Food Sci. Biotechnol. 24(4): 1487-1494 (2015)
DOI 10.1007/s10068-015-0192-y

RESEARCH ARTICLE

In vitro Antioxidant Activities of Proanthocyanidins Extracted from the Lotus Seedpod and Ameliorative Effects on Learning and Memory Impairment in Scopolamine-induced Amnesia Mice

Juan Xiao, Shuyi Li, Yong Sui, Xiaopeng Li, Qian Wu, Ruifen Zhang, Mingwei Zhang, Bijun Xie, and Zhida Sun

Received October 23, 2014; revised January 4, 2015; accepted February 8, 2015; published online August 31, 2015

Abstract The antioxidant activity of proanthocyanidins extracted from the lotus seedpods (LSPC) *in vitro* and ameliorative effects on memory impairment induced using scopolamine in mice were studied. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability, oxygen radical absorbance capacity, and cellular antioxidant activity assays revealed a potent LSPC antioxidant activity. Y-maze and step-down avoidance testing showed that LSPC administration at 30, 60, and 90 mg/kg ameliorated memory impairment. LSPC improved glutathione peroxidase and superoxide dismutase activities, inhibited activities of monoamine oxidase-B, total nitric oxide synthase, neuronal nitric oxide synthase, and acetylcholinesterase, and had no influence on inducible nitric oxide synthase and nitric oxide levels in the brain. LSPC ameliorated scopolamine-induced memory impairment based on improvement of the antioxidant system and cholinergic activity, which may be associated with potent antioxidant ability.

Keywords: proanthocyanidin, lotus, scopolamine, memory, antioxidant

Yong Sui, Xiaopeng Li, Qian Wu, Bijun Xie, Zhida Sun (✉)
College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, China
Tel: +86-27-87283201; Fax: +86-27-87282966
E-mail: sunzhida@sina.com

Juan Xiao, Ruifen Zhang, Mingwei Zhang
Key Laboratory of Functional Foods, Ministry of Agriculture/Guangdong Key Laboratory of Agricultural Products Processing, Sericultural & Agri-Food Research Institute Guangdong Academy of Agricultural Sciences, Guangzhou 510610, Guangdong, China

Shuyi Li
College of Food Science and Engineering, Wuhan Polytechnic University, Wuhan 430023, Hubei, China

Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease concomitant with progressive cognitive impairment, including memory loss and learning disturbances (1). Oxidative stress and a cholinergic deficit have emerged as important mechanisms in AD pathogenesis, in addition to inammation, and Aβ formation and accumulation (1,2). Oxidative stress arises due to an imbalance between production of free radicals and an antioxidant defense (2). Excess free radicals are highly prone to react with lipids, proteins, DNA, and RNA, which leads to oxidative damage that has been implicated in aging and a number of human diseases, including AD and other related neurological diseases (2-4). Nervous tissue, including the brain, is extremely susceptible to free radical damage due to an abundance of inspired oxygen, easily oxidizable poly-unsaturated fatty acids, redoxactive transition metal ions, and a relatively weak antioxidant defense system (3,4). Many studies have demonstrated that increased amounts of lipids, proteins, and DNA oxidation products are present in AD, which strengthens the hypothesis that free radical-mediated oxidative damage plays a critical role in the pathogenesis of AD and establishes oxidative damage as an early event in AD pathogenesis that can be treated as a therapeutic target (5,6).

Increased oxidative damage in the aging process may be due to a deficiency of antioxidants (3,5). Moreover, reactive oxidative species act as a secondary messenger in inammation, which is an important mechanism in AD pathogenesis (7). Reactive oxygen metabolites affect binding of ligands to membrane receptors, such as muscarinic cholinergic receptors, which indicates that oxidative stress induces cholinergic dysfunction (8). For these reasons, use of antioxidants may be helpful in treatment of AD (1,3). It has

been demonstrated that chronic administration of antioxidants restores age-related impairment in spatial learning and memory (9,10). A cholinergic deficit is a major neuropathological feature that is strongly associated with memory loss, and closely related to the severity of cognitive impairment in AD patients (11). The neurotransmitter acetylcholine (ACh) undergoes a hydrolytic reaction in the presence of acetylcholinesterase, which blocks cholinergic neurotransmission (10). Currently, the most accepted treatment for AD is administration of AChE inhibitors, such as tacrine, donepezil, rivastigmine, and galantamine for activation of ACh synthesis (11,12). However, these AChE inhibitors are helpful to only a subset of patients and cause the side effects of nausea, diarrhea, and vomiting. For these reasons, new drugs without adverse effects are urgently required (12).

Proanthocyanidins, a class of polyphenolic compounds, are widely distributed in plants and have attracted increasing attention in the fields of nutrition, health, and medicine largely due to strong antioxidant properties and other biological activities (13). Recent studies have demonstrated that proanthocyanidins act as anti-aging agents for protection against memory deficits, and extend the life span (14,15). The lotus seedpod, a part of *Nelumbo nucifera* Gaertn, has been used as a traditional Chinese medicine for more than 1,000 years for anti-aging, improved learning and memory abilities, soothing of nerves, and hemostasia (16). Proanthocyanidins, the main active ingredients in lotus seedpods (LSCP), have demonstrated effective scavenging of the superoxide anion and the hydroxyl radicals *in vitro* (17), and amelioration of cognitive deficits in SAMP8 and cognitively impaired aged rats (9,10). In addition, mixtures of LSPC and bilobalide (or L-cysteine) have been demonstrated to improve behavioral performances in scopolamine-induced amnesia mice (18,19). However, the ameliorative effects of LSPC on scopolamine-induced memory impairment and the antioxidant mechanism have not been studied.

