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Cognitive-enhancing and antioxidant activities of iridoid glycosides from *Scrophularia buergeriana* in scopolamine-treated mice

Eun Ju Jeong, Ki Yong Lee, Seung Hyun Kim, Sang Hyun Sung, Young Choong Kim *

College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, Seoul 151-742, Republic of Korea

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ABSTRACT

The cognitive-enhancing activities of *E*-harpagoside and 8-*O*-*E*-*p*-methoxycinnamoylharpagide (MCA-Hg) isolated from *Scrophularia buergeriana* were evaluated in scopolamine-induced amnesic mice by the Morris water maze and by passive avoidance tests. *E*-harpagoside and MCA-Hg significantly improved the impairment of reference memory induced by scopolamine in the Morris water maze test. The mean escape latency, the mean path length and swimming movement were also improved by both compounds. In passive avoidance test, *E*-harpagoside and MCA-Hg (2 mg/kg body weight, p.o.) significantly ameliorated scopolamine-induced amnesia by as much as 70% of the level found in normal control mice. Donepezil, an acetylcholinesterase inhibitor and the most widely used drug for AD treatment was employed as a positive control. The activity of acetylcholinesterase was inhibited significantly by *E*-harpagoside or MCA-Hg within the cortex and hippocampus to a level similar to that observed in mice treated with donepezil (2 mg/kg body weight, p.o.). Moreover, treatment with *E*-harpagoside or MCA-Hg to scopolamine-induced amnesic mice significantly decreased *TBARS level* which was accompanied by an increase in the activities or contents of glutathione reductase, SOD and reduced GSH. We believe these data demonstrate that *E*-harpagoside or MCA-Hg exerted potent cognitive-enhancing activity through both anti-acetylcholinesterase and antioxidant mechanisms.

1. Introduction

Alzheimer's disease is a progressive neurodegenerative disorder common in people over 65 years of age. Alzheimer's disease results from neurodegeneration characterized by the deposition of amyloid plaques, development of neurofibrillary tangles, inflammation, and neuronal loss in specific regions of the forebrain. The most notable symptom of Alzheimer's disease is progressive memory loss followed by a general cognitive decline (Giacobini, 2002). Although multiple neurotransmitter systems appear to be affected in Alzheimer's disease, an important neuropathological feature that appears as an early marker for Alzheimer's disease is degeneration and functional impairment within the cholinergic system (Giacobini, 2003). Neuronal loss in the basal forebrain particularly within the septohippocampal acetylcholinergic systems involved in learning and memory processes constitutes a pathological hallmark of Alzheimer's disease. Currently, the treatment for Alzheimer's disease is the administration of acetylcolinesterase inhibitors that increase the availability of acteylcholine at cholinergic synapses. Such acetylcholinesterase inhibitors as physostigmine, tacrine and donepezil antagonize the negative effects of scopolamine on spatial memory in various behavioral tests (Braida et al., 1996; Bejar et al., 1999; Dawson et al., 1991).

Scopolamine, a muscarinic antagonist that induces central cholinergic blockade, produces a reversible and well-described impairment in both (i) maintaining attention; and (ii) both processing of information and the acquisition of new knowledge in rodents (Bejar et al., 1999; Ohno and Watanabe, 1996; Zhang and O'Donnell, 2000) and in human (Jones et al., 1991; Wesnes et al., 1991). The cognitive deterioration observed resembles the memory disturbances seen in Alzheimer's disease. Hence, the scopolamine-induced amnesic mouse has been used as an experimental model for Alzheimer's disease (Beatty et al., 1986; Ebert and Kirch, 1998; Kopolman and Corn, 1988).

Recently, El-Sherbiny et al. (2003) reported that memory impairment in the scopolamine-induced animal model is associated with increased oxidative stress within rat brain. Jimenez-Jimenez et al. (2006) demonstrated increased oxidation of lipids, proteins and deoxyribonucleic acid, alterations in mitochondrial function and a possible role of amyloid beta and its precursor protein in oxidative reactions in experimental models of Alzheimer's disease. Moreover, strong evidence supporting the involvement of oxidative damage in neurodegenerative disease has been suggested by various clinical studies (Cruz et al., 2003; Floyd, 1999, Schlz et al., 2000). It is known

Abbreviations: CMC, carboxy methyl cellulose; TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase.

* Corresponding author. San 56-1, Shillim-Dong, Gwanak-Gu, Seoul, 151-742, Republic of Korea. Tel.: +82 2 880 7842; fax: +82 2 888 2933.
E-mail address: youngkim@snu.ac.kr (Y.C. Kim).

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that oxidative damage may serve as an early event initiating cognitive disturbances and the pathological features observed in Alzheimer's disease (Ding et al., 2007).

