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In vitro and in vivo comparisons of the effects of the fruiting body and mycelium of *Antrodia camphorata* against amyloid β-protein-induced neurotoxicity and memory impairment

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Abstract *Antrodia camphorata* is a particular and precious medicinal mushroom, and its fruiting body was found to provide more efficient protection from oxidative stress and inflammation than its mycelium because of its higher content of triterpenoids, total phenols, and so on. In the previous in vitro studies, the mycelium of *A. camphorata* is proven to provide strong neuroprotection in neuron cells and suggested to have the potential of protection against neurotoxicity of amyloid β-protein (Aβ) known as the risk factor toward Alzheimer's disease (AD) development. However, the in vivo study and the comparison study with the fruiting body have not yet been investigated. This study compared the effect of the fruiting body and mycelium of *A. camphorata* on alleviating the Aβ40-induced neurocytotoxicity in the in vitro Aβ-damaged neuron cell model (PC-12 cell treated with Aβ40) and memory impairment in the in vivo AD animal model induced with a continuous brain infusion of Aβ40. In the results of in vitro and in vivo studies, the fruiting body possessed stronger anti-oxidative and anti-inflammatory abilities for inhibiting neurocytotoxicity in Aβ40-treated PC-12 cells and Aβ40 accumulation in Aβ40-infused brain than mycelium. Moreover, hyperphosphorylated tau (*p*-tau) protein expression, known as an important AD risk factor, was suppressed by the treatment of fruiting body rather than that of mycelium in the in vitro and in vivo studies. These comparisons supported the reasons why the fruiting body resulted in a more significant improvement effect on working memory ability than mycelium in the AD rats.

Keywords Alzheimer's disease · Amyloid · *Antrodia camphorata* · Fruiting body · Mycelium

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Introduction

The main characteristic of the brains of Alzheimer's disease (AD) patients is the accumulation of senile plaques around the neuron cells. The main component of these plaques is a peptide called amyloid β-peptide (Aβ), which is composed of 39 to 43 amino acids. A large Aβ production causes Aβ aggregation and eventually leads to the formation of senile plaques. The Aβ oligomers act on synapses and further activate glial cells and astrocytes, eventually leading to progressive synapse and nerve damage. They then disrupt neuronal ion homeostasis, cause oxidative damage, and alter the activities of kinases and phosphatases, leading to neurofibrillary tangles. This then causes a deficiency of neurotransmitters, general loss of neural functions, and death of

neural cells, which all eventually lead to the development of Alzheimer's disease (Christen 2000; Smith et al. 2000). Many researches have revealed the correlation between anti-oxidative ability and the prevention of Alzheimer's disease. The aggregated Aβ fibrils were proven to stimulate the formation of free radicals, reactive oxygen species, and proinflammatory factors and then further damage neuron cell via oxidative stress. Many antioxidants such as vitamin E, garlic acid, and ginkgo leaf extract are proven to block Aβ-induced oxidative stress in many previous studies (Christen 2000; Smith et al. 2000). Insoluble fibrillar Aβ plaques and neurofibrillary deposits of hyperphosphorylated tau proteins are the diagnostic lesions of AD. Aβ and tau are proven to synergistically impair the oxidative phosphorylation system in triple transgenic AD mice (Rhein et al. 2009). Furthermore, the soluble Aβ dimers are found to directly induce tau hyperphosphorylation and neuritic degeneration (Jin et al. 2011). Therefore, suppressing Aβ deposition, tau phosphorylation, and oxidative stress is an important goal for breaking the occurrence of serious AD pathology.

Antrodia camphorata, a medicinal mushroom, is a species of fungus unique to Taiwan, possessing numerous healthy characteristics and functional metabolites. This fungus forms a fruit body that causes brown rot on *Cinnamomum kanehirai* Hay (Andersson et al. 2001; Chen et al. 1995). The physiologically active substances of *A. camphorata* are yet to be identified. The active substances found in *A. camphorata* include polysaccharide, ergosterol, and triterpenoids (Chen et al. 2008; Lee et al. 2002; Shen et al. 1997; Yu et al. 2009). Triterpenoids are considered as one of the most biologically active components in recent years. For example, the oxygenated triterpenoids found in fungi which were isolated from *Ganoderma lucidium* have biological activities including inhibition of cholesterol synthesis, inhibition of angiotensin-converting enzyme, and inhibition of platelet aggregation (Wang et al. 1991). However, more and more studies have indicated that *A. camphorata* is able to express neuroprotective effect in cell model (Chen et al. 2006; Huang et al. 2005; Lu et al. 2008). Aβ is known to be highly correlated with the formation of AD. Aβ accumulation in the brain causes oxidative stress and inflammation, neuron damage, and memory and learning deficit (Christen 2000; Sastre et al. 2006). *A. camphorata* has been proven to possess fairly beneficial antioxidative, anti-inflammatory, and neuroprotective effects and therefore should also be effective in suppressing Aβ accumulation.

Previous studies have proven the neuroprotective effects of *A. camphorata* (Chen et al. 2006; Huang et al. 2005; Lu et al. 2008) and its potential to be developed into a functional food for prevention of AD. However, no studies have targeted the fruiting body and mycelium via in vivo experiments to evaluate and compare their improvement effects on memory and learning ability, as well as AD risk factors. Therefore, this study subjects the fruiting body and mycelium of *A. camphorata* to in vitro PC-12 cell model and in vivo AD animal model induced with continuous brain infusion of Aβ40 as the methods to compare their neuroprotective properties against Aβ40-induced neurotoxicity, memory impairment, and learning deficit. In the in vitro evaluation tests, water extract and ethanol extract of fruiting body and mycelium were used to evaluate their antioxidative and anti-inflammatory effects, as well as neuroprotective effects against Aβ40. In the in vivo animal test, Aβ40 was continuously infused into the brains of animal models to evaluate and compare the effects of fruiting body and mycelium on the improvement of memory and learning abilities and the inhibition of the AD risk factors, including thiobarbituric acid-reactive substances (TBARS), reactive oxygen species (ROS), *p*-tau, and β-secretase for beta-site amyloid precursor protein cleaving enzyme (BACE) expression, as well as Aβ40 accumulation.

Materials and methods

Chemicals

Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Difco Co. (Detroit, MI, USA). Ethanol (95%) was purchased from Taiwan Tobacco and Liquor Co. (Taipei, Taiwan). RPMI 1640 medium, horse serum, and fecal bovine serum were purchased from Gibco BRL Co. (Grand Island, NY, USA). Aβ40 was purchased from Tocris Bioscience Co. (Ellisville, MO, USA). Glucose, fructose, maltose, 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin–Ciocalteau agent was purchased from Panreac Quimina S.A. (Barcelona, Spain). Gallic acid was purchased from Scharlau Chemie S.A. (Barcelona, Spain). Phenol, sulfuric acid, and sodium carbonate were purchased from Merck Co. (Darmstadt, Germany). The fruiting body of *A. camphorata* grown on the root of wild *C. kanehirai* Hay was kindly provided by Professor Jyh-Jye Wang.

Microorganism and cultures

A. camphorata BCRC 35396 was purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan). *A. camphorata* was maintained on PDA at 28 °C and transferred to fresh medium for 20-day intervals. Seed cultures were prepared by transferring a loopful of colony from the

PDA agar slant into a 500-mL Hinton flask containing 100 mL medium (24 g/L PDB, 20 g/L glucose). The cultures were incubated at 100 rpm and 28 °C for 7 days. After that, 5 mL seed culture broth was transferred to 100 mL submerged cultured medium. *A. camphorata* mycelium was cultured under the basic condition of 500-mL Hinton flask containing 100 mL medium (24 g/L PDB, 20 g/L glucose) at 28 °C and 100 rpm for 14 days.

Preparation of the water extract and ethanol extract of mycelium and fruiting body

The dry fruiting body and mycelium were extracted by 95% ethanol at 37 °C and 100 rpm for 6 h and then centrifuged at 1,500×*g* for 10 min in triplicate. After lyophilization of supernatant, the dried extract was resolved to a suitable concentration by water or ethanol for the assay.