This study was aimed at estimation of the antioxidant activity of LSPC *in vitro* based on assays of the DPPH radical scavenging ability, the oxygen radical absorbance capacity (ORAC), and the cellular antioxidant activity (CAA), and investigation of the effects of LSPC on scopolamine-induced memory impairment in mice based on Y-maze and step-down avoidance testing. Changes in the brain antioxidant system and the cholinergic system were also determined.

Materials and Methods

Chemicals and reagents AB-8 resin was obtained from Chemical Plant of Nankai University (Tianjin, China). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, quercetin, 2',7'-dichlorofluorescin diacetate, fluorescein disodium salt, 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) and potassium phosphate buffer (PBS, pH 7.4) were purchased from Gibco (Carlsbad, CA, USA). HepG2 human liver cancer cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), monoamine oxidase-B (MAO-B), AChE, nitric oxide synthase (NOS), and nitric oxide (NO) testing reagent kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Scopolamine hydrobromide injections were purchased from Xuzhou Ryen Pharm Co. Ltd. (Xuzhou, China).

Preparation of LSPC Mature LSCP of *Nelumbo nucifera* cv. Number 2 Wuhan plant were harvested from the Honghu District in Hubei, China in late July of 2010 and arrived in the laboratory within 24 h of harvest. Seedpods were kept in a refrigerator (BCD-186; Siemens, Berlin and Munich, Germany) at –20ºC prior to extraction. LSCP were identified by Prof. Xueming Ni from the Department of Botany, Wuhan Plant Institute of the Chinese Academy of Science in Wuhan, Hubei province. LSCP (100 g) were subjected to extraction using boiling distilled water (1 L) (AJY-6000-U Aquapro Water Purifier; Chongqing Yi-yang Enterprise Development Co., Ltd., Chongqing, China) for 1 h, then the filtrate was applied to a column (60 cm×4.0 cm i.d.; Wuhan Aisipei Scientific Instrument Co., Ltd., Wuhan, China) packed with AB-8 resin (Chemical Plant of Nankai University). The resin was washed with distilled water (10 L) (Chongqing Yi-yang Enterprise Development Co., Ltd.) to remove sugar and low Mw materials, then eluted using a 70% aqueous ethanol (0.6 L) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The eluant was concentrated using a rotary evaporator (RE111; Buchi Labortechnik, Flawil, Switzerland) at 30ºC, then lyophilized by a Freeze Dryers (ALPHA 1-4LD; Marin Chris, Osterode, Germany) to recover LSPC (3.8 g) with a purity of 98.7%, as measured following the method reported by Porter *et al.* (20). LSPC was kept in a refrigerator (BCD-186; Siemens) at –20ºC. Based on electrospray ionization mass spectrometry analysis, LSPC was comprised of monomers, dimers, and tetramers of proanthocyanidins, in which catechin and epicatechin are the base units. The amount of dimers was greatest (17).

DPPH radical scavenging ability The scavenging effect of LSPC on DPPH radicals was evaluated following the method of Sahin and Samli (21) with modification. Briefly, 2 mL of an LSPC solution was added to 2 mL of a DPPH

solution (0.2 mmol/L) (Sigma-Aldrich) in a test tube. Each analysis was performed in triplicate. After incubation in the dark at 37ºC for 30 min, the absorbance of each solution was determined by UV-vis spectrophotometer (UV1750; Shimadzu, Kyoto, Japan) at 517 nm.

ORAC assay An ORAC assay was carried out according to the method reported by Wolfe and Liu (22). Briefly, 20 μL of an LSPC solution, Trolox (6.25-50 μmol/L) (Sigma-Aldrich) in a 75 mmol/L PBS (Gibco), or PBS (Gibco) was added to triplicate wells in a black 96-well microplate (Packard Instrument Company, Meriden, CT, USA). Then, fluorescein disodium salt (Sigma-Aldrich) (200 μL, 0.96 μmol/L in PBS; Gibco) was added to each well. The plate was kept at 37ºC for 20 min in an Infinite® M200 PRO plate reader (Tecan Austria GmbH, Grödig, Austria). Subsquently, AAPH (Sigma-Aldrich) (20 μL, 119 mmol/L in PBS; Gibco) was added to all wells except for a blank well. The fluorescence value was measured at 485 nm (excitation spectra) and 520 nm (emission spectra) for 35 cycles (4.5 min/cycle) using an Infinite® M200 PRO plate reader (Tecan Austria GmbH). LSPC ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the Fluorescein decay curve and expressed as ìmol of Trolox equivalents (TQ)/mg of LSPC.

CAA assay The CAA value was determined according to the method reported by Wolfe and Liu (22). Briefly, HepG2 cells were seeded at a density of 6×10^4 in 100 μL of DMEM (Gibco) per well on a black 96-well microplate (Packard Instrument Company). After 24 h of incubation in CO_2 incubator (Hera cell 240i; Thermo Scientific, Hudson, NH, USA) at 37ºC, the growth medium was removed and cells were washed with PBS (Gibco). Cells were treated in triplicate for 1 h with 100 μL of a standard (quercetin; Sigma-Aldrich) or LSPC with 2',7'-dichlorofluorescin diacetate (Sigma-Aldrich) (25 μmol/L) in DMEM (Gibco). Then, AAPH (Sigma-Aldrich) at 600 μmol/L in 100 μL of DMEM (Gibco) was added to every treated well, and immediately the microplate was read using an Infinite® M200 PRO plate reader (Tecan Austria GmbH) at 37ºC. The fluorescence value was recorded at 485 nm (excitation spectra) and 538 nm (emission spectra) for 12 cycles (5 min/cycle). CAA values were calculated following the method reported by Wolfe and Liu (22) and expressed as μg of quercetin equivalents (QE)/μg of LSPC.

