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Protective effect of the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol on scopolamine-induced memory impairment and the inhibition of acetylcholinesterase activity

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Abstract Magnolol, honokiol, and obovatol are well-known bioactive constituents of the bark of *Magnolia officinalis* and have been used as traditional Chinese medicines for the treatment of neurosis, anxiety, and stroke. We recently isolated novel active compound (named 4-*O*-methylhonokiol) from the ethanol extract of *Magnolia officinalis*. The present study aimed to test two different doses of ethanol extracts of *Magnolia officinalis* (5 and 10 mg/kg/mouse, p.o., 1 week) and 4-*O*-methylhonokiol (0.75 and 1.5 mg/kg/mouse, p.o., 1 week) administered for 7 days on memory impairment induced by scopolamine (1 mg/kg body weight i.p.) in mice. Memory and learning were evaluated using the Morris water maze and the step-down avoidance test. Both the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol prevented memory impairment induced by scopolamine in a dose-dependent manner. The ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol also dose-dependently attenuated the scopolamine-induced increase of acetylcholinesterase (AChE) activity in the cortex and hippocampus of mice, and

inhibited AChE activity in vitro with IC_{50} (12 nM). This study, therefore, suggests that the ethanol extract of *Magnolia officinalis* and its major ingredient, 4-*O*-methylhonokiol, may be useful for the prevention of the development or progression of AD.

Keywords Ethanol extract of *Magnolia officinalis* · 4-*O*-methylhonokiol · Alzheimer's disease · Memory · Acetylcholinesterase

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Introduction

Alzheimer's disease (AD), a type of progressive dementia, is a neurodegenerative disorder characterized by a progressive cognitive decline resulting from selective neuronal dysfunction, synaptic loss, and neuronal cell death [1–5]. Numerous medications are undergoing testing for the treatment of the dementia associated with AD, however, there is no known cure for AD. Studies of patients with AD revealed the depleted levels of acetylcholine in an AD patient's brain. Centrally acting cholinergic drugs have been reported to increase the regional cerebral blood flow of acetylcholine in the brain regions affected by AD [6]. Thus, the maintaining of the acetylcholine level in the brain is important for the cure of AD patients. One method for this is blocking the activity of acetylcholinesterase (AChE), the enzyme-degrading acetylcholine. The cholinesterase inhibitors (AChEI) donepezil hydrochloride, galantamine hydrobromide, and rivastigmine tartrate are the current approved drugs for the treatment of AD patients [7]. However, AChEI presents some limitations, such as their short half-lives and excessive side effects caused by the activation of peripheral cholinergic systems, as well as hepatotoxicity, which is the most frequent and important

side effect of these drug therapies [8–11]. For this reason, alternative and complementary therapies need to be developed. Thus, novel AChE inhibitors from plant sources could be valuable alternatives in the context of the treatment of AD. Several studies have shown the neuroprotective and/or cognition-enhancing properties of natural products and their components using different animal models [12–18].

Magnolol, honokiol, and obovatol are well-known bioactive constituents of the bark of *Magnolia officinalis*. Magnolol and honokiol have been known to have various pharmacological activities, such as anti-inflammatory, antibacterial, and anti-allergic activities, and have also been used as traditional Chinese medicines for the treatment of neurosis, anxiety, stroke, fever, and headache [19]. Honokiol was known to promote a potassium-induced release of acetylcholine in a rat hippocampal slice [20] and to enhance neurite sprouting [21]. In addition, magnolol and honokiol exhibited an AChE inhibitory property in rat spleen microsomes and human polymorphonuclear leukocytes [22]. Moreover, we recently found that obovatol has high anti-anxiety activity [23] and anti-AChE activity (unpublished data). Among various constituents of the ethanol extract of *Magnolia officinalis*, we have isolated a major compound (40–50%) identified as 4-*O*-methylhonokiol that has not demonstrated any pharmacological activities. Therefore, in this study, we investigated whether the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol could inhibit the memory impairment induced by scopolamine through the inhibition of AChE.

Materials and methods

Animals

Male ICR mice weighing 25–30 g (Samtako, Gyeonggi-do, Korea) were maintained in accordance with the guidelines of the National Institute of Toxicological Research of the Korea Food and Drug Administration for the care and use of laboratory animals. Animals were housed three per cage, allowed access to water and food ad libitum, and maintained on a 12-h light/dark cycle regulated at 23°C room temperature. The experiments were performed at least 1 week after their arrival in individual home cages. Sixty mice were used for the water maze test and another 60 mice were used for the step-through test. Ten mice per group were used for each of the memory tests; Group 1: control, Group 2: scopolamine-treated, Group 3: scopolamine with 5 mg/kg ethanol extract, Group 4: scopolamine with 10 mg/kg ethanol extract, Group 5: scopolamine with 0.75 mg/kg 4-*O*-methylhonokiol, Group 6: scopolamine with 1.5 mg/kg 4-*O*-methylhonokiol. The mice used for the water maze test were used for the assay of AChE activity.

