Ameliorative effects of *Acanthopanax trifoliatus* on learning and memory deficits in olfactory bulbectomized mice

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1. Introduction

Acanthopanax is a plant species that possess ginseng-like activities and is known as a "ginseng-like herb" (Huang, 1999, Bucci, 2000). The roots and stem bark of *Acanthopanax* plants have also been used as tonics and sedatives, as well as in the treatment of rheumatism and diabetes (Perry and Metzger, 1981a, Perry and Metzger, 1981b). A Thai traditional plant that belongs to this species is *Acanthopanax trifoliatus* or phak paem which is used in the folk medicines of Southeast Asia as a drug with ginseng-like activity ((Perry and Metzger, 1981a, Perry and Metzger, 1981b, Chi, 1997, Petelot, 1954). Moreover, young leaves and the shoots are popularly consumed with Northern Thai traditional cuisine as vegetables (Petelot, 1954). From its ethnomedical uses as a ginseng-like herb, *A. trifoliatus* has potential for the treatment of degenerative diseases and cognitive dysfunction. Our previous works revealed that extracts from young leaves, root and root bark exhibited strong *in vitro* antioxidant activity tested by 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay and inhibitory effect to lipid peroxidation of rat brain homogenate tested by thiobarbituric reactive substances (TBARS) method (Sithisarn and Jarikasem, 2009). Moreover, leaf extract from *A. trifoliatus* significantly exhibited anti-inflammatory and anti-anxiety effects in animal models (Sithisarn et al., 2009, Sithisarn et al., 2010). Phytochemical investigation by HPLC-MS suggested that leaf decoction extract contained phenolics and flavonoids (Sithisarn et al., 2009). Since the extracts from *A. trifoliatus* showed antioxidant, anti-inflammatory and anti-anxiety properties, it is interesting to investigate the efficacy of these extracts on learning and memory impairments, the possible mechanisms of action in olfactory bulbectomized mice (OBX mice) which was previously reported as animal model of Alzheimer's disease.

2. Materials and Methods

2.1. Materials

2.1.1. Animals

The overall study was conducted according to the experimental protocols described in Fig. 1

and 2. Male ddY mice (Japan SLC Inc., Shizuoka, Japan) were obtained at the age of 9 weeks old and housed with a 12-h light/dark cycle (lights on: 07:30–19:30) at 22 ± 1°C. Food and water were available ad libitum. The animals were habituated to the laboratory animal room for at least 1 week before surgery. The behavioral experiments were performed during the light phase from 9:00 to 18:00. The present studies were conducted in accordance with the Guiding Principles (NIH publication #85-23, revised in 1985) for the Care and Use of Animals and were approved by the Institutional Animal Use and Care Committee in the University of Toyama.

2.1.2. Plant extract preparation

The leaves of *Acanthopanax trifoliatus* were collected from Sunpathong district, Chiang Mai province, Thailand in 2010. The plant samples were identified by Mr.Winai Supatanakul, botanist of Thailand Institute of Scientific and Technological Research. The voucher specimens were deposited at the same place (AT11001). The leaves were cleaned, dried in a hot air oven (60°C) and powdered with electronic mill (20 mesh sieve) then were boiled with distilled water (1:10 w/v) for 3 h and filtered. The filtrate was taken to dryness by lyophilization to yield dried leaf decoction extracts (YD). The extract was previously standardized using high performance liquid chromatography (HPLC) for quantitative analysis of phenolic and flavonoid components and *in vitro* antioxidant activity tests for analysis of biological activities (Sithisarn and Jarikasem, 2009, Sithisarn et al., 2011).

2.2. Methods

2.2.1. Surgical operation

OBX of mice was conducted according to a previous report (Hozumi et al., 2003, Yamada et al., 2011). Briefly, the mice were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and fixed on stereotactic instruments (Narishige, Tokyo, Japan). The skull covering the bulbs was exposed by skin incision, 1% lidocaine solution was used as local anesthetic then a 1mmburr hole was drilled. The bilateral bulbs were aspired through a syringe and the cavity of the bulbs was filled with hemostatic gelatin sponge. After completing behavioral studies, all the animals were sacrificed and the operated lesion was verified visually. The data from animals with less than 70% of removal or with no intact cortex was excluded from data analysis. Sham operation was performed in a similar way without removal of the bulb. At the end of experiments, the olfactory bulbs of sham group mice were confirmed to be intact.

