



Anti-amnesic activity of neferine with antioxidant and anti-inflammatory capacities, as well as inhibition of ChEs and BACE1

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ABSTRACT

Aims: The multifunctional potential of neferine derived from the embryo of *Nelumbo nucifera* seeds for the age-related neurodegenerative disorders, in vivo anti-amnesic activities and in vitro cholinesterases (ChEs)- and β -site APP cleaving enzyme 1 (BACE1)-inhibitory activities, as well as anti-inflammatory and antioxidant activities were investigated.

Main methods: In vivo anti-amnesic activities were performed via the passive avoidance, Y-maze, and Morris water maze tasks in a scopolamine-induced amnesia model. The cell-free antioxidant capacities were evaluated by in vitro scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals, and peroxynitrite (ONOO⁻), as well as inhibitory activities against nitric oxide (NO[•]), superoxide anion (\bullet O₂⁻), lipid peroxidation, and ONOO⁻-mediated tyrosine nitration. The intracellular antioxidant capacities were also determined via inhibitory activities of lipopolysaccharide (LPS)-induced NO[•] generation and NF- κ B activation in RAW 264.7 cells.

Key findings: Neferine showed significant improvement in cognitive impairment in scopolamine-induced amnesia animal models and moderate inhibitory activities in ChEs and BACE1 assays. In addition, it exhibited notable scavenging activities against DPPH, ABTS, NO[•], and \bullet O₂⁻ radicals, as well as ONOO⁻. Neferine also demonstrated remarkable inhibitory activity against lipid peroxidation and protein nitration in cell-free antioxidant assays and moderate inhibitory activity of NO[•] generation with exceptional suppression of NF- κ B activation in cell-based assays.

Significance: The results demonstrate that the anti-amnesic effect of neferine may be mediated via antioxidant and anti-inflammatory capacities, as well as inhibition of ChEs and BACE1.

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Introduction

Neferine is a major bisbenzylisoquinoline alkaloid derived from the embryos of *Nelumbo nucifera* Gaertn., along with liensinine and isoliensinine (Liu et al. 2009). This abundant alkaloid in the green embryos is reported to possess a variety of biological activities, including anti-arrhythmia, anti-hypertensive (Qian 2002; Gu et al. 2009), relaxant (Chen et al. 2007, 2008), anti-diabetic (Pan et al. 2009), cholinesterase inhibitory (Xiong and Zeng 2003), sedative (Sugimoto et al. 2008), and anti-multidrug resistance (Cao et al. 2004). Previous reports revealed that neferine inhibited Cu²⁺-mediated LDL oxidation and rabbit platelet

aggregation (Feng et al. 1998; Yu and Hu 1997). In particular, the embryos have been used widely in traditional Chinese medicine for nervous disorders, insomnia, high fevers with restlessness, relieving cough pulmonary fibrosis, and cardiovascular diseases such as hypertension and arrhythmia (Sridhar and Bhat 2007). Recently, Oh et al. (2009) demonstrated that *N. nucifera* semen extract showed improvement of cognitive impairment in scopolamine-induced amnesia model.

Alzheimer's disease (AD) is an age-related neurodegenerative disease and the most frequent and predominant cause of dementia in the elderly, concomitant with the presence of senile plaques and neurofibrillary tangles, a decrease in cholinergic transmission, psycho-behavior disturbances, and progressive cognitive impairments, including memory loss and learning disturbances (Scarpini et al. 2003; Parihar and Hemnani 2004). In particular, amnesic cognitive impairment commonly occurs in the aging process and sometimes results in AD

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(Gavett et al. 2009). Most pharmacological research of AD has focused on two major aspects (Parihar and Hemnani 2004): recovery of cholinergic transmitters levels via acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitors (Rao et al. 2007); inhibition of amyloid β ($A\beta$) formation and aggregation via β -site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1; β -secretase) inhibitors (Vassar 2002; Yan et al. 1999). Interestingly, age-related neurodegenerative disorders are highly implicated in not only severe neuronal cell damage and decline in neurotransmitters but also increase in inflammation and oxidative stress (Orhan and Aslan 2009). Additional research has revealed that lipid peroxidation, including thiobarbituric acid reactive substances (TBARS) and 4-hydroxy-2-trans-nonenal (HNE), and protein oxidation/nitration, such as protein carbonyls and 3-nitrotyrosine, can act as biomarkers for the neuronal oxidative stress of AD (Markesbery 1997; Butterfield et al. 2007, 2001; Torrealles et al. 1999). Their mechanisms of memory-enhancing action may predominantly involve a combination of anti-inflammatory, antioxidant, and neuroprotective roles in signal transduction pathways rather than the chemical antioxidant properties alone (Rossi et al. 2008; Fan et al. 2005; El-Sherbiny et al. 2003). Since AD is highly related to cholinergic deficits, $A\beta$ toxicity, inflammation, and intracellular oxidative stress, simultaneous study of ChEs- and BACE1-inhibitory, anti-inflammatory, antioxidant, and anti-amnesic activities is a worthy approach for the development of promising anti-AD agents.

Therefore, this lab investigated the in vivo anti-amnesic activities, in vitro ChEs- and BACE1-inhibitory activities, in vitro antioxidant activities, and intracellular anti-inflammatory activities of neferine. Considering the results of these above experiments, we will evaluate the possibility of neferine as a therapeutic or preventive agent for amnesia and oxidative stress-related neurodegenerative disorders.

Materials and methods

Cells and animals

The mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Rockville, MD, USA). Cell culture medium and all of the other associated materials were purchased from Gibco BRL Life Technologies (Gaithersburg, MD, USA). In order to obtain rat liver homogenates for lipid peroxidation, this lab followed the Guidelines for Care and Use of Laboratory Animals as approved by Pukyong National University. All experiments were performed using male ICR (CD-1) mice weighing 28–30 g (6-week-old), purchased from the Orient Co., Ltd., a branch of Charles River Laboratories (Seoul, Korea). Animal treatment and maintenance for anti-amnesic effects were conducted in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea.

Reagents

DPPH, ABTS, electric-eel AChE (EC 3.1.1.7), horse-serum BChE (EC 3.1.1.8), xanthine oxidase (XOD) from bovine milk, acetylthiocholine iodide (ACh), butyrylthiocholine chloride (BCh), 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), sodium nitroprusside dihydrate, xanthine (2,6-dihydroxypurine), 2-thiobarbituric acid (TBA), bovine serum albumin (BSA), 4,5-diaminofluorescein diacetate (DAF-2), L-2-amino-3-mercapto-3-methylbutanoic acid (L-penicillamine), L-ascorbic acid, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (Carboxy-PTIO) potassium salt, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), allopurinol, galantamine, 4-methylumbelliferyl phosphate (MUP), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate (tacrine, THA), scopolamine, and lipopolysaccharide

(LPS) from *Escherichia coli* were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The BACE1 FRET assay kit (β -Secretase) was purchased from the PanVera Co. (Madison, WI, USA). Dihydrorhodamine 123 (DHR 123) were of high quality and were purchased from Molecular Probes (Eugene, OR, USA), and 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT) and peroxyxynitrite ($ONOO^-$) from Cayman Chemicals Co. (Ann Arbor, MI, USA). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA). Anti-nitrotyrosine, clone 1A6 (mouse-monoclonal primary antibody, IgG2b) and anti-mouse IgG, HRP conjugate (goat, polyclonal secondary antibody) were purchased from Millipore Co. (Temecula, CA, USA), and polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was purchased from Millipore Co. (Billerica, MA, USA). Supersignal[®] West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). All chemicals and solvents used were purchased from E. Merck, Fluka, and Sigma-Aldrich Co., unless stated otherwise.