Animals Four-week-old male Kunming mice (20±2 g) were obtained from Hubei Research Center of Experimental Animals, Hubei, China. Animals were housed 5 per cage with *ad libitum* access to food and water, kept under a controlled temperature of 22±1ºC, a humidity of 55-60%, and a 12 h light/12 h dark cycle throughout the experimental period. All experimental procedures involving animals were conducted in accordance with the animal care and use guidelines of Wuhan General Hospital of Guangzhou Military Command Animal Care and Use Committee (SYXK, Hubei, 2008-0007).

Treatments Mice were randomly divided into 5 groups with 10 mice in each group. The groups were (1) a control group (CON), (2) a vehicle scopolamine control group (SCOP), (3) a low dose LSPC group (L-LSPC, 30 mg/kg), (4) a middle dose LSPC group (M-LSPC, 60 mg/kg), and (5) a high dose LSPC group (H-LSPC, 90 mg/kg). The initial body weights of the mice were not significantly ($p>0.05$) different between the 5 groups. After acclimatization for 3 days, mice in L-LSPC, M-LSPC, and H-LSPC groups were administered 30, 60, and 90 mg/kg of LSPC, respectively, at a dosage of 0.1 mL/10 g of body weight via oral gavage daily for 20 days. The dosage was based on previous experimental work (9,10,19). LSPC solutions for L-LSPC (3 mg/mL), M-LSPC (6 mg/mL), and H-LSPC (9 mg/mL) group mice were freshly prepared in 0.9% saline every day, and administration began at 9:00 a.m. Animals, except in the CON group, were given scopolamine (3 mg/kg, i.p.) to induce memory impairment 30 min before a training course. CON group received 0.9% saline (i.p.) in the same manner as above.

Behavioral procedures At the end of the treatment period the learning and memory capabilities of mice was studied using Y-maze testing following the method described by Gong *et al.* (9) with modification. Briefly, each mouse was placed at the end of an arm of the Y-maze to adapt for 5 min without electric shock, then the mouse ran to the safe arm under the stimulus of an electric shock and stayed in the safe arm for adaptation to conditions for 30 s. Subsequently, another electric shock was applied following the sequence ABCABC (A, B, and C represent three arms of Y-maze). Trials were performed 35 times during a training course then, 24 h after the end of training, the number of errors in 10 tests was recorded as the memory ability.

Seven days after Y-maze testing, the learning and memory abilities of mice were tested using step-down avoidance testing, as described by Gong *et al.* (9). Briefly, a mouse was placed in the testing box to adapt for 3 min without electric shock. Then, the mouse was placed on the platform (safe area). A step-down from the platform initiated an intermittent electric shock (1 s, 0.3 A) and the mouse was forced to escape back up onto the platform. The number of errors (mouse step-down from the platform and receiving a shock) during 5 min was recorded. After 24 h, the mouse was placed on the platform for retention testing. The

latency (step-down onto the grid with all 4 paws for the first time) and the number of errors within 5 min were measured as learning performance.

Biochemical analysis Following step-down avoidance testing, mice were killed using cervical dislocation. The whole brain was dissected, weighed (BL3100 electronic balance; Sartorius, Göttingen, Germany) and stored at −80ºC (DW-86L388V ultra-low temperature refrigerator; Haier, Qingdao, China). Before biochemical analysis, brain tissue was homogenized with ice-cold 0.9% saline by a motor homogenizer (IKA T10; IKA Labortechnik, Staufen, Germany) at 1500 rpm for 10 s at 0ºC. The homogenate was centrifuged (TGL-16G-A high-speed centrifuge; Shanghai Jiapeng Technology Co., Ltd., Shanghai, China) at 2,054×*g* for 10 min at 4ºC and the supernatant was used for biochemical analysis. Activities of GSH-Px, SOD, MAO-B, AChE, and NOS, and contents of NO and protein in brain tissue were determined following manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute).

Statistical analysis Data were expressed as a mean± standard deviation (SD) and analyzed using a one-way analysis of variance (ANOVA) followed by Duncan's test if significant differences between groups were identified. Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) at $p<0.05$.

Results and Discussion

Antioxidant activity of LSPC *in vitro* The antioxidant activity of LSPC was evaluated using a DPPH radical scavenging ability assay, an ORAC assay, and a CAA assay *in vitro*. ORAC and CAA assays were used herein the first time for determination of the antioxidant activities of LSPC. The scavenging effects of LSPC against the DPPH radical were observed over a concentration range of 2-80 μg/mL (Fig. 1). The scavenging rate rose sharply in a concentration range of 2-20 μg/mL, increased slowly over a concentration range of 20-40 μg/mL, then remained constant at 92% over a concentration range of 40-80 μg/mL. The median effective concentration (EC_{50}) (defined as the dose required for a 50% inhibition for LSPC or quercetin) of LSPC against the DPPH radical was 9.10±0.11 μg/mL. The ORAC value of LSPC was 52.15±1.42 μmol of TQ/mg. The CAA value of LSPC was 89.20±2.54 μg of QE/μg, and EC_{50} value was 9.03±0.69 μg/mL, which was close to the EC_{50} value of quercetin (6.43±0.84 μg/mL). Ling *et al.* (17) reported that LSPC effectively scavenged the superoxide anion and hydroxyl radicals *in vitro* with EC_{50} values of 17.6 and 10.5 μg/mL, respectively. Thus, LSPC had a strong antioxidant activity *in vitro*.