2.2.2. Drug administration

Tacrine (THA, 2.5 mg/kg, i.p.) was kindly supplied by Eisai Co. Ltd. (Tokyo, Japan) and imipramine (IM, 10 mg/kg, i.p.) were separately dissolved in 0.9% normal saline solution and intraperitoneally administered. Sham group of mice and OBX control group mice were orally administered water. Leaf extract of *A. trifoliatus* was dissolved in water and given per orally at daily doses of 250–500 mg/kg. On a behavioral testing day, either THA or YD was administered 1 h before the testing.

2.2.3. Behavioral study

2.2.3.1. Modified Y-maze test

Forty mice were randomly divided into four groups of ten mice. Each group of mice was orally administrated with distilled water as a control group, THA (2.5 mg/kg, i.p.) was used as a standard drug, and *A. trifoliatus* leaf extract in 2 different doses of 250 and 500 mg/kg, p.o., respectively. A modified version of the Y-maze test has been reported by Yamada et al. (2011). The apparatus used for the modified Y-maze test consists of black polypropylene walls with 3 arms each 40cm long, 12cm wide at the top, 3 cm wide at the bottom, 18cm high. This test is a two-trial task with a sample phase trial and a test phase trial that are separated by an inter-trial interval. In the sample phase trial, each mouse was individually placed in the maze with one of the 3 arms closed. The animals were allowed to freely explore the other 2 arms for 5 min. Thirty min after the sample phase trial, the animal was again placed in the maze with all 3 arms opened, and allowed to explore the arms freely. The closed arm that is opened in the test phase trial is defined as the new arm. The animal behavior was video-recorded for later analysis. Percent time spent in the new arm and numbers of total arm entries were analyzed by Smart® system.

2.2.3.2. Novel object recognition test (ORT)

This test is based on the tendency of mice to discriminate a familiar from a new object. On the day before the test, mice were individually habituated to an open-field box (35x 35x50 cm) for 10 min, and performance of the animals was analyzed automatically by the Smart® system. The total distance exploring the arena was used to determine the locomotor activity. The ORT consists of a sample phase trial and a test phase trial. During the sample phase trial, two objects of the same material were placed in a symmetric position in the center of the chamber for 10 min. thirty minute after the sample phase trials, one of the objects was replaced by a novel object, and exploratory behavior was again analyzed for 5 min. After each session, objects were thoroughly cleaned with 70% ethanol to prevent odor recognition. Exploration of an object was defined as rearing on the object or sniffing it at a distance of less than 2 cm. Successful recognition of a previously explored object was reflected by preferential exploration of the novel object. Discrimination of spatial novelty was assessed by comparing the difference between time of exploration of the novel and familiar.

2.2.3.3. Tail suspension test

Tail suspension test (TST) is a widely used model for assessing antidepressant effect. Applied from Shioda et al. (2010), mice were subjected to the short-term inescapable stress of being suspended by the tail that will develop an immobile posture. Using another group of mice as indicated in protocol#2 (Fig 2), 3 mice were separately suspended 50 cm above the floor in a chamber by adhesive tape placed approximately 2 cm from the tip of the tail. Immobility time was recorded during a 8 min period using Smart® system.

2.2.4. Quantitative real-time polymerase chain reaction (PCR)

To analyze changes in expression levels of choline acetyltransferase (ChAT) and muscarinic M1 receptor mRNA in the brain, the animals were killed by decapitation after completing the behavioral studies. The brain was removed immediately and the hippocampus were dissected out and kept at − 80°C until use. Quantitative PCR was conducted as previously described (Zhao et al, 2011). Briefly, total RNA was extracted from the hippocampus using Sepazol® (Nacalai Tesque, Kyoto) according to the manufacturer's instructions. First-strand cDNA synthesis was conducted using oligo (dT) primers and M-MLV Reverse Transcriptase® (Invitrogen, Rockville, MD, USA) in a total volume of 20 μl. The reaction was performed at 25°C for 10 min and heated at 37°C for 60 min and 98°C for 5 min before cooling to 4°C. DNA corresponding to the RNA was used as a template for real-time PCR. Quantitative realtime PCR was carried out using Fast SYBR Green Master Mix (Applied BioSystems, Foster City, CA, USA) in a StepOne Real-time PCR System (Applied BioSystems). Melting curve analysis of each gene was performed every time after amplification was completed. Standard curves of the log concentration of each gene vs. cycle threshold were plotted to prove negative linear correlations.

