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Timosaponin AIII, a saponin isolated from *Anemarrhena asphodeloides*, ameliorates learning and memory deficits in mice

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ABSTRACT

Anemarrhena asphodeloides Bunge (AA, family Liliaceae), which primarily contains xantones, such as mangiferin, and steroidal saponins, such as timosaponin AIII and sarsasapogenin, has been used as an antipyretic, anti-inflammatory, anti-diabetic, anti-platelet aggregation, and anti-depressant agent in traditional Chinese medicine. In the present study, the memory-enhancing effects of these saponins were investigated in scopolamine-treated mice. Among saponins, timosaponin AIII (TA3) significantly reversed the scopolamine-induced deficits in a passive avoidance test and in the Morris water maze test. TA3 also increased hippocampal acetylcholine levels in scopolamine-treated mice and dose-dependently inhibited acetylcholinesterase (AChE) activity (IC_{50} value, 35.4 μM). When TA3 (50 mg/kg) was orally administered to mice and its blood concentration was measured by liquid chromatography and tandem mass spectrometry, the C_{max} of TA3 occurred 4–6 h after TA3 treatment. The memory-enhancing effect of TA3 was greater when it was administered 5 h before the acquisition trial than 1 h before. Scopolamine treatment in mice increased brain levels of TNF-α and IL-1β expression. However, treatment with TA3 and scopolamine inhibited the increase of TNF-α and IL-1β expression. These results suggest that scopolamine may cause learning and memory deficits that are further complicated by inflammation. TA3 also inhibited the activation of NF-κB signaling in BV-2 microglia and in SK-N-SH neuroblastoma cells induced with TNF-α or scopolamine. Nevertheless, TA3 may ameliorate memory deficits, mainly by inhibiting AChE.

1. Introduction

Alzheimer's disease (AD) is a progressive degenerative disease of the brain that is characterized by deterioration of memory and cognitive functions (Ingkaninan et al.). Cholinergic neurons in the central nervous system (CNS) degenerate in patients with AD, and senile dementia correlates with this functional loss (Davies and Maloney, 1976; Perry et al., 1978). Inflammation, as well as cholinergic neuron degeneration, may play a critical role in the pathogenesis of the degenerative changes and cognitive impairments of AD. Proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), are upregulated in the AD brain (Dickson et al., 1993; Griffin et al., 1995; Rothwell et al., 1997). These cytokines may play a role in several events in the pathological cascade of AD (Eikelenboom et al., 1994). Indeed, chronic treatment with NSAIDs reverses the cognitive deficits in scopolamine-treated mice (Jain et al., 2002). Nevertheless, treatments have focused on increasing brain cholinergic activity *via* acetylcholinesterase (AChE) inhibitors or cholinergic agonists; in fact, a selective AChE inhibitor, donepezil, has been approved to treat mild AD (Doody, 1999; Whitehouse et al., 1981). However, the resulting adverse effects associated with these agents have limited their use (Blazer et al., 1983). Medicinal plants may provide valuable alternatives with fewer side effects for patients with memory impairments.

Anemarrhena asphodeloides Bunge (AA, family Liliaceae), which primarily contains xantones, such as mangiferin, and steroidal saponins, such as timosaponin AIII and sarsasapogenin, has been used as an antipyretic, anti-inflammatory, anti-diabetic, anti-platelet aggregator, and anti-depressant agent in traditional medicine in China, Japan, and Korea (Dong and Han, 1991; Iida et al., 1999; Oh et al., 2007; Takahashi et al., 1985). Its saponin mixture improves learning and memory in rats with dementia induced by amyloid β-peptide (Ouyang et al., 2005). Sarsasapogenin can also alleviate depression (Ren et al., 2006) and improve memory by elevating muscarinic acetylcholine receptor density in the brain (Hu et al., 2005).

During an herbal medicine screening program for compounds to improve memory impairment, we purified four saponins from AA. Among these four steroidal saponins and the previously reported sarsasapogenin, timosaponin AIII (TA3, Fig. 1) most potently improved learning and memory deficits in scopolamine-treated memory-deficient mice. Therefore, we investigated the memory-enhancing

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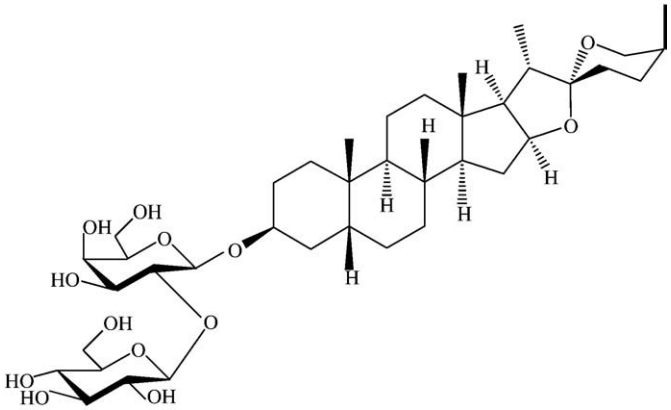

Fig. 1. Structure of timosaponin AIII (TA3).

effects of TA3 in scopolamine-treated mice in the passive avoidance test and Morris water maze test. In addition, its anti-inflammatory effects and inhibitory effects on scopolamine and TNF-α in BV-2 and SK-N-SH cells were also examined.

2. Methods and materials

2.1. Chemicals

Tacrine (9-amino-1, 2, 3, 4-tetrahydroacridine hydrochloride), scopolamine hydrobromide, acetylthiocholine (ATCh), 5,5′-dithiobis-[2-nitrobenzoic acid] (DTNB), AChE (electric eel type VI-S), radioimmunoprecipitation assay (RIPA) lysis buffer, dimethylsulfoxide (DMSO), and sarsasapogenin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Recombinant human TNF-α was purchased from R&D Systems, Inc. (Minneapolis, MN, U.S.A.). Antibodies against NF-κB (pp65 and p65) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). The protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). Enzyme-linked immunosorbent assay (ELISA) kits were from Pierce Biotechnology, Inc. (Rockford, IL, U.S.A.). Immobilon-P nylon membrane was from Millipore (Billerica, MA, U.S.A.). The enhanced chemiluminescence (ECL) immunoblot system was from Pierce Co. (Rockford, IL, U.S.A.). All other materials were of the highest grade available.