E-Harpagoside, 8-O-(*E*-*p*-methoxycinnamoyl)harpagide (MCA-Hg), *E*-cinnamic acid (CA), and *E*-*p*-methoxycinnamic acid (MCA); the four main bioactive constituents of *Scrophularia buergeriana* root have been reported to have neuroprotective activity against glutamate-induced neurotoxicity in primary culture of rat cortical cells. MCA showed significant antioxidative effect suppressing the overproduction of nitric oxide and cellular peroxide in glutamate-injured neurons (Kim et al., 2002b). Indeed, MCA-Hg also attenuated the decrease of GSH contents as well as inhibiting the formation of nitric oxide and cellular peroxide in cultured cortical neurons injured with glutamate (Kim et al., 2003a).

In our previous work, MCA showed significant anti-amnesic activity in mice with amnesia induced by scopolamine (Kim et al., 2003b). Hence, we attempted to examine whether *E*-harpagoside and MCA-Hg, which showed similar neuroprotective mechanism to MCA with antioxidant property, could mitigate the memory deficit induced by scopolamine in mice using passive avoidance and the Morris water maze tests. To elucidate any potential cognitive-enhancing activity of MCA-Hg and *E*-harpagoside and to ascertain the mechanism, we assessed the effects of these compounds on acetylcholinesterase in the cortex and hippocampus of scopolamine-treated mice. In addition, the effects of these compounds on the levels of TBARS, brain glutathione (GSH), and the activities of glutathione peroxidase, glutathione reductase and superoxide dismutase (SOD) within the brain were evaluated.

2. Materials and methods

2.1. Experimental animals

Male ICR mice (Harlan Sprague–Dawley; 4-weeks-old; Orient. CO. LTD, Gyunggi, Korea), weighing 25–30 g, were used after a one week adaptation period (20 to 23 °C; 12 h light cycle from 09:00 to 21:00; food, Agribrand Purina Korea, and water *ad libitum*). All experiments (viz., the Morris water maze test, passive avoidance test) and the method used for euthanasia were according to the guidelines of the Committee on Care and Use of Laboratory Animals of the Seoul National University. The number and batch of experimental animals used in behavioral tests and biochemical assays are indicated in Table 1.

2.2. Compounds or drugs

E-harpagoside and MCA-Hg were isolated from *S. buergeriana* root. A total extract of *S. buergeriana* and its methylene chloride, 90% methanol and *n*-hexane fractions were prepared by the procedure as described previously. *E*-harpagoside and MCA-Hg were isolated from the 90% methanol fraction and identified by spectral analysis (Kim et al., 2002a). Donepezil (Aricept®) was used as a positive control. *E*-harpagoside, MCA-Hg and donepezil were administered orally to mice 90 min before the scopolamine treatment. Scopolamine was purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, U.S.A) and given subcutaneously 30 min before behavioral tests.

Table 1
Experimental animals used for behavioral tests and biochemical assays

Groups	Experiments
I–VII	Morris water maze test
I, II, IV, VI, VII	Antioxidant assay (followed by the Morris water maze test I)
I, II, IV, VI, VII	TBARS assay (followed by the Morris water maze test II)
I, II, IV, VI, VII	Passive avoidance test (a new batch of animals)
I, IV, VI, VII	AChE activity assay (a new batch of animals)

10 animals were randomized into each group and were treated as below.
Group I: CMC treatment (normal control group).
Group II: CMC and scopolamine treatment.
Group III: *E*-harpagoside (1 mg/kg body weight, p.o.) and scopolamine treatment.
Group IV: *E*-harpagoside (2 mg/kg body weight, p.o.) and scopolamine treatment.
Group V: MCA-Hg (1 mg/kg body weight, p.o.) and scopolamine treatment.
Group VI: MCA-Hg (2 mg/kg body weight, p.o.) and scopolamine treatment.
Group VII: Donepezil (2 mg/kg body weight, p.o.) and scopolamine treatment.