Materials

The bark of *Magnolia officinalis Rehd. et Wils.* was dried in the shade at room temperature and stored in a dark, cold room until use. The air-dried bark of *Magnolia officinalis Rehd. et Wils.* (3 kg) was cut into pieces and extracted twice with 95% (v/v) ethanol (four times as much as the weight of the dried plants) for 3 days at room temperature. After filtration through the 400-mesh filter cloth, the filtrate was filtered again through filter paper (Whatman Grade No. 5) and concentrated under reduced pressure. The combined extract (450 g) was suspended in H_2O and the aqueous suspension was extracted with *n*-hexane, ethyl acetate, and *n*-BuOH, respectively. The *n*-hexane layer was evaporated to dryness to give a residue (70 g), which was chromatographed on silica gel with *n*-hexane:ethyl acetate (9:1) gradient to yield a crude fraction that included 4-*O*-methylhonokiol. This fraction was repeatedly purified by silica gel chromatography using *n*-hexane:ethyl acetate as the eluent to obtain pure 4-*O*-methylhonokiol (Fig. 1a). 4-*O*-methylhonokiol was identified by ^{1}H-NMR and ^{13}C-NMR. The results of the NMR data are as follows and are in agreement with previously published data [24]. ^{1}H-NMR (400 MHz, CDCl$_3$): δ 3.36 (2H, d, $J = 7$ Hz, H-7), 3.44 (2H, d, $J = 7$ Hz, 7'-H), 3.89 (3H, s, OMe), 5.05–5.14 (5H, m, H-9, H-9', OH), 5.93–6.07 (2H, m, H-8, H-8'), 6.92 (1H, d, $J = 7$ Hz, Ar-H), 6.97 (1H, d, $J = 8$ Hz, Ar-H), 7.04–7.08 (2H, m, Ar-H), 7.24–7.31 (2H, m, Ar-H). ^{13}C-NMR (100 MHz, CDCl$_3$): δ 34.5 (C-7), 39.6 (C-7'), 55.8 (OMe), 111.2 (C-3'), 115.7 (C-4'), 115.8 (C-9), 116.1 (C-9'), 128.0 (C-1'), 128.1 (C-6), 129.0 (C-3), 129.2 (C-1), 130.0 (C-5), 130.4 (C-6'), 130.7 (C-2), 132.4 (C-5'), 136.7 (C-8), 138.0 (C-8'), 151.0 (C-2'), 157.2 (C-4). The ethanol extract of *Magnolia officinalis* contained 16.6% 4-*O*-methylhonokiol, followed by 16.5% honokiol and 12.9% magnolol, and 42–45% others.

Passive avoidance performance test

The passive avoidance performance test is widely accepted as a simple and rapid method for memory testing [24]. The passive avoidance response was determined using a 'step-through' apparatus (Med Associates Inc., Vermont, USA) that consisted of an illuminated and a dark compartment (each of dimensions 20.3 × 15.9 × 21.3 cm) adjoining each other through a small gate with a grid floor, 3.175-mm stainless steel rod set 8 mm apart. On a training trial, the ICR mice were placed in the illuminated compartment facing away from the dark compartment. When the mice moved completely into the dark compartment, it received an electric shock (1 mA, 3 s duration). Then, the mice were returned to their home cage. Twenty-four hours later after the training trial, the