Isolation of neferine

The embryos of *N. nucifera* (2.0 kg) were refluxed in methanol (MeOH) for 3 h (3.0 L \times 3). The total filtrate was then concentrated to dryness in vacuo at 40 °C in order to render the MeOH extract (543.5 g). This extract was then suspended in distilled water (H_2O) and successively partitioned with methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) to yield CH_2Cl_2 (13.8 g), EtOAc (2.2 g), and *n*-BuOH (58.3 g) fractions, respectively, as well as a H_2O residue (469.0 g). Repeated chromatography of the CH_2Cl_2 fraction (13.8 g) over a Si gel column (120 mm i.d.) with benzene–EtOAc–diethylamine (7:2:1, isocratic) afforded the neferine (920 mg). The neferine was characterized and identified by spectroscopic methods, including ESI-MS, 1H and ^{13}C NMR, as well as through comparison with published data (Yang and Zhou 2004). The spectral data are as follows and the structure is shown in Fig. 1.

ESI-MS(positive module): 625 [M + H]⁺, 1H NMR (400 MHz, $CDCl_3$): δ 6.88 (2H, d, J = 8.4 Hz, H-11/H-15), 6.81 (1H, d, J = 8.1 Hz, H-14'), 6.66 (3H, d, J = 8.2 Hz, H-15'/H-12/H-14), 6.62 (1H, s, H-5), 6.51 (1H, m, H-11'), 6.49 (1H, s, H-5'), 6.35 (1H, s, H-8), 5.98 (1H, s, H-8'), 3.79 (3H, s, 6-OCH₃), 3.76 (3H, s, 7'-OCH₃), 3.70 (3H, s, 13-OCH₃), 3.60 (2H, m, H-1/H-1'), 3.52 (3H, s, 6'-OCH₃), 3.15–2.95 (4H, m, H-3/H-3'/H-4/H-4'), 3.04 (1H, dd, J = 12.0, 5.0 Hz, H-9), 2.98 (1H, dd, J = 12.0, 5.3 Hz, H-9'), 2.83–2.53 (8H, m, H-3/H-3'/H-4/H-4'/H-9/H-9'), 2.47 (3H, s, H-2), 2.43 (3H, s, H-2'); ^{13}C NMR (100 MHz, $CDCl_3$): δ 157.7 (C-13), 148.8 (C-6), 147.1 (C-7'), 146.2 (C-6'), 145.5 (C-13'), 144.7 (C-12'), 142.7 (C-7), 131.6 (C-10'), 131.3 (C-10), 130.8 (C-8a), 130.3 (C-4a), 130.3 (C-11), 130.3 (C-15), 129.0 (C-4a'), 125.5 (C-8a'), 125.1 (C-11'), 119.8 (C-8), 119.1 (C-15'), 115.6 (C-14'), 113.3 (C-12), 113.3 (C-14), 112.2 (C-5), 111.0 (C-5'), 110.9 (C-8'), 64.6 (C-1'), 64.3 (C-1), 55.7 (7'-OCH₃), 55.6 (6'-OCH₃), 55.3 (6-OCH₃), 55.0 (13-OCH₃), 47.0 (C-3), 46.5 (C-3'), 42.5 (C-2), 42.3 (C-2'), 40.5 (C-9), 39.7 (C-9'), 26.0 (C-4), 25.0 (C-4'). See Table 1.

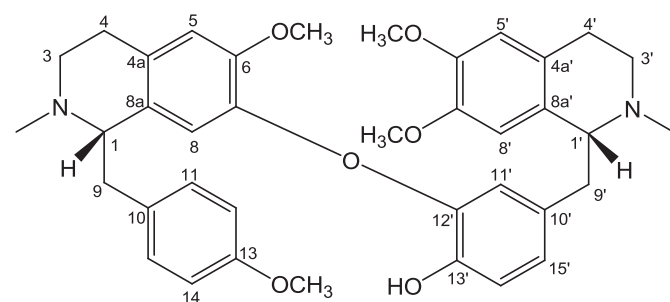


Fig. 1. Structure of neferine.

Table 1
¹H and ¹³C NMR spectroscopic data of neferine in CDCl₃.

No	¹ H	¹³ C	No	¹ H	¹³ C
1	3.60 m	64.3	1'	3.60 m	64.6
3	3.15–2.95 m	47.0	3'	3.15–2.95 m	46.5
4	2.83–2.53 m		4'	2.83–2.53 m	
	3.15–2.95 m	26.0		3.15–2.95 m	25.0
	2.83–5.53 m			2.83–2.53 m	
4a		130.3	4a'		129
5	6.62 s	112.2	5'	6.49 s	110.0
6		148.8	6'		146.2
7		142.7	7'		147.1
8	6.35 s	119.8	8'	5.98 s	110.9
8a		130.8	8a'		125.5
9	3.04 dd, <i>J</i> = 12.0, 5.0	40.5	9'	2.98 dd, <i>J</i> = 12.0, 5.3	39.7
	2.83–2.53 m			2.83–2.53 m	
10		131.3	10'		131.6
11	6.88 d, <i>J</i> = 8.4	130.3	11'	6.51 m	125.1
12	6.66 d, <i>J</i> = 8.2	113.3	12'		144.7
13		157.7	13'		145.5
14	6.66 d, <i>J</i> = 8.2	113.3	14'	6.81 d, <i>J</i> = 8.1	115.6
15	6.88 d, <i>J</i> = 8.4	130.3	15'	6.66 d, <i>J</i> = 8.2	119.1
N-CH ₃	2.47 s	42.5	N-CH ₃	2.43 s	42.3
6-OCH ₃	3.79	55.3	7'-OCH ₃	3.79	55.7
13-OCH ₃	3.79	55.0	6'-OCH ₃	3.52	55.6

Passive avoidance test in scopolamine-induced amnesia model

Male ICR (CD-1) mice weighing 28–30 g were used for passive avoidance. A one-week adaptation period (22 to 25 °C, 12 h light cycle from 06:00 to 18:00) for passive avoidance performance was carried out in two, identical, light and dark square boxes. When the mice entered the dark compartment, the door closed and an electrical foot shock (0.5 mA/30 g body weight) for a time period of 3 s was delivered through the stainless steel rods. For acute treatment experiments, the mice received neferine (control, 0, 1.25, 2.5, 5.0, 10 mg/kg body weight, p.o.). After 30 min, amnesia was induced in mice with scopolamine (1.0 mg/kg body weight, dissolved in distilled water, i.p.). Mice which went through dark compartment from illuminated room within 60 s were used for experiments. Twenty-four hours after the trial, the mice were again placed in the light compartment. The latency time to enter the dark compartment was measured. THA (10 mg/kg, p.o.) was used as the positive control.

Y-maze task in scopolamine-induced amnesia model

The Y-maze is a three-arm horizontal maze (40-cm long and 3-cm-wide with 12-cm-high walls) in which the arms are symmetrically disposed at 120° angles from each other. The maze floor and walls were constructed from dark opaque polyvinyl plastic as previously described (Kim et al. 2006). The mice were initially placed within one arm, and the sequence (ABCCAB) and number of arm entries were recorded manually for each mouse over an 8-min period. An actual alternation was defined as entries into all three arms on consecutive choices (ABC, CAB, or BCA, but not BAB). Maze arms were thoroughly cleaned between tasks to remove residual odors. One hour after the final administration of neferine (10 mg/kg, p.o.) or vehicle, mice were gently placed in the maze. The percentage of alternations was defined according to the following equation: % Alternation = [(Number of alternations) / (Total arm entries - 2)] × 100. The number of arm entries served as an indicator of locomotor activity. THA (10 mg/kg, p.o.) was used as the positive control.