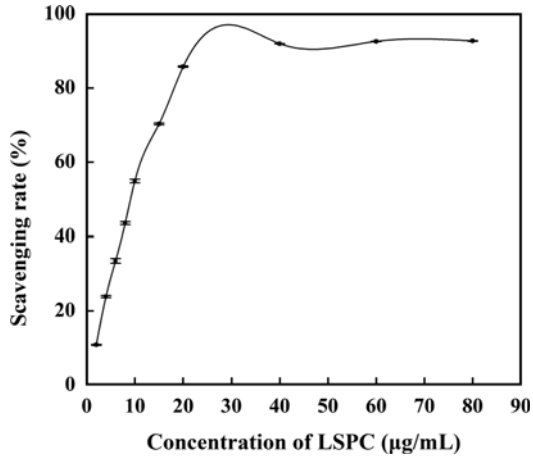

Fig. 1. The scavenging ability of LSPC against the DPPH Radical. Data are expressed as a mean±SD (n=3).

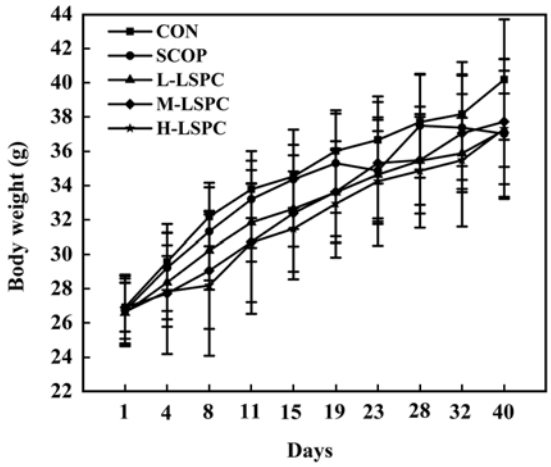

Fig. 2. Effects of LSPC (30, 60, and 90 mg/kg) on body weights of scopolamine-induced memory impaired mice. All data are expressed as a mean±SD (n=10). CON, SCOP, L-, M-, and H-LSPC are control, vehicle scopolamine control, low, middle, and high dose LSPC groups, respectively. Comparisons of mean values were performed using a one-way ANOVA.

Effects of LSPC on body weight There were no significant ($p>0.05$) differences in body weights among the 5 treatment groups on the same day during 40 days of treatment (Fig. 2). There were also no significant ($p>0.05$) differences in body weights among the 5 groups before LSPC treatment. Gong *et al.* (9) reported that administration of 90 mg/kg of LSPC for 2.5 and 3 months increased the body weight in SAMP8 rats, compared with age-matched SAMR1 rats. The results in this study and reported results of Gong *et al.* (9) cannot be directly compared due to differences in treatment times and experimental subjects. Short-term administration of LSPC apparently had no effect on changes in body weights of mice.

Effects of LSPC on Y-maze testing The Y-maze is regarded as an indicator of spatial short-term memory

(9,12). Effects of LSPC on Y-maze testing are shown in Fig. 3A. SCOP group mice exhibited a significantly (p<0.05) increased number of errors, compared with CON group mice, which verified that cognitive deficits induced by scopolamine were apparent in mice and that memory was closely associated with the central cholinergic system (9, 19,21). However, the numbers of errors were significantly (p<0.05) reduced by all LSPC treatments *vs*. SCOP group mice. In addition, M-LSPC and H-LSPC treatments normalized the scopolamine-induced increase in the number of errors. Gong *et al.* (9) reported that LSPC improved the behavioral performances of SAMP8 rats in Y-maze testing. Thus, LSPC effectively ameliorated a scopolamine-induced memory impairment.

Effects of LSPC on step-down avoidance testing Step-down avoidance testing can be used to evaluate the memory retention ability (7,18,19). Scopolamine resulted in a significant (p<0.05) increase in the number of errors (Fig. 3B) in step-down avoidance testing, and a significant (p<0.05) decrease in the step-down latency, compared with CON group mice (Fig. 3C), consistent with previous studies (7,18,19) and with the results of Y-maze testing in this study. Significantly (p<0.05) reduced error numbers and significantly (p<0.05) increased step-down latency times for all 3 LSPC treatment groups was observed, compared with SCOP group mice. Moreover, the number of errors in testing trials was decreased to the level exhibited by CON group mice by all LSPC treatments. LSPC treatments at 30 and 90 mg/kg normalized the decrease in the step-down latency, suggesting that LSPC exerted a cognition-improvement effect, in agreement with previous reports that LSPC ameliorated cognitive deficits in SAMP8 rats and cognitively impaired aged rats (9,10).