PC12 cell culture and MTT assay for cell viability

The rat pheochromocytoma cell line, PC12, was purchased from Bioresource Collection and Research Center (BCRC 60048, Hsinchu, Taiwan). PC12 cells were grown in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, and 50 U/mL penicillin in a humified incubator aerated with 95% air and 5% CO_2 at 37 °C. Anti-Aβ-induced neurocytotoxicity ability was determined using MTT assay (Lee et al. 2008). In brief, the cells were seeded in 96-well culture plates at a density of 5×10^4 cells/well and adhered for 24 h. The culture medium was removed and changed to serum-free RPMI 1640 medium, and the cells were incubated with Aβ40 (1.2 μM) in the presence or absence of the water and ethanol extract of mycelium and fruiting body for 48 h. After treatment, 50 μL of MTT (250 ng/mL in serum-free medium) was added to the cells for 4 h and 50 μL of DMSO was added to dissolve the formed formazan crystals at room temperature for 10 min in the dark. The absorbance was measured at 595 nm by ELISA reader (Sunrise, Tecan Trading AG, Switzerland) and used to calculate the cell viability.

Antioxidative ability assay

Antioxidative ability assay was determined using DPPH free radical scavenging effect (Aniya et al. 1999). In brief, the presence or absence of 150 μL ethanol extract or water extract of mycelium or fruiting body was added to 50 μL of freshly prepared ethanol solution of DPPH (0.1 mM). The absorbance was measured at 492 nm exactly 30 min by ELISA reader. The lower the absorbance, the more potent is the DPPH scavenging effect. The reducing power was determined using the method proposed by a previous study (Oyaizu 1986).

Total polysaccharide, total triterpenoids, and anti-oxidative substances analysis

The polysaccharide of fruiting body or mycelium was precipitated by 95% ethanol at 4 °C for 24 h. The precipitated polysaccharides were collected by centrifugation (3,000×*g*, 15 min) and dried. The polysaccharide was resolved to a suitable concentration by distilled water. The polysaccharide concentration was analyzed according to Dubois et al. (1951). Total triterpenoids were measured using high-performance liquid chromatography according to the previous study (Chang et al. 2006). The total phenol concentration of extract was analyzed according to Folin–Ciocalteu method as described by the previous study (Cliffe et al. 1994). In brief, 0.25 mL extract or standard (gallic acid) was added with 2.25 mL of distilled water, 0.5 mL of the Folin–Ciocalteu stock reagent, and 1.0 mL of Na_2CO_3 reagent (75 g/L) to the mixture at room temperature for 30 min. The absorbance was measured at 765 nm by ELISA reader. Total flavonoids concentration was analyzed according to Zhu et al. (2004). Half milliliter of extract or standard (rutin) was mixed with 2 mL of distilled water and 0.15 mL 5% $NaNO_2$ for 6 min and then mixed with 0.15 mL 10% $AlCl_3$ for 6 min. After adding 2 mL 4% NaOH and 0.2 mL ddH_2O and mixing for 15 min, the mixture was measured at 510 nm.

Animals' grouping and experiment schedule

Male Wistar rats (weighing 280–320 g) were obtained from Laboratory Animal Center of National Taiwan University, College of Medicine. They were kept in a temperature-controlled room (23 °C) under a 12 L/12D cycle (lights on at 6:00) and were given free access to food and water. In the experiment, 30 rats were randomly divided to five groups. Two groups of the rats were infused i.c.v. with vehicle solution (NOR group) or Aβ40 solution (Aβ group) without oral administration of test materials. The other groups were treated with Aβ infusion as well as orally administrated with lovastatin (1.51 mg/kg/day) (LS group), onefold dosage mycelium of *A. camphorata* (79.45 mg/kg/day) (mycelium group), or onefold dosage fruiting body of *A. camphorata* (79.45 mg/kg/day) (fruiting body group). The dosage of *A. camphorata* or lovastatin is calculated in accordance with Boyd's formula of body surface area as recommended by the FDA (Food and Drug Administration) (Boyd 1935; Lee et al. 2006). Feeding rat with *A. camphorata* fruiting body or mycelium at a onefold dosage per day corresponds to supplementing the daily diet with that at 3.5 g for an adult. In addition, the dosage of lovastatin for rat used at 1.51 mg/kg per day corresponds to the daily diet including 20 mg lovastatin for an adult.

The experiment schedule of AD animal model was shown as follows. Aβ40 infusion on day 0 was continued for 28 days and lovastatin, *A. camphorata* fruiting body or mycelium in suspension was orally administrated to the rat from the 1st day to the 28th day. The behavioral test was started on the 19th day. The passive avoidance task was carried out from the 19th day to the 21st day. Subsequent reference memory task, probe test, and working memory task were started on the 22nd day, the 24th day, and the 25th day. On the 28th day, rats were sacrificed and brain tissues were collected for the measurement of TBARS, ROS levels, *p*-tau, BACE protein expression, and Aβ40 accumulation.

Surgery for i.c.v. Aβ40 infusion

Rats were anesthetized with sodium pentobarbital (50 mg/kg BW, i.p.). The left skull was exposed and drilled (relative to the bregma: 0.8 mm posterior, 1.4 mm lateral) according to the atlas of Paxinos and Watson (2005) using a stereotaxic frame (Narishige, Tokyo, Japan). Aβ40 was prepared in the vehicle solvent of 35% (*v*/*v*) acetonitrile plus 0.1% (*v*/*v*) trifluoroacetic acid (pH 2.0). The osmotic mini-pump (2004, Durect Co., Cupertino, CA, USA) used to result in an animal model of AD with impaired memory was filled with Aβ40 solution or the vehicle solution. The outlet of infusion cannula was inserted 4.0 mm into the left ventricle and attached to the skull with dental cement, and then the mini-pump was quickly implanted into the backs of the rats. Aβ solution of 234 μL contained in the osmotic pump was continuously infused into the left ventricle by 0.28 μL/h for 28 days. Finally, the total amount infused was approximately 4.9–5.5 nmol Aβ40 (Lee et al. 2007).

Apparatus of water maze

The Morris water maze task was used to evaluate the memory and learning ability from the 22nd day to the 27th day (Lee et al. 2007). A black circular tank (diameter, 140 cm; height, 45 cm) was used as the apparatus of water maze in which a movable escape platform (diameter, 10 cm; height, 25 cm) was located inside the tank. The tank was filled to a height of 27.5 cm with water of temperature at approximately 23 °C; thus, the surface of the platform was 2.5 cm below the surface of the water. The circular tank was divided into four quadrants (I, II, III, and IV), and a position with equal distance from center and edge in the middle of each quadrant was marked for the location of platform. The water tank was located in a test room with many cues external to the maze. The room had adjustable indirect light, and a camera was set at the ceiling above the center of the water tank. The position of the cues remained unchanged throughout the water maze task.

Morris water maze task

According to the procedure of our previous study (Lee et al. 2007), reference memory test was carried out from the 22nd day to the 24th day and included four continuous trials per day. Probe test was immediately carried out after the 12th training trial of reference memory task on the 24th day. Working memory test was performed from the 25th day to the 27th day and consisted of five trials per day.

Preparation of brains

After completing the behavioral studies, the rats were anesthetized with sodium pentobarbital (65 mg/kg BW, i.p.), and blood was collected; the cerebral cortex and hippocampus were separated from the whole brain on ice, blotted gently with filter paper to remove blood and extraneous tissue fragments, then flash-frozen with liquid nitrogen, and stored at −80 °C until use. Hippocampus and cortex tissues (100 mg) were crushed with an amalgam mixer (UT-1600, Sharp, Osaka, Japan), suspended in 1.0 mL of ice-cold Tris saline (50 mM Tris–HCl, pH, 7.6, 0.15 M NaCl) buffer containing 1% (*v*/*v*) Triton X-100 and protease inhibitor cocktail, and then sonicated for 30 s. The homogenate was centrifuged at 100,000×g for 30 min and the supernatant was used for TBARS and ROS assay. Regarding protein extraction for immunoblotting, the tissue (100 mg) was homogenated in 1.0 mL of lysis buffer (1% Triton X-100, 20 mM Tris, pH 7.5, 100 mM NaCl, 40 mM NaF, 0.2% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, and 1 mM Na_3VO_4) and subjected to brief sonication (10 s). The homogenate was centrifuged at 100,000×g for 30 min and the supernatant was used for immunoblotting assay.

Determination of TBARS and ROS levels in hippocampus and cortex

Hippocampus and cortex homogenates were centrifuged at 4,000×g for 15 min and the supernatant was used for neurochemical assay. TBARS level was determined by the method of thiobarbituric acid colorimetric analysis, and the optical density value was measured at 532 nm (Ohkawa et al. 1979). In the measurement of ROS, homogenates were added to 96-well plates, and NBT reduction was measured by absorbance at 550 nm in triplicate (Lee et al. 2007).