Morris water maze test in scopolamine-induced amnesia model

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),

according to the method of Kim et al. (2006). The tank was placed in a dimly lit, soundproof test room with various visual cues. The pool was conceptually divided into quadrants, with a white platform (6 cm in diameter and 29 cm high) 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 60 s swim training in the absence of the platform. During the four subsequent days, the mice were given four trials per day with the platform in place. When the mice located the platform, it was permitted to remain on it for 10 s. If the mice 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 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, the 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 sessions, mice were subjected to a probe trial session in which the platform was removed from the pool and mice 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. Neferine (10 mg/kg, p.o.) or THA (10 mg/kg, p.o.) as a positive control was given 1 h prior to the first trial session every consecutive day. Memory impairment was induced in mice with scopolamine (1.0 mg/kg, i.p.) 30 min after treatment with neferine. The control group received a 10% Tween 80 solution only.

In vitro ChEs enzyme assay

Inhibitory activities of the ChEs were measured using the spectrophotometric method developed of Ellman et al. (1961). The ACh and BCh were used as the substrates to assay the inhibitions of AChE and BChE, respectively. The reaction mixture contained: 140 µl of sodium phosphate buffer (pH 8.0); 20 µl of neferine (final conc., 100 µM); and 20 µl of either AChE or BChE solution, which were mixed and incubated for 15 min at room temperature. Neferine and the positive control (galantamine) were dissolved in 10% analytical grade DMSO. The reactions were initiated with the addition of 10 µl of DTNB and 10 µl of either ACh or BCh, respectively. The hydrolysis of ACh or BCh was monitored by following the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm for 15 min, which resulted from the reaction of DTNB with thiocholine, released by the respective enzymatic hydrolysis of either ACh or BCh. All reactions were

performed in triplicate and recorded in 96-well microplates, using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Percent inhibition was calculated from $(1 - S/E) \times 100$, where E and S were the respective enzyme activities without and with the tested sample, respectively. The ChEs inhibitory activity was expressed in terms of the IC₅₀ value (μM required to inhibit the hydrolysis of the substrate, ACh or BCh, by 50%), as calculated from the log-dose inhibition curve.

In vitro BACE1 enzyme assay

The assay was carried out according to the supplied manual with select modifications. Briefly, a mixture of 10 μl of assay buffer (50 mM sodium acetate, pH 4.5), 10 μl of BACE1 (1.0 U/ml), 10 μl of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 μl of neferine (final conc., 100 μM), dissolved in 10% DMSO, was incubated for 60 min at 25 °C in the dark. The proteolysis of two fluorophores (Rh-EVNLDAEFK-Quencher) by the BACE1 was monitored by a formation of the fluorescent donor (Rh-EVNL) that increases in fluorescence wavelengths at 530–545 nm (excitation) and 570–590 nm (emission), respectively. Fluorescence was measured with a microplate spectrofluorometer (Molecular Devices). The mixture was irradiated at 545 nm and the emission intensity recorded at 585 nm. The percent inhibition (%) was obtained by the following equation: % Inhibition = $[1 - (S_{60} - S_0) / (C_{60} - C_0)] \times 100$, where C₆₀ is the fluorescence of the control (enzyme, buffer, and substrate) after 60 min of incubation, C₀ the initial fluorescence of the control, S₆₀ the fluorescence of the tested samples (enzyme, sample solution, and substrate) after 60 min of incubation, and S₀ the initial fluorescence of the tested samples. To allow for the quenching effect of the samples, the sample solution was added to reaction mixture C, and any reduction in fluorescence by the sample was then investigated. The BACE1 inhibitory activity of neferine was expressed in terms of the IC₅₀ value (μM required to inhibit the proteolysis of the substrate, BACE1, by 50%), as calculated from the log-dose inhibition curve. Quercetin was used as the positive control.

Assay for cell viability

Neferine cytotoxicity was evaluated with the MTT assay. The RAW 264.7 cells were seeded onto a 96-well plate at a density of 1.0×10^4 cells per well and incubated at 37 °C for 24 h. The cells were then treated with various concentrations of neferine. After additional 24 h incubation at 37 °C, 100 μl of MTT (0.5 mg/ml in PBS) was added to the wells and incubation continued for another 3 h. The resulting color was assayed at 540 nm using a microplate spectrophotometer (Molecular Devices).

Assay for inhibition of cellular nitric oxide formation

The macrophage RAW 264.7 cell line was incubated in Dulbecco's modified Eagle's medium (DMEM) at 37 °C under 5% CO₂ humidified air. The cells (1.0×10^5 cells/well in a 24-well plate with 500 μl of culture medium) were pretreated with neferine for 2 h and incubated for 18 h with LPS (1.0 $\mu\text{g}/\text{ml}$). After incubation, the nitrite concentration of the supernatants (100 $\mu\text{l}/\text{well}$) was measured by adding 100 μl of Griess reagents (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). To quantify the nitrite concentration, standard nitrite solutions were prepared and the absorbance of the mixture determined using a microplate spectrophotometer (Molecular Devices) at 540 nm. An inducible nitric oxide synthase inhibitor (AMT) was used as the positive control.

Assay for inhibition of NF- κ B activation by reporter gene assay

The RAW 264.7 cells harboring the pNF- κ B-SEAP-NPT reporter construct were plated at a density of 1.0×10^5 cells/well in a 24-well plate with 500 μl of culture medium and incubated for 24 h. The cells

were then pre-treated with neferine for 2 h before stimulation with LPS (1.0 $\mu\text{g}/\text{ml}$) for 16 h. Supernatants were heated at 65 °C for 6 min and reacted in the SEAP assay buffer [2.0 M diethanolamine, 1.0 mM MgCl₂, 500 μM MUP] in darkness at 37 °C for 1 h. The fluorescence from adduct of the SEAP/MUP reaction was measured in relative fluorescence units (RFU) using a microplate spectrofluorometer (Molecular Devices) at excitation and emission wavelengths of 360 and 449 nm. A select inhibitor of NF- κ B (TPCK) was used as the positive control.

DPPH radical scavenging activity

The DPPH radical scavenging activity was evaluated using the method of Blois (1958), with slight modification. Neferine and DPPH were dissolved in MeOH. One hundred sixty microliters of neferine at various concentrations (final conc., 100 μM) were added to 40 μl DPPH solution (1.5×10^{-4} M). After mixing gently and standing at room temperature for 30 min, the optical density of the reactant was measured at 520 nm using a microplate spectrophotometer (Molecular Devices). The antioxidant activity of neferine was expressed in term of IC₅₀ values (μM , required to inhibit DPPH radical formation by 50%), which was calculated from the log-dose inhibition curve. L-Ascorbic acid was used as the positive control.

Trolox equivalent antioxidant capacity (TEAC)

This assay was based on the ability of different substances to scavenge ABTS radical cation (ABTS^{•+}) as compared to the positive control trolox: the method developed by Re et al. (1999). To oxidize colorless ABTS to blue-green ABTS^{•+}, a 7 mM ABTS stock solution was mixed with 2.45 mM potassium persulfate (1:1, v/v) and left at the room temperature in the dark for 12–16 h until the reaction was complete and the absorbance was stable. The blue/green ABTS^{•+} solution was diluted in ethanol (EtOH) to an absorbance of 0.70 ± 0.02 at 734 nm for measurement. The photometric assay was conducted on 180 μl of the ABTS^{•+} solution and 20 μl of neferine dissolved in EtOH solution (final conc., 100 μM) that was stirred for 30 s. The optical density was measured at 734 nm after 2 min using a microplate spectrophotometer (Molecular Devices). The antioxidant activities of neferine were calculated by determining the decrease in absorbance at different concentrations using the following equation: $E = [(A_c - A_t) / A_c] \times 100$, where A_t and A_c are the absorbance of with and without neferine, respectively. Trolox and L-ascorbic acid were used as the positive controls. The TEAC results are expressed as IC₅₀ values (μM), trolox equivalent (TE), and L-ascorbic acid equivalent (AE).