Effects of LSPC on the brain antioxidant system The effects of free radicals on memory impairment and many neurodegenerative diseases, such as AD and other related neurological diseases, have attracted attention in recent years (2,3). It is well known that free radical-mediated oxidative damage is approved in AD pathogenesis and that antioxidants have served as useful treatments in clinical AD trials (1,5). Scopolamine-induced amnesia animals have been widely used for preliminary evaluation of potential cognition-enhancing activities of herbal and other agents (7,12,18,19,23). Scopolamine induces cognitive deficits not only by inducing a cholinergic deficit (7,12,19,24,25), but also by inducing oxidative stress (7,12,19,23-25). Moreover, Van der Vliet and Bast (8) reported that reactive oxygen metabolites affect binding of ligands to membrane receptors, such as muscarinic cholinergic receptors, indicating that oxidative stress induces cholinergic dysfunction. Recent studies have reported that herbal extracts reverse scopolamine-induced amnesia via antioxidant mechanisms, including free radical scavenging and ROS/RNS inhibitory activities (7,12,19,23-25). Thus, the action mechanism of LSPC was

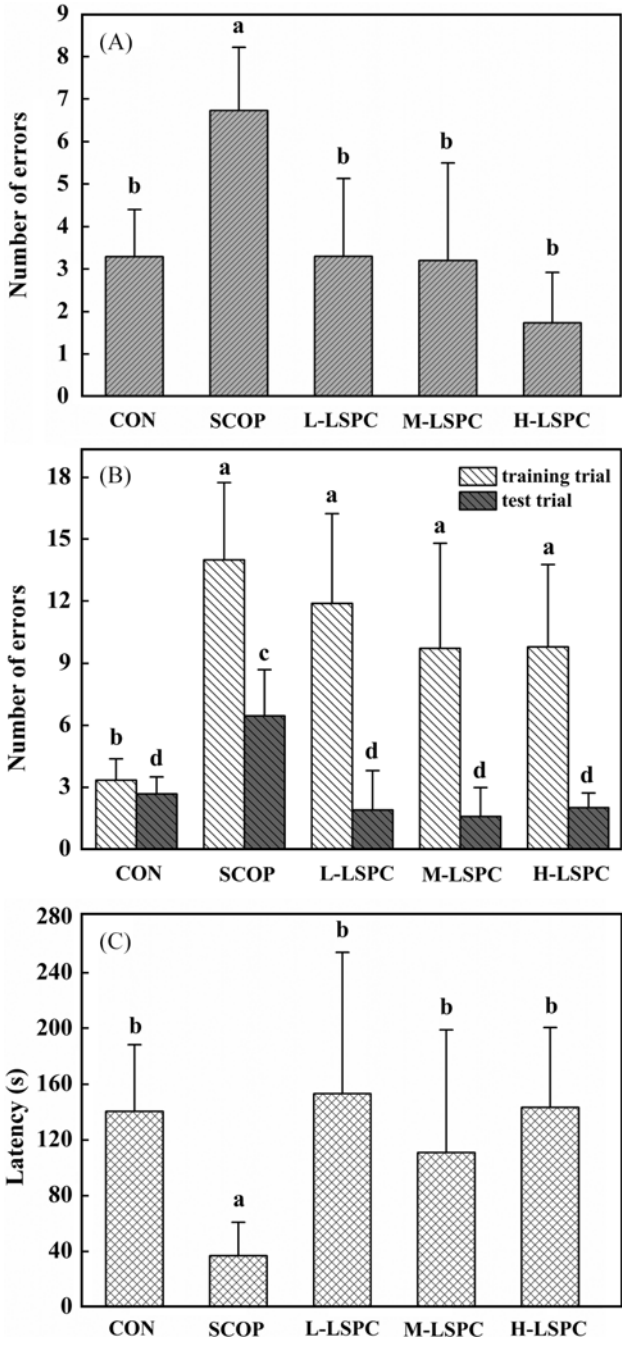

Fig. 3. Effects of LSPC (30, 60, and 90 mg/kg) on behavioral performance of scopolamine-induced memory impaired mice. (A): Number of errors in Y-maze testing; (B): Number of errors in step-down avoidance testing; (C): Latency in step-down avoidance testing. Values are expressed as a mean±SD (n=10). CON, SCOP, L-, M-, and H-LSPC are control, vehicle scopolamine control, low, middle, and high dose LSPC groups, respectively. Comparisons of mean values were performed using a one-way ANOVA followed by Duncan's test. Means with different superscripts above columns are significantly different (p<0.05).

evaluated mainly from the perspective of oxidative stress, including activities of GSH-Px, SOD, MAO-B, and NOS, and the NO level.

The GSH-Px activity for SCOP group mice was significantly (p<0.05) lower than for CON group mice (Table 1). The SOD activity of SCOP group mice was reduced by 6.3%, compared with CON group mice. GSH-Px and SOD, both endogenous antioxidant enzymes, contribute to the cellular defenses against free radical damage in the brain (4). The decline in GSH-Px activity induced by scopolamine confirmed that scopolamine-induced amnesia is associated with the antioxidant system (7,12,24). However, LSPC treatments at 30, 60, and 90 mg/kg significantly (p<0.05) increased the GSH-Px activity, compared with all SCOP group mice. Moreover, no significant (p>0.05) differences in GSH-Px activity were found between mice of all 3 LSPC groups and CON group mice.

Scopolamine caused an increase in MAO-B activity (1.16× with respect to CON group mice). MAO-B activity was significantly (p<0.05) reduced by LSPC treatments at 60 and 90 mg/kg, compared with SCOP group mice, and compared with CON group mice. H-LSPC group mice exhibited a significant (p<0.05) increase in the SOD activity, compared with SCOP group mice. Nevertheless, in this study, the brain SOD activity in SCOP group mice was not altered significantly (p>0.05), compared with CON group mice. Similarly, El-Sherbiny *et al.* (23) reported that there was no significant difference in brain SOD activity between scopolamine-treated and control groups, perhaps because SOD activity was not significantly different between different brain regions of AD patients, and declined significantly only in the cerebellum, frontal cortex, and hippocampus, compared with age-matched controls (26).