Immunoblotting of hyperphosphorylated tau, BACE, and Aβ40

Protein concentration was determined by bicinchoninic acid method. The samples were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. After blocking in a gelatin-NET solution, blots were incubated

with polyclonal hyperphosphorylated tau (*p*-tau) antibody (1:1,000) and monoclonal BACE antibody (1:1,000) at room temperature for 1 h. Then, bands were incubated with specific horse radish peroxidase-conjugated secondary antibodies (1:100,000) at room temperature for 1 h and visualized by enhanced chemiluminescence substrate with UVP AutoChemi Image system (UVP Inc., Upland, CA, USA). Protein loading was evaluated by anti-actin antibody (1:3,000). Band intensities were quantified using UVP LabWork 4.5 software (UVP Inc.).

Immunohistochemical stain of Aβ40 in hippocampus

The brain tissue was fixed in 10% formalin at pH 7.4. Brains were blocked, and serial 35-μm-thick frozen sections cut on a sledge microtome were collected sequentially and without interruption into wells. Brain sections were processed for immunohistochemical stain with monoclonal Aβ40 antibody according to our previous study (Lee et al. 2007).

Statistical analysis

Data are expressed as means ± standard deviation. Analysis of variance by Duncan's test and Pearson's product–moment correlation coefficient test were determined using SPSS version 10.0 software (SPSS Institute, Inc., Chicago, IL, USA). Differences with $p<0.05$ were considered as statistically significant.

Results

Effects of total flavonoids, total phenols, polysaccharides, and triterpenoids of the *A. camphorata* fruiting body and mycelium

The total flavonoids and total phenols are regarded as the typical antioxidants of *A. camphorata*. Moreover, polysaccharides and triterpenoids are the important functional ingredients of mycelium and fruiting body, respectively. Results (as shown in Table 2) indicate that the fruiting body contained significantly higher levels of total phenols and total triterpenoids than mycelium ($p< 0.01$), but higher total polysaccharides were produced by mycelium than by fruiting body ($p< 0.01$). However, fruiting body and mycelium had similar levels in the concentration of total flavonoids.

DPPH radical scavenging ability of the fruiting body, mycelium water, and ethanol extracts

Antioxidative ability is highly correlated with AD prevention (Christen 2000); hence, screening extracts with high antioxidative ability can help obtain an AD prevention substance. The free radical scavenging ability of DPPH was applied as an evaluation indicator. Table 1 compares the DPPH radical scavenging ability of mycelium water extract (M-WE), mycelium ethanol extract (M-EE), fruiting body water extract (F-WE), and fruiting body ethanol extracts (F-EE) of the *A. camphorata*. Results indicate a dose-dependent response in these two types of extracts, but both fruiting body and mycelium expressed more DPPH radical scavenging ability in their water extracts (F-WE and M-WE) than their ethanol extracts (F-EE and M-EE) using the same concentration. Furthermore, more DPPH radical scavenging abilities were performed by the F-WE rather than M-WE, implying that fruiting body may have more antioxidative effect than mycelium.

Effects of the fruiting body, mycelium water, and ethanol extracts on Aβ40-induced cytotoxicity in PC-12 cells

Water and ethanol extracts of the *A. camphorata* fruiting body and mycelium were used to treat PC-12 cells simultaneously with Aβ40. Figure 1 shows that Aβ40 treatment induced the cytotoxicity and lowered cell viability to 40%. However, the cell viabilities were increased with the increasing concentrations of M-WE, F-WE, and F-EE but not M-EE. The rescue effects of M-WE, F-WE, and F-EE were as stronger as lovastatin (known as compound for repressing Aβ40 cytotoxicity) when compared at the same concentration. At a concentration of 1.25 ppm, M-WE, F-WE, and F-EE displayed improved inhibitory effects on Aβ40 cytotoxicity than M-EE. The results suggest that fruiting body had more effect on inhibiting Aβ40 cytotoxicity than mycelium, especially their ethanol extracts.

Effects of the fruiting body, mycelium water, and ethanol extracts on Aβ40-induced oxidative stress and inflammation in PC-12 cells

The effects of the *A. camphorata* fruiting body and mycelium on repressing Aβ40-induced oxidative stress and inflammation in PC-12 cells are shown in Fig. 2. After the treatment of Aβ40, the TBARS levels in PC-12 cells were significantly increased to 27.5 times of the control group ($p<0.05$). However, such an increase was significantly reversed by the treatment of water and ethanol extracts of the fruiting body and mycelium ($p<0.05$). At a concentration of 1 ppm, the inhibitory effects of Aβ40 oxidative stress in a top–down order were F-WE, F-EE, M-WE, and finally M-EE. Regarding Aβ40-induced ROS levels in PC-12 cell, the addition of Aβ40 resulted in the significant increase of ROS levels by 1.55 times ($p<0.05$) in PC-12 cells, but which were reversed by

Table 1 DPPH free radical scavenging effect between water and ethanol extracts of *A. camphorata* fruiting body and mycelium

Sample concentration (μg/mL)	DPPH free radical scavenging effect (%)				
	Mycelium		Fruiting body		Vitamin E
	Water extract	Ethanol extract	Water extract	Ethanol extract	
1	0.45±0.99 a	1.84±6.94 a	5.09±2.26 a	1.20±3.72 a	6.95±0.79 a
25	2.75±0.36 a	2.34±2.78 a	19.23±2.50 b	3.65±4.50 a	31.05±0.32 c
50	9.53±2.67 a, b	4.61±1.79 a	34.54±1.58 c	4.79±3.21 a	31.79±0.22 b, c
100	11.39±2.11 b	8.13±1.43 a, b	48.35±2.75 d	5.61±0.32 a	38.53±0.37 c
150	21.49±2.11 c	13.10±1.50 b	72.02±2.75 e	6.60±2.99 a	52.69±0.01 d
200	24.45±3.24 b	18.81±1.11 a, b	69.36±1.12 c	14.45±2.82 a	93.94±0.06 d
250	33.03±4.42 b	20.27±2.92 a	73.26±1.14 c	15.37±4.11 a	92.73±0.10 d
300	45.76±3.65 b	21.73±2.69 a	76.61±0.39 c	21.01±1.76 a	92.70±0.32 d

Data are presented as means ± SD (n=4). Mean values within each row with different letters are significantly different (p<0.05)