2.3. Cognitive measurements

2.3.1. Morris water maze test

A spatial memory test was performed by the method of Morris (1984) with minor modification as described in our previous reports (Kang et al., 2003; Kim et al., 1999; Kim et al., 2003b). The Morris water maze is a white circular pool (100 cm in diameter and 45 cm in height) with a featureless inner surface. The circular pool was filled with water in which 500 ml of milk had been mixed to a height of 30 cm (20±1 °C). The pool was divided into four quadrants of equal area. A white platform (6 cm in diameter and 29 cm in height) was centered in one of the four quadrants of the pool and submerged 1 cm below the water surface so that it was invisible at water level. The continuous location of each swimming mouse, from the start position to the platform, was monitored by a video tracking system (Smart 2.5). In the water maze experiments, the day prior to the experiment was dedicated to swim training for 60 s in the absence of the platform. In the days following, the mice were given two trial sessions each day for four consecutive days. During each trial, the escape latencies of mice were recorded. This parameter was averaged for each session of trials and for each mouse. Once the mouse located the platform, it was permitted to remain on it for 10 s. If the mouse did not locate the platform within 120 s, it was placed on the platform for 10 s and then removed from the pool by the experimenter. The mouse was given two daily trials for four consecutive days with an inter-trial interval of 20 min. The point of entry of the mouse into the pool and the location of the platform for escape remained unchanged between trials 1 and 2 but was changed each day thereafter. The decrease in escape latency from day to day in trial 1 represents long-term memory or reference memory, while that from trial 1 to trial 2 represents short-term memory or working memory. Ten mice were used per treatment. Mice were treated with 0.5%-carboxy methyl cellulose (CMC) or test compounds (MCA-Hg and *E*-harpagoside) (1 and 2 mg/kg body weight, p.o.) given before the training trial, respectively. After 90 min, amnesia was induced in mice with scopolamine (1 mg/kg body weight, dissolved in normal saline) given subcutaneously. All mice were tested for spatial memory 30 min after the scopolamine treatment.

2.3.2. Passive avoidance test

Training for and testing of passive avoidance performance were carried out in two identical light and dark square boxes (Gemini San Diego Inc., USA) as described in our previous reports (Kang et al., 2003; Kim et al., 1999; Kim et al., 2003b). The mice were initially placed in the light chamber and 10 s later the door between compartments was opened. When mice entered the dark compartment, the door automatically closed and an electrical foot shock (0.1 mA/10 g body weight) lasting 2 s was delivered through the stainless steel rods (one trial training). Ten mice were used per treatment. Mice received CMC or test compounds (MCA-Hg and *E*-harpagoside; 2 mg/kg body weight, p.o.), respectively, 120 min before the training trial. After 90 min, amnesia was induced in mice with scopolamine (1 mg/kg body weight, dissolved in normal saline) given subcutaneously. Twenty-four h after the training trial, the mice were again placed in the light compartment. The escape latency to enter the dark compartment was measured. If the mice

did not enter the dark compartment within 180 s, the experiment was stopped.

2.4. TBARS assay

Following the Morris water maze test, the mouse was anesthetized, decapitated and the whole brain was rapidly dissected under standard conditions at 4 °C; the tissue was then homogenized in 0.02 M phosphate buffer (pH 7.4) at a concentration of 10% (w/v). The homogenate was diluted to 5% (w/v) and resuspended with a hand homogenizer. The homogenate was incubated at 37 °C. Four-ml of homogenate was taken for MDA measurement by the thiobarbituric acid reaction (Buege and Aust, 1978; Stocks et al., 1974). Two-ml of trichloroacetic acid (28% w/v in 0.25 N HCl) was added to 4 ml of the homogenate followed by centrifugation. Then, the 4 ml of supernatant was combined with 1 ml of thiobarbituric acid (1% w/v in 0.25 N HCl) and boiled for 15 min to allow for chromophore development. The absorbance was read at 535 nm using a spectrophotometer. The MDA content was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (Buege and Aust, 1978).

2.5. Antioxidant assay

Following the Morris water maze test, mouse was euthanized. The cerebral cortex and hippocampus were dissected rapidly under standard conditions at 4 °C and homogenized in 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged for 30 min at 3000 ×g at 4 °C and the supernatant (cytosolic and mitochondrial fractions) was collected for assessing antioxidative enzyme activity and GSH content. The activity of SOD was determined according to the method of McCord and Fridovich (1969) by the xanthine–xanthine oxidase reaction. Oxidized glutathione (GSSG) reductase activity was measured according to the method of Carlberg and Mannervik (1975) based on the reduction of GSSG by GSSG reductase in the presence of NADPH. The activity of glutathione peroxidase was determined by quantifying the rate of oxidation of GSH to GSSG by cumene hydroperoxide, a reaction catalyzed by GSH peroxide (Flohe and Gunzler, 1984). Values shown are the mean±S.D. Protein concentration was determined using a bicinchoninic acid (BCA) kit with bovine serum albumin as a standard (Smith et al., 1985). Total GSH in the supernatant was determined

