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Silkworm Pupae Protect Against Alzheimer's Disease

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