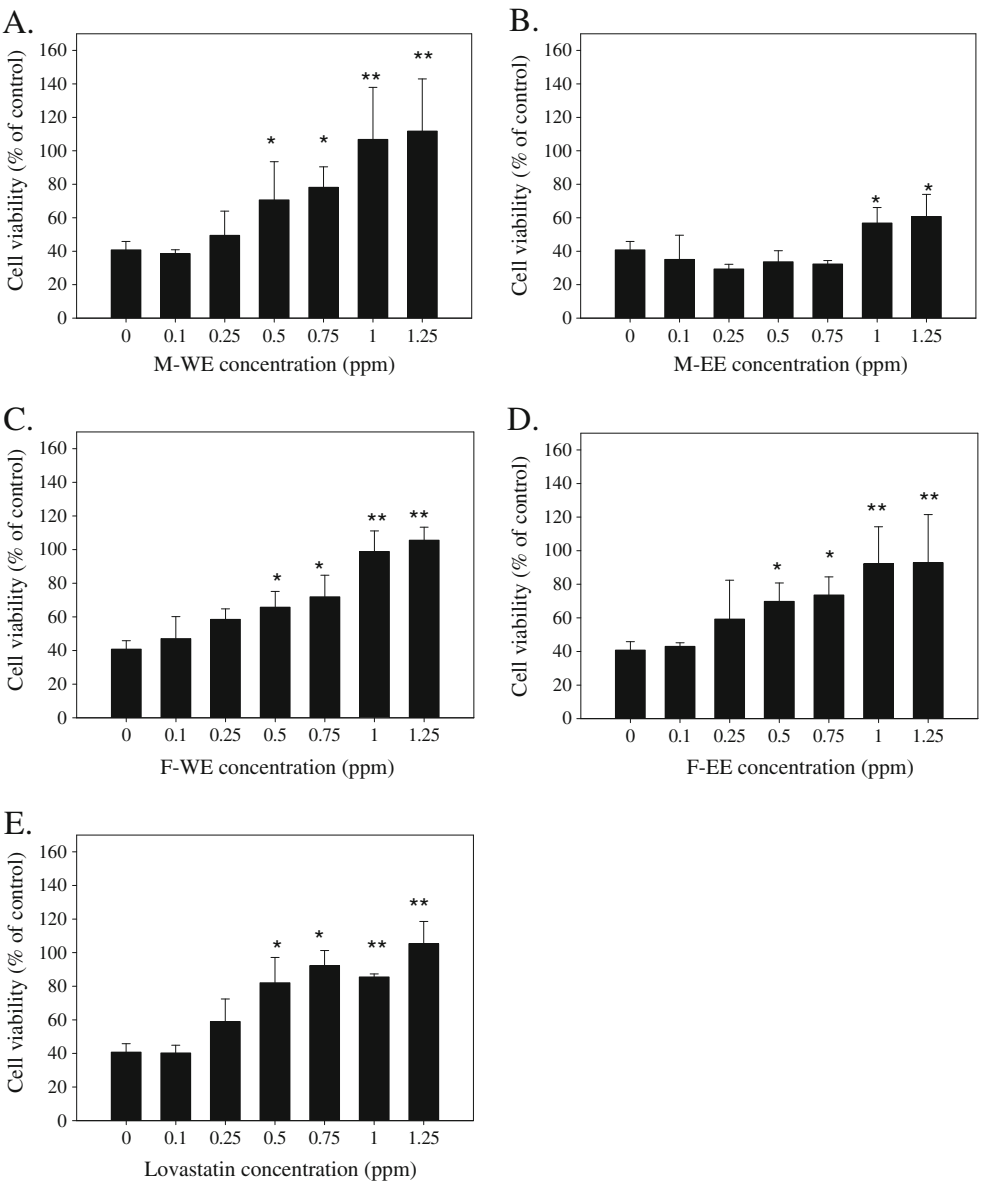

Fig. 1 Effects of ethanol extract and water extract of the fruiting body and mycelium of *A. camphorata* on the Aβ40-induced neurotoxicity in PC12 cells. Aβ40-induced cell death was evaluated with MTT assay. PC12 cells (3×10^6) were treated with an increasing concentration of the water extract of mycelium (*M-WE*) (**a**), the ethanol extract of mycelium (*M-EE*) (**b**), the water extract of fruiting body (*F-WE*) (**c**), the ethanol extract of fruiting body (*F-EE*) (**d**), and lovastatin (**e**) in the serum-free medium (100 μL) containing 1.2 μM Aβ40 fibrils for 48 h. The data are means ± SD expressed as percentage of control values (n=3). A statistical comparison was made using ANOVA, followed by Duncan's test. *p<0.05, **p<0.01 versus control group

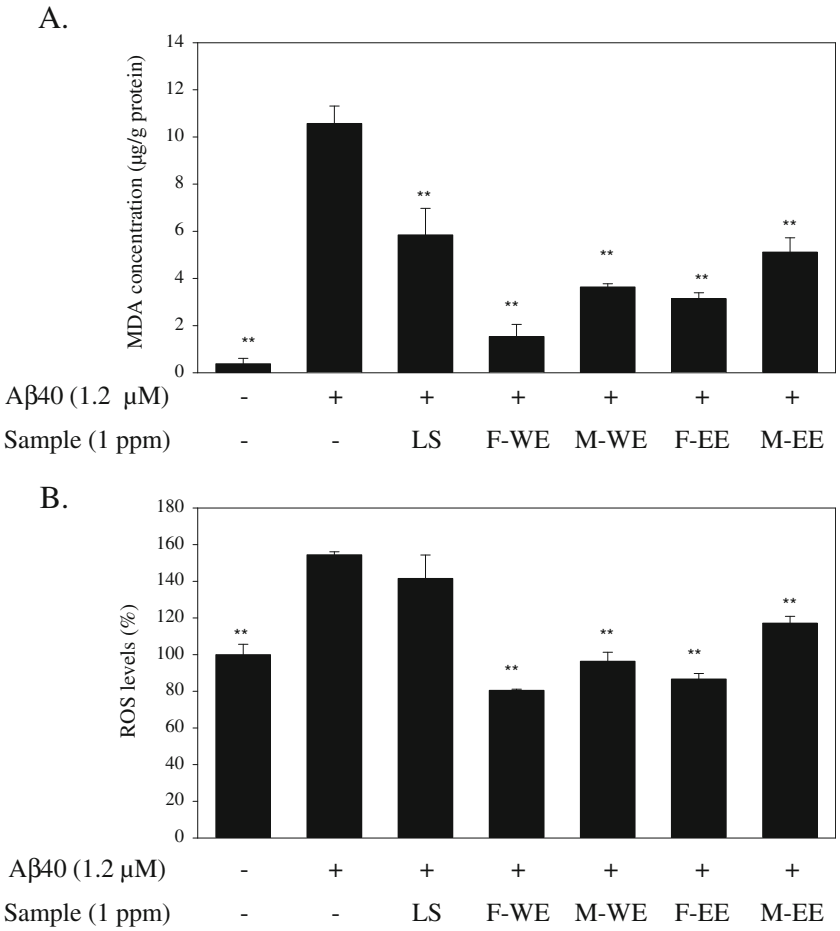

Fig. 2 Effects of ethanol extract and water extract of the fruiting body and mycelium of *A. camphorata* on the expression of MDA (**a**) and ROS (**b**) in Aβ40-treated PC12 cells. PC12 cells (3×10^6) were treated with the water extract of mycelium (*M-WE*), the ethanol extract of mycelium (*M-EE*), the water extract of fruiting body (*F-WE*), the ethanol extract of fruiting body (*F-EE*), and lovastatin in the serum-free medium (100 μL) containing 1.2 μM Aβ40 fibrils for 48 h. The data are means ± SD expressed as percentage of control values (n=3). A statistical comparison was made using ANOVA, followed by Duncan's test. *p<0.05, **p<0.01 versus control group

treatment with the fruiting body and mycelium extracts (1 ppm). At a concentration of 1 ppm, the inhibitory ability against Aβ40-induced ROS levels was as follows: 74.0% (F-WE), 67.8% (F-EE), 58.1% (M-WE), and 37.3% (M-EE), respectively. Therefore, the above-mentioned results show that fruiting body has more effect against Aβ40-induced oxidative stress and inflammatory response than mycelium.

Effects of the fruiting body, mycelium water, and ethanol extracts on phosphorylated tau protein expression in Aβ40-treated PC-12 cells

p-Tau is regarded as an important marker for the evaluation of AD pathology development (Mulder et al. 2010). The effects of the *A. camphorata* fruiting body and mycelium on the expression of *p*-tau protein in Aβ40-treated PC-12 cells are shown in Fig. 3. The expression of *p*-tau protein in PC-12 cells was significantly increased by the treatment of Aβ40. F-WE and F-EE resulted in a more significant effect on suppressing *p*-tau protein expression than M-WE and M-EE, suggesting that the fruiting body has more potential to suppress the expression of Aβ40-induced risk factor.

Effects of fruiting body and mycelium on memory and learning ability of Aβ40-infused AD rats

The effects of the *A. camphorata* fruiting body and mycelium on improvement of memory and learning ability in Aβ40-infused AD rats are shown in Fig. 4. Reference memory test is an indicator for the evaluation of long-term memory ability. The time required for test animals to reach the resting platform from the water maze starting point served as an indicator for reference memory test evaluation. Memory training results from the second time to the ninth time (as shown in Fig. 4a) indicate that Aβ40-infused AD rats (Aβ group) took longer to reach the resting platform than memory rats in the NOR group. However, after feeding *A. camphorata*-fermented products, the AD rats took less time finding the platform. Both fruiting body group and mycelium group needed less time than the Aβ group, but their improvement effect has no significant difference.