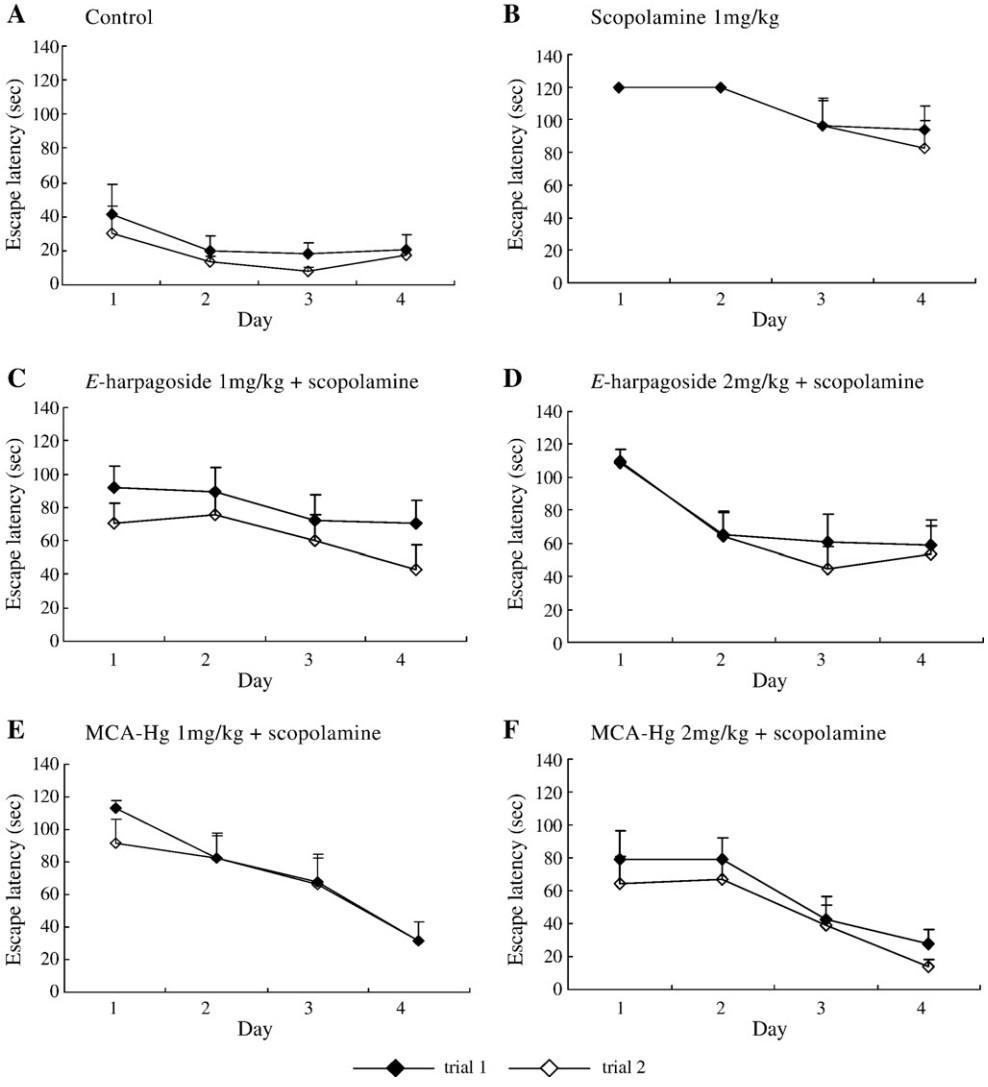

Fig. 1. The enhancing effects of *E*-harpagoside and MCA-Hg on spatial memory impairment induced by scopolamine in mice. Mice were given two sessions of trials each day for 4 consecutive days. The swimming time required for mouse to escape to the platform was recorded in each day. Each day, mice were treated with MCA-Hg or *E*-harpagoside (1 or 2 mg/kg body weight, p.o.). After 90 min of the treatment, amnesia was induced by scopolamine (1 mg/kg body weight, s.c.). All mice were tested for spatial memory 30 min after the injection of scopolamine. The values shown are the mean escape latency±S.E.M. Each group represents 10 animals. (A) Normal control group. (B) Scopolamine-treated group. (C) *E*-harpagoside (1 mg/kg body weight, p.o.), (D) *E*-harpagoside (2 mg/kg body weight, p.o.), (E) MCA-Hg (1 mg/kg body weight, p.o.), (F) MCA-Hg (2 mg/kg body weight, p.o.)-treated group 90 min before the scopolamine injection.

spectrophotometrically using the enzymatic cycling method (Tietz, 1969).

2.6. Acetylcholinesterase activity determination

Mice were treated with CMC or test compounds (MCA-Hg and *E*-harpagoside; 2 mg/kg body weight, p.o.). Ten mice were used per treatment. Acetylcholinesterase activity was measured by the method of Ellman et al. (1961) with slight modification (Cheng and Tang, 1998). The mice were euthanized 40 min after treatment and the brains were removed. The cerebral cortex and hippocampus, respectively was dissected out from the brain. Both parts of the brain were rapidly homogenized, respectively, with sodium phosphate buffer (0.1 mM, pH 7.4). Each homogenate was preincubated for 5 min at 37 °C with 0.1 mM tetraisopropyl pyrophosphoramide (TPPA), a selective inhibitor of butyrylcholinesterase. For the determination of acetylcholinesterase activity, a reaction mixture that contained 470 µl sodium phosphate (0.1 mM, pH 8.0), 167 µl of 4% 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and 33 µl of homogenate was incubated at 37 °C for 5 min. Then, 280 µl of 1 mM acetylcholine iodide solution was added to the reaction mixture. After incubation for 3 min at 37 °C, the reaction was terminated by adding 50 µl of 2 mM neostigmine. The acetylcholinesterase activity was calculated as the optical density (OD) value per mg protein (see above).

