a recent study showed that 2, 3, 5, 4′-tetrahydroxystilbene-2-O-β-D-glucoside, an active component extracted from *P. multiflorum*, exerts anti-AD properties by protecting synaptic structure and function in Aβ42-injected rats. Consistently, in the present study, we also found the neuroprotective effects of 2, 3, 5, 4′-tetrahydroxystilbene-2-O-β-D-glucoside, in our *in vivo* and *in vitro* systems. Neuroprotective effects of *R. glutinosa* have also been reported in several previous studies. Moreover, catalpol, an iridoid glucoside isolated from the root of *R. glutinosa*, protected primary cultured cortical neurons from the Aβ42-induced damage, through a mitochondrial-dependent caspase pathway. It is of interesting to test whether
catalpol also shows similar effect in our Drosophila models. Volatile oil of C. sativum has been used for the relief of pain, anxiety, flatulence, loss of appetite, and convulsions in traditional medicine; it also increased anxiolytic, antidepressant-like behaviors and ameliorates Aβ42-induced memory impairment by attenuation of the oxidative stress in the rat hippocampus. However, the molecular components which are responsible for the neuroprotective activity is needed to be isolated. Collectively, the results of previous and present studies evidenced the protective effect of P. multiflorum, R. glutinosa, and C. sativum on Aβ toxicity. However, little is known about the molecular mechanisms by which these plant extracts suppressed Aβ-induced neuronal damage. Biochemical approaches, to find the bioactive components, and genomic study, to isolate the responsive genes, are needed to reveal these mechanisms.

On the other hand, N. jatamansi and S. commixta have not been associated with the treatment of AD so far. Interestingly, in this study, the neuroprotective activity was found only in the stem of S. commixta, but not in its leaf and fruit. This result suggested that different part of same plant would have different bioactive properties. In traditional medicine, S. commixta and N. jatamansi has been used for several inflammatory diseases and as tranquilizer, respectively. Recent studies have reported that they possess antioxidant and anti-inflammatory properties. Oxidative damage and neuroinflammation are fundamental to the progression of AD; therefore, these plants would provide neuroprotective activity through their antioxidative and anti-inflammatory functions. Supporting this idea, we found that S. commixta intake significantly reduced ROS levels as well as rescuing the AD-like phenotypes in Drosophila. Although the benefit of antioxidant therapy is under debate, oxidative damage in proteins, lipids, DNA, and carbohydrates is closely associated with AD development. Several studies have shown that antioxidants supplementation significantly delayed AD progression in patients with moderately severe impairment from AD, and
rescued distorted neurite trajectories and cognitive defects in AD mouse models.\textsuperscript{57,58} In addition, based on pathological evidence of neuroinflammation in AD and epidemiological evidence of AD sparing in individuals consuming non-steroidal anti-inflammatory drugs, anti-inflammatory therapy is believed to be a promising approach for treating AD.\textsuperscript{59} Therefore, \textit{S. commixta}, which is a source of various antioxidants and anti-inflammatory components, is a promising medicinal plant to treat AD as well as inflammatory diseases.

In this study, we established an \textit{in vivo} screening system by using \textit{Drosophila} AD models to isolate neuroprotective medicinal plants. Our results identified five plants, \textit{C. sativum}, \textit{N. jatamansi}, \textit{P. multiflorum}, \textit{R. glutinosa}, and \textit{S. commixta}, which have neuroprotective activities against Aβ42 cytotoxicity out of 23 traditional medicinal plants. The beneficial effects of \textit{P. multiflorum} and \textit{S. commixta} extracts were further confirmed in both \textit{Drosophila} and mammalian cell AD models. Based on these results, we propose that \textit{P. multiflorum} and \textit{S. commixta} are promising sources of drugs for AD treatment.
Acknowledgement

This paper was written as part of Konkuk University's research support program for its faculty on sabbatical leave in 2011.

[Conflict of Interest]

The authors declare no conflict of interest.