Effects of LSPC on activities of total nitric oxide synthase (TNOS), inducible nitric oxide synthase (iNOS), and neuronal nitric oxide synthase (nNOS), and the NO level, are shown in Table 2. Scopolamine caused increases in activities of TNOS and nNOS, and the NO level (1.10×, 1.12×, and 1.17× with respect to CON group mice, respectively). Significantly (p<0.05) reduced TNOS activity was observed in all LSPC group mice, compared with SCOP group mice. However, LSPC treatments at 30 and 90 mg/kg significantly (p<0.05) inhibited TNOS activity in comparison with CON group mice. Compared with SCOP group mice, the nNOS activity was significantly (p<0.05) reduced by LSPC treatments at 30 and 90 mg/kg). Additionally, there were no significant (p<0.05) differences in brain iNOS activities and NO levels among all 5 treatment groups.

As a free radical, NO reacts with superoxide radicals to form peroxynitrite and results in neurotoxicity and cell apoptosis under an abnormal physiological status, which is involved in the pathogenesis of AD and Parkinson's disease (27). Previous study has shown that administration of LSPC for 3 months resulted in NO inhibition in the

Table 1. Effects of LSPC on brain SOD, GSH-Px, MAO-B, and AChE activities in scopolamine-induced memory impaired mice[1)]

Group	GSH-Px	SOD	MAO-B	AChE
	U/mg protein			
CON	59.75±3.35^{b}	57.27±2.89^{ab}	3.52±0.81^{a}	0.67±0.03^{a}
SCOP	53.07±2.35^{a}	53.70±1.65^{a}	4.08±1.55^{a}	0.70±0.02^{a}
L-LSPC	61.56±4.09^{b}	54.08±4.74^{ab}	3.64±0.89^{a}	0.52±0.06^{b}
M-LSPC	57.80±1.42^{b}	57.06±3.48^{ab}	2.17±1.10^{b}	0.56±0.08^{b}
H-LSPC	57.88±3.70^{b}	58.88±3.83^{b}	2.22±0.88^{b}	0.51±0.04^{b}

[1)]Values are expressed as a mean±SD (n=10). CON, SCOP, L-, M-, and H-LSPC are control, vehicle scopolamine control, low, middle, and high dose LSPC groups, respectively. Comparisons of mean values were performed using a one-way ANOVA followed by Duncan's test. Means in the same column with different superscripts are significantly different (p<0.05).

Table 2. Effects of LSPC on brain NOS activity and the NO level in scopolamine-induced memory impaired mice[1)]

Group	TNOS	iNOS	nNOS	NO
	U/mg protein			μmol/g of protein
CON	0.97±0.17^{ab}	0.37±0.05	0.61±0.18^{ab}	0.63±0.15
SCOP	1.07±0.11^{a}	0.38±0.15	0.68±0.15^{a}	0.74±0.25
L-LSPC	0.71±0.15^{c}	0.28±0.09	0.42±0.20^{b}	0.61±0.13
M-LSPC	0.85±0.23^{bc}	0.29±0.07	0.56±0.26^{ab}	0.60±0.32
H-LSPC	0.68±0.17^{c}	0.25±0.11	0.43±0.11^{b}	0.40±0.33

[1)]Values are expressed as a mean±SD (n=10). CON, SCOP, L-, M-, and H-LSPC are control, vehicle scopolamine control, low, middle, and high dose LSPC groups, respectively. Comparisons of mean values were performed using a one-way ANOVA, followed by Duncan's test if significant differences between groups existed. Mean values in the same column with different superscripts are significantly different (p<0.05).

SAMP8 rat brain, but a 2 month administration of LSPC did not (9). Additionally, a 6 month administration of LSPC exhibited an inhibitory effect on the NO level in the hippocampus and cerebral cortex in cognitively impaired aged rats (28). Thus, the results of this study did not contradict previously reported results, and indicated that short term administration of LSPC had no influence on the NO level.

In the central nervous system, NO is produced during catalysis of NOS, which contains at least the 3 distinct isoforms of endothelial NOS (eNOS), nNOS, and iNOS (29). nNOS and eNOS are constitutive isoenzymes (cNOS), and nNOS is the predominant isoform in the brain accounting for more than 95% of the cNOS activity. It has been reported that nNOS is largely responsible for NO production and is related to learning and memory function (30), sleep deprivation recovery (29), and neuronal death in neurodegenerative disorders (31). Recent study has found that aging is associated with enhancement of nNOS activity, a lowering of eNOS activity, and no alteration in iNOS activity (32). However, NOS inhibition showed memory-enhancing and neuroprotection effects (28,33,34). In this study, LSPC exhibited inhibitory effects against TNOS and nNOS activities, but had no influence on iNOS activity, in agreement with previous reports (9,32). Thus, LSPC improved the learning and memory abilities of mice via nNOS inhibition.

This study demonstrated that LSPC has potent antioxidant activities *in vitro* and exhibited a protective effect against oxidative damage induced by scopolamine. Moreover, LSPC has been reported to scavenge the superoxide anion and hydroxyl radicals effectively *in vitro* (17), and to inhibit oxidative damage in the brain and serum of SAMP8 rats and cognitively impaired aged rats (9,10). Thus, the protective effect of LSPC may be due to an antioxidant property that protects brain cells that are otherwise susceptible to oxidative stress.