2.7. Statistical analysis

Data for the Morris water maze and passive avoidance tests were expressed as mean ± S.E.M. The activity of acetylcholinesterase and the levels of antioxidant values were expressed as mean ± S.D. Passive avoidance latencies, acetylcholinesterase activity and antioxidant values were analyzed by one-way ANOVA. Morris water maze latencies were analyzed by two-way ANOVA with the day as one variable and the treatment as a second. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

3. Results

3.1. Cognitive-enhancing effects of E-harpagoside and MCA-Hg in scopolamine-induced amnesia

The efficacy of *E*-harpagoside and MCA-Hg in enhancing cognition after impairment of spatial memory via scopolamine was evaluated through the Morris water maze test. The *escape latency* for finding the platform as described in Methods is depicted in Fig. 1. Control mice rapidly learned the location of the platform from their first training day and reached stable latencies by day 2 (Fig. 1A). By contrast, mice treated with scopolamine (1 mg/kg body weight) failed to find the platform until given the maximum time limit, 120 s, on days 1 and 2. Though the escape latency was slightly shortened on days 3 and 4, eight of ten mice failed to find the platform during the four training days (Fig. 1B). Amnesic mice treated with MCA-Hg or *E*-harpagoside demonstrated a significantly shortened interval to find the platform as compared to mice given scopolamine alone. In amnesic mice treated with *E*-harpagoside at a dose of 1 mg/kg, the mean escape latency of 91.5 ± 13.1 s observed on day 1 fell to 70.8 ± 13.0 s by day 4. In amnesic mice treated with *E*-harpagoside at a dose of 2 mg/kg, the escape latency was reduced more rapidly to 65.5 ± 13.7 s on day 2 and maintained at this latency for the following days (Fig. 1C, D). Amnesic mice treated with MCA-Hg showed a further reduction in escape latency when compared to mice treated with *E*-harpagoside. In amnesic mice treated with MCA-Hg at 1- or 2-mg/kg, the escape latencies were shortened to 31.7 ± 11.4 s and 28.0 ± 8.4 s, respectively, on day 4 (Fig. 1E, F). However, a statistically significant reduction in escape latencies from trial 1 to trial 2 was not observed in either *E*-harpagoside or MCA-Hg treated amnesic mice.

In treatment with *E*-harpagoside, the effects on amnesic mice were significant with respect to *treatment* ([trial 1]: $F(3, 144)=39.4$, $P<0.0001$; [trial 2]: $F(3, 144)=38.0$, $P<0.0001$), with respect to *days* ([trial 1]: $F(3, 144)=7.2$, $P<0.001$; [trial 2]: $F(3, 144)=8.4$, $P<0.0001$) but with no effect with respect to *day-treatment interaction* ([trial 1]: $F(9, 144)=0.6$, $P<1.0$; [trial 2]: $F(9, 144)=1.4$, $P<0.5$). Similarly, in treatment with MCA-Hg, the effects were also significant with respect to *treatment* ([trial 1]: $F(3, 144)=42.9$, $P<0.0001$; [trial 2]: $F(3, 144)=45.1$, $P<0.0001$), with respect to *days* ([trial 1]: $F(3, 144)=14.1$, $P<0.0001$; [trial 2]: $F(3, 144)=13.0$, $P<0.0001$) but with no effect with respect to *day-treatment interaction* ([trial 1]: $F(9, 144)=1.7$, $P<0.2$; [trial 2]: $F(9, 144)=1.3$, $P<0.3$). In track movement analysis, the swimming paths of amnesic mice treated with MCA-Hg or *E*-harpagoside were all simplified, which correlated well with the decreased escape latencies (data not shown).

The effects of MCA-Hg or *E*-harpagoside on the scopolamine-induced memory deficit were further evaluated using passive avoidance test (Table 2). The step through latency was shortened in mice treated with scopolamine (1 mg/kg body weight s.c.) as compared to that of normal control mice. Treatment of amnesic mice with MCA-Hg or *E*-harpagoside significantly decreased the latency to a level of 70% of normal control mice. At a dose of 2 mg/kg, the efficacies of *E*-harpagoside and MCA-Hg were comparable to the positive control which received donepezil.