The online version of this article contains supplementary materials.
References


42) Cioanca O, Hriteu L, Mihasan M, Trifan A, Hancianu M. Inhalation of coriander volatile oil increased anxiolytic-antidepressant-like behaviors and decreased


**Figure legends**

**Fig. 1.** Five plant extracts ameliorated the decreased survival rate of *Drosophila AD* models. (A) The effects of medicinal plant extract intake on the decreased survival rate in *Aβ42*-expressing flies (*elav>*Aβ42). 0.1 μg/mL donepezil and 50 μg/mL KSOP1009 were used for positive controls. All data are compared with vehicle (ethanol)-treated *Aβ42*-expressing flies and expressed as the mean ± standard error (SE) (***p<0.001, **p<0.01, *p<0.05; Student’s *t*-test; *n*≥120). (B) Intake of 2, 3, 5, 4’-tetrahydroxystilbene-2-O-β-D-glucoside improved the decreased survival rate of *Aβ42*-expressing *Drosophila* AD models. All data are compared with vehicle (distilled water)-treated control and expressed as the mean ± SE (***p<0.001, NS, not significant; Student’s *t*-test; *n*=250). The genotypes of the samples are CTL (*w^{118}* ) and *elav>*Aβ42 (*UAS-Aβ42/UAS-Aβ42; elav-GAL4/elav-GAL4*). THSG, 2, 3, 5, 4’-tetrahydroxystilbene-2-O-β-D-glucoside.

**Fig. 2.** Medicinal plant extracts suppressed the Aβ42-induced reduction of eye size in *Drosophila AD* models. (A-G) The effects of medicinal plant extract intake on the Aβ42-induced reduction of eye size. (H-I) For positive controls, 0.1 μg/mL donepezil and 50 μg/mL KSOP1009 were used. (J) Graph showing the relative size of *Drosophila* adult eyes compared to vehicle (ethanol)-treated Aβ42-expressing flies. All data are expressed as the mean ± SE (***p<0.001, **p<0.01; Student’s *t*-test; *n*≥15). The genotypes of the samples are (A) GMR-GAL4 (*GMR-GAL4/GMR-GAL4* ) and (B-I) GMR>33770 (*GMR-GAL4, 33770/GMR-GAL4, 33770* ). EtOH, ethanol.

**Fig. 3.** Isolation of 2, 3, 5, 4’-tetrahydroxystilbene-2-O-β-D-glucoside and lyoniresinol 3α-O-β-D-xylopyranoside as marker compounds from *P. multiflorum* and *S. commixta*.
extracts, respectively. (A, B) High-pressure liquid chromatograms of 2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-D-glucoside (A), and extract of *P. multiflorum* (B). Arrows indicate 2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-D-glucoside. (C, D) High-pressure liquid chromatograms of lyoniresinol 3a-O-β-D-xylopyranoside (C) and extract of *S. commixta* (D). Arrows indicate lyoniresinol 3a-O-β-D-xylopyranoside. The insets show molecular structures of 2, 3, 5, 4'-tetrahydroxystilbene-2-O-β-D-glucoside (A) and lyoniresinol 3a-O-β-D-xylopyranoside (C).

**Fig. 4.** *P. multiflorum* and *S. commixta* extracts ameliorated neurological phenotypes of *Drosophila* AD models. (A-D) The effects of *P. multiflorum* and *S. commixta* extracts intake on survivability (A, B) and pupariation timing (C, D) during development of wild-type flies (*w*¹¹¹⁸). (E-H) Intake of *P. multiflorum* and *S. commixta* extracts improved the delayed pupariation time (E, F) and decreased climbing ability (G, H) of Aβ42-expressing *Drosophila* AD models. All data are compared with vehicle (ethanol)-treated control and expressed as the mean ± SE (***p***<0.001, **p***<0.01, *p***<0.05, NS, not significant; Student’s *t*-test; A-D, *n*≥200; E, F, *n*≥150; G, H, *n*=80). The climbing abilities are represented as boxes and whisker plots. The boxes represent the 25th and 75th percentiles with median value (solid black line). The whiskers represent data range. The genotypes of the samples are wild-type (*w*¹¹¹⁸) and *elav>*Aβ42 (*UAS-Aβ42/UAS-Aβ42; elav-GAL4/elav-GAL4*). AEL, after egg laying; EtOH, ethanol; P5 and P50, 5 and 50 µg/mL *P. multiflorum* extracts, respectively; S5 and S50, 5 and 50 µg/mL *S. commixta* extracts, respectively; D, 0.1 µg/mL donepezil; K, 50 µg/mL KSOP1009.