Fig. 1 Chemical structure of 4-*O*-methylhonokiol (**a**) and experimental scheme (**b**)
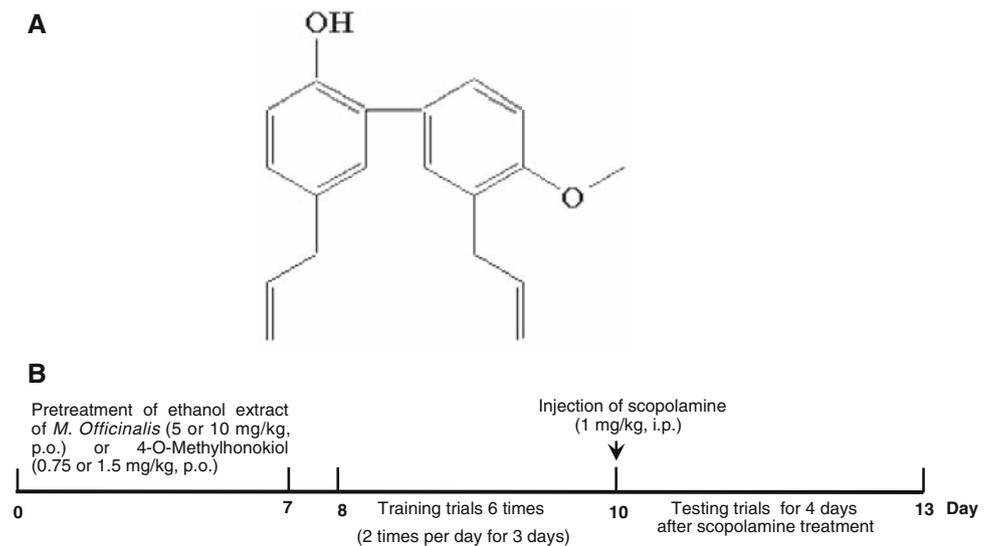
mice were placed in the illuminated compartment and the latency period to enter the dark compartment defined as 'retention.' The time when the mice entered the dark compartment was recorded and described as step-through latency. Scopolamine (1 mg/kg) was injected intraperitoneally (i.p.) into mice 30 min before the retention trial. The retention trials were set at a limit of 180 s of cut-off time.

Water maze test

The water maze test is a widely accepted method for memory testing, and we performed this test following the method described by Morris et al. [25]. Maze testing was performed by the SMART-CS (Panlab, Barcelona, Spain) program and equipment. A circular plastic pool (height 35 cm, diameter 100 cm) was filled with milky water and kept at 22–25°C. An escape platform (height 14.5 cm, diameter 4.5 cm) was submerged 0.5–1 cm below the surface of the water in position. On training trials, the mice were placed in a pool of water and allowed to remain on the platform for 10 s, and then returned to the home cage. The mice that did not find the platform within 60 s were placed on the platform for 10 s. Twenty-four hours after the last training trial (six training trials, 2 times/day for 3 days), the mice were given the test trial. Scopolamine (1 mg/kg, i.p.) was injected into mice 30 min before the test trial. They were allowed to swim until they discovered the escape platform. The escape latency, escape distance, swimming speed, and swimming pattern of each mouse was monitored by a camera above the center of the pool connected to the SMART-LD program (Panlab, Barcelona, Spain).

AChE activity assay

After behavior testing, the animals were perfused with PBS under inhaled chloroform anesthetization. The brains were immediately collected in the same manner and frozen stored at −20°C, and separated into cortical and hippocampal regions. Brain tissues were homogenized with PBS and lyzed by 60 min of incubation on ice. The lysate was centrifuged at 15,000 rpm for 15 min and the supernatant was used for the assays. AChE activity was determined by Ellman's method [26]. Briefly, 5 μl of sample was mixed with 200 μl of reaction buffer (0.5 mM PBS pH 7.4) containing 0.02% DTNB (5,5′-dithio-bis-2-nitrobenzoic acid), 0.02% acetylcholine, and 0.1 mM isoOMPA (tetra-isopropyl pyrophosphoramide). The activity of the enzyme was determined after 5 min of incubation at 37°C and stopped with 1 mM BW284c51, a potent selective inhibitor of AChE. The reaction mixture was converted to yellow color. The optical density was measured at 405 nm and then expressed as units of the quantity (μM) of the acetylcholine that were hydrolyzed to thiocholine per 1 min per mg. Specific activity was standardized by the amount (mg) of protein of the sample (μM/min/mg protein).

To test the in vitro AChE activity, the whole mouse brain was homogenized in a glass Teflon homogenizer containing ten volumes of homogenization buffer (12.5 mM sodium phosphate buffer, pH 7.0, 400 mM NaCl) and then centrifuged at $1{,}000 \times g$ for 10 min at 4°C. The supernatant was used as an enzyme source for the assay. Aliquots of diluted 4-*O*-methylhonokiol were then mixed with reaction buffer and reacted at room temperature for 5 min and the activity was determined by the same method as described above. The concentrations of 4-*O*-methylhonokiol required to

inhibit AChE activity by 50% (IC_{50}) were calculated using enzyme inhibition dose response curves. Tacrine was used as a positive control.