2.2. Isolation of steroidal saponins (timosaponin AIII) from A. asphodeloides

The rhizomes of *A. asphodeloides* were purchased from KyungDong Market, Seoul, Korea, and taxonomically identified by Dr. Nam-Jae Kim, East–West Research Institute, Kyung Hee Medical Center, Kyung Hee University (Seoul, Korea). A voucher specimen (KHOP061102) was deposited at the Herbarium of Kyung Hee University, Seoul, Korea. The pulverized rhizomes of AA (1 kg) were extracted three times with 70% EtOH under boiling water bath. The EtOH extracts were combined and evaporated to dryness under reduced pressure to yield 84.7 g. This extract was fractionated with ethyl acetate and BuOH to give an EtOAc-soluble fraction (12.5 g) and a BuOH-soluble fraction (50 g), respectively. The EtOAc fraction (12.5 g) was separated by silica gel column chromatography and eluted with a gradient of EtOAc in hexane (FE1–FE7). FE2 was further separated by silica gel column chromatography eluted with a linear gradient of hexane–EtOAc (95:1, 1 L) and CH_2Cl_2–MeOH–H_2O (7:1:0.5, 1 L) to afford compound 1 (35 mg).

The BuOH fraction was then separated by column chromatography on silica gel and eluted with a gradient of MeOH in CH_2Cl_2. A total of 6 fractions (FB1–FB6) were collected on the basis of thin layer chromatography (TLC) behavior. FB2 (18 g) and FB3 (25 g) were subjected to chromatography on a silica gel column with a gradient of CH_2Cl_2/MeOH/H_2O (7:1:0.5, 7:2:0.5, and 7:3:1) to afford compound 2 (112 mg) (Fig. 1). Fraction FB3 was further subjected to chromatography on a silica gel column eluted with CH_2Cl_2/MeOH/H_2O 7:3:1 to afford four subfractions (FB5-1 to FB5-4). Fraction FB5-3 was further subjected to semi-preparative HPLC (35% CH_3CN in 50% MeOH at a flow rate of 7 ml/min over 60 min, GS-320 column, 30×500 mm, Japan Analytical Instrument) to afford compound 3 (120 mg) and compound 4 (222 mg). The structures of all compounds were elucidated by chemical and spectroscopic means. The spectral data of each compound was compared with published data (Meng et al., 1998; Niwa et al., 1988; Saito et al., 1994).

β-Sitosterol glucoside (1) White amorphous powder: FABMS: m/z 575.9 $[M]^-$.

Timosaponin AIII (2) White amorphous powder: FABMS: m/z 763.5 $[M + Na]^+$.

Timosaponin BII (3) White amorphous powder: FABMS: m/z 919.6 $[M]^-$.

Timosaponin BIII (4) White amorphous powder: FABMS: m/z 926.2 $[M + Na]^+$.

2.3. Animals

Male ICR mice weighing 28–30 g were purchased from the Orient Co., Ltd., a branch of Charles River Laboratories (Seoul); all experiments were performed according to the guidelines of the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea. The mice were housed 5 or 6 per cage, allowed access to water and food *ad libitum*, and maintained at an ambient temperature of 22±1 °C with 50±10% humidity and a 12-h diurnal light cycle (lights on 07:00–19:00 h) prior to testing. All behavioral experiments were carried out in a room adjacent to the housing room under the same ambient conditions.

2.4. Mouse behavioral experiments

2.4.1. Passive avoidance test

The step-through passive avoidance test was performed in identical illuminated and dark chambers, according to the method of Kim et al. (2007). The illuminated compartment (20×20×20 cm) contained a 100 W bulb, and the floor of the non-illuminated compartment (20×20×20 cm) was composed of 2-mm stainless steel rods spaced 1 cm apart. These compartments were separated by a guillotine door (5×5 cm). For the acquisition trial, mice were initially placed in the illuminated compartment and the door between the two compartments was opened 10 s later. When the mice entered the dark compartment, the door closed automatically and an electrical foot shock (0.5 mA) of 3-s

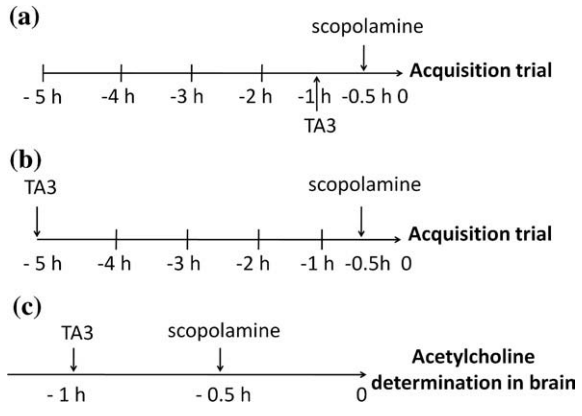

Fig. 2. Experimental protocols for passive avoidance test (a) and acetylcholine determination (b).

duration was delivered through the stainless steel rods. Each group contained ten mice. One hour or 5 h before the acquisition trial, mice received each test agent (10, 20 or 40 mg/kg, *p.o.*) (Fig. 2-a and b). One hour before the acquisition trial, mice received tacrine (10 mg/kg, *p.o.*) as a positive control. Memory impairment was induced by scopolamine treatment (1 mg/kg, *i.p.*) 0.5 h or 4.5 h after the administration of test agents, tacrine, or 10% Tween 80 solution. Control animals were administered 10% Tween 80 solution alone. Twenty-four hours after the acquisition trial, the mice were again placed in the illuminated compartment for retention trials. The time taken for a mouse to enter the dark compartment after the door opened was measured as the latency time in both acquisition and retention trials, with a maximum of 300 s.