Effects of LSPC on brain AChE activity A cholinergic deficit is a major neuropathological feature that is strongly associated with memory loss, and closely related to the severity of cognitive impairment in AD patients and animals (11,12,19). Cholinergic enhancers ameliorated AD symptoms and are the current main therapy for AD (12). As a cholinergic antagonist known to interfere with acetylcholine transmission in the central nervous system, scopolamine leads to cognitive impairment based on induction of a cholinergic deficit (12,18,19). In this study, brain AChE activity was measured as a marker for a cholinergic deficit. Scopolamine caused an increase in brain AChE activity (1.05× with respect to CON group mice) (Table 1). Many studies have demonstrated that AChE activity is increased in the whole brain, in the hippocampus, and the cerebral cortex of scopolamine-induced mice, and an increase in AChE activity accompanies memory impairment (12,19). However, several studies have reported that scopolamine results in a small increase in whole brain AChE activity with memory impairment (24,25). AChE is the enzyme that catalyzes hydrolysis of the ACh in cholinergic neurons and, thus, plays a key role in blocking cholinergic neurotransmission (35). A small increase in brain AChE activity can apparently lead to memory impairment, and a cholinergic deficit is associated with memory loss (11,35). Significantly ($p<0.05$) reduced AChE activities were observed in all LSPC treatment group mice, compared with SCOP group mice, and no significant ($p>0.05$) differences were found among 3 LSPC group mice (Table 1), which indicated that the cognition-enhancing activity of LSPC is related to a decline in brain AChE activity.

In conclusion, LSPC ameliorated scopolamine-induced memory impairment in mice. The action mechanism of LSPC was based on improvement of the brain antioxidant system due to enhancement of GSH-Px and SOD activities, inhibition of MAO-B, TNOS, and nNOS activities, and amelioration of brain cholinergic activity due to AChE inhibition, which may be associated with a potent antioxidant ability. LSPC may be useful for treatment of learning and memory impairments caused by aging and AD.

Acknowledgments This work was supported by the National Natural Science Foundation of China (NSFC, No. 31071633, No. 31301459) and The Innovation Capacity Construction Special Project of the Main Scientific Research Organization of Guangdong Province (2011).

Disclosure The authors declare no conflict of interest.