Table 2
The enhancing effects of *E*-harpagoside and MCA-Hg on memory impairment induced by scopolamine in mice

Groups	Step through latency (s) (% of control)
Control	177.8 ± 0.6 (100%)
Scopolamine	27.0 ± 1.9[a] (15.2%)
Scopolamine + *E*-harpagoside	129.8 ± 4.4[c] (73.0%)
Scopolamine + MCA-Hg	130.1 ± 4.2[b] (73.4%)
Scopolamine + Donepezil	129.1 ± 5.1[c] (72.6%)

E-harpagoside, MCA-Hg or donepezil was treated 90 min before the scopolamine injection at a dose of 2 mg/kg body weight. The values shown are the mean escape latency ± S.E.M. for animals to stay in the light compartment. Each group represents 10 animals. Results significantly differ from the values of normal control group: [a]$P<0.0001$ and scopolamine only-treated group: [b]$P<0.01$, [c]$P<0.0001$.

Table 3
The effects of *E*-harpagoside and MCA-Hg on AChE activity within the cortex and hippocampus of mice

Groups	U/mg protein	
	Cortex	Hippocampus
Control	0.4089 ± 0.1204	1.2265 ± 0.1452
E-harpagoside	0.2594 ± 0.0287[b]	0.8900 ± 0.0486[c]
MCA-Hg	0.2574 ± 0.0888[b]	0.8305 ± 0.1655[b]
Donepezil	0.2885 ± 0.0427[a]	0.8611 ± 0.0439[c]

The values shown are the mean U/mg protein ± S.D. Results significantly differ from the values of normal control group: [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$.

Table 4
The effects of *E*-harpagoside and MCA-Hg on TBARS level in the brain of scopolamine-induced amnesic mice

Groups	MDA (nM/wet mg)
Control	22.03 ± 4.97
Scopolamine	32.55 ± 3.07[a]
Scopolamine + *E*-harpagoside	18.06 ± 1.56[b]
Scopolamine + MCA-Hg	16.77 ± 3.38[b]
Scopolamine + Donepezil	21.37 ± 4.47[b]

The values shown are the mean optical nM/mg protein ± S.D. Results significantly differ from the values of normal control group. [a]$P<0.01$ and scopolamine-only treated group: [b]$P<0.001$.

Table 5
The effects of *E*-harpagoside and MCA-Hg on glutathione peroxidase, glutathione reductase and SOD activities within the cortex and hippocampus of scopolamine-induced amnesic mice

Groups	Glutathione peroxidase (μmol NADPH oxidized/min/mg protein)		Glutathione reductase (μmol NADPH oxidized/min/mg protein)		SOD (μmol NADPH oxidized/min/mg protein)	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
Control	0.063±0.010	0.125±0.021	26.754±1.839	15.392±1.084	10.431±0.736	6.564±1.238
Scopolamine	0.055±0.008	0.123±0.009	19.736±0.567[b]	11.110±1.432[a]	7.173±0.809[b]	3.929±0.485[b]
Scopolamine+*E*-Harpagoside	0.059±0.014	0.139±0.005	20.730±1.872	14.038±0.731[d]	8.241±0.858[c]	7.151±0.753[e]
Scopolamine+MCA-Hg	0.067±0.010	0.142±0.005[d]	26.936±1.615[e]	15.009±0.915[e]	7.692±1.286	8.175±0.741[e]
Scopolamine+Donepezil	0.068±0.006	0.134±0.009	19.756±2.755	14.986±1.261[d]	8.934±0.693[d]	6.548±0.513[e]

The activity of each enzyme was measured as described in Materials and methods. Each value represents the mean±S.D. Results significantly differ from the values of normal control group: [a]$P<0.001$, [b]$P<0.0001$ and scopolamine only-treated group: [c]$P<0.05$, [d]$P<0.01$, [e]$P<0.001$.

3.2. Acetylcholinesterase inhibitory effects of MCA-Hg and E-harpagoside

Since acetylcholinesterase inhibitors are known to antagonize scopolamine-induced amnesia (Braida et al., 1996; Dawson et al., 1991), the effects of MCA-Hg and *E*-harpagoside on acetylcholinesterase activities within the cortex and hippocampus of mice were evaluated (Table 3). At a dose of 2 mg/kg, acetylcholinesterase activity in the cortex and hippocampus was significantly inhibited by 36% and 27%, respectively, by *E*-harpagoside, and by 36% and 33% by MCA-Hg when compared to normal, controls. The statistics are shown in Table 3.