2.4.2. Morris water maze test

The Morris water maze is a circular pool (90 cm in diameter and 45 cm in height) with a featureless inner surface. The pool was filled to a depth of 30 cm with water containing 500 ml of milk (20 ± 1 °C) as described by Kim et al. (2007). The tank was placed in a dimly lit, soundproof test room with various visual cues. The pool was conceptually divided into quadrants, and a white platform (6 cm in diameter and 29 cm high) was placed in one of the pool quadrants and submerged 1 cm below the water surface so that it was not visible at water level. The first experimental day was dedicated to swimming training for 60 s in the absence of the platform. During the four subsequent days, the mice underwent four trials per day with the platform in place. When a mouse located the platform, it was permitted to remain on the platform for 10 s. If the mouse did not locate the platform within 60 s, it was placed on the platform for 10 s. The animal was taken to its home cage and was allowed to dry under an infrared lamp after each trial. The time interval between each trial was 30 s. During each trial, the time taken to find the hidden platform (latency) was recorded using a video camera-based Ethovision System (Nodulus, Wageningen, The Netherlands). For each training trial, mice were placed in the water facing the pool wall in different pool quadrants, with a variable order each day. One day after the last training trial session, mice were subjected to a probe trial session in which the platform was removed from the pool and mice were allowed to swim for 60 s to search for it. A record was kept of the swimming time in the pool quadrant where the platform had previously been placed. TA3 (40 mg/kg, *p.o*) or tacrine (10 mg/kg, *p.o.*; positive control) was given 1 h before the first trial session every consecutive day. Each group contained ten mice. Memory impairment was induced in mice with scopolamine (1 mg/kg, *i.p.*) at 30 min after treatment with TA3. The control group received 10% Tween 80 solution only.

2.5. Analysis of acetylcholine in brain

TA3 (40 mg/kg, *p.o.*) was orally administered to the mice, and scopolamine (1 mg/kg, *i.p.*) was intraperitoneally injected 0.5 h after TA3 administration (Fig. 2-c). Each group consisted of five mice. The mice were sacrificed by cervical dislocation 30 min after scopolamine administration. The brains were removed from the skulls and dissected into several regions. The hippocampus was stored at −70 °C until used in experiments. The hippocampal region was thawed at room temperature prior to acetylcholine analysis. The samples were homogenized and deproteinized using 100% methanol. After the samples were centrifuged (14,000 ×*g*, 4 °C) for 10 min, the resulting supernatant was used for acetylcholine analysis by LC-MS/MS.

Acetylcholine was analyzed by a Waters 2795 Alliance system (Waters, Milford, MA, USA) equipped with an automatic sample injector: column, Shiseido capcell pak UG 120 CN (50×2.0 mm I.D., S-5 μm) maintained at 40 °C; mobile phase, 10 mM ammonium formate, pH 3.09 (pH adjusted with formic acid), in 100% methanol; flow rate, 0.2 ml/min; injection volume, 10 μl. MS experiments were performed on Micromass Quattro API. The mass spectrometer was operated in electrospray positive ionization mode (cone voltage: 19 V). Multiple reaction monitoring (MRM) data acquisition for the transition mass Ach (*m/z* 146 → 87) was achieved with a collision energy of 15 eV and a dwell time of 0.50 s. Nitrogen (approximately 550 l/h) was used for the nebulizing and desolvation gases, and the desolvation temperature was 350 °C.

2.6. Acetylcholinesterase activity assay

AChE activity was measured using Ellman's coupled enzyme assay (Ellman et al., 1961). The reaction mixture consisted of 125 μl of 3 mM DTNB, 25 μl of 15 mM ATCh, 50 μl of 50 mM Tris–HCl, pH 8.0, and 25 μl of test agents in a microplate. The mixture was pre-incubated for 10 min, and then 25 μl AChE (0.226 U/ml) was added before scanning at 405 nm for 10 min in a microplate reader, Model Biotek μQuant MQX200 (Winooski, VT, U.S.A.). Enzyme activity was calculated as a percentage compared to buffer without any inhibitor (Ingkaninan et al., 2000).

2.7. Pharmacokinetic study of timosaponin AIII in mice

The mice were divided into 10 groups of three mice per group. Each mouse was orally administered TA3 at a dose of 50 mg/kg. The mice were exsanguinated from the heart at 0, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h after TA3 treatment, one group at a time. The blood of each mouse was centrifuged for 10 min at 4000 ×*g*. The supernatant was deproteinized with acetonitrile/MeOH for analysis on the Agilent G6410 Triple Quadrupole Mass Spectrometer from Agilent Technologies, Inc. (Santa Clara, CA, USA) equipped with an automatic sample injector. TA3 was analyzed on a ZORBAX XDB-C18 column (Rapid Resolution HT 2.1×50 mm, i.d., 1.8 μm, Agilent) using a mobile phase of 0.1% aqueous formic acid and acetonitrile at a flow rate of 0.3 ml/min. The column was maintained at 40 °C during HPLC analysis and the injection volume was 5 μl. MS experiments were performed on Micromass Quattro API. The mass spectrometer was operated in electrospray positive ionization mode (cone voltage: 19 V) with multiple reaction monitoring (MRM) data acquisition for the transitions mass. The ESI/MS source was set as follows: capillary temperature 350 °C; spray voltage 5 kV; capillary voltage 4 kV; gas flow rate 10 l/min. The flow rate of the nebulizer gas (nitrogen) was 5 l/min. Spectra were acquired in positive-ion mode. TA3 (*m/z* 758 → 579, 579 → 417) was analyzed with a collision energy of 15 eV and a dwell time of 0.50 s. Nitrogen (approximately 550 l/h) was used for the nebulizing and desolvation gases, and the desolvation temperature was 350 °C.

2.8. Assay of TNF-α and IL-1β in brain by enzyme-linked immunosorbent assay (ELISA)

For the ELISA of TNF-α and IL-1β, the hippocampus was homogenized in 1 ml of ice-cold lysis RIPA buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% phosphatase inhibitor cocktail and a protease inhibitor cocktail. The lysate was centrifuged (14,000 ×*g*, 4 °C) for 15 min, and the supernatant was transferred to 96-well ELISA plates. The TNF-α and IL-1β concentrations were then determined using commercial ELISA kits.

2.9. Cell cultures and treatment

The immortalized murine BV-2 cell line and SK-N-SH neuroblastoma cells were maintained in DMEM (GIBCO BRL, Life Technologies, Inc., NY, USA) supplemented with 10% fetal bovine serum and 1% antibiotics. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and split once every 2 days. BV-2 cells were treated with TA3 (2.5, 5 and 10 μM) or tacrine (5 μM) for 1 h before the addition of 10 ng/ml TNF-α for 90 min *in vitro*. SK-N-SH cells were treated with TA3 and tacrine for 1 h before the addition of TNF-α (10 ng/ml) for 5 min. TA3 and tacrine were dissolved in DMSO before addition to cells. DMSO concentration never exceeded 0.1%.