2.2.5. Neurochemical study

2.2.5.1. *Ex vivo measurement of cholinesterase activity in the brain*

After completing the behavioral experiments, all mice were decapitated and the frontal cortices were dissected out and kept at −80°C until use. Determination of cholinesterase activity was performed on the basis of the colorimetric method as previously described (Ellman et al., 1961). Briefly, the frozen cortex was weighed and homogenized in 10-times volume of 0.1 M phosphate buffer (pH 7.4) containing 1% Triton-X-100. After centrifugation at 15,000 × g at 4°C for 20 min, the clear supernatants were collected and served as the enzyme source. Cholinesterase activity was determined in 10 μl aliquots of homogenates (run as triplicates) in 96-well flat-bottom microplates. The reaction was started by adding 8 μl of 10 mM 5,5´-dithiobis-2-nitrobenzoic acid (DTNB), 16 μl of 7.5 mM acetylthiocholine (ATCI), and 201 μl of 0.1 M sodium phosphate buffer (pH 8.0). The spectrophotometric absorption at 405 nm during a 3 min incubation period at 25°C was quantitatively measured using a microplate reader (Sunrise Classic; TECAN Japan, Kawasaki) and expressed as nmol ACh hydrolyzed/min/mg tissue.

2.2.5.2. In vitro measurement of cholinesterase activity in the brain

The assay for measuring AChE activity was modified from the assay described by Ellman et al. (1961) and Ingkaninan et al. (2003). Cholinesterase activity was determined in 10μl aliquots of homogenates in 96-well flat-bottom microplates using frontal cortex supernatants as the enzyme source. As same as the *ex vivo* experiment, the reaction was started by adding 8 μl of 10 mM 5,5´-dithiobis-2-nitrobenzoic acid (DTNB), 16 μl of 7.5 mM acetylthiocholine (ATCI), 10 μl of YD or THA, and 146 μl of 0.1 M sodium phosphate buffer (pH 8.0). The spectrophotometric absorption at 405 nm during a 3 min incubation period at 25°C was quantitatively measured using a

microplate reader (Sunrise Classic; TECAN Japan, Kawasaki). The velocities of the reactions were measured. Enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer without any inhibitor. Inhibitory activity was calculated from 100 subtracted by the percentage of enzyme activity. Every experiment was done in triplicate.

2.3. Data analysis

The results were expressed as the mean ± SEM. The data obtained from the behavioral tests and neurochemical experiments were analyzed by pair and unpaired student's *t*-test or one-way analysis of variance (ANOVA) followed by a post hoc multiple comparison test (Dunnett's method) as appropriate. Differences of $P < 0.05$ were considered as significant. The analysis was conducted using SigmaStat® ver 3.5 (SYSTAT Software Inc., Richmond, CA, USA).

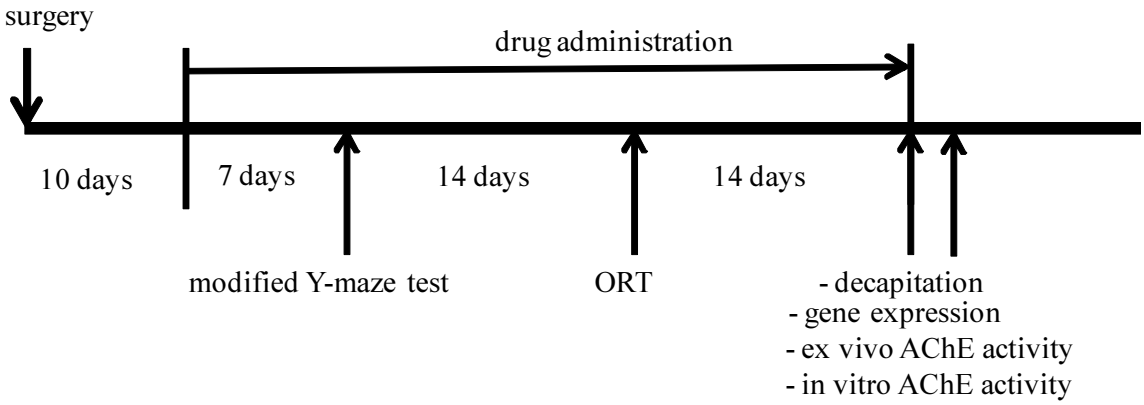

Fig. 1 Schematic drawing of experimental schedule protocol#1
After one week of acclimatization, the ddY mice were randomly divided into 4 groups of 10 mice. All mice (except mice in sham group) were subjected for OBX surgery. Ten days after the surgery, the drug administration was started. Modified Y-maze and object recognition tests were performed 1 and 3 weeks after starting drug administration, respectively. Quantitative real-time polymerase chain reaction and neurochemical studies were done after decapitation of all mice.

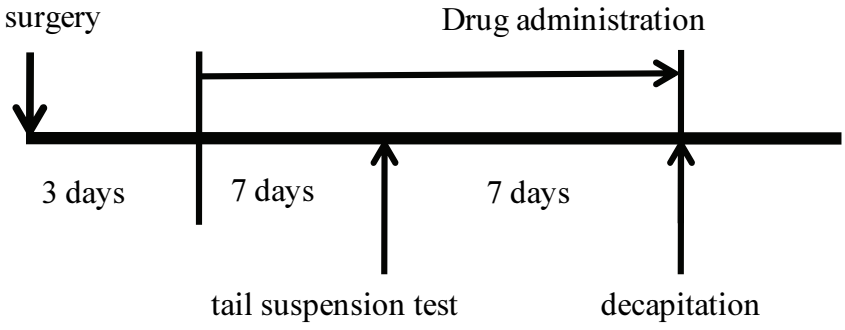

Fig. 2 Schematic drawing of experimental schedule protocol#2
After one week of acclimatization, the ddY mice were randomly divided into 4 groups of 6 mice. All mice (except mice in sham group) were subjected for OBX surgery. Three days after the surgery, the drug administration was started. Tail suspension test was performed 7 days after starting drug administration.

3. Results

3.1. Modified Y-maze test

As shown in Fig. 3, percentage time spent of the vehicle-treated sham mice visiting the new arm (49.30 ± 2.19%) was significantly higher than the chance level of 36.77 ± 3.05% indicating a preference for new arms over the familiar arms. OBX mice that were administered THA (2.5 mg/kg, i.p) or YD (250 and 500 mg/kg. p.o.) 1 hour before the experiment spent significantly longer time exploring new arm than the vehicle-treated OBX group (50.26 ± 2.75, 46.97 ± 2.18 and 54.13 ± 3.52%, respectively).

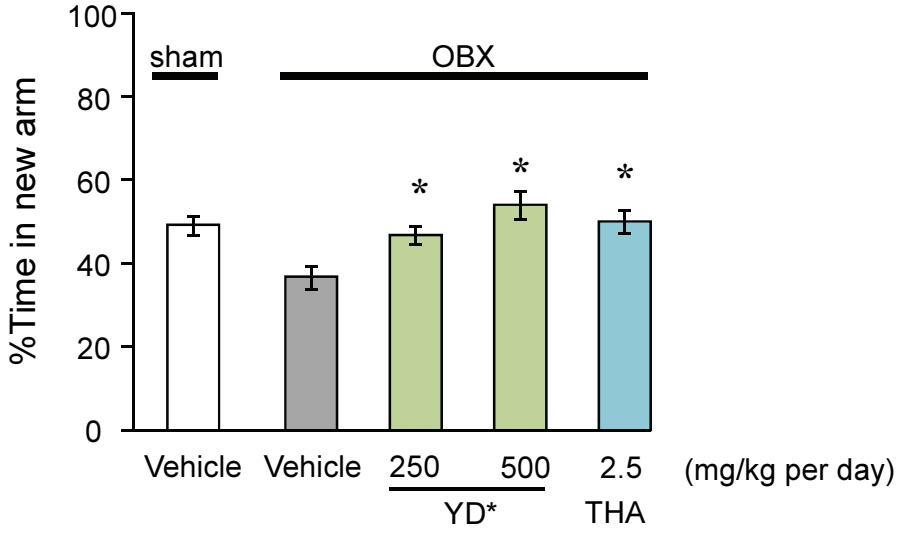

Fig. 3 Evaluation of the modified Y-maze test of OBX-induced spatial working memory deficit mice using reference drug, tacrine or *A. trifoliatus* leaf extract (YD). Surgical operation-naïve mice (OBX) were orally administered with distilled water 60 min before sample phase trial while tacrine (2.5 mg/kg) and YD (250 and 500 mg/kg) were administered by intra-peritoneal injection and oral administration, respectively (n=10). Each data column represents the mean ± S.E.M. **P*<0.05 compared with vehicle-treated OBX group (one-way ANOVA, Dunnett's method).

3.2. Novel object recognition test (ORT)

As shown in Fig. 4A, there was no significantly difference in locomotor activity determined as total distance between vehicle-treated OBX and sham mice groups (6383.07 ± 566.87 and 5834.80 ± 289.14 cm, respectively). Moreover, daily administration of THA (2.5 mg/kg, i.p.) or YD (250 and 500 mg/kg, p.o.) for 1 week before the experiments showed no effect on locomotor activity (total distance of 5514.95 ± 299.20, 5688.09 ± 284.13 and 5791.06 ± 196.18 cm, respectively).

In sample phase, no mice groups showed significantly differences in time spent exploring each identical object. There also no significant difference in total time spent exploring two objects between sham and OBX groups (Fig. 4B). In test phase, the sham group spent a significantly longer time exploring the new object than exploring the familiar one (P<0.05) while the vehicle-treated OBX group showed the deficit of the novel object recognition performance as shown in Fig. 4C. THA (2.5 mg/kg, i.p.) and YD (250 and 500 mg/kg, p.o.)- treated mice groups spent significantly longer time exploring the new object than exploring the familiar object (P<0.01).

3.3. Tail suspension test (TST)

The duration of immobility in TST was measured 10 days after OBX. The duration of immobility in vehicle-treated OBX mice significantly increased (25.18 ± 1.72 s) compared with that observed in sham-operated group (11.47 ± 2.13 s) (Fig. 5). One week of daily administration of imipramine (10 mg/kg, i.p.) or YD (500 mg/kg, p.o.) significantly reduced the duration of immobility (6.48 ± 3.85 and 3.86 ± 2.64 s, respectively) while mice in THA administered group (2.5 mg/kg, i.p.) showed no reducing effect to the immobility (17.44 ± 3.75 s).

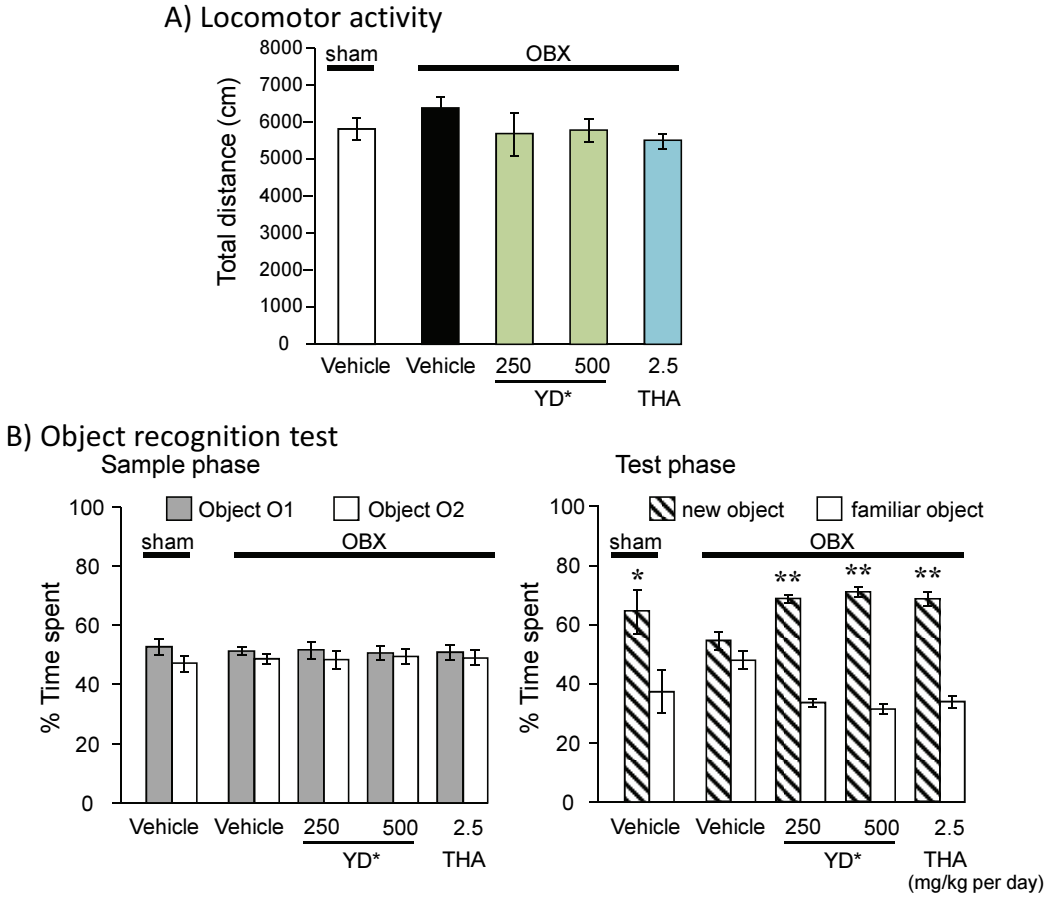

Fig. 4 Effects of tacrine and YD on object recognition deficits in OBX mice in sample phase (B) and test phase (C) while data of locomotor activities is shown in (A). Each datum represents the mean ± S.E.M. (n=10). Each data column represents the mean ± S.E.M. *P<0.05 and **P<0.01 vs. time spent exploring a familiar object (paired t-test).

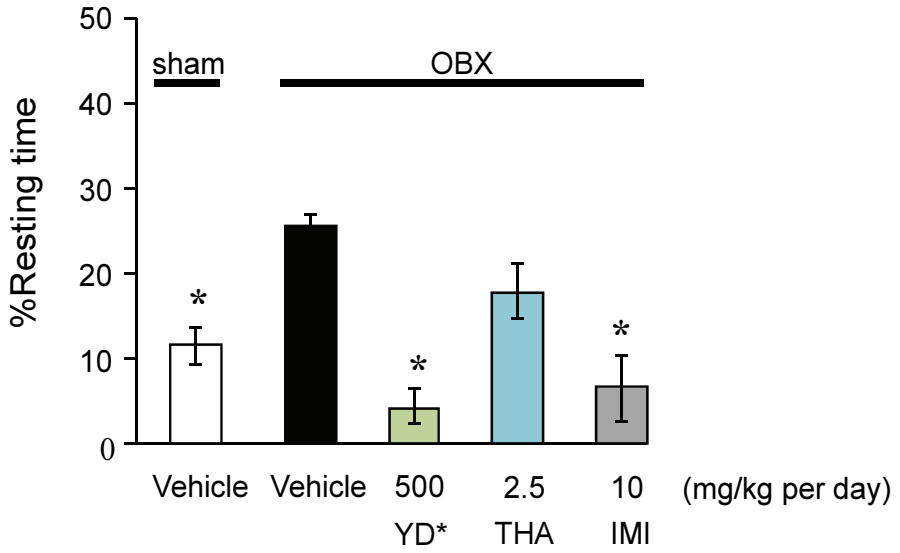

Fig. 5 Effects of administration with tacrine (2.5 mg/kg, i.p.), imipramine (10 mg/kg, i.p.) and YD (500 mg/kg, p.o.) on OBX-induced depressive behavior in the TST. Each data column represents the mean ± S.E.M. *P<0.05 vs. % resting time in vehicle-treated OBX mice (one-way ANOVA, Dunnett's method).

3.4. Quantitative real-time polymerase chain reaction (PCR)

It was found that the expression levels of M1 receptor and ChAT mRNAs in the hippocampus of vehicle-treated OBX mice was significant lower compared with sham-operated group (0.23 ± 0.04 and 0.41 ±0.03, respectively for M1 receptor mRNA and 0.12 ± 0.02 and 0.56 ±0.28, respectively for ChAT mRNA). However, the expression levels of M1 receptor and ChAT mRNAs in hippocampus were up-regulated in OBX mice treated with THA (2.5 mg/kg, i.p.) or YD (250 and 500 mg/kg, p.o.). The expression levels of M1 receptor mRNA in hippocampus in THA and YD (250 and 500 mg/kg, p.o.) were 0.35 ± 0.02, 0.52 ± 0.07 and 1.78 ± 0.64, respectively (Fig. 6A) while the expression levels of ChAT mRNA in hippocampus in THA and YD (250 and 500 mg/kg, p.o.) were 0.59 ± 0.11, 1.15 ± 0.58 and 2.06 ± 0.39, respectively (Fig. 6B).

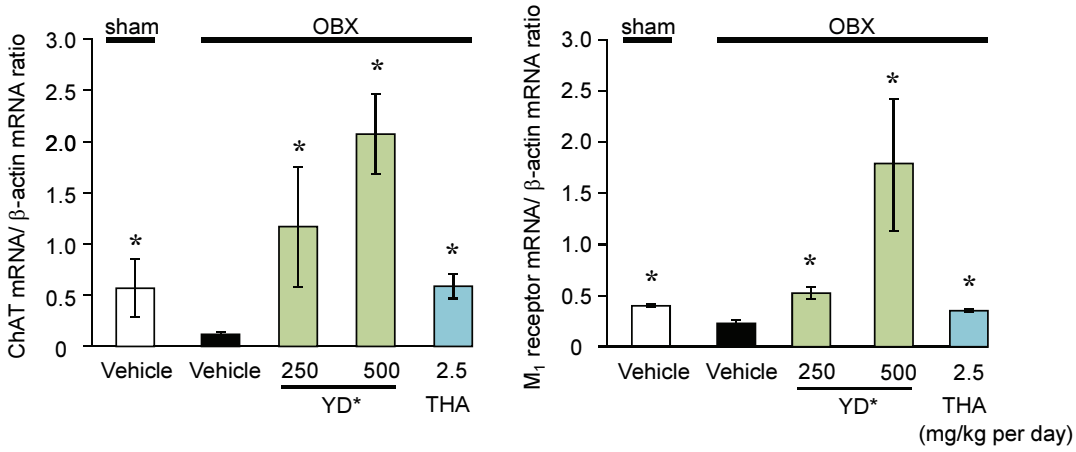

Fig. 6 Effects of tacrine and YD on M1 receptor (A) and ChAT (B) mRNA expression levels in the hippocampus of OBX mice. Each data column represents the mean ± S.E.M. *P<0.05 vs. mRNA expression levels in vehicle-treated OBX mice (one-way ANOVA, Dunnett's method).

3.5. *Ex vivo* measurement of cholinesterase activity in the brain

The activity of acetylcholinesterase (AChE) in the cerebral cortex was measured in mice treated with YD (500 mg/kg, p.o.) or THA (2.5 mg/kg, i.p.). As shown in Fig. 7, it was found that the activity of cortical AChE in YD- and THA-treated groups were significantly reduced compared with the activity measured in vehicle-treated mice (2.27 ± 0.08 and 2.21 ± 0.21 nmol/min/mg tissue, respectively).

3.6. *In vitro* measurement of cholinesterase activity in the brain

YD (100 μg/ml) and THA (0.05 – 5 μg/ml) were tested for AChE inhibitory activity using Ellman's colorimetric method in 96-welled microplate. At concentration of 100 μg/ml, YD showed low inhibitory activity (5.01 ± 0.37%) while THA showed high inhibitory effect with IC_{50} less than 50 ng/ml. Inhibitory effects of THA are shown in Table 1.

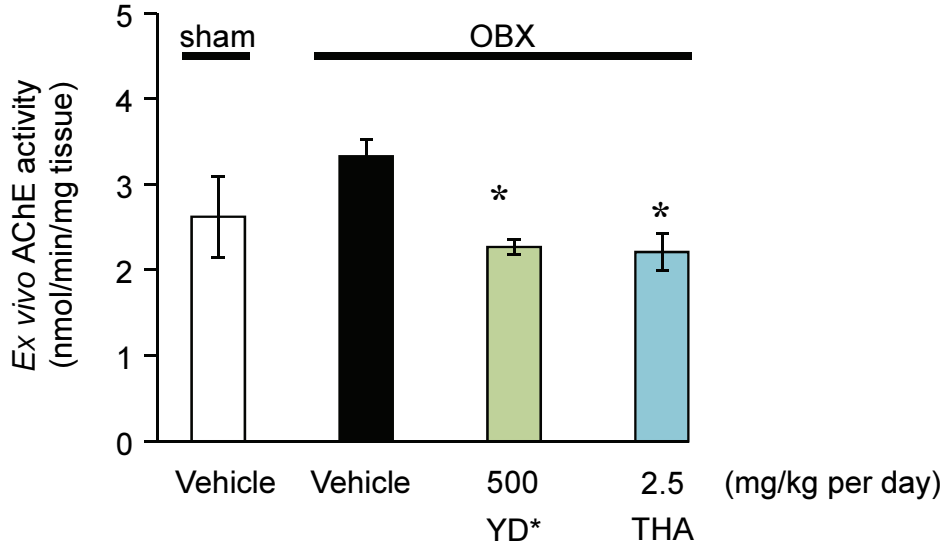

Fig. 7 Effects of YD and tacrine on *ex vivo* acetylcholinesterase activity in OBX mice. *P<0.05 vs. acetylcholinesterase activity in vehicle-treated OBX mice (one-way ANOVA, Dunnett's method).

Concentration (μg/ml)	% inhibition
0.05	77.14 ± 0.60
0.25	90.56 ± 0.25
0.5	93.67 ± 0.13
2.5	95.94 ± 0.06
5	95.76 ± 0.42

Table 1 *In vitro* inhibitory effects of THA on acetylcholinesterase activity in the mouse brain homogenate.

Discussion

The effects of *Acanthopanax trifoliatus* leaf extract (YD) on OBX induced short term memory deficit were investigated. The results showed that YD treatment could ameliorate the impairments via restoring the function of the central cholinergic system down-regulated by OBX. In modified Y-maze test, it was found that OBX operation impaired spatial working memory performance in mice. Treatments of THA (2.5 mg/kg, i.p.) and YD (250 and 500 mg/kg, p.o.) ameliorated spatial working memory performance indicated by the increasing of total time spent exploring new arm. The ameliorative effect of YD on memory deficit was further studied using novel object recognition test. In sample phase trial, no significant difference in total time spent exploring two identical objects was observed between sham and OBX groups, indicating no differences in ability to recognize objects between animals. In test phase, the results showed that mice in sham group spent more time exploring new object, while vehicle treated OBX mice showed no total time difference between familiar and new object indicating the impairment of non-spatial object recognition memory. Administration of YD (250 and 500 mg/kg, p.o.) and THA (2.5 mg/kg, i.p.) could significantly ameliorate OBX induced object recognition deficit suggesting the ability of YD in the therapy of cognitive deficits in human in the future. From the quantitative real-time PCR, the results revealed that mice in vehicle-treated OBX group exhibited down-regulated expression levels of both ChAT and M1 muscarinic mRNAs in hippocampus, a brain region responsible for spatial learning and memory performance (Zhao et al, 2011) indicating the deficit of cognitive performance and pathological changes in central cholinergic systems. Our findings agree with previous reports (Hozumi et al, 2003 and Yamada et al, 2011).

Administration of YD (250 and 500 mg/kg, p.o.) and THA (2.5 mg/kg, i.p.) showed up-regulate effects to the expression levels of ChAT and muscarinic M1 receptor in OBX mice suggests that YD can protect cognitive function against degeneration of central cholinergic system caused by OBX via the reversal of hippocampal expression of ChAT and muscarinic M1 receptor mechanisms. Moreover, the similar action between YD and THA was found in *ex vivo* acetylcholinesterase activity test. Indeed, the present results demonstrated that YD could significantly reduce the activity of AChE as well as THA. However, *in vitro* test using hippocampal tissue as an enzyme source revealed that only THA showed strong inhibitory effect to AChE while YD exhibited low effect. Taken together, the present results suggest that there could be other mechanisms or pathway affecting the effect of YD to hippocampal acetylcholinesterase activity. Cholinergic system was reported to implicate in depressive behavior (Shioda et al, 2003). There was a significant reduction of hippocampal neurogenesis in OBX mice (Harkin et al, 2003, Jaako-Movits and Zharkovsky, 2005). YD has significantly antidepressant activity in OBX mice. However, the mechanisms of action are still unknown. The possible mechanisms could be the effects to AChE and ChAT which are presynaptic markers of cholinergic neurons or enhancement to neurogenesis in OBX mice. From all the results, YD showed improving effects on spatial working memory and non-spatial short term memory

in OBX mice which could mediate by the up regulation of the expression levels of ChAT and muscarinic M1 receptor mRNA and the inhibitory effects to acetylcholine esterase. Moreover, YD also showed *in vivo* antidepressant effect in OBX mice. From these results, it is likely that YD is beneficial to treatment of depression and improvement cognitive impairment related to Alzheimer's disease and that the effects of YD may be mediated by improving dysfunction of central cholinergic systems.

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