Probe tests were performed immediately following the last reference memory test on day 24. The resting platform was removed after the reference memory test ended, and the time test animals spent wandering in the quadrant where the resting platform was placed served as the memory–learning indicator of the probe test. Swimming paths helped determine

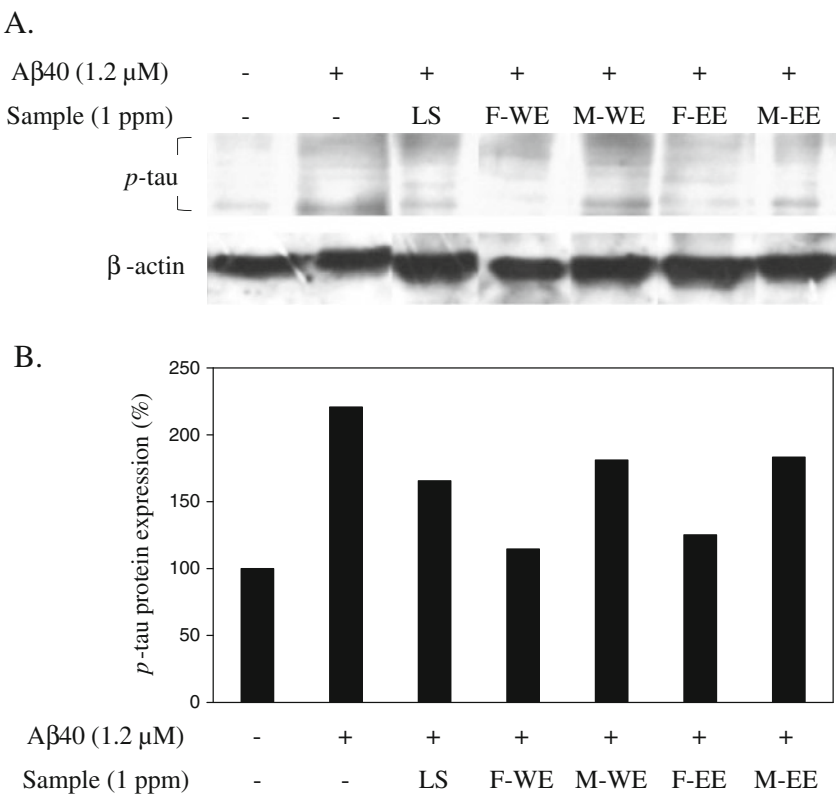

Fig. 3 Effects of ethanol extract and water extract of the fruiting body and mycelium of *A. camphorata* on the expression of phosphorylated tau protein in Aβ40-treated PC12 cells. PC12 cells (3×10^6) were treated with the water extract of mycelium (*M-WE*), the ethanol extract of mycelium (*M-EE*), the water extract of fruiting body (*F-WE*), the ethanol extract of fruiting body (*F-EE*), and lovastatin in the serum-free medium (100 μL) containing 1.2 μM Aβ40 fibrils for 48 h. Phosphorylated tau protein expressions were visualized using immunoblotting (**a**) and quantified using image J software (**b**)

the actual memory and learning performances of the test animals in the probe test. Figure 4b indicates that, in the spatial learning task, the Aβ group searched for the target quandrant without a sense of direction or target, and their swimming paths spanned the entire pool area. The NOR group, LS group, mycelium group, and fruiting body group, however, directly entered the target quadrant and spent more time wandering in the target region. Results (Fig. 4c) show that the Aβ group took less time searching by 29.1% in the target quadrant than the NOR group ($p<0.05$). The mycelium group and fruiting body group took 29.77% and 39.69% at least longer searching time in the target quadrant than the Aβ group, respectively, indicating that the mycelium and fruiting body could improve memory and learning abilities in AD rats. According to the results of the probe test, improved long-term memory and learning abilities of the mycelium group were similar as in the fruiting body group.

The working memory test is a method to evaluate short-term memory and learning abilities. Results (as shown in Fig. 5**)** indicate that the Aβ group spent more time searching by 36.93% for the resting platform than the NOR group ($p<0.05$). However, fruiting body group and mycelium group displayed superior improvement in memory and learning abilities, which were 46.49% and 33.77% shorter than the Aβ group ($p<0.05$), respectively. The LS group displayed searching time 23.18% shorter than the Aβ group ($p<0.05$), but the effects were not as significant as those of the fruiting body group. According to the aforementioned memory and learning test results, the fruiting body group displayed more improvement effect than the mycelium group in the short-term memory ability rather than in the long-term memory ability.

Effects of fruiting body and mycelium on brain oxidative stress and inflammation in Aβ brain infusion rats

Aβ has been proven to induce oxidative stress and inflammation in the brains of AD patients (Christen 2000; Smith et al. 2000). Therefore, inhibiting Aβ-induced oxidative stress and inflammation is regarded as the important indicator in AD medication development. The effects of fruiting body and mycelium on Aβ-induced TBARS levels in the cortex and hippocampus are shown in Fig. 6a. After brain infusion of Aβ, the TBARS levels in the cortex and hippocampus significantly increased by two times ($p<0.05$) and 8.3 times ($p<0.01$) as compared with the NOR group, respectively; this increase in TBARS levels was effectively lowered through feeding fruiting body and mycelium ($p<0.05$). As for ROS levels, results (as shown in Fig. 6b) indicate that the ROS levels in hippocampus but not in cortex were significantly increased by 147.6% ($p<0.05$). The brain Aβ infusion-induced ROS levels in hippocampus were significantly reduced by daily feeding of *A. camphorata*-fermented fruiting body ($p<0.01$), but not mycelium ($p>0.05$), as compared with Aβ group. Orally administrating with lovastatin (LS group) was still default to alleviate raised ROS levels in hippocampus.

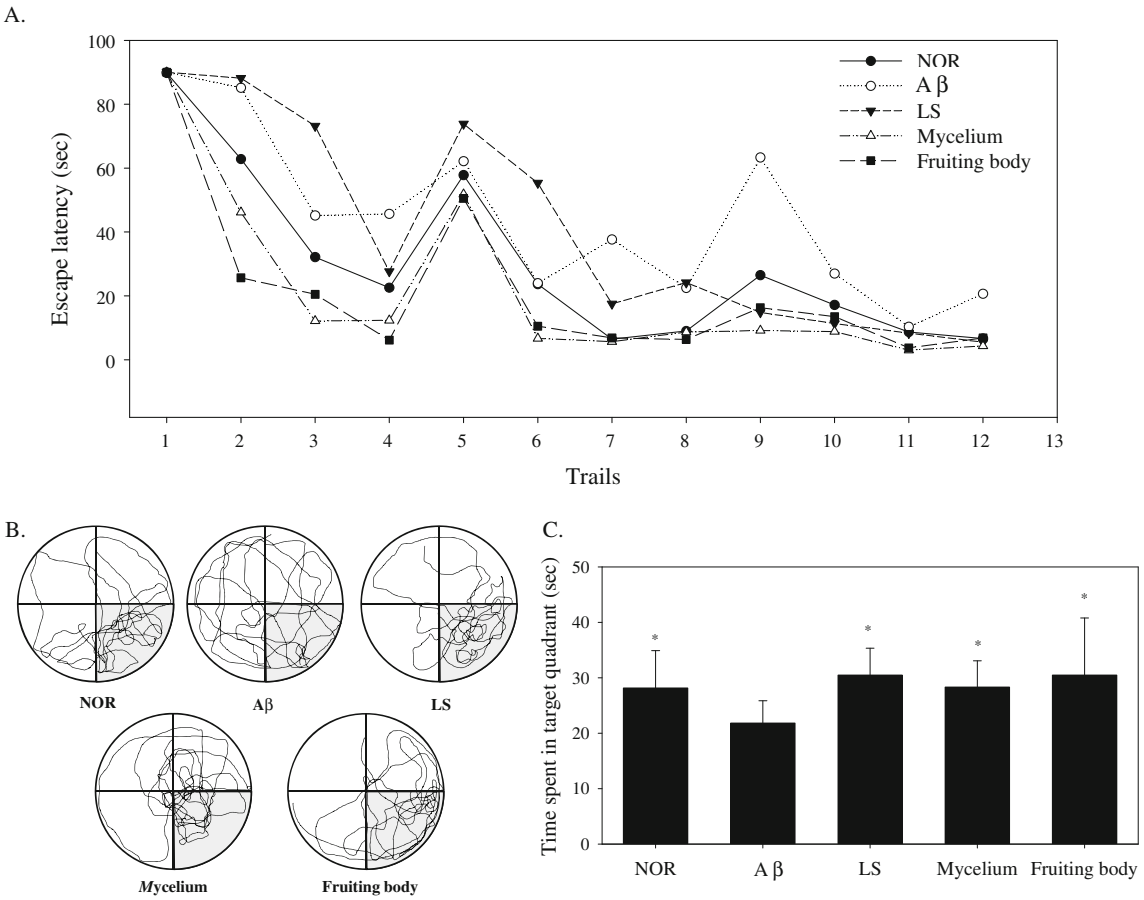

Fig. 4 Effect of the fruiting body and mycelium of *A. camphorata* on performance in the training trials of reference memory task (**a**), in the probe trial (**b**), and in the swimming pathway (**c**) of the water maze task in the Aβ40-infused rats. The reference memory task was carried out from day 22 to day 24. After the reference memory task, the probe trial was accomplished immediately on day 24. Data are presented as means ± SD (n=6). *p<0.05 versus Aβ group