Statistics

The data were analyzed using GraphPad Prism 4 software (Version 4.03, GraphPad Software, Inc.). The data are presented as mean ± standard error (SE). The homogeneity of variances was assessed using a Bartlett test. If variances were homogeneous, differences between groups and treatment were assessed by one-way or two-way analysis of variance (ANOVA). If the P-value in the ANOVA test was significant, the differences between pairs of means were assessed by Dunnet's test. One-way ANOVA was used to analyze data for AChE, while data obtained from the water Morris maze (swimming distance, escape latency, and average speed) were analyzed using two-way ANOVA. When variances were not homogeneous, the Kruskal–Wallis test was used to assess differences between groups for nonparametric analyses. Data from the step-through avoidance test (latency) were analyzed using the nonparametric tests mentioned above, followed by Dunnet's test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Effect of the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol on the memory impairment induced by scopolamine

Escape latency (s) of animals treated with scopolamine (1 mg/kg, i.p.) was decreased (12 ± 5.5 s, $P < 0.01$) in comparison with the control group (28 ± 7.3 s), indicating that scopolamine induced memory impairment. On the other hand, the escape latency of animals pretreated with 5 or 10 mg/kg of ethanol extract of *Magnolia officinalis* for 1 week showed significant preventive effect on memory impairment compared to that of scopolamine-treated animals (24 ± 7.1 s, $P > 0.05$ or 19 ± 6.1 s, $P < 0.05$) (Fig. 2a). Tacrine (3 mg/kg) also showed a significant preventive effect against scopolamine-induced memory impairment (25 ± 1.9 s, $P > 0.05$). The escape latency of animals administered with 0.75 or 1.5 mg/kg 4-*O*-methylhonokiol was approximately 2–3 times longer than that of scopolamine-treated animals, and showed significant preventive effect on memory impairment (28 ± 1.8 s, $P < 0.01$ or 33 ± 8.7 s, $P < 0.01$) (Fig. 2b).

To further examine the memory-enhancing activity of the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol, we determined the improvement of the spatial memory function of these compounds using the Morris water maze. The mice were pretreated continuously with the ethanol extract of *Magnolia officinalis* (5 or 10 mg/kg into drinking water) and 4-*O*-methylhonokiol (0.75 or 1.5 mg/kg into drinking water) for 1 week, and then the animals were given the training trial. After finishing the training trial for 3 days (2 times/day, six times training), scopolamine (1 mg/kg, i.p.) was then injected into the mice to induce memory impairment. Two-way ANOVA revealed an effect of the number of days ($F_{3, 144} = 6.981$, $P = 0.0002$) and the treated groups ($F_{3, 144} = 6.956$, $P = 0.0002$) on the escape latency, and an effect of the number of days ($F_{3, 144} = 6.254$, $P < 0.0001$) and the treated groups ($F_{3, 144} = 9.423$, $P = 0.0005$) on the escape distance.

The escape latency on the next day of training to the platform was about 450 ± 127 cm and 19 ± 3 s. Scopolamine-treated mice arrived slower at the location of the platform compared to the controls (885 ± 47 cm, $P < 0.05$; 42 ± 2 s, $P < 0.05$). The escape latency of animals pretreated with 5 or 10 mg/kg ethanol extract of *Magnolia officinalis* for 1 week showed a memory-enhancing effect. The escape latency of animals pretreated with 5 or 10 mg/kg ethanol extract of *Magnolia officinalis* for 1 week and subsequently treated with scopolamine was shown to be approximately 2.5 or 2.4 times shorter (354 ± 40 cm, $P < 0.05$; 16 ± 3 s, $P < 0.05$ or 361 ± 50 cm, $P < 0.05$; 17 ± 2 s, $P < 0.05$) than that of scopolamine-treated animals determined at 4 day after the training trial (Fig. 3a).

There was also shown to be a significant memory-improving effect by the treatment with 4-*O*-methylhonokiol. Elevated escape latency and escape distance by scopolamine was inhibited by the time (third and fourth day) and treatment (1.5 mg/kg). An amount of 1.5 mg/kg of 4-*O*-methylhonokiol significantly reduced the escape latency (s) ($F_{3, 39} = 5.983$, $P < 0.05$ and $P < 0.01$ at the third and fourth days after training, respectively) and distance (cm) ($F_{3, 39} = 4.93$, $P < 0.05$ and $P < 0.01$ at the third and fourth days after training, respectively) (Fig. 3b). The average speed was not affected by any of the treatments during all days of the evaluation (data not shown).