Assay for ONOO⁻ scavenging activity

The ONOO⁻ scavenging activity was assessed by the modified Kooy's method, which involved the monitoring of highly fluorescent rhodamine 123, which was rapidly produced from non-fluorescent DHR 123 in the presence of ONOO⁻ (Kooy et al. 1994). In brief, the rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM DTPA. The final DHR 123 concentration was 5 μM . The assay buffer was prepared prior to use and placed on ice. Neferine was dissolved in 10% DMSO (final conc., 100 μM). The background and final fluorescent intensities were measured 5 min after treatment with and without the addition of authentic ONOO⁻ (10 μM), dissolved in 0.3 N sodium hydroxide. The fluorescence intensity of the oxidized DHR 123 was evaluated using a microplate spectrofluorometer (Bio-Tek Instruments Inc., Winooski, VT, USA) at excitation and emission wavelengths of 480 and 530 nm, respectively. The values of ONOO⁻ scavenging activity were calculated as the final fluorescence intensity minus the background fluorescence, via the detection of DHR 123 oxidation. L-Penicillamine was used as the positive control.

Assay for NO• scavenging activity

The NO• scavenging activity was determined by the modified method of Kim et al. (2004) using DAF-2 monitoring. The DAF-2, as a specific NO• indicator, selectively traps NO• between two amino groups within the molecule and yields triazolofluorescein that emits green fluorescence when excited at 490–495 nm. In brief, a stock solution of DAF-2 (4.5 mM) was diluted in 50 mM potassium phosphate buffer (pH 7.4, 1/45-fold); sodium nitroprusside, as a NO• donor, was also dissolved (final conc., 2.0 mM). Neferine was dissolved in 10% DMSO (final conc., 100 μM). The reaction was initiated by addition of the diluted DAF-2 solution (final conc., 25 μM) to the mixed solution of sodium nitroprusside and neferine in a 96-well black microplate. The fluorescence intensity caused by the reaction of DAF-2 with NO• was evaluated using a microplate spectrofluorometer (Bio-Tek Instruments Inc.) at excitation and emission wavelengths of 495 and 515 nm, respectively. Carboxy-PTIO was used as the positive control.

Assay for •O₂⁻ scavenging activity

The inhibition of XOD activity was evaluated by measuring the formation of uric acid from xanthine (Park et al. 2008). The assay mixture consisted of 50 μl of neferine (final conc., 5.50 μg/ml) and 350 μl of xanthine (1.0 mM) as the substrate. Xanthine and XOD were dissolved in 50 mM potassium phosphate buffer (pH 7.4) with 0.25 mM EDTA. The reaction mixtures were preincubated at room temperature for 2 min and the reaction initiated by addition of 400 μl of XOD (50 mU/ml). The mixtures (800 μl) were incubated at 37 °C for 30 min. The reaction was terminated by addition of HCl (100 μl, 5.0 M). The production of uric acid was determined with an UV/Visible spectrophotometer (Amersham Biosciences, Piscataway, New Jersey, USA) at 295 nm. Allopurinol was used as the positive control.

Assay for in vitro lipid peroxidation

The rats were sacrificed and the livers removed. The liver was homogenized in 5.0 ml of 0.9% sodium chloride and then diluted up to 10 ml with the same solution. A mixture of the liver homogenate (0.3 ml) was then mixed with 0.3 ml of aqueous 8.1% sodium dodecyl sulfate (SDS). The SDS/homogenate mixture was then split between two test tubes, one with and one without 0.1 ml of neferine (final conc., 500 μg/ml). The mixtures were incubated at 37 °C for 2 h. After addition of 1.5 ml of 20% acetic acid and 1.0 ml of 1.2% TBA solution, the test solutions were boiled at 100 °C for 30 min and then cooled to room temperature. The solutions were centrifuged at 2500 rpm for 15 min and the absorbance of the upper layer measured with a UV/Visible spectrophotometer (Amersham Biosciences) at 532 nm (Igarashi and Ohmura 1995). One TBA unit corresponded to 0.1 optical density at 532 nm and was calculated to a TBA value per g liver weight. L-Ascorbic acid was used as the positive control.

Inhibition of ONOO⁻-mediated tyrosine nitration

In order to examine the inhibition of ONOO⁻-induced BSA nitration, 2.5 μl of neferine (final conc., 6.25, 12.5, and 25 μM) dissolved in 10% EtOH (v/v) was added to 95 μl of BSA (0.5 mg protein/ml) and then mixed with 2.5 μl of ONOO⁻ (200 μM). After incubation with shaking at 37 °C for 20 min, the mixed sample was added to Bio-Rad gel buffer in a ratio of 1:1 and boiled for 5 min to denature the proteins. The total protein equivalent for the reactant was separated on 10% SDS-polyacrylamide minigel at 80 V for 30 min, followed by 100 V for 1 h, and then transferred to a PVDF membrane at 80 V for 110 min in a wet transfer system (Bio-Rad, Hercules, CA, USA). The membrane was immediately placed in a blocking solution (5% non fat dry milk in TBS-Tween buffer (w/v), Bio-Rad TBS, and 0.1% Tween-20, pH 7.4) at room temperature for 1 h. The membrane was washed three times (10 min

in TBS-Tween buffer and incubated with a monoclonal anti-nitrotyrosine antibody (5% non fat dry milk), diluted 1:2000 in TBS-Tween buffer at 4 °C overnight. After two more washings in TBS-Tween buffer (10 and 5 min), the membrane was incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody from sheep (5% w/v non fat dry milk), and diluted 1:2000 in TBS-Tween buffer at room temperature for 1 h. After two washings in TBS-Tween buffer (10 and 5 min), the antibody labeling was visualized with a supersignal west pico chemiluminescent substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions, and exposed to X-ray film (Kodak, Rochester, NY, USA). Pre-stained blue protein markers were used for molecular-weight determination.

Statistics

Results were expressed as the mean ± S.E.M. In the passive avoidance task, acquisition and retention latencies were evaluated using the Kruskal–Wallis nonparametric test followed by Dunn's post hoc test. In the Y-maze task and Morris water maze task, data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test. In particular, group differences in the escape latency of the training trial session in the Morris water maze task were analyzed using two-way ANOVA with repeated measures. Differences were considered significant at a level of $P < 0.05$.

Results

Isolation of neferine

Neferine was isolated from the CH₂Cl₂ fraction (13.8 g) through repeated column chromatography. The neferine was characterized and identified by spectroscopic methods, including ESI-MS, ¹H and ¹³C NMR (Table 1). Since the observed spectral data were superimposed on published data (Yang and Zhou 2004) and there are no additional peaks to impurity, an isolated compound was determined as neferine and used in further in vitro and in vivo works.

Effect of neferine in the scopolamine-induced memory impairment model

In order to determine the improvement of AD-derived cognitive impairment, such as memory loss and learning disturbance, the in vivo anti-amnesic activity of neferine was evaluated via the passive avoidance, Y-maze, and Morris water maze tasks with the scopolamine-treated animal model. During acquisition trials in the passive avoidance test, latency times remained the same among the experimental groups with administration of neferine with scopolamine (Fig. 2). For retention trials, however, it revealed significant differences in latency times by neferine. As shown in Fig. 2, the step-through latency time (s) of the scopolamine-treated mice was significantly shorter than that of the vehicle-treated control mice ($P < 0.001$) in the passive avoidance task. After THA administration in scopolamine-treated mice (positive control group), step-through latency was significantly higher than that of the scopolamine-treated group ($P < 0.001$). Moreover, the shorter step-through latencies induced by scopolamine were dose-dependently and significantly attenuated by neferine administration (1.25, 2.5, 5.0, and 10 mg/kg) versus the scopolamine-treated group ($P < 0.05$). Therefore, neferine is suggested to possess the long-term memory improving effect, which was superior to that of THA (10 mg/kg) in the passive avoidance test.