References

1. Roberson ED, Mucke L. 100 years and counting: Prospects for defeating Alzheimer's disease. Science 314: 781-784 (2006)
2. Reddy VP, Zhu X, Perry G, Smith MA. Oxidative stress in diabetes and Alzheimer's disease. J. Alzheimers Dis. 16: 763-774 (2009)
3. Devasagayam TPA, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS, Lele RD. Free radicals and antioxidants in human health: Current status and future prospects. J. Assoc. Physician. I. 52: 794-804 (2004)
4. Reiter RJ. Oxidative processes and antioxidative defense mechanisms in the aging brain. FASEB J. 9: 526-533 (1995)
5. Bonda DJ, Wang XH, Perry G, Nunomura A, Tabaton M, Zhu XW, Smith MA. Oxidative stress in Alzheimer disease: A possibility for prevention. Neuropharmacology 59: 290-294 (2010)
6. Markesbery WR, Kryscio RJ, Lovell MA, Morrow JD. Lipid peroxidation is an early event in the brain in amnestic mild cognitive impairment. Ann. Neurol. 58: 730-735 (2005)
7. 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. 588: 78-84 (2008)
8. Van der Vliet A, Bast A. Effect of oxidative stress on receptors and signal transmission. Chem.-Biol. Interact. 85: 95-116 (1992)
9. Gong YS, Liu LG, Xie BJ, Liao YC, Yang EL, Sun ZD. Ameliorative effects of lotus seedpod proanthocyanidins on cognitive deficits and oxidative damage in senescence-accelerated mice. Behav. Brain Res. 194: 100-107 (2008)
10. Xu JQ, Rong S, Xie BJ, Sun ZD, Zhang L, Wu HL, Yao P, Zhang YJ, Zhang XP, Liu LG. Rejuvenation of antioxidant and cholinergic systems contributes to the effect of procyanidins extracted from the lotus seedpod ameliorating memory impairment in cognitively impaired aged rats. Eur. Neuropsychopharm. 19: 851-860 (2009)
11. Kopelman MD, Corn TH. Cholinergic 'blockade' as a model of cholinergic depletion: a comparison of the memory deficits with those of Alzheimer-type dementia and the alcoholic korsakoff syndrome. Brain 111: 1079-1110 (1988)
12. Kwon S-H, Lee H-K, Kim J-A, Hong S-I, Kim H-C, Jo T-H, Park Y-I, Lee C-K, Kim Y-B, Lee S-Y, Jang C-G. Neuroprotective effects of chlorogenic acid on scopolamine-induced amnesia via anti-acetylcholinesterase and anti-oxidative activities in mice. Eur. J. Pharmacol. 649: 210-217 (2010)
13. Aron PM, Kennedy JA. Flavan-3-ols: Nature, occurrence and biological activity. Mol. Nutr. Food Res. 52: 79-104 (2008)
14. Lee YA, Cho EJ, Yokozawa T. Oligomeric proanthocyanidins improve memory and enhance phosphorylation of vascular endothelial growth factor receptor-2 in senescence-accelerated mouse prone/8. Brit. J. Nutr. 103: 479-489 (2010)
15. Yokozawa T, Lee YA, Zhao Q, Matsumoto K, Cho EJ. Persimmon oligomeric proanthocyanidins extend life span of senescence-accelerated mice. J. Med. Food 12: 1199-1205 (2009)
16. Li SZ. Fruits. p. 1893. In: Compendium of Materia Medica. Liu HR (ed). People's Medical Press, Beijing, China (1982)
17. Ling ZQ, Xie BJ, Yang EL. Isolation, characterization, and determination of antioxidative activity of oligomeric proanthocyanidins from the seedpod of *Nelumbo nucifera* Gaertn. J. Agr. Food Chem. 53: 2441-2445 (2005)
18. Zhang L, Xu JQ, Rong S, Xie BJ, Sun ZD, Zhang YJ, Liu LG. The mixture of procyanidins extracted from the lotus seed pod and bilobalide ameliorates scopolamine-induced memory impairment in mice. Neurosci. Bull. 25: 203-208 (2009)
19. Xiao J, Sui Y, Li SY, Wu Q, Yang T, Xie BJ, Sun ZD. Combination of proanthocyanidins extracted from lotus seedpod and L-cysteine ameliorates memory impairment induced by alcohol and scopolamine in mice. Eur. Food Res. Technol. 236: 671-678 (2013)
20. Porter LJ, Hrstich LN, Chan BG. The conversion of proanthocyanidins and prodelphinidins to cyanidins and delphinidin. Phytochemistry 25: 223-230 (1986)
21. Sahin S, Samli R. Optimization of olive leaf extract obtained by ultrasound-assisted extraction with response surface methodology. Ultrason. Sonochem. 20: 595-602 (2013)
22. Wolfe KL, Liu RH. Structure-activity relationships of flavonoids in the cellular antioxidant activity assay. J. Agr. Food Chem. 56: 8404-8411 (2008)
23. El-Sherbiny DA, Khalifa AE, Attia AS, Eldenshary EED. *Hypericum perforatum* extract demonstrates antioxidant properties against elevated rat brain oxidative status induced byamnestic dose of scopolamine. Pharmacol. Biochem. Be. 76: 525-533 (2003)
24. Lee M-R, Yun B-S, Zhang D-L, Liu L, Wang Z, Wang C-L, Gu L-J, Wang C-Y, Mo E-K, Ly S-Y, Sung C. Effect of aqueous antler extract on scopolamine-induced memory impairment in mice and antioxidant activities. Food Sci. Biotechnol. 19: 655-661 (2010)
25. Lee M-R, Yun B-S, Zhang D-L, Liu L, Wang Z, Wang C-L, Gu L-J, Wang C-Y, Mo E-K, Ly S-Y, Sung C. Anti-amnesic effect of *chong-myung-tang* on scopolamine-induced memory impairments in mice. J. Ethnopharmacol. 132: 70-74 (2010)
26. Chen L, Richardson JS, Caldwell JE, Ang LC. Regional brain activity of free radical defense enzymes in autopsy samples from patients with Alzheimer's disease and from nondemented controls. Int. J. Neurosci. 75: 83-90 (1994)
27. Ali AK, Banks WA, Kumar VB, Shah GN, Lynch JL, Farr SA, Fleegal-DeMotta MA, Morley JE. Nitric oxide activity and isoenzyme expression in the senescence-accelerated mouse P8 model of Alzheimer's disease: Effects of anti-amyloid antibody and antisense treatments. J. Gerontol. A-Biol. 64: 1025-1030 (2009)
28. Xu JQ, Rong S, Xie BJ, Sun ZD, Deng QC, Bao W, Wang D, Yao P, Huang FH, Liu LG. Changes in the nitric oxide system contribute to effect of procyanidins extracted from the lotus seedpod ameliorating memory impairment in cognitively impaired aged rats. Rejuv. Res. 14: 33-43 (2011)
29. Kalinchuk AV, Stenberg D, Rosenberg PA, Porkka-Heiskanen T. Inducible and neuronal nitric oxide synthases (NOS) have complementary roles in recovery sleep induction. Eur. J. Neurosci. 24: 1443-1456 (2006)
30. Zoubovsky SP, Pogorelov VM, Taniguchi Y, Kim S-H, Yoon P, Nwulia E, Sawa A, Pletnikov MV, Kamiya A. Working memory deficits in neuronal nitric oxide synthase knockout mice: Potential impairments in prefrontal cortex mediated cognitive function. Biochem. Bioph. Res. Co. 408: 707-712 (2011)
31. Przedborski S, Jackson-Lewis V, Yokoyama R, Shibata T, Dawson VL, Dawson TM. Role of neuronal nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. P. Natl. Acad. Sci. USA 93: 4565-4571 (1996)
32. Domek-Łopaciñska KU, Strosznajder JB. Cyclic GMP and nitric oxide synthase in aging and Alzheimer's disease. Mol. Neurobiol. 41: 129-137 (2010)
33. Boultadakis A, Pitsikas N. Effects of the nitric oxide synthase inhibitor L-NAME on recognition and spatial memory deficits produced by different NMDA receptor antagonists in the rat. Neuropsychopharmacol. 35: 2357-2366 (2010)
34. Yang Z-J, Carter EL, Torbey MT, Martin LJ, Koehler RC. Sigma receptor ligand 4-phenyl-1-(4-phenylbutyl)-piperidine modulates neuronal nitric oxide synthase/postsynaptic density-95 coupling mechanisms and protects against neonatal ischemic degeneration of striatal neurons. Exp. Neurol. 221: 166-174 (2010)
35. Giacobini E. Cholinesterases: New roles in brain function and in Alzheimer's disease. Neurochem. Res. 28: 515-522 (2003)