3.3. Antioxidant effects of E-harpagoside and MCA-Hg on scopolamine-induced oxidative stress

To further elucidate the biochemical mechanism of anti-amnesic activity of MCA-Hg and *E*-harpagoside in brain tissue, we measured their antioxidant effects on lipid peroxidation, on the activities of antioxidant enzymes, and on cellular GSH levels. Administration of scopolamine resulted in a significant increase of TBARS in the mouse brain (Table 4), while the activities of glutathione reductase and SOD were reduced (Table 5). The activity of glutathione peroxidase was virtually unchanged by administration of scopolamine. Treatment of amnesic mice with *E*-harpagoside and MCA-Hg significantly reduced TBARS levels. The reduction in glutathione reductase activity in cortex and hippocampus induced by scopolamine was also reversed by treatment with *E*-harpagoside or MCA-Hg. While the change in glutathione peroxidase activity in hippocampus was not significant statistically after treatment with MCA-Hg, it was increased to some extent. Treatment with *E*-harpagoside or MCA-Hg significantly restored the activity of SOD in amnesic hippocampus. The SOD activity in response to treatment with *E*-harpagoside or MCA-Hg was even greater than that seen in normal control mice.

The effects of the two compounds on GSH levels in amnesic mice are shown in Table 6. Scopolamine administration resulted in a depletion of reduced GSH both in the cortex and hippocampus. Treatment with *E*-harpagoside or MCA-Hg significantly restored the level of reduced GSH both in the cortex and hippocampus.

4. Discussion

The present study was designed to examine the cognitive-enhancing activities of MCA-Hg and *E*-harpagoside on amnesic mice using the Morris water maze and passive avoidance tests. Scopolamine interferes with memory and cognitive function, and subsequently causes impairment of reference (= long-term) and working (= short-term) memories. In this study, mice were given scopolamine to induce memory impairment at a dose of 1 mg/kg; this dose has been reported to have no effect on acquisition latency or swimming ability and appears to be dissociated from drug-induced hyperactivity (Bejar et al., 1999; Dawson et al., 1991; Wanibuchi et al., 1994).

The simultaneous analysis for a distinction between reference and working memory is well established through the Morris water maze test (Morris, 1984). In our experiments, normal control mice exhibited well-formed reference memory. By contrast, mice given scopolamine exhibited neither reference nor working memory. Both *E*-harpagoside and MCA-Hg improved the amnesic deficits in reference memory but not in working memory. The prolongation in escape latency induced by scopolamine was significantly and gradually decreased over the four testing days. Considering the escape latencies determined, and especially as observed on day 4, it seems likely that MCA-Hg is more effective in improving impaired spatial memory induced by scopolamine than is *E*-harpagoside.

Both *E*-harpagoside and MCA-Hg also mitigated the memory deficits induced by scopolamine in mice as measured in passive avoidance test. Considering that donepezil was able to ameliorate cognitive activity to a level of 72% of that in normal control mice at a dose of 2 mg/kg, and comparing this level to our results, we suggest that the potencies of *E*-harpagoside and MCA-Hg appear to be comparable to donepezil.

If we compare the efficacy of MCA-Hg to *E*-harpagoside, while no significant difference was observed between the two iridoids in passive avoidance test, MCA-Hg showed the stronger activity in the Morris water maze test. As the Morris water maze test is well established in detecting changes within the central cholinergic system and especially influences on spatial memory (Becker et al., 2000; Fibriger, 1991; Lydon and Nakajima, 1992; Upchurch and Wehner,

Table 6
The effects of *E*-harpagoside and MCA-Hg on GSH level within the cortex and hippocampus of scopolamine-induced amnesic mice

Groups	Total GSH (nmol/mg protein)		Reduced GSH (nmol/mg protein)		GSSG/total GSH ratio	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
Control	14.252±1.066	9.966±1.456	11.226±1.726	9.647±1.268	0.265±0.019	0.032±0.005
Scopolamine	11.051±0.737[a]	7.323±1.521[a]	6.037±1.033[a]	6.905±1.326[a]	0.457±0.062[a]	0.174±0.026[a]
Scopolamine+*E*-harpagoside	13.222±1.158[c]	10.377±0.912[d]	11.571±1.056[d]	9.918±0.825[c]	0.125±0.021[d]	0.044±0.010[d]
Scopolamine+MCA-Hg	13.510±0.386[c]	10.338±0.990[d]	12.155±0.402[d]	9.889±0.872[c]	0.100±0.014[d]	0.043±0.005[d]
Scopolamine+Donepezil	13.550±0.577[d]	10.036±0.554[d]	12.223±0.537[d]	9.573±0.455[c]	0.098±0.005[d]	0.043±0.004[d]

GSH level was measured as described in Materials and methods. Each value represents the mean±S.D. Results significantly differ from the value of normal control group: [a]$P<0.0001$ and scopolamine only-treated group: [b]$P<0.05$, [c]$P<0.01$, [d]$P<0.001$.