With respect to the improvement of short-term memory, the effects of neferine on alternation behavior were observed in the Y-maze task (Fig. 3A and B). As shown in Fig. 3A, spontaneous alternation of scopolamine-treated mice was significantly lower than that of vehicle-treated control mice ($P < 0.05$), and the lowered spontaneous alternation induced by scopolamine was significantly ameliorated by treatment with neferine (5 or 10 mg/kg, $P < 0.05$). Moreover, the effect of neferine

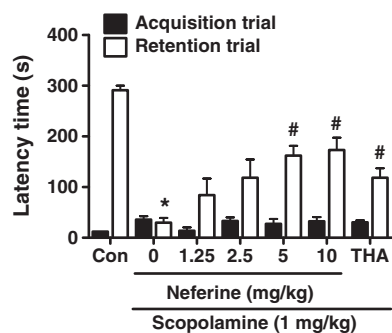


Fig. 2. Effect of neferine on scopolamine-induced cognitive impairment by the passive avoidance task. Mice were administered neferine (1.25, 2.5, 5.0, and 10 mg/kg, p.o.) or vehicles (same volume of a 10% Tween 80 solution) or THA (10 mg/kg, p.o.) 1 h prior to the acquisition trial. Memory impairment was induced by scopolamine treatment (1.0 mg/kg, i.p.) and acquisition trials were carried out 30 min after a single scopolamine treatment. At 24 h after the acquisition trials, retention trials were carried out for 5 min. Data represent mean \pm S.E.M ($n = 10$) * $P < 0.05$ versus vehicle control group, # $P < 0.05$ versus the scopolamine-treated group.

on spontaneous alternation behavior was similar to that of THA (10 mg/kg). In addition, the number of arm entries was similar across all experimental groups, showing that general locomotor activity was not affected by neferine treatments (Fig. 3B).

In order to evaluate the effect of hippocampal-dependent spiral learning and long-term memory of neferine, the Morris water maze task was performed (Fig. 4). As shown in Fig. 4A, the escape latency (s) of scopolamine-treated mice was significantly longer than that of vehicle-treated control mice ($P < 0.05$). However, the escape latency was significantly reduced by administration of neferine (5 or 10 mg/kg), as compared with that of THA (10 mg/kg), indicating normal learning abilities. As illustrated in Fig. 4B and C, the swimming time in search of the missing platform (%) and the crossing number of platform location in scopolamine-treated mice were significant decreased by that of vehicle-treated control mice ($P < 0.05$), reflecting the scopolamine-induced memory impairment; however, the lower swimming time in target (%) (5 or 10 mg/kg) and crossing number (10 mg/kg) were significant increased ($P < 0.05$) by treatment of neferine, which were similar to those of THA (10 mg/kg), indicating the recovery to normal memory capacity by neferine. In addition, the swim speed (~ 26.5 cm/s) was similar across all experimental groups, showing that general locomotor activity was not affected by neferine treatments (Fig. 4D).

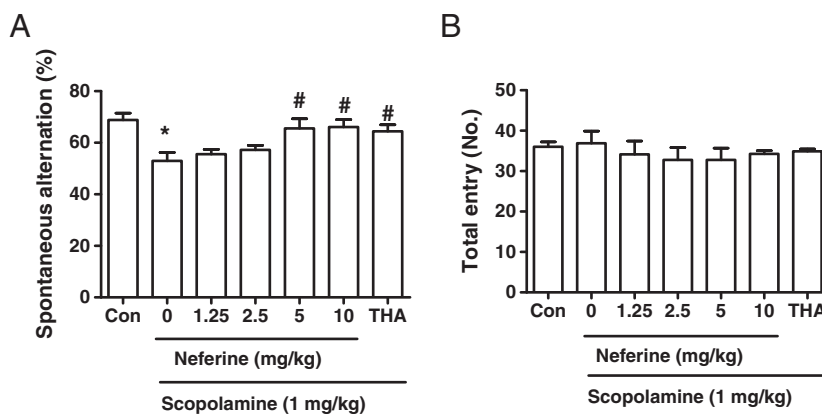


Fig. 3. Effect of neferine on scopolamine-induced cognitive impairment by the Y-maze task. One hour before the test trial, the mice were administered neferine (1.25, 2.5, 5, or 10 mg/kg, p.o.) or THA (10 mg/kg, p.o.) as a positive control. Memory impairment was induced by scopolamine (1 mg/kg, i.p.), and the test carried out 30 min later. Spontaneous alternation behavior (A) and numbers of arm entries (B) during 8-min sessions were measured. Data represent mean \pm S.E.M. ($n = 10$) * $P < 0.05$ versus the vehicle control group, # $P < 0.05$ versus the scopolamine-treated group.

In vitro inhibitory activities of neferine against BACE1 and ChEs

Since AD pathogenesis is potentially attributed to acetylcholine decline and A β formation (Parihar and Hemnani 2004), the anti-Alzheimer effects of neferine were evaluated by inhibition of BACE1 and ChEs. Neferine exhibited AChE- and BChE-inhibitory activities in a concentration-dependent manner with IC₅₀ values of 14.19 ± 1.46 and 37.18 ± 0.59 μ M, respectively. In the BACE1 assay, neferine also showed moderate inhibitory activities with an IC₅₀ value of 15.48 ± 0.20 μ M. These results indicate that neferine may reverse scopolamine-induced cognitive impairment through inhibition of enzymes implicated in Alzheimer's disease (Table 2).

In vitro inhibitory activities of neferine against intracellular oxidative stress and inflammation

In addition to both cholinergic deficit and A β formation and accumulation, inflammation and oxidative stress have emerged as important mechanisms in AD pathogenesis (Butterfield et al. 2001; Sastre et al. 2008). The effects of neferine on markers of neuroinflammation were assessed in cell-based assays that measures LPS-induced NO \bullet formation and NF- κ B activation in RAW 264.7 cells.

The cytotoxic effects of neferine on RAW 264.7 cells were first measured in the *in vitro* cell-based assays. Although data was not shown, neferine did not significantly affect cell viability at concentrations up to 30 μ M. However, neferine showed marginal cytotoxicity at a higher concentration of 50 μ M (IC₅₀ value of 48.16 μ M), therefore, neferine injections were determined at a concentration range of 1 to 30 μ M in subsequent experiments.

To assess the effect of neferine on LPS-induced NO \bullet production in RAW 264.7 cells, the cells were treated with LPS for 18 h after treatment in the presence or absence of neferine (0, 1.0, 5.0, 10, 20, and 30 μ M) for 2 h. The amount of nitrite, a stable metabolite of NO \bullet , was used as an indicator of NO \bullet production in the medium. During the 18 h incubation with LPS (1.0 μ g/ml), NO \bullet production in RAW 264.7 macrophages increased dramatically (Fig. 5A). As demonstrated in Fig. 5A, neferine showed concentration-dependent inhibition of NO \bullet production with an IC₅₀ value of 30.54 μ M (10.4, 30.4, and 51.3% inhibition at 10, 20, and 30 μ M, respectively). As a positive control, AMT also showed significant inhibitory effect with an inhibition% of 91.7 upon NO \bullet production at a concentration of 10 μ M.