Effects of fruiting body and mycelium on BACE and *p*-tau protein expression in Aβ brain infusion rats

Both *p*-tau protein and BACE are the key factors for the development of AD pathology (Mulder et al. 2010). The expression levels of *p*-tau protein and BACE in the hippocampus and cortex are shown in Fig. 7. In the Aβ group, BACE and *p*-tau protein expression levels in the hippocampus and cortex were bort increased significantly due to a 28-day continuous infusion of Aβ40. Significant lowering effects on *p*-tau protein expression in hippocampus and cortex were performed by administrating fruiting body but not mycelium. This trend was similar to the results of in vitro cell tests. Furthermore, lovastatin was also default to results in the reduction of *p*-tau protein expression. Concerning the BACE expression, lovastatin performed significant inhibitory effects in BACE expression as compared with Aβ group, which was similar to the result of a previous study (Lee et al. 2010). However, the Aβ-induced BACE expression in the hippocampus and cortex were not significantly affected by administrating fruiting body or mycelium. Therefore, the modulation of *A. camphorata* for AD prevention should not be expressed by inhibiting protein expression of *p*-tau rather than that of BACE, and fruiting body performed more effect than its mycelium.

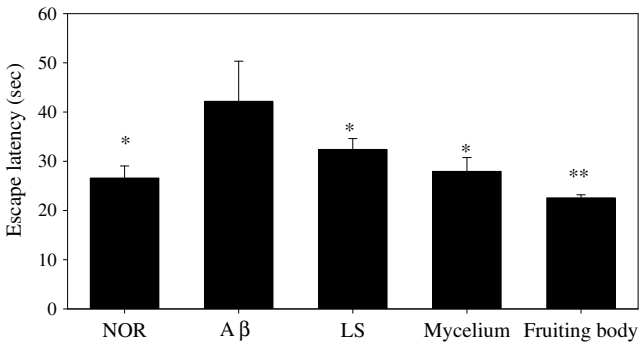

Fig. 5 Effect of the fruiting body and mycelium of *A. camphorata* on performance in the training trials of working memory task in the Aβ40-infused rats. The working memory task was carried out from day 25 to day 27. Data are presented as means ± SD (n=6). *p<0.05 versus Aβ group

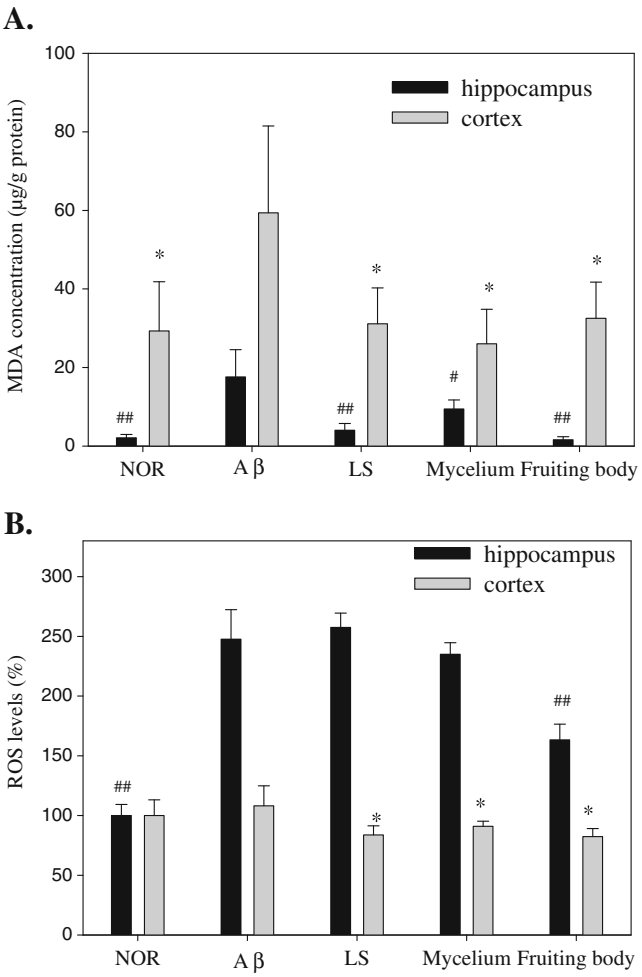

Fig. 6 **a**, **b** Effects of the fruiting body and mycelium of *A. camphorata* on the TBARS and ROS levels in the hippocampus and cortex of Aβ40-infused rats. Data are presented as means ± SD (n=6)

Effects of fruiting body and mycelium on Aβ40 protein accumulation in Aβ brain infusion rats

Aβ40 accumulation was the most important factor in the development of AD pathology. However, memory impairment and learning deficit are more and more serious with the accumulation of Aβ in brain (Mulder et al. 2010). After a 28-day continuous infusion of Aβ40 in brain, results of both immunoblotting and immunohistochemistry stain (as shown in Fig. 8) indicate that, compared to the NOR group, the Aβ group accumulated higher levels of Aβ40 deposition in the cortex. Though the LS group displayed less Aβ40 accumulation in the cortex than the Aβ group, a large amount of Aβ40 accumulation was still found. Feeding fruiting body or mycelium significantly reduced Aβ40 accumulation levels, but the mycelium group performed slightly lower effects in reducing Aβ40 accumulation than the fruiting body group.

Discussion

Aβ has been proven to be the primary factor of memory and learning deficiencies in AD patients (Hashimoto et al. 2005; Stephan and Phillips 2005). Infusion of Aβ40 into rat brain ventricles has been successful in establishing animal models (Stephan and Phillips 2005). AD model animals continuously accumulated Aβ40 in brain by a daily brain infusion of Aβ40, resulting in deficient memory and learning abilities. However, lowering Aβ40 accumulation is regarded as the most powerful and direct way to alleviating the Aβ40-induced memory impairment and learning deficit (Lee et al. 2007; Lee et al. 2010). According to previous studies and the results of this study, infused Aβ40 deposit and further accumulation cause oxidative stress and inflammatory response in brain; moreover, the Aβ40-induced oxidative stress and inflammatory response would aggravate more Aβ40 accumulation which causes more serious damages. In addition, Aβ40 also promotes BACE expression and *p*-tau protein expression. Increasing BACE expression results in the stimulation of APP cleavage toward the formation of Aβ, and high *p*-tau protein expression causes neuron damage. Therefore, the expression of these risk factors is highly associated with memory and learning abilities.

This study is aimed to evaluate the effect of the fruiting body and mycelium of *A. camphorata* on alleviating the Aβ40-induced memory impairment and learning deficit. According to the results of this study, two important results, Morris water maze memory test and Aβ40 accumulation, were found to be significantly alleviated by oral administration with fruiting body and mycelium. The Morris water maze is a primary method to evaluate memory and learning abilities. In such tests, the reference memory test and working memory test serve as evaluation bases for long- and short-term learning abilities, respectively. In this study, fruiting body and mycelium were proven to reduce Aβ40-induced memory impairment and learning deficit, but the administration with fruiting body resulted in more improvement in short-term memory than that with mycelium. However, this study tries to clarify how the fruiting body alleviated the Aβ40-induced memory impairment and learning deficit and had more effect on working memory ability than mycelium according to a comprehensive consideration of the results of in vitro and in vivo tests.