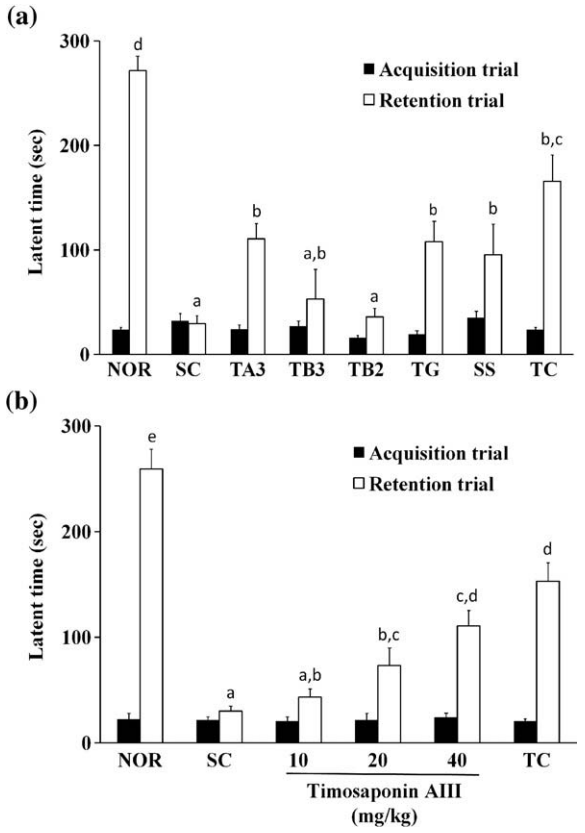

Fig. 3. Effect of timosaponin AIII and other steroidal saponins isolated from AA on scopolamine-induced memory deficits mice in the passive avoidance task. (a) Timosaponin AIII (TA3), timosaponin BII (TB2), timosaponin BIII (TB3), β-sitosterol glucoside (TG) or sarsasapogenin (SS) (40 mg/kg, *p.o.*), tacrine (TC, 10 mg/kg, *p.o.*) or vehicle (equal volume of 10% Tween 80) were administered to mice 1 h before acquisition trials. (b) TA3 (10, 20, 40 mg/kg, *p.o.*) was administered to mice 1 h before qcquisition trials. In all experiments, memory deficits were induced by a single scopolamine (SC) treatment (1 mg/kg, *i.p.*). Normal group (NOR) was treated with vehicle alone instead of scopolamine and test agents. Acquisition trials were performed 30 min after scopolamine treatment. At 24 h after acquisition trials, the retention trials were carried out for 5 min. Data represent mean ± S.E.M. ($n = 10$). ^{a,b,c,d,e}Items with the same letter in retention trial are not significantly different ($P > 0.05$).

2.10. Immunoblot

After stimulation with scopolamine or TNF-α whole cell lysates were prepared with ice-cold lysis RIPA buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM sodium chloride, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% phosphatase inhibitor cocktail and a protease inhibitor cocktail. Cell lysates were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to an Immobilon-P nylon membrane. NF-κB (pp65 and p65) was analyzed using the corresponding antibodies as previously reported (Lee et al., 2008). Immunodetection was carried out using an enhanced chemiluminescence detection kit.

2.11. Statistics

Values are expressed as means ± S.E.M. For the passive avoidance and Morris water maze test, data were analyzed by a Kruskal–Wallis non-parametric ANOVA test. If the results were significant, each treatment group was compared using the Tukey's *post hoc* test. Statistical significance was set at $P < 0.05$. For the microplate assay, data were analyzed by one-way analysis of variance (ANOVA). If the results were significant, each group was compared using the Dunnett's *post hoc* test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of steroidal saponins isolated from AA on scopolamine-induced memory impairment

During an herbal medicine screening program for agents to improve memory impairment, we selected AA, from which we isolated four steroidal saponins to investigate their effects in scopolamine-treated memory-deficient mice in the passive avoidance test. The step-through latency of scopolamine-treated mice was significantly shorter than that of vehicle-treated control mice (Fig. 3-a, $P < 0.05$). Of the tested steroidal saponins, TA3 most potently improved memory deficits.

Scopolamine-induced deficits in the passive avoidance test are dependent on long-term memory. When the effect of TA3 on scopolamine-induced deficits in the passive avoidance test was tested (Fig. 3-b, $P < 0.05$), TA3 increased the scopolamine-induced reduction in step-through latency by 17% (10 mg/kg), 28% (20 mg/kg), and 43% (40 mg/kg). During the acquisition trial, no differences in latent time were observed. As a positive control, tacrine (10 mg/kg) restored the step-through latency to 59% of the control group, consistent with previously published data (Bejar et al., 1999).

In the Morris water maze test, scopolamine treatment produced longer escape latencies in the training trials than the control treatment (Fig. 4-a). TA3 (40 mg/kg) significantly shortened this latent escape time, as did tacrine. Treatment with TA3 and tacrine also significantly