The NF- κ B transcription factor has been evidenced to play an important role in LPS-induced expression of pro-inflammatory genes, including iNOS, COX-2, and various cytokines. These directly and

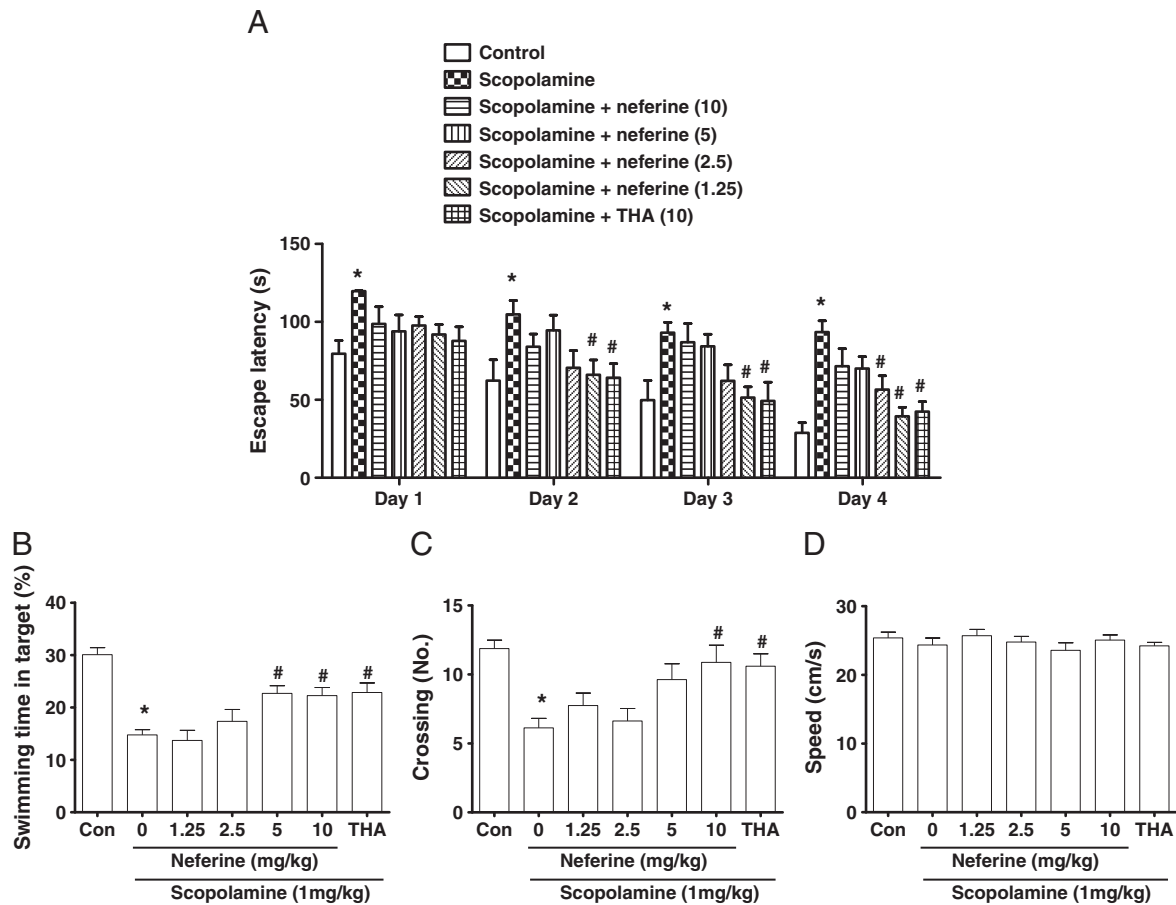


Fig. 4. Effects of neferine on scopolamine-induced cognitive impairment by the Morris water maze task. Escape latency was the mean value of each trial sessions for four consecutive days (A). Swimming time (B) in the target quadrant, crossing number (C) within previously platform existed zone, and swimming speed (D) were measured during the probe trial session. 60 min before first training trial in each day, neferine (1.25, 2.5, 5, or 10 mg/kg, p.o), tacrine (THA, 10 mg/kg, p.o), or their vehicles was administered to mice. The training trials and the probe trial were composed as described in the text. Data represent mean \pm S.E.M. ($n = 7-8$ /group) * $P < 0.05$ versus vehicle control group, # $P < 0.05$ versus the scopolamine-treated group.

indirectly lead to increased cellular oxidative stress as well as A β formation and accumulation (Xie and Nathan 1994; Sastre et al. 2008). To investigate the molecular mechanism of neferine-mediated inhibition of neuroinflammation and oxidative damage, NF- κ B transcription activity was measured using a reporter gene assay system. The RAW 264.7 cells were transfected stably with NF- κ B-SEAP-NPT plasmid, containing four copies of the κ B sequence fused to SEAP as the reporter (Moon et al. 2001). The LPS treatment of the transfected cells for 16 h increased the SEAP expression about 3.8-fold over basal levels (Fig. 5B). The pretreatment of cells with neferine (1.0, 5.0, 10, and 15 μ M) significantly inhibited LPS-induced SEAP expression in a concentration-dependent manner with an IC₅₀ value of 8.20 μ M (SEAP expression: 61.1% at 5.0 μ M, 57.1% at 10 μ M, 34.4% at 15 μ M). As a positive control, TPCK also showed significant inhibitory effect on NF- κ B transcription activity with an inhibition% of 76.7 at a concentration of 10 μ M.

Table 2
Inhibitory Activities of Neferine against BACE1 and ChEs.

Compounds	BACE1 ^a	AChE ^b	BChE ^c
Neferine	15.48 \pm 0.20	14.19 \pm 1.46	37.18 \pm 0.59
Quercetin ^d	3.17 \pm 0.12		
Galantamine ^e		0.03 \pm 0.00	3.26 \pm 0.07

^{a-c}IC₅₀ (μ M). ^{d,e}Positive controls. Each value represents the mean \pm S.E.M.

In vitro antioxidant activities of neferine

Since A β , a crucial factor of brain aging in AD patients, has been reported to accelerate lipid peroxidation and protein nitration and induce extensive formation of ROS/RNS and free radicals (Butterfield et al. 2001; Rossi et al. 2008), the inhibitory activities against TBARS and 3-nitrotyrosine formation and the scavenging activities against DPPH, ABTS radicals, ONOO⁻, NO[•], and \bullet O₂⁻ of neferine were evaluated.

As shown in Table 3, neferine showed potent scavenging activities against DPPH and ABTS radicals with respective IC₅₀ values of 17.07 \pm 0.56 and 3.49 \pm 0.34 μ M, as compared with a positive control, L-ascorbic acid, with an IC₅₀ value of 21.89 \pm 0.41 μ M. In addition, neferine exhibited good scavenging activities against ONOO⁻, NO[•], and \bullet O₂⁻, with respective IC₅₀ values of 6.92 \pm 0.95, 3.07 \pm 0.10, and 3.91 \pm 0.04 μ M, as compared with positive controls, L-penicillamine, carboxy-PTIO, and allopurinol, with respective IC₅₀ values of 0.62 \pm 0.95, 1.10 \pm 0.25, and 0.98 \pm 0.28 μ M. Since lipid peroxidation and protein nitration might at least in part play important roles as biomarkers in neuronal oxidative stress, the inhibitory activities of neferine on lipid peroxidation and 3-nitrotyrosine production were determined. Since 3-nitrotyrosine is a major product resulting from the reaction of proteins (BSA) and ONOO⁻, the inhibitory activity of neferine against ONOO⁻-induced tyrosine nitration was determined via Western blot analysis using the 3-nitrotyrosine antibody (Fig. 6). The results indicated that pretreatment of neferine (6.25, 12.5, and 25 μ M) significantly inhibited 3-nitrotyrosine in a concentration-dependent manner.