Effect of the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol on the activities of AChE induced by scopolamine

In animals treated with scopolamine (1 mg/kg, i.p.), the AChE activity was significantly increased in comparison with the control group, and in animals pretreated with the ethanol extract of *Magnolia officinalis* (5 or 10 mg/kg) for 1 week and subsequently treated with scopolamine (1 mg/kg, i.p.), the AChE activity was significantly ($P < 0.05$)

Fig. 2 Inhibitory effect of the
ethanol extract of *Magnolia*
officinalis (**a**) and 4-*O*-
methylhonokiol (**b**) on memory
impairment induced by
scopolamine in the passive
avoidance test. Mice were
administered the ethanol extract
of *Magnolia officinalis* at 5.0
and 10.0 mg/kg and 4-*O*-
methylhonokiol at 0.75 and
1.5 mg/kg for 1 week into
drinking water, and then the
training trial was given on the
last day. After 24 h, the passive
avoidance test was performed.
Scopolamine (i.p., 1 mg/kg)
was treated 30 min before the
test trial as a positive control.
Each value is mean ± standard
error (SE) from ten animals.
$^{\#}P < 0.05$ showed a significant
difference compared with the
controls. $*P < 0.05$ showed a
significant difference compared
with the scopolamine-treated
controls
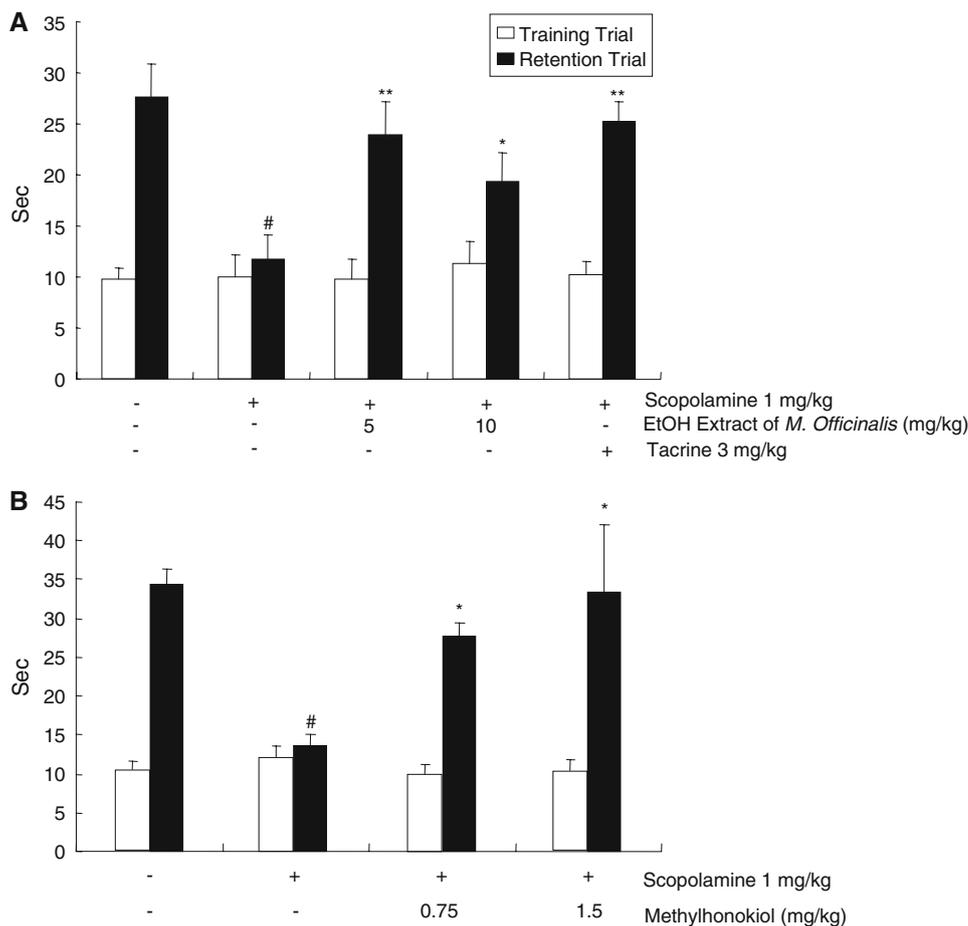
suppressed in both the hippocampus and cortex in a dose-
dependent manner (Fig. 4a). In animals pretreated with 4-
O-methylhonokiol (0.75 or 1.5 mg/kg), the AChE activity
was also significantly ($P < 0.05$) suppressed in the hippo-
campus and cortex in a dose-dependent manner (Fig. 4b).
By in vitro testing, 4-*O*-methylhonokiol also inhibited
AChE activity in a concentration (1 nM–100 μM)-depen-
dent manner, with IC_{50} values of 12 nM. The IC_{50} value of
tacrine (positive control) was 135.4 nM.