**Fig. 5.** *P. multiflorum* and *S. commixta* extracts repressed cell death in the brains of *Drosophila* and mammalian cell AD models. (A) The acridine orange (AO)-stained brains
showing the reduction of Aβ42-induced cell death in of *P. multiflorum* or *S. commixta* extract-fed larvae. (B) Graph showing the relative number of AO-positive cells per brain in the larva fed with medium containing the indicated extract. The effects of *P. multiflorum* and *S. commixta* extracts on Aβ42-induced cell death were examined in different doses (5 and 50 μg/mL). All data are compared with vehicle-treated Aβ42-expressing flies and expressed as boxes and whisker plots (***p<0.001; Student’s *t*-test; B, *n*=18). The boxes represent the 25th and 75th percentiles with median value (solid black line). The whiskers represent data range. The genotypes of the samples are CTL (*w*1118) and *elav>*Aβ42 (*UAS-Aβ42/UAS-Aβ42; elav-GAL4/elav-GAL4*). D, 0.1 μg/mL donepezil; K, 50 μg/mL KSOP1009. (C, D) The effects of *P. multiflorum* (C) and *S. commixta* (D) extracts on the viability of SH-SY5Y cells with or without 25 μM Aβ25-35 treatment. Cell viabilities were measured by MTT assay. For each group with or without Aβ25-35 treatment, the data are compared with vehicle-treated control and expressed as the mean ± SE (***p<0.001, **p<0.01, *p<0.05, NS, not significant; Student’s *t*-test; n=3). EtOH, ethanol; P5 and P50, 5 and 50 μg/mL *P. multiflorum* extracts, respectively; S5 and S50, 5 and 50 μg/mL *S. commixta* extracts, respectively; D, 5 μM donepezil; K, 10 μg/mL KSOP1009; THSG, 0.1 μg/mL 2, 3, 5, 4’-tetrahydroxystilbene-2-O-β-D-glucoside.

**Fig. 6.** *P. multiflorum* and *S. commixta* extracts intake reduced ROS levels in Aβ42-expressing flies, but did not affect Aβ42 accumulation or JNK and EGFR signal transduction pathways. (A) *In vivo* detection of ROS with dihydroethidium (DHE) in the eye imaginal discs of ethanol (EtOH)-, *P. multiflorum* ethanol extract (P50)- or *S. commixta* extract (S50)-fed *Drosophila* larvae (stage L3). (B) Graph showing the relative number of DHE-positive signals per eye imaginal disc in the larva fed with medium containing the indicated extract. All data are expressed as boxes and whisker plots.
(***p<0.001; Student’s t-test; n≥17). (C) The free radical scavenging effects of *P. multiflorum* and *S. commixta* extracts in different doses (0.1, 1, 5, 12.5, 25, 50, or 100 μg/mL; n=3). Ascorbic acid was used for a positive control. All data are expressed as the mean ± SE. (D) Representative images showing that the effects of *P. multiflorum* and *S. commixta* extracts intake on the TDP-43-induced rough eye phenotype (n=15). (E-G) Representative images of Aβ42-immunostained eye imaginal discs of *Drosophila* larvae (E), and relative Aβ42 protein levels in the heads of Aβ42-expressing flies (GMR>33770) fed with medium containing *P. multiflorum* (F) or *S. commixta* (G) extract. All data are expressed as boxes and whisker plots (NS, not significant; Student’s t-test; n=6). (H, I) Representative images of *Drosophila* adult eyes showing the effects of *P. multiflorum* and *S. commixta* extracts intake on defective eye generation in flies overexpressing hep\textsuperscript{CA}, a constitutively active form of JNKK (H), or EGFR (I). No significant effects were observed in both cases. The genotypes of the samples are GMR-GAL4 (GMR-GAL4/GMR-GAL4), GMR>33770 (GMR-GAL4, 33770/GMR-GAL4, 33770), GMR>TDP-43 (GMR-GAL4/UAS-TDP-43), sev>hep\textsuperscript{CA} (sev-GAL4/sev-GAL4; UAS-hep\textsuperscript{CA}/MKRS), and GMR>EGFR (GMR-GAL4/UAS-EGFR). EtOH, ethanol; P50, 50 μg/mL *P. multiflorum* extract; S50, 50 μg/mL *S. commixa* extract; hep, hemipterous; sev, sevenless.
Fig. 1. Liu et al.,
Fig. 2. Liu et al.,
Fig. 3. Liu et al.,
**Biological and Pharmaceutical Bulletin Advance Publication**