Oxidative stress and inflammatory response induced by brain Aβ infusion are considered as primary factors in memory damage (Smith et al. 2000). According to the result, functional ingredients of *A. camphorata* should contribute anti-oxidative and anti-inflammatory abilities to inhibit Aβ-induced neuron damages. In previous studies, numerous instances of alleviating memory and learning deficit exist in animal models by counteracting oxidative

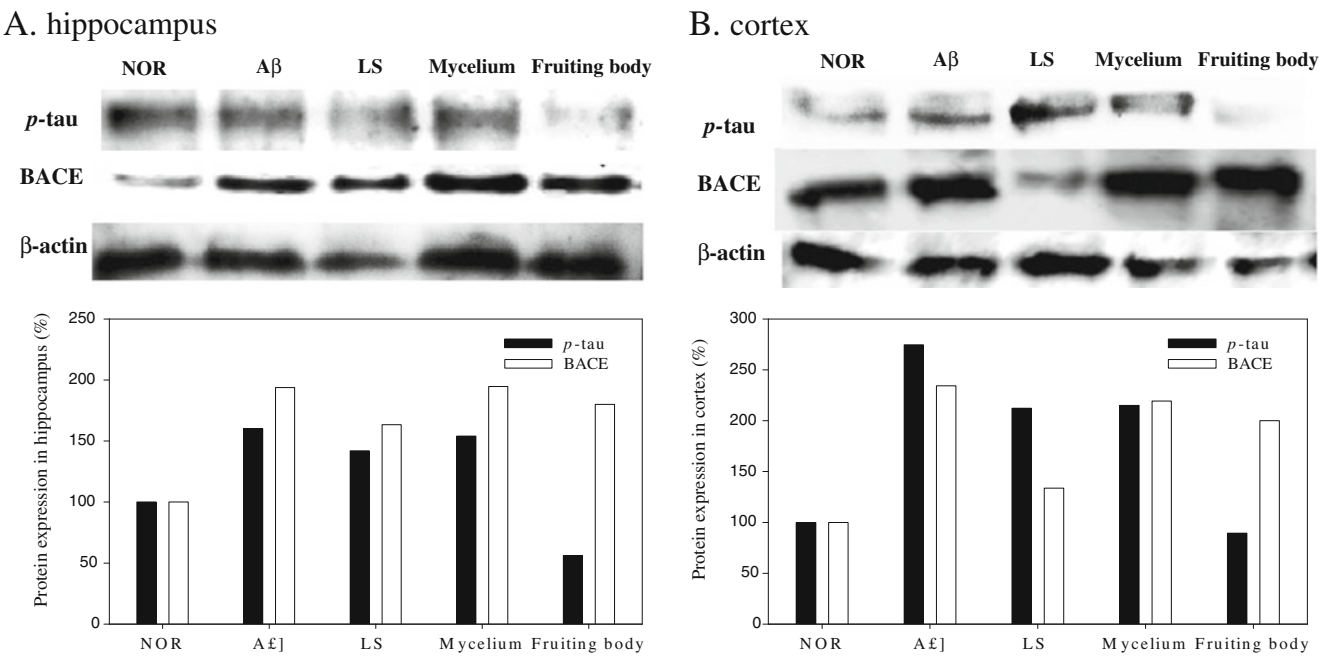

Fig. 7 Effects of the fruiting body and mycelium of *A. camphorata* on the phosphorylated tau protein and BACE protein expression in the hippocampus and cortex of Aβ40-infused rats. Target protein expressions were visualized using immunoblotting (**a**) and quantified using image J software (**b**). Data are presented as means ± SD (n=6)

stress, as is the case with grape seed extracts and *Rosa laevigata* (Choi et al. 2006; Long et al. 2006). However, *A. camphorata* products were also proven as anti-oxidative and anti-inflammatory agents, in which its fruiting body, solid fermentation products, and mycelium extracts inhibit microglia cell inflammation caused by inflammatory factors, such as lipopolysaccharides, interferon-gamma, and Aβ (Liu et al. 2007). Zhankuic acid in mycelium reduce the neutrophil-released ROS and Mac-1 (CD11b/CD18), thus attaining anti-inflammatory effects (Shen et al. 2004). In vitro and in vivo tests of this study both revealed that *A. camphorata* fruiting body and mycelium both provide anti-oxidative and anti-inflammatory effects for attaining Aβ40-induced TBARS and ROS levels.

The in vitro test of this study applied the cell model of PC-12 cells to evaluate the effects of hydrophilic and hydrophobic extracts of fruiting body and mycelium to inhibit Aβ-induced cytotoxicity. Fruiting body and mycelium had a similar inhibitory effect of Aβ-induced cytotoxicity in water extract but not in ethanol extract. The ethanol extract of fruiting body had more Aβ40 cytotoxicity inhibitory effects than that of mycelium. According to previous studies (Lee et al. 2010; Lee et al. 2008), the inhibitory effect of Aβ40 cytotoxicity was possibly contributed from the inhibitory effects of Aβ40-induced oxidative stress and inflammatory response. *A. camphorata* has also been proven to contain anti-oxidants such as polyphenol compounds. The previous studies used fruiting body and mycelium extracts to prevent damage of free radicals to endothelial cells with their anti-oxidative effects (Hseu et al. 2002; Hseu et al. 2008; Hsiao et al. 2003). In this study, the result trends of the inhibitory effect of Aβ40 cytotoxicity were similar to that of the inhibitory effects of Aβ40-induced TBARS levels and ROS levels, in which ethanol extract of mycelium performed the weakest inhibitory effect on these Aβ40-induced damages in the extracts of fruiting body or mycelium. These trends are also visible in animal model tests for reducing AD memory-damaging effects. In vivo results indicate that fruiting body is more effective in inhibiting Aβ40-induced TBARS levels and ROS levels than mycelium, in the hippocampus, possibly due to the difference in their anti-oxidative abilities.

A composition analysis revealed a higher amount of anti-oxidative substances (total flavonoids and total phenols) in the fruiting body than the mycelium. Previous studies have shown that polyphenols and flavonoids lower Alzheimer's Aβ production and suppress Aβ-induced neurotoxicity in the in vitro and in vivo studies (Heo et al. 2004; Li et al. 2004; Paris et al. 2011). The results of DPPH scavenging ability test indicated that water extracts of *A. camphorata* were significantly superior to ethanol extracts. This is possibly due to the abundance of anti-oxidative substances in polar extracts, including total flavonoids and total phenols. Mycelium resulted in a weaker effect on anti-oxidative effect and the Aβ40 cytotoxicity inhibitory effects than fruiting body, even though higher polysaccharides were found in mycelium rather than fruiting body. The greater effectiveness of the fruiting body compared to the mycelium