Discussion

Alzheimer's disease (AD) is a progressive and eventually
fatal disease of the brain, and it is the cause of more than
50% of dementia patients developing in the population
older than 60 years of age [27–30]. A deficiency in ace-
tylcholine is probably the single most common cause of
declining memory [20]. AChE modulates acetylcholine to
proper levels by degradation, thus, excessive AChE activ-
ity leads to constant acetylcholine deficiency, causing
memory and cognitive impairments in AD [16]. Currently,
AChE inhibitors (AChEI) are the first group of drugs for

AD treatment. Several AChEI have been approved by the
FDA for the treatment of AD, such as tacrine (Cognex®),
donepezil (Aricept®), rivastigmine (Exelon®), and galan-
tamine (Reminyl®) [10]. However, these drugs have
limitations in their usage, such as their short half-lives and
excessive side effects caused by the activation of peripheral
cholinergic systems, as well as hepatotoxicity [8, 9, 11].
Thus, a complementary therapeutic strategy is required.
Neuroprotective and/or cognition-enhancing properties of
natural products and their components using different
animal models have been suggested as alternative therapies
in several studies [12–18].

In this study, the ethanol extract of *Magnolia officinalis*
and its major ingredient, 4-*O*-methylhonokiol, recovered
the memory impairment induced by scopolamine, and its
effect may be related to the ability of AChE inhibition. The
step-through latency, which was reduced by scopolamine
treatment, was recovered to 60–70% of the vehicle-treated
control group. The Morris water maze learning task was
used to assess hippocampal-dependent spatial learning
ability [25, 31] and long-term memory [25]. Impairment in
the long-term memory was observed in the scopolamine-
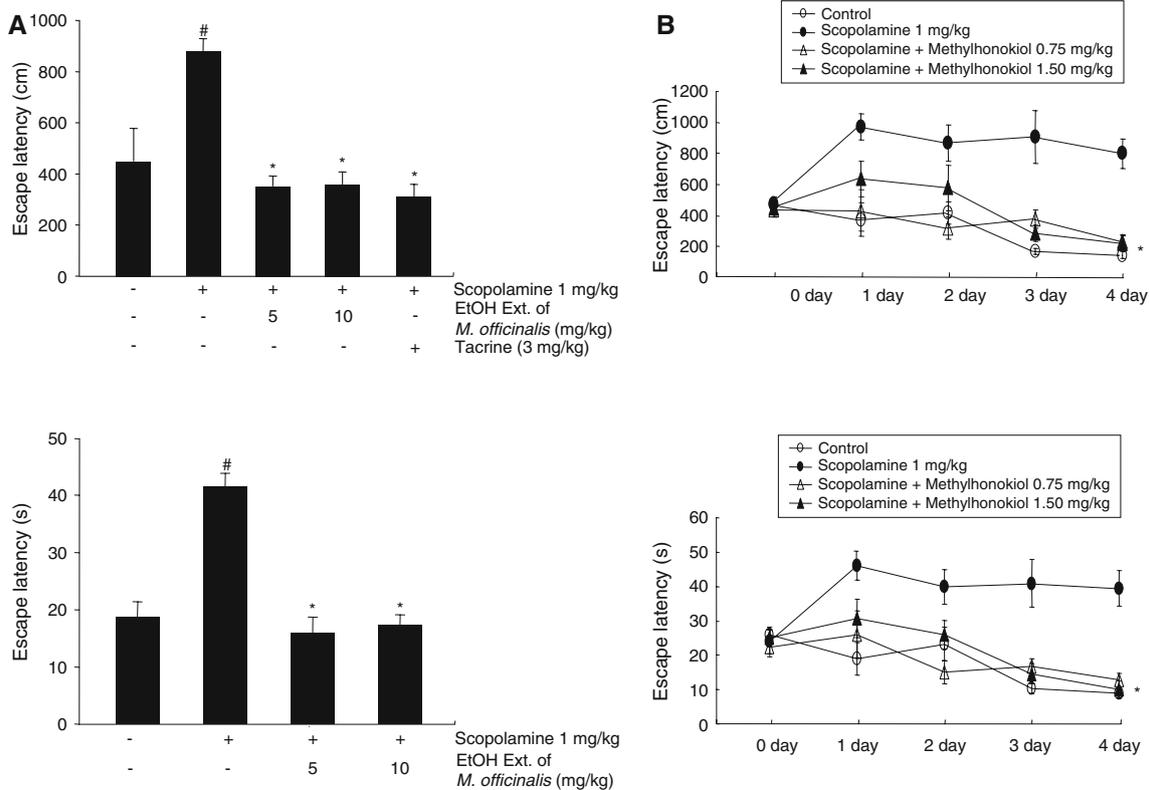treated group. However, the ethanol extract of *Magnolia*