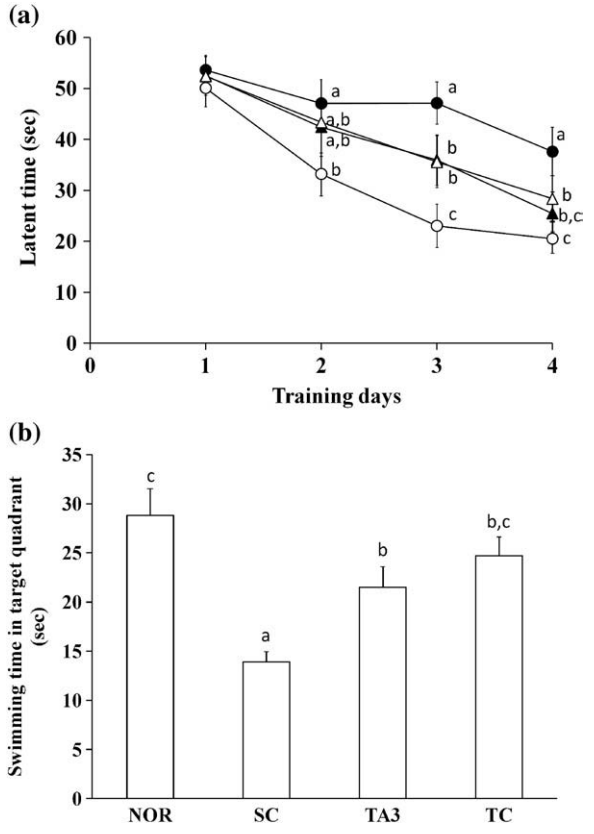

Fig. 4. Effects of timosaponin AIII on scopolamine-treated mice in the Morris water maze task. (a) Effect of TA3 on escape latency in the training trial sessions. TA3 (40 mg/kg, *p.o.*), tacrine (TC, 10 mg/kg, *p.o.*) or vehicle (same volume of 10% Tween 80) was administered to mice 60 min before the training trial session. Memory impairment was induced by scopolamine treatment (1 mg/kg, *i.p.*). Open circle and SC, scopolamine alone; closed triangle and TA3, TA3 (40 mg/kg, *p.o.*); open triangle and TC, tacrine (10 mg/kg, *p.o.*); closed circle and NOR, vehicle alone (10% Tween 80). (b) Effect of TA3 on swimming time spent in the target quadrant with platform. The training trial and the probe trial sessions were performed as described in the Methods and materials. Data represent mean ± S.E.M. ($n = 10$). ^{a,b,c,d,e}Items with the same letter in each training day (a) and swimming time spent in the target quadrant (b) are not significantly different ($P > 0.05$).

ameliorated the decrease in swimming time in the platform quadrant induced by scopolamine during the probe trial (Fig. 4-b).

3.2. Inhibitory effect of TA3 against AChE activity

To understand the memory-improving mechanism of TA3, we measured the hippocampal acetylcholine levels of scopolamine-treated mice with, and without, TA3 (Fig. 5-a). Scopolamine treatment reduced the level of acetylcholine in the hippocampus by 39.2%. TA3 (20, 40 mg/kg, *p.o.*) potently inhibited this reduction of acetylcholine in scopolamine-treated mouse brain. The inhibitory effect of TA3 was comparable to that of tacrine, which was used as a positive control ($P>0.05$).

We also measured the inhibitory effect of TA3 against AChE activity. TA3 inhibited AChE activity in a dose-dependent manner, with an IC_{50} value of 35.4 μM (Fig. 5-b).

3.3. Analysis of timosaponin AIII absorbed into mice serum

To confirm the memory-enhancing effect of TA3 in mice, we measured TA3 levels in blood after oral administration of TA3 using liquid chromatography/tandem mass spectrometry (LC-MS/MS) (Fig. 6-a). TA3 blood levels were highest between 4 h and 6 h after TA3 administration. The area under the serum concentration time curve

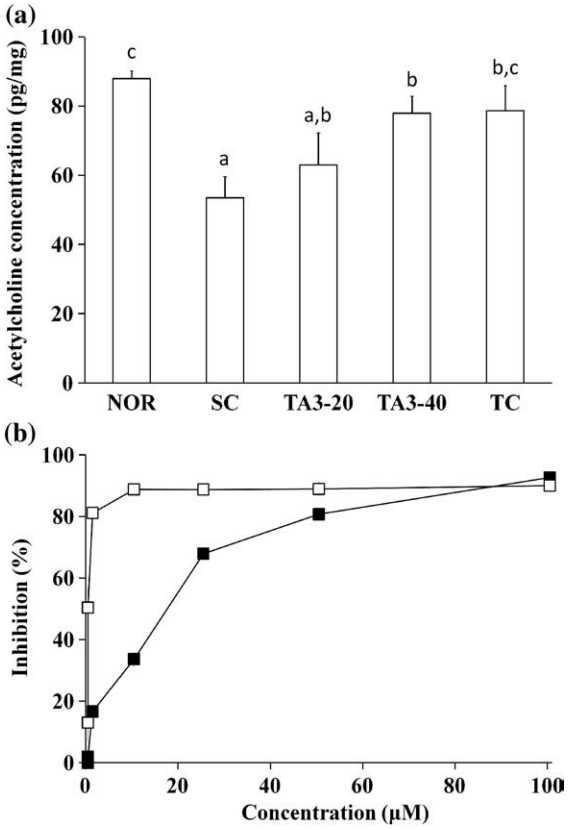

Fig. 5. Effects of timosaponin A3 on acetylcholine levels in scopolamine-treated mice and on acetylcholinesterase activity. (a) Effect of timosaponin AIII (TA3) on the acetylcholine level in mouse brain. We measured the level of acetylcholine in the hippocampus of scopolamine-treated mouse. The mice were administered TA3 (TA3-20, 20 mg/kg TA3; TA3-40, 40 mg/kg TA3, *p.o.*) and tacrine (TC, 10 mg/kg, *p.o.*) 30 min before scopolamine (SC, 1 mg/kg, *i.p.*) treatment. Normal group (NOR) was treated with vehicle alone instead of scopolamine and test agents. (b) Inhibitory effect of TA3 on acetylcholinesterase activity. Open square, tacrine; closed square, TA3. Inhibition is expressed as percent inhibition of enzyme activity compared with the medium control and presented as mean ± S.E.M. ($n=5$). [a,b,c]Items with the same letter are not significantly different ($P>0.05$).