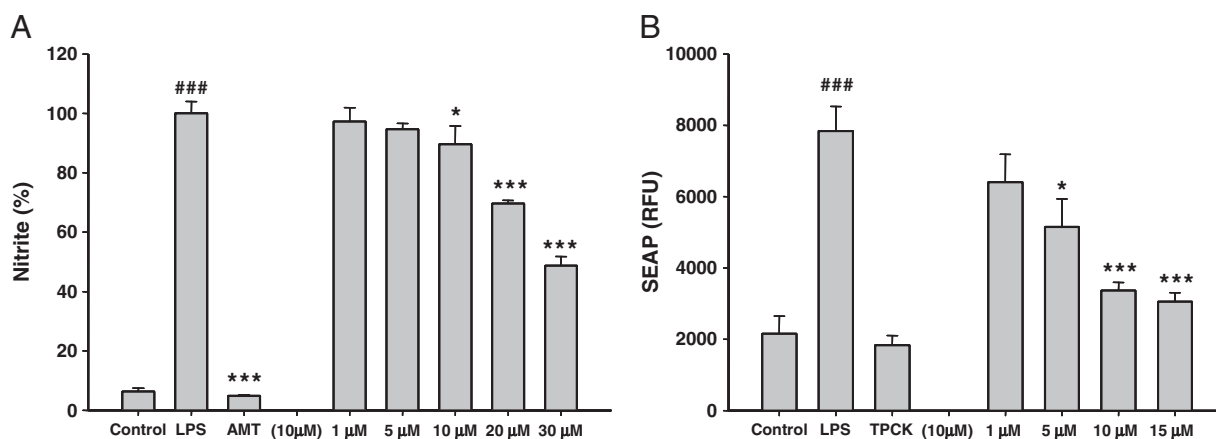


Fig. 5. Anti-inflammatory effect of neferine in RAW 264.7 cells. (A) Effect of neferine was performed on LPS-induced nitrite formation in RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of neferine for 2 h and LPS (1.0 μg/ml). After an 18 h incubation, the amount of nitrite in the culture supernatants was measured by the Griess reaction assay, as described in the Materials and methods. The values are expressed as the mean ± S.E.M. of three individual experiments. *** $P < 0.001$ indicates significant differences from the unstimulated control group. * $P < 0.05$ and *** $P < 0.001$ indicate significant differences from the LPS-treated group. (B) Effect of neferine was performed on LPS-induced NF-κB activation in RAW 264.7 cells. RAW 264.7 cells harboring the NF-κBSEAP-NPT reporter construct were pretreated with the indicated concentrations of neferine for 2 h and treated with LPS (1.0 μg/ml). After a 16 h incubation, the SEAP activity was measured in RFU, using a microplate fluorometer. The values are expressed as the mean ± S.E.M. of three individual experiments. *** $P < 0.001$ indicates significant differences from the unstimulated control group. * $P < 0.05$ and *** $P < 0.001$ indicate significant differences from the LPS-treated group.

Discussion

AD is an age-related neurodegenerative disorder that predominantly affects the elderly. Cholinesterases (ChEs) inhibitors have been intriguing targets in the pharmaceutical industry. Although some ChEs inhibitors (physostigmine, tacrine, rivastigmine, and donepezil) can improve the cognitive dysfunction of AD as symptomatic treatments, several limitations exist with usage due to low bioavailability in oral administration, permeability through the blood brain membrane, pharmacokinetic parameters, and hepatotoxicity (Scarpini et al. 2003; Sugimoto 2008). There has been extensive evidence that formation and accumulation of Aβ and BACE1 activity might be highly associated with inflammation (Sastre et al. 2008) and oxidative stress and/or nitrosative stress (Butterfield et al. 2001). Pursuant to lipid peroxidation and protein oxidation/nitration, ONOO⁻, formed by the in vivo reaction of nitric oxide (NO•) and superoxide anion (•O₂⁻), has been implicated in Aβ aggregation, with high levels of Aβ augmenting ONOO⁻ generation in the brain of AD patients (Butterfield et al. 2001). Inflammation might also relate to ROS generation, which induces NF-κB activation, followed by enhancing the level of cytokines, and the expression of iNOS and COX-2 (Sastre et al. 2008). Several natural antioxidants have ameliorated Aβ-induced oxidative stress and improved cognitive impairment in aged rats. Their mechanisms of cognitive impairment improvement may be complicated and diversified approach will be needed; a combination of anti-inflammatory, antioxidant, and neuroprotective roles in signal transduction pathways. Thus, the multifunctional effects of neferine for the age-related neurodegenerative disorder were evaluated by in vivo anti-amnesic activities in scopolamine-induced cognitive impairment animal models, in vitro ChEs- and BACE1-inhibitory activities, in vitro antioxidant, and cellular anti-inflammatory activities.

In order to evaluate anti-amnesic activities, the passive avoidance, Y-maze, and Morris water maze tasks were generally used as indicators of long-term memory, short-term memory, and learning abilities in scopolamine-induced cognitive impairment animals. As a result, neferine significantly improved scopolamine-induced cognitive impairments in three amnesic models (Figs. 2–4). Since scopolamine, a muscarinic receptor antagonist, can pass through the blood brain barrier and cause memory loss and cognitive dysfunction in animals by blocking cholinergic neurotransmission, most AChE inhibitors might be effective in scopolamine-damaged memory loss through the increase of acetylcholine levels in cholinergic synapses (Bejar et al. 1999). Given that scopolamine-induced cognitive impairment has also been reported to be associated with neuronal oxidative stress (El-Sherbiny et al. 2003), additional plausible mechanisms might be involved in the significant in vivo anti-amnesic effects of neferine. Also, extracellular senile plaques resulting from Aβ aggregation and accumulation is physiopathologically characterized as AD (Scarpini et al. 2003; Parihar and Hemnani 2004). In particular, Aβ fragments bearing the 11 amino acids (25–35) are reported to produce free radicals and extensive oxidative stress (Liu et al. 2009) and induce memory deficits (Stepanichev et al. 2004, 1998), followed by high in vitro and in vivo cytotoxicity (Pike et al. 1995; Maurice et al. 1996; Liu et al. 2009). Therefore, the inhibitory activity against BACE1, a crucial enzyme for Aβ formation, was evaluated. Similar to the ChEs inhibition results, the BACE1 inhibitory activity of neferine were good (Table 2). However, based upon the remarkable effects of neferine in the in vivo anti-amnesic model, the ChEs and BACE1 inhibitory activities were lower than expected (Table 2), indicating that the ameliorative activities of neferine upon memory dysfunction are not only dependent on its ChEs and BACE1 inhibitory activities. Xiong and Zeng (2003) demonstrated that the reactivation of

Table 3
In vitro IC₅₀ values (μM) of antioxidant activities of neferine.

Compounds	DPPH ^a	TEAC (ABTS) ^b	TE ^l	AE ^m	TBARS ^c	ONOO ^d	NO• ^e	•O ₂ ^f
Neferine	17.07 ± 0.56	3.49 ± 0.34	2.2	1.7	52.00 ± 0.00	6.92 ± 0.95	3.07 ± 0.10	3.91 ± 0.04
L-Ascorbic acid ^g	21.89 ± 0.41	5.82 ± 0.72		1.0	50.48 ± 0.03			
L-Penicillamine ^l						0.62 ± 0.00		
Carboxy-PTIO ^l							1.10 ± 0.25	
Allopurinol ^l								0.98 ± 0.28
Trolox ^k		7.78 ± 0.17	1.0					

^{a-f}IC₅₀ (μM); ^{g-k}positive controls; ^c50% inhibition concentration of lipid peroxidation; ^ltrolox equivalent (TE); ^mtrolox equivalent (TE) values. Each value represents the mean ± S.E.M.

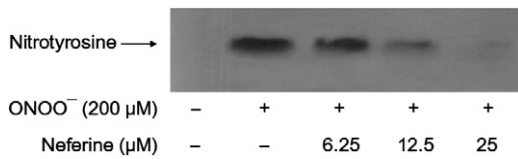


Fig. 6. Inhibitory activity of neferine on the protein nitration of BSA by ONOO⁻. The mixture of neferine, BSA, and ONOO⁻ were incubated with shaking at 37 °C for 30 min; this reactant was resolved by electrophoresis in 10% polyacrylamide gel.

organophosphorus ester pesticide poisoned rabbit AChE by neferine is due to dissociation of phosphoryl group from the poisoned/or aged enzyme (i.e., phosphorylase) by its active oxime. But in our result we examined the inhibitory effect of neferine on ChEs from the results of in vitro experiment using the electric-eel AChE enzyme. Furthermore, there are several reports of inhibitory effects by *tert*-isoquinoline alkaloids having neferine-like structure.