Fig. 3 Inhibitory effect of the ethanol extract of *Magnolia officinalis* (**a**) and 4-*O*-methylhonokiol (**b**) on memory impairment induced by scopolamine in the water maze test. Mice were administered the ethanol extract of *Magnolia officinalis* at 5.0 and 10.0 mg/kg and 4-*O*-methylhonokiol at 0.75 and 1.5 mg/kg for 1 week into drinking water. The mice were then given training trials six times (2 times/day), and the test was performed 24 h after the last training trial. Scopolamine (i.p., 1 mg/kg), as a positive control, was treated 10 min before the first test trial, as shown in the Fig. 1b. Each value is the mean ± SE from ten animals. [#]$P < 0.05$ showed a significant difference compared with the controls. [*]$P < 0.05$ showed a significant difference compared with the scopolamine-treated controls

officinalis and 4-*O*-methylhonokiol also significantly shortened the escape latencies prolonged by the scopolamine treatment. At the probe trial session, the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol improved the swimming time and distance within the zone of the platform to the control level. Collectively, these behavioral studies suggest that the ethanol extract of *Magnolia officinalis* improves the long-term memory in an amnestic mouse model induced by scopolamine treatment, and 4-*O*-methylhonokiol may be a major active ingredient.

To confirm the mechanism of the action of the ethanol extract of *Magnolia officinalis* and its major ingredient, 4-*O*-methylhonokiol, their inhibitory activity on AChE was assessed in vivo. It is well known that the memory-improving effect of the anti-amnestic effect of tacrine and donepezil is due to the inhibition of AChE in the brain. The present data showed that the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol inhibited AChE activity in a dose-dependent manner in both the cortex and hippocampus. Thus, it is likely that the memory-improving effect of the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol may be, at least in part, mediated by

the AChE inhibition. AChEI, such as tacrine and donepezil, causes severe hepatic toxicity, nausea, vomiting, etc., so these drugs must be used carefully. The efficacy of the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol is superior compared with conventional drugs such as tacrine (Table 1). In the present study, we found that 5 or 10 mg/kg of ethanol extract of *Magnolia officinalis* or 0.75 or 1.5 mg/kg of 4-*O*-methylhonokiol has a similar inhibitory effect to 3 mg/kg tacrine on scopolamine-induced memory impairment as well as the inhibition of AChE activity in vivo. The inhibitory effect on the AChE activity may be related to the inhibitory effect of 4-*O*-methylhonokiol on the expression of AChE. We observed that the elevated expression of AChE by scopolamine in both the cortex and hippocampus was decreased by the treatment with 4-*O*-methylhonokiol (data not shown). In the in vivo study, however, we did not observe clear dose-dependent prevention on the memory impairment. These unexpected data (similar dose effects) may be caused by the non differential effect of 4-*O*-methylhonokiol by these two doses with only two times the difference, thus, additional increased or decreased doses
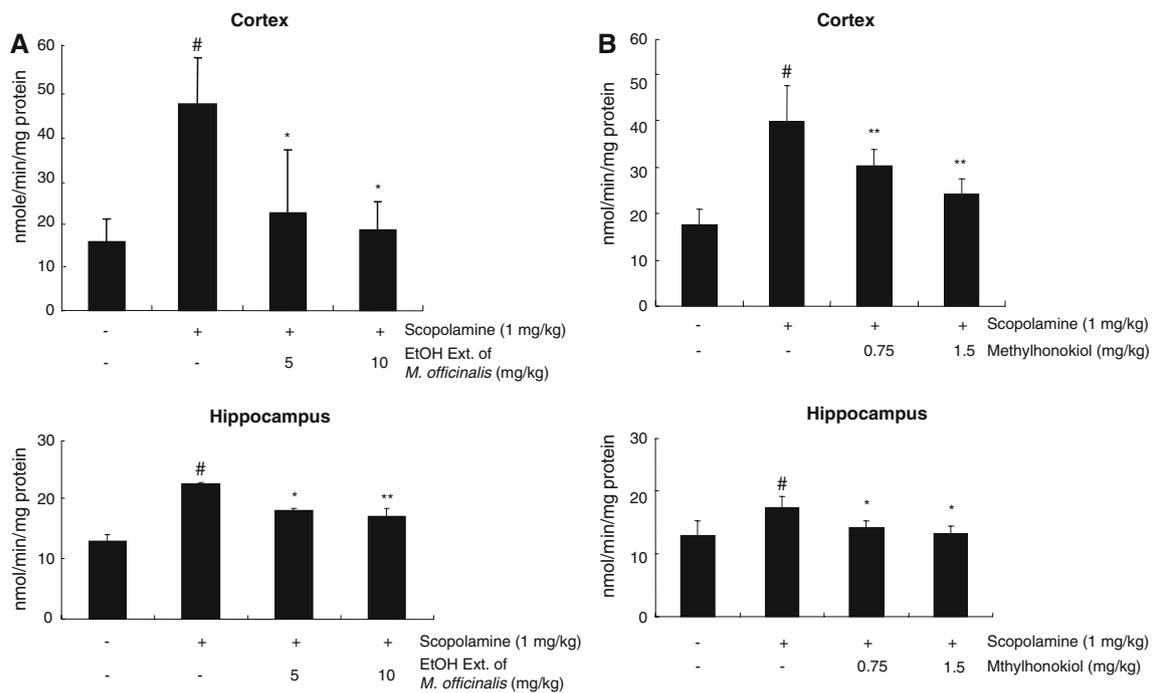
Fig. 4 Inhibitory effect of the ethanol extract of *Magnolia officinalis* (**a**) and 4-*O*-methylhonokiol (**b**) on the AChE activity in the cortex and hippocampus of scopolamine-induced mice. Mice were treated with the ethanol extract of *Magnolia officinalis* (5 and 10 mg/kg) and 4-*O*-methylhonokiol (0.75 and 1.5 mg/kg) for 1 week into drinking water, and then the AChE activity was measured. Each value is the mean ± SE from ten animals. $^{\#}P < 0.05$ showed a significant difference compared with the controls. $^{*}P < 0.05$ showed a significant difference compared with the scopolamine-treated controls