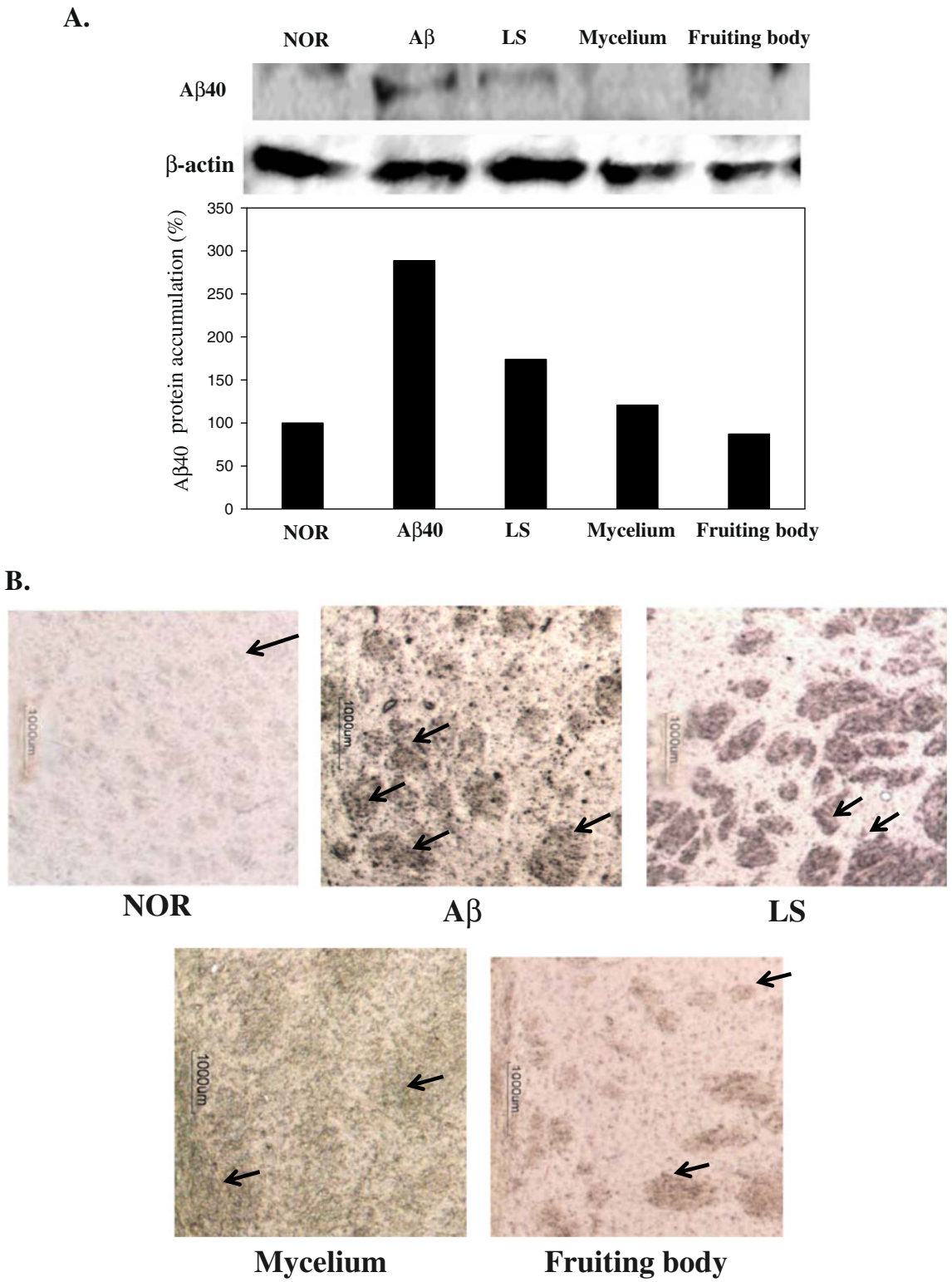

Fig. 8 Effects of the fruiting body and mycelium of *A. camphorata* on the Aβ40 accumulation in the cortex of Aβ40-infused rats. Aβ40 accumulation was visualized using immunoblotting (**a**) and immunohistochemical stain (**b**). Immunohistochemical stain was carried out using the non-biotin hydrogen peroxidase kit. The Aβ40 accumulation in cortex was monitored by microscope examination and presented as the brown dye (*arrows*) in the graph. Data are presented as means ± SD ($n=6$)

is possibly due to the presence of substances, such as anti-oxidants (total flavonoids, total phenols, etc.).

In addition to the anti-oxidants, higher levels of triterpenoids were found in fruiting body than mycelium (Table 2),

Table 2 The contents of total flavonoids, total phenols, total polysaccharides, and total triterpenoids in the fruiting body and mycelium of *A. camphorata*

Sample	Total flavonoids (μg/g)	Total phenols (μg/g)	Total polysaccharides (μg/g)	Triperpenoids (%)
Mycelium	57.7±0.2 a	460±1 a	3,449±178 b	100±12 a
Fruiting body	59.5±4.5 a	3,513±24 b	63±51 a	231±18 b

The data are the means ± SD expressed as percentage of control values (n=3). Mean values within each row with different letters are significantly different (p<0.05)

which may be one of the key factors to explain why fruiting body had more effect on alleviating the Aβ40-induced memory impairment than mycelium. In many studies, triterpenoids such as ursolic acid were proven to express potent neuroprotective effect by inhibiting the binding of Aβ to CHO-CD36 cells in a dose-dependent manner. The ursolic acid effect reached a plateau at ≈20 μM, with a maximal inhibition of 64%. Ursolic acid also blocked binding of Aβ to microglial cells and subsequent ROS production (Wilkinson et al. 2011). In another study, the effect of the triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid-methylamide (CDDO-MA) is used to improve memory and to decrease amyloid plaques in Tg19959 mice. CDDO-MA significantly improves spatial memory retention and reduced plaque burden, Aβ42 levels, microgliosis, and oxidative stress in Tg19959 mice (Dumont et al. 2009). Chen et al. (2006) isolated and purified the functional compounds from fruiting body of *A. camphorata* (Chen et al. 2006) and found that, at concentrations between 5 and 20 μM, the following five compounds, 19-hydroxylabda-8(17)-en-16,15-olide, 11E-dien-16,15-olide, 11E-dien-16,15-olide, 13-dien-16,15- olide, and 14-deoxy-11,12- didehydroandrographolide, prevented Aβ-induced neurocytotoxicity to the extent of 39.2%, 35.0%, 36.7%, 30.6%, and 27.0%, respectively. Therefore, triterpenoids should be the important functional ingredient with neuroprotective physiological activities for the prevention of Aβ-induced memory deficit.

As for the effect of *A. camphorata* on the expression of AD risk factors, the production pathway of Aβ, an AD risk factor, is primarily through excision by BACE and subsequently γ-secretase. Enhancing either BACE expression or enzyme activity results in an increase of Aβ levels; therefore, most studies regarding Aβ inhibition in the brain focus on inhibiting BACE expression or enzyme activity (Scott and Laake 2001). Lovastatin, a cholesterol synthesis inhibitor, is proven to suppress BACE expression levels via its cholesterol-lowering effect (Lee et al. 2007; Lee et al. 2008). However, the inhibition of BACE expression was default to be performed by administrating fruiting body or mycelium. Lower brain Aβ levels in fruiting body or mycelium group did not result from inhibiting BACE expression.

Tau proteins, the proteins for stabilizing microtubules, are abundant in neurons of the central nervous system. When defective tau proteins no longer stabilize microtubules properly, hyperphosphorylation of the tau protein (*p*-Tau) is common in neurons and involved in the pathogenesis of Alzheimer's disease. (Ballatore et al. 2007). Abnormal phosphorylation of tau proteins results in the self-assembly of tangles of paired helical filaments and straight filaments that combine with Aβ and Apo E to form neural tangles; these hyperphosphorylated tau proteins (*p*-tau protein) accumulate to form paired helical filaments that further aggregate into neurofibrillary tangles (Iqbal and Grundke-Iqbal 2007). In the cortex and hippocampus, fruiting body performed significant effect on inhibiting *p*-tau protein expression as compared to Aβ group, and its inhibitory effect was also significantly higher than the mycelium. *p*-Tau protein expression between fruiting body group and mycelium group has the most significant difference in all comparisons of risk factors expression, which should be an important reason why minimum Aβ accumulation and memory impairment were also performed in the fruiting body group. Although *A. camphorata* products were proven to perform neuroprotective effect, mediating the expression of *p*-tau protein has never been studied. *p*-Tau protein is proven to mediate neurodegeneration and suggested as the important risk factor for the development of AD pathology (Ballatore et al. 2007). Although the functional ingredients of fruiting body were not clear in this study, they possibly exist in both hydrophobic extract (F-EE) and hydrophilic extract (F-WE) according to *p*-tau protein expression in the in vitro cell test (Fig. 3). Current studies have yet to clarify whether *A. camphorata* fruiting body inhibits *p*-tau protein expression successfully, and therefore the aforementioned innovative results may contribute significantly to future studies.

According to the above-mentioned descriptions, the metabolite compositions of fruiting body and mycelium were regarded to significantly influence the effect of AD prevention. However, the mycelium, a status of exponential phase of *A. camphorata*, was cultured under a submerged state. The polysaccharide content is usually produced at this culture mode and with increasing biomass (Chang et al. 2006). The fruiting body grown on the root of wild *C. kanehirai* Hay is a mature status of *A. camphorata*. In general, the fungus is able to produce more secondary metabolites during the mature phase (Chang et al. 2006). Therefore, the

fruiting body included higher triterpenoids contents, but not polysaccharide contents, and further performed higher effect of AD prevention than mycelium.

The aforementioned results, supporting previous studies, indicate that Aβ40 causes oxidative stress and inflammation in the brain, consequently leading to Aβ40 accumulation. Furthermore, the accumulated Aβ40 stimulated the expression of *p*-tau protein involved in the development of neuron damage. In this study, in vivo Aβ40 infusion or in vitro Aβ40 treatment both caused neuron damage and death but which were reversed by the treatment of *A. camphorata* mycelium or fruiting body. Moreover, the improvement effect on working memory ability was higher in fruiting body group than in mycelium group, suggesting that the reason resulted from the significant difference in the expression of oxidative stress and inflammation, as well as *p*-tau protein. These higher protection contributed from fruiting body further resulted in lower Aβ40 accumulation in brain. Although higher triterpenoids and total phenol levels were found in fruiting body than mycelium, the functional ingredients in fruiting body were unclear in this study, which should be investigated in future studies.

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