**Fig. 5. Liu et al.**

**A**

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Fig. 6. Liu et al.,
Table 1. The list of tested medicinal plant extracts and their effects of on the survival rate of $\alpha\beta_{42}$-expressing Drosophila AD models.

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<tr>
<th>Botanical name</th>
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<th>Survival rate % $[\text{elav} &gt; \alpha\beta_{42}]$</th>
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<td>5 $\mu$g/mL (n)</td>
<td>50 $\mu$g/mL (n)</td>
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<td>Artemisia annua Linné (Asteraceae)</td>
<td>Herba</td>
<td>48.33 ± 5.52 (160)</td>
<td>47.50 ± 9.36 (160)</td>
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<td>Arctium sativum Linné (Apiaceae)</td>
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<td>55.00 ± 2.28 (160) *</td>
<td>60.62 ± 2.13 (160) **</td>
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<td>Dimocarpus longan Loureiro (Sapindaceae)</td>
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<td>41.66 ± 3.96 (120)</td>
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<td>Dioscorea batatas Decaisne (Dioscoreaceae)</td>
<td>Rhizome</td>
<td>37.50 ± 1.59 (160)</td>
<td>42.50 ± 5.50 (160)</td>
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<tr>
<td>Drynaria fortunei Smith (Polypodiaceae)</td>
<td>Rhizome</td>
<td>50.83 ± 5.06 (120)</td>
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<td>Fritillaria cirrhosa D. Don (Liliaceae)</td>
<td>Bulbus</td>
<td>48.33 ± 2.20 (120)</td>
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<td>Houttuynia cordata Thunberg (Saururaceae)</td>
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<td>Phalaris arundinacea Linné (Poaceae)</td>
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<td>Polygonon multiflorum Thunberg (Polygonaceae)</td>
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<td>Polygonatum sibiricum F. Delaroche (Asparagaceae)</td>
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<td>Donepezil (0.1 $\mu$g/mL)</td>
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<td>KSOP1009 (50 $\mu$g/mL)</td>
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Survival rates of the $A\beta 42$-expressing flies ($elav>A\beta 42$) fed with each extract were compared with that of control flies with a same genotype that fed with fly medium containing vehicle (ethanol). The survival rate of the control was $43.75 \pm 2.39\%$ (n=160). All data are compared with vehicle-treated control and expressed as the mean ± standard error (SE) (**$P < 0.01$, *$P < 0.05$; Student’s $t$-test). 0.1 $\mu$g/mL donepezil and 50 $\mu$g/mL KSOP1009 were used for positive controls. The significantly increased values are denoted in bold. The genotype of the samples is $elav>A\beta 42$ ($UAS-A\beta 42/ UAS-A\beta 42; elav-GAL4/ elav-GAL4$).