Table 1 Inhibitory activities of acetylcholinesterase (AChE)

Compound	Concentration	Inhibition (%)
4-*O*-methylhonokiol	0.1 nM	0.4 ± 0.1
	1 nM	10.43 ± 1.43
	10 nM	47.4 ± 2.54
	100 nM	68.11 ± 7.64
	1 μM	78.11 ± 4.34
	10 μM	84.15 ± 2.60
Tacrine	1 nM	1.14 ± 1.24
	10 nM	9.62 ± 2.32
	100 nM	46.54 ± 3.86
	1 μM	54.71 ± 7.74
	10 μM	61.41 ± 2.94
	100 μM	59.97 ± 5.31

Efficacy of inhibition was expressed as percent inhibition versus the control values (100%). Values represent mean ± SD ($n = 5$)

could be tested in further studies. We are currently undertaking another investigation using AD transgenic mice with three different doses with three times the difference among the dosing, since the present memory prevention effects were likely to be saturated, and the effect was similar to the effect of tacrine. In in vitro acetylcholine esterase, 4-*O*-methylhonokiol (IC$_{50}$: 12 nM) showed more than ten times stronger inhibitory effect than tacrine (IC$_{50}$: 135.4 nM). Moreover, the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol originate from a natural plant, and, thus, side effects could be relatively smaller. In fact, 4-week oral treatment (up to 300 mg/kg) of the ethanol extract of *Magnolia officinalis* to mice and rats did not show any toxicities in the liver, kidney, intestine, or cardiovascular and central nervous systems, and the animals did not have any high cholinergic symptoms (data not shown). Similar effects of other ingredients isolated from *Magnolia officinalis*, such as honokiol and magnolol, were reported to increase acetylcholine release in freely moving rats, and these effects may be related to the inhibitory effects on the AChE activity [20, 32]. Therefore, from the above behavioral and biochemical data, it can be concluded that the ethanol extract of *Magnolia officinalis* has an ability to improve or ameliorate spatial long-term memory impairment, in part, via enhancement of the cholinergic nervous system, and 4-*O*-methylhonokiol may be one of major components of the memory-improving effect of *Magnolia officinalis*. It is also noteworthy that cognition-enhancing agents activate cholinergic transmission via an agonistic or antagonistic effect on GABA$_A$/benzodiazepine receptor [33–35], and GABA$_A$/benzodiazepine receptor complex

controls acetylcholine release [36]. We previously found that obovatol, a similar compound isolated from *Magnolia officinalis*, has high affinity of GABA$_A$/benzodiazepine receptor [23]. Therefore, it can also be speculated that the GABA$_A$/benzodiazepine receptor ligand properties of 4-*O*-methylhonokiol may also be related to the ameliorating effects on scopolamine-induced memory impairment. Further studies are needed to clarify these points. We are currently attempting to identify the mechanisms of the interactions between 4-*O*-methylhonokiol and GABA$_A$/benzodiazepine receptors. Taken together, these data suggest that the ethanol extract of *Magnolia officinalis* and 4-*O*-methylhonokiol may be useful leading compounds in anti-AD drug development.

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