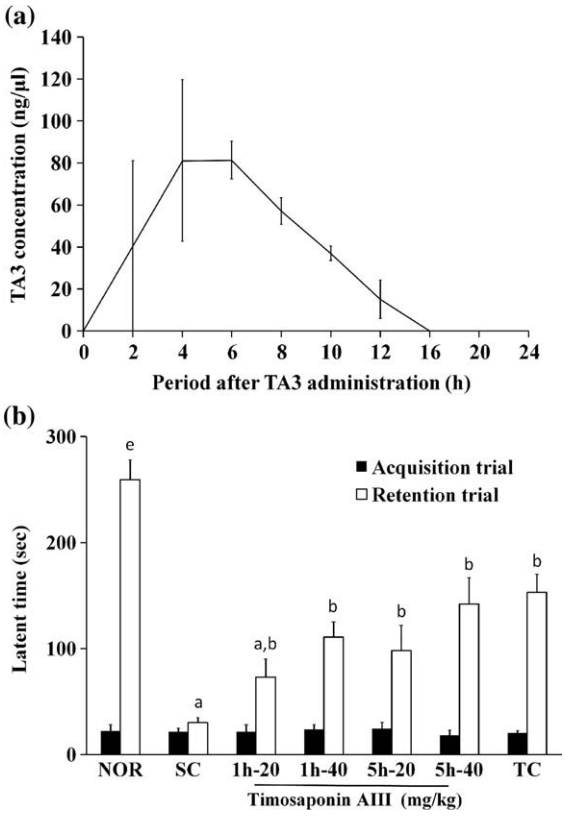

Fig. 6. The absorption of orally administered timosaponin AIII into the blood of mice and its memory-enhancing effect. (a) Absorption of orally administered timosaponin AIII into the blood. TA3 (50 mg/kg) was orally administered to mice. Bars represent standard deviation ($n=3$). (b) Memory-enhancing effect of TA3 orally administered 30 min or 4.5 h before scopolamine treatment in mice for the passive avoidance test. TA3 (20 and 40 mg/kg, *p.o.*) was administered 1 h or 5 h before acquisition trials for scopolamine-treated mice. Memory deficits were induced by a single scopolamine (SC) treatment (1 mg/kg, *i.p.*) 30 min and 4.5 h after TA3 treatment. Normal group (NOR) was treated with vehicle alone instead of scopolamine and test agents. Acquisition trials were performed 30 min after scopolamine treatment. At 24 h after acquisition trials, the retention trials were carried out for 5 min. Data represent mean ± S.E.M. (n-α and IL-1β expression in the brains of scopolamine-treated mice. TA3 (20 and 40 mg/kg, *p.o.*) was administered 1 h and 5 h before acquisition trials for scopolamine-treated mice. NOR, vehicle alone; SC, control (scopolamine alone treated); T20, 20 mg/kg of TA3; T40, 40 mg/kg of TA3; TC, 10 mg/kg of tacrine. Data represent mean ± S.E.M. ($n=5$). [a,b,c,d]Items with the same letter are not significantly different ($P>0.05$).

(AUC) and the peak concentration (C_{max}) of TA3 were 588 ± 76.9 ng/μl h and 104.7 ± 20.7 ng/μl, respectively.

3.4. Memory-enhancing effect of TA3 orally administered 1 h or 5 h before the acquisition trial

To investigate the relationship between blood concentration and the memory-enhancing effect of TA3, we orally administered TA3 to mice, injected scopolamine intraperitoneally 0.5 h or 4.5 h after TA3 administration, and examined the effect in the passive avoidance test 0.5 h after scopolamine injection. TA3 administered (20 and 40 mg/kg) 5 h before the acquisition trial was more effective than that administered 1 h before (Fig. 6-b, $P<0.05$). The memory-enhancing effect of TA3 orally administered 5 h before the acquisition trial was comparable to that of tacrine (10 mg/kg, *p.o.*) administered 1 h before the acquisition trial as a positive control ($P>0.05$).

3.5. Expression of proinflammatory cytokines IL-1β and TNF-α in scopolamine-stimulated mice

To evaluate whether scopolamine caused inflammation in the brain and memory deficits in mice, hippocampal expression levels of the proinflammatory cytokines, TNF-α and IL-1a, in scopolamine-treated

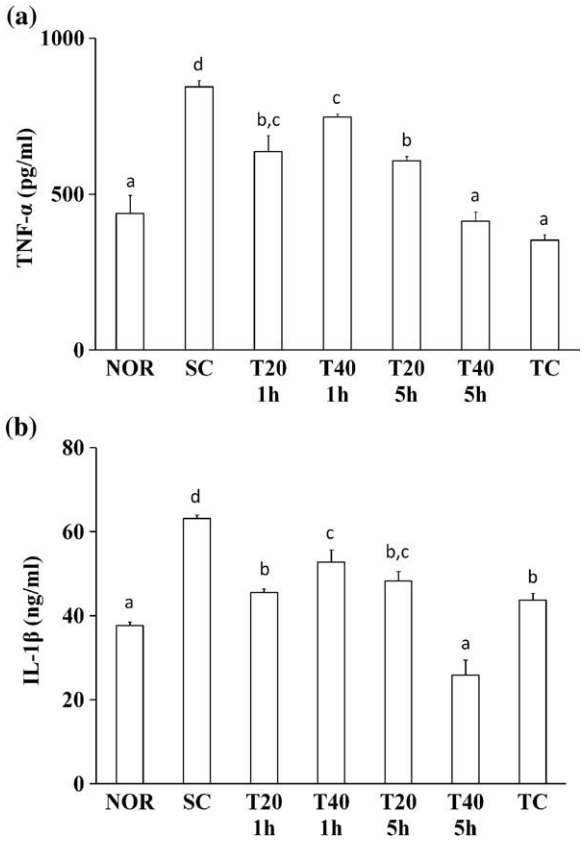

Fig. 7. Inhibitory effects of TA3 on TNF-α and IL-1β expression in the brains of scopolamine-treated mice. TA3 (20 and 40 mg/kg, *p.o.*) was administered 1 h and 5 h before acquisition trials for scopolamine-treated mice. NOR, vehicle alone; SC, control (scopolamine alone treated); T20, 20 mg/kg of TA3; T40, 40 mg/kg of TA3; TC, 10 mg/kg of tacrine. Data represent mean ± S.E.M. ($n = 5$). [a,b,c,d]Items with the same letter are not significantly different ($P > 0.05$).

mice were examined. Scopolamine significantly increased protein expression of IL-1β and TNF-α by 1.7- and 1.9-fold, respectively (Fig. 7). When TA3 was orally administered to scopolamine-treated mice at doses of 20 and 40 mg/kg, it potently inhibited the effect of scopolamine on IL-1β and TNF-α expression by 22% and 57% and by 27% and 50%, respectively. Oral administration of TA3 5 h before scopolamine was more effective than administration 1 h before. Its inhibitory potency was comparable to that of tacrine.

3.6. Effect of TA3 on NF-κB activation in scopolamine- and TNF-α-activated cells

To understand the anti-inflammatory mechanism of TA3, we investigated the effect of TA3 on transcription factors for the proinflammatory cytokines TNF-α and IL-1β in BV-2 microglial and SK-N-SH neuroblastoma cells activated by scopolamine and TNF-α. TNF-α activated NF-κB in both cell types. Treatment with TA3 significantly decreased the scopolamine- or TNF-α-induced NF-κB activation (pp65) in BV-2 microglial cells (Fig. 8-a, b). TA3 also significantly reduced TNF-α-induced NF-κB (pp65) activation, but not scopolamine-induced activation, in SK-N-SH neuroblastoma cells (Fig. 8-c, d).