1987), the *E*-*p*-methoxycinnamoyl moiety in MCA-Hg may be found to be responsible for improving spatial memory.

It has been demonstrated that impairments in learning, memory and behavior observed in patients with dementia are caused, at least in part, by changes within the cholinergic system (Blokland, 1995; Fodale et al., 2006). It has been demonstrated by previous animal and human studies that learning and memory can be modified by drugs affecting the central cholinergic system (Bartus et al., 1982; Collerton, 1986). Cholinergic transmission is terminated mainly by acetylcholine hydrolysis via the enzyme acetylcholinesterase. This enzyme is essential in maintaining the normal function of the nervous system, since it rapidly terminates the action of acetylcholine released into the synapse. It is currently believed that the action of this enzyme could affect the underlying processes in Alzheimer's disease (Ballard et al., 2005). Consequently, acetylcholinesterase has been a potential target for treatment of Alzheimer's disease and for prevention strategies. This is why we evaluated the effects of MCA-Hg and *E*-harpagoside on acetylcholinesterase activity and correlated these activities with their anti-amnesic activities.

E-harpagoside and MCA-Hg inhibited acetylcholinesterase activity significantly in the hippocampus by 36% as compared to normal control mice. In this regard, the compounds showed a similar level of inhibition on acetylcholinesterase to donepezil. The memory-improving action of donepezil in scopolamine-induced amnesia could be explained, in part, by neurochemical changes in the brain. Donepezil reversed the scopolamine-induced memory deficit in which available synaptic acetylcholine was increased via inhibition of the degradative enzyme, acetylcholinesterase (Giacobini, 2002). Our data suggest that the ameliorating effects on memory of these iridoid compounds could be explained, at least in part, by their inhibition on acetylcholinesterase activity within the hippocampus.

Many clinical studies have reported strong evidence that oxidative stress is involved in the pathogenesis of Alzheimer's disease (Lovell et al., 1995; Marcus et al., 1998; Sano et al., 1997). In these reports, the activities of glutathione peroxidase and glutathione reductase were found to be elevated or changed in those brains showing severe Alzheimer's disease pathology; this likely reflects a protective response to increased peroxidation within the brain. As such, the progression of neurodegenerative diseases was found to be inhibited by free radical scavengers and antioxidant agents. Recently, El-Sherbiny et al. (2003) reported that memory impairment induced by acute scopolamine administration in rats is associated with altered levels of GSH in the brain and with the activities of antioxidant enzymes. In our experimental conditions, scopolamine administration resulted in a significant increase in TBARS, an important marker for lipid peroxidation, and in a reduction in both glutathione reductase and SOD activities in the cortex and hippocampus of amnesic mice. The administration of *E*-harpagoside and MCA-Hg produced a significant fall in TBARS, and restored the activities of glutathione reductase and SOD in both cortex and hippocampus of mice. Although, the activity of glutathione peroxidase was not changed by the administration of scopolamine, treatment with *E*-harpagoside and MCA-Hg elevated the activity of glutathione peroxidase to a level higher than that found in normal control mice. Glutathione peroxidase reduces toxic radicals using GSH as a substrate, subsequent to the oxidation of GSH to GSSG. GSSG is in turn reduced again to GSH by glutathione reductase at the expense of NADPH, forming a redox cycle (Lu, 1999). We suggest that the restoration of the activities of glutathione peroxidase and glutathione reductase by these iridoid compounds might promote scavenging of free radicals using recycled GSH from GSSG. Consistent with this hypothesis regarding the activity of glutathione peroxidase and glutathione reductase, we showed that the depletion of brain GSH which normally accompanies scopolamine administration was restored significantly by treatment with MCA-Hg or *E*-harpagoside. The most remarkable effects of *E*-harpagoside and MCA-Hg are the increased activity of SOD in the hippocampus. Treatment with *E*-harpagoside or MCA-Hg elevated SOD activity significantly higher than that found in normal control mice.

The present study demonstrated that *E*-harpagoside and MCA-Hg both possess potent antioxidant activities. They scavenge ROS and exert a protective effect against oxidative damage induced by scopolamine by diminishing the reduction in the activities of glutathione reductase and SOD. The effects of *E*-harpagoside and MCA-Hg on antioxidant enzymes were significant, especially with regard to SOD in the hippocampus. The potent anti-amnesic effects of *E*-harpagoside and MCA-Hg might result, in part, from the reduction in reactive oxygen species by the retention of SOD.

In conclusion, *E*-harpagoside and MCA-Hg showed potent cognitive-enhancing activities by the inhibition of acetylcholinesterase activity, and by the regulation of the antioxidant system. As such, *E*-harpagoside and MCA-Hg might offer a useful therapeutic choice in either the prevention or the treatment of Alzheimer's disease.

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