As mentioned above, neferine is a major *tert*-bisbenzylisoquinoline alkaloid derived from the embryos of *N. nucifera*, which have been used widely in traditional Chinese medicine for nervous disorders, insomnia, high fevers with restlessness, relieving cough pulmonary fibrosis/mic, and cardiovascular diseases such as hypertension and arrhythmia (Sridhar and Bhat 2007). There is some evidence on anti-Alzheimer effects of tertiary alkaloids: physostigmine, rivastigmine, and galantamine are reported to enhance cognitive impairment due to their potent ChEs inhibitory activities (Shu 1998; Meunier et al. 2006; Woodruff-Pak et al. 2001). Interestingly, the antioxidant and anti-inflammatory activities of neferine might play a partly role in anti-amnesic effect.

In addition to both cholinergic deficit and A β formation and accumulation, inflammation and oxidative stress have emerged as important mechanisms in AD pathogenesis (Butterfield et al. 2001; Sastre et al. 2008). In particular, A β toxicity could be mediated through the induction of an inflammatory response. Numerous researchers have speculated that intraneuronal A β toxicity (A β 40 and A β 42) and secretases (α -secretase and β -secretase) might play important roles in the neuroinflammatory responses, concomitant with the activation of NF- κ B as a crucial transcription factor and the release of inflammatory mediators, including COX-2, iNOS, cytokines, as well as the formation of reactive oxygen intermediates and NO \bullet (Butterfield et al. 2001; Sastre et al. 2008). Macrophages are potent secretory cells that release such inflammatory mediators (Laskin and Pendino 1995), induced by many cellular stimuli, including LPS, cytokines, and oxidants (Kopp and Ghosh 1995; Pahl 1999). Based on the close relationship between inflammation and AD, the anti-inflammatory activity of neferine was determined via cell-based assays, including inhibitory activities of LPS-induced NO \bullet generation and NF- κ B activation in RAW264.7 cells. As a result, neferine strongly inhibited NF- κ B activation rather than NO \bullet generation in RAW264.7 cells (Fig. 5), indicating that suppression of NF- κ B activation might be an indispensable factor in anti-amnesic activity.

Through A β cascade and inflammatory pathways, free radicals and ROS/RNS, including ONOO⁻, NO \bullet , and \bullet O $_2^-$, consequently produce protein nitration and lipid peroxidation in cells and conversely excessive oxidative stress produces severe A β formation and inflammation. Therefore, the scavenging/inhibitory activities against free radicals and ROS/RNS of neferine were evaluated via the cell-free based DPPH, ABTS, ONOO⁻, NO \bullet , \bullet O $_2^-$, TBARS assays, as well as the cell-based ONOO⁻

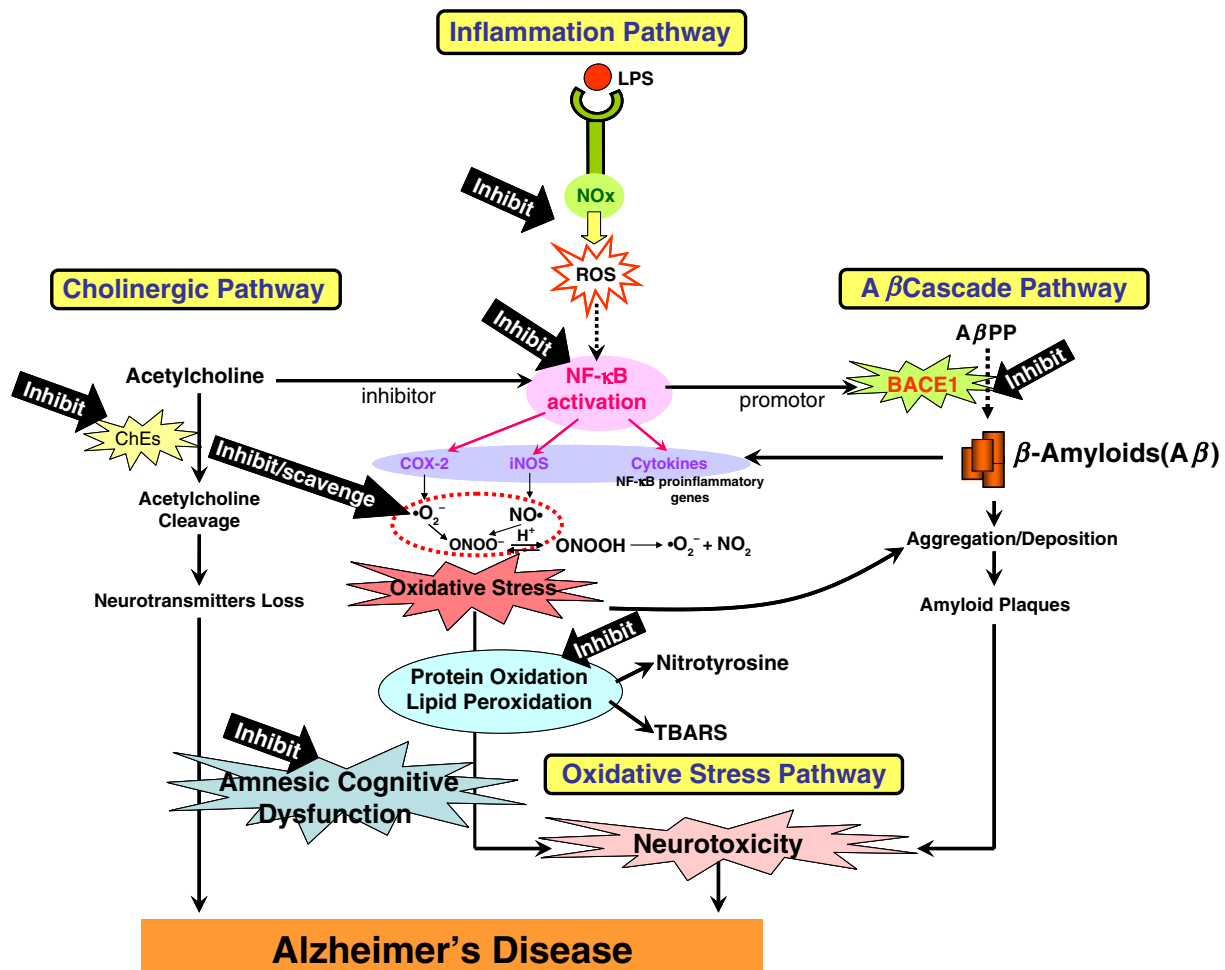


Fig. 7. Plausible multifunctional actions of neferine in an age-related neurodegenerative disorder.

mediated tyrosine nitration assay. From these assays, neferine showed remarkable antioxidant activities (Table 2 and Fig. 6). In particular, the results revealed that neferine significantly inhibited the formation of 3-nitrotyrosine and TBARS as important biomarkers in neuronal oxidative stress, indicating that the antioxidant capacities of neferine might participate in anti-amnesic activity.

Conclusions

Neferine, a major alkaloid from the embryo of *N. nucifera* seeds, exerted significant cognitive improvement in scopolamine-induced amnesia animal models. The anti-amnesic and/or anti-AD effects of neferine might be related to its moderate inhibitory activities against ChEs and BACE1, antioxidant activities, including free radical scavenging and ROS/RNS inhibitory activities, and anti-inflammatory activities in the intracellular NO• and NF-κB assays (Fig. 7). The present findings demonstrate that neferine might possess potential as a therapeutic or preventive agent for amnesia and oxidative stress-related neurodegenerative disorders via anti-amnesic and inhibitory activities of ChEs and BACE1, and its antioxidant capacities.

Conflict of interest statement

None.

Acknowledgements

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