4. Discussion

Previous studies have shown that *A. asphodeloides* (AA) attenuates learning deficits caused by brain damage and aging in rodents (Hu et al., 2005; Li et al., 2007; Ouyang et al., 2005). Of the constituents isolated from AA, sarsasapogenin and timosaponin BII enhanced learning and memory in amyloid β-peptide (25–35)- or scopolamine-induced dementia in rats (Ouyang et al., 2005; Hu et al., 2005). In the present study, sarsasapogenin also improved learning and memory. We isolated TA3 from AA as a novel memory-enhancing agent. In the Morris water maze and passive avoidance tests, TA3 potently and dose-dependently reversed the behavioral changes in scopolamine-induced memory impairment. TA3 also inhibited AChE activity in vitro. Many AChE inhibitors, such as sesquiterpenes, glabridin and alkaloids isolated from natural products, as well as a synthetic donepezil, improved memory and increased Ach levels in brain (Hornick et al., 2008; Hung et al., 2008; Cui et al., 2008). E-harpagoside, which was isolated from *Scrophularia buergeriana* as a cognitive-enhancing constituent, reduced AChE in the brains of scopolamine-induced amnesic mice (Jeong et al., 2008). Similarly, TA3 also led to a recovery of brain acetylcholine levels decreased by scopolamine treatment (Mishima et al., 2000), an effect that was comparable to that of tacrine. These results suggest that TA3 may improve scopolamine-induced hippocampal long-term memory deficits by inhibiting AChE. Orally administered TA3 was absorbed into the blood of mice. Its C_{max} in the blood occurred between 4 and 6 h after oral administration. Its memory-enhancing effect was also more potent 5 h before scopolamine treatment than 1 h before. These results suggest that the memory-enhancing effect of orally administered TA3 may be dependent on its absorption from the intestine to the blood.

Cholinergic neurons in the central nervous system (CNS) degenerate in a manner that correlates with functional loss in patients with AD and senile dementia (Davies and Maloney, 1976; Perry et al., 1978). TNF-α and

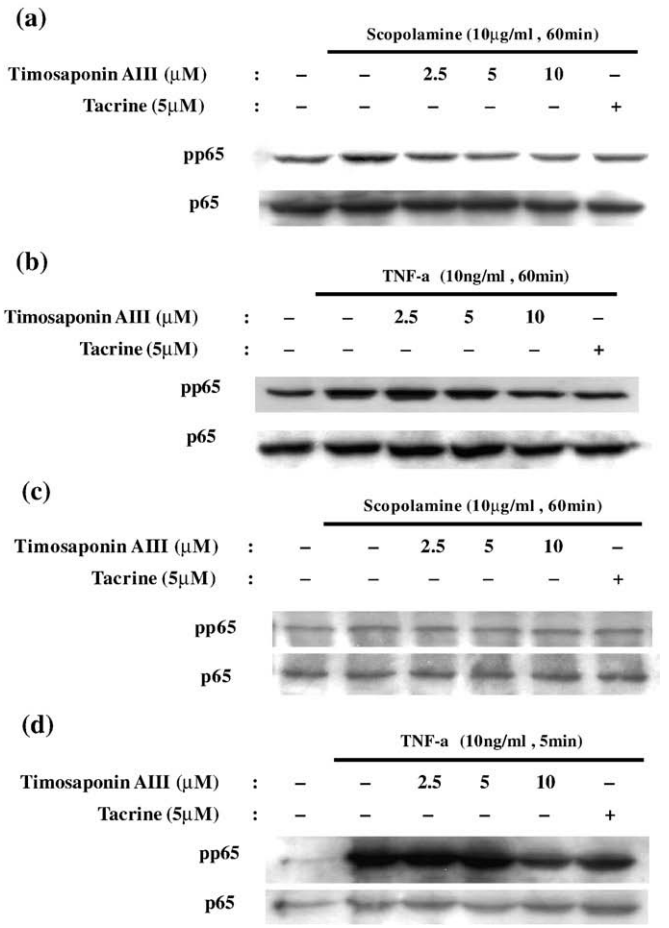

Fig. 8. Inhibitory effects of TA3 on NF-κB activation in BV-2 microglia (a, b) and SK-N-SH neuroblastoma (c, d) cells induced with TNF-α and scopolamine. BV-2 and SK-N-SH cells (5×10^5 cells) were treated with scopolamine (10 μg/ml) for 60 min or with TNF-α (10 ng/ml) for 5 min. Two microliters of the test agents (TA3, 2.5, 5 and 10 μM; tacrine, 5 μM) were incubated with BV-2 and SK-N-SH cells for 60 min before treatment with scopolamine or TNF-α. Immunoblotting for NF-κB (pp65 and p65) was performed. The normal and control groups were incubated with vehicle alone.

IL-1β are upregulated in the AD brain (Dickson et al., 1993; Griffin et al., 1995) and these cytokines may play a role in several events constituting the pathological cascade in AD (Eikelenboom et al., 1994). These results suggest that inflammatory reactions may be related to the pathogenesis of the degenerative changes and cognitive impairments associated with this disease (Rothwell et al., 1997). In the present study, scopolamine treatment caused learning and memory deficits, as well as increases of the proinflammatory cytokines TNF-α and IL-1β. Scopolamine also activated the transcription factor NF-κB (pp65), which regulates the expression of proinflammatory cytokines (Aggarwal, 2003) in BV-2 microglia cells; however, scopolamine did not activate NF-κB in SK-N-SH neuroblastoma cells. Thus, scopolamine may weakly cause cell injury and activate phagocytic cells, particularly BV-2 cells, and subsequent inflammatory reactions. TNF-α also activated NF-κB (pp65) in BV-2 microglia and SK-N-SH neuroblastoma cells. These findings suggest that scopolamine may cause learning and memory deficits through cholinergic neuronal injury complicated by inflammation. TA3 inhibited the increase of proinflammatory cytokines in scopolamine-treated mice, as well as NF-κB (pp65) activation in scopolamine- or TNF-α-stimulated BV2 microglia and SK-H-SH neuroblastoma cells. These results suggest that TA3 may inhibit the inflammation in scopolamine-stimulated mice by inhibiting NF-κB (pp65) activation.

Finally, TA3 may improve memory deficits mainly through AChE inhibition rather than through the inhibition of inflammatory cytokine expression via inhibition of transcription factor NF-κB activation.

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References

Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev 2003;3:745–56.
Bejar C, Wang RH, Weinstock M. Effect of rivastigmine on scopolamine-induced memory impairment in rats. Eur J Pharmacol 1999;383:231–40.
Blazer 2nd DG, Federspiel CF, Ray WA, Schaffner W. The risk of anticholinergic toxicity in the elderly: a study of prescribing practices in two populations. J Gerontol 1983;38:31–5.
Cui YM, Ao MZ, Li W, Yu LJ. Effect of glabridin from *Glycyrrhiza glabra* on learning and memory in mice. Planta Med 2008;74:377–80.
Davies P, Maloney AJ. Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet 1976;2:1403.
Dickson DW, Lee SC, Mattiace LA, Yen SH, Brosnan C. Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. Glia 1993;7:75–83.
Dong JX, Han GY. A new active steroidal saponin from *Anemarrhena asphodeloides*. Planta Med 1991;57:460–2.
Doody RS. Clinical profile of donepezil in the treatment of Alzheimer's disease. Gerontology 1999;45(Suppl 1):23–32.
Eikelenboom P, Zhan SS, van Gool WA, Allsop D. Inflammatory mechanisms in Alzheimer's disease. Trends Pharmacol Sci 1994;15:447–50.
Ellman GL, Courtney KD, Andres Jr V, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
Griffin WS, Sheng JG, Roberts GW, Mrak RE. Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution. J Neuropathol Exp Neurol 1995;54:276–81.
Hornick A, Schwaiger S, Rollinger JM, Vo NP, Prast H, Stuppner H. Extracts and constituents of *Leontopodium alpinum* enhance cholinergic transmission: brain ACh increasing and memory improving properties. Biochem Pharmacol 2008;76:236–48.
Hu Y, Xia Z, Sun Q, Orsi A, Rees D. A new approach to the pharmacological regulation of memory: sarsasapogenin improves memory by elevating the low muscarinic acetylcholine receptor density in brains of memory-deficit rat models. Brain Res 2005;1060:26–39.
Hung TM, Na M, Dat NT, Ngoc TM, Youn U, Kim HJ, et al. Cholinesterase inhibitory and anti-amnesic activity of alkaloids from *Corydalis turtschaninovii*. J Ethnopharmacol 2008;119:74–80.
Iida Y, Oh KB, Saito M, Matsuoka H, Kurata H, Natsume M, et al. Detection of antifungal activity in *Anemarrhena asphodeloides* by sensitive BCT method and isolation of its active compound. J Agric Food Chem 1999;47:584–7.
Ingkaninan K, de Best CM, van der Heijden R, Hofte AJ, Karabatak B, Irth H, et al. High-performance liquid chromatography with on-line coupled UV, mass spectrometric and biochemical detection for identification of acetylcholinesterase inhibitors from natural products. J Chromatogr 2000;872:61–73.
Jain NK, Patil CS, Kulkarni SK, Singh A. Modulatory role of cyclooxygenase inhibitors in aging- and scopolamine or lipopolysaccharide-induced cognitive dysfunction in mice. Behav Brain Res 2002;133:369–76.
Jeong EJ, Lee KY, Kim SH, Sung SH, Kim YC. Cognitive-enhancing and antioxidant activities of iridoid glycosides from *Scrophularia buergeriana* in scopolamine-treated mice. Eur J Pharmacol 2008;588:78–84.
Kim DH, Jeon SJ, Son KH, Jung JW, Lee S, Yoon BH, et al. The ameliorating effect of oroxylin A on scopolamine-induced memory impairment in mice. Neurobiol Learn Mem 2007;87:536–46.
Lee HS, Han SY, Bae EA, Huh CS, Ahn YT, Lee JH, et al. Lactic acid bacteria inhibit proinflammatory cytokine expression and bacterial glycosaminoglycan degradation activity in dextran sulfate sodium-induced colitic mice. Int Immunopharmacol 2008;8:574–80.
Li TJ, Qiu Y, Yang PY, Rui YC, Chen WS. Timosaponin B-II improves memory and learning dysfunction induced by cerebral ischemia in rats. Neurosci Lett 2007;421:147–51.
Meng Z, Xu S, Meng L. Timosaponins E1 and E2. Acta Pharm Sin 1998;33:693–6.
Mishima K, Iwasaki K, Tsukikawa H, Matsumoto Y, Egashira N, Abe K, et al. The scopolamine-induced impairment of spatial cognition parallels the acetylcholine release in the ventral hippocampus in rats. J J Pharm 2000;84:163–73.
Niwa A, Takeda O, Ishimaru M, Nakamoto Y, Yamasaki K, Kohda H, et al. [Screening test for platelet aggregation inhibitor in natural products. The active principle of *Anemarrhenae* rhizoma]. Yakugaku Zasshi 1988;108:555–61.
Oh JK, Hyun SY, Oh HR, Jung JW, Park C, Lee SY, et al. Effects of *Anemarrhena asphodeloides* on focal ischemic brain injury induced by middle cerebral artery occlusion in rats. Biol Pharm Bull 2007;30:38–43.
Ouyang S, Sun LS, Guo SL, Liu X, Xu JP. Effects of timosaponins on learning and memory abilities of rats with dementia induced by lateral cerebral ventricular injection of amyloid beta-peptide. Di 1 jun yi da xue xue bao. Acad J First Med Coll PLA 2005;25:121–6.
Perry EK, Tomlinson BE, Blessed G, Bergmann K, Gibson PH, Perry RH. Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. Br Med J 1978;2:1457–9.
Ren LX, Luo YF, Li X, Zuo DY, Wu YL. Antidepressant-like effects of sarsasapogenin from *Anemarrhena asphodeloides* BUNGE (Liliaceae). Biol Pharm Bull 2006;29:2304–6.
Rothwell N, Allan S, Toulmond S. The role of interleukin 1 in acute neurodegeneration and stroke: pathophysiological and therapeutic implications. J Clin Invest 1997;100:2648–52.
Saito S, Nagase S, Ichinose K. New steroidal saponins from the rhizomes of *Anemarrhena asphodeloides* Bunge (Liliaceae). Chem Pharm Bull 1994;42:2342–5.
Takahashi M, Konno C, Hikino H. Isolation and hypoglycemie activity of anemarans A, B, C and D, glycans of *Anemarrhena asphodeloides* rhizomes1. Planta Med 1985;51:100–2.
Whitehouse PJ, Price DL, Clark AW, Coyle JT, DeLong MR. Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis. Ann neurol 1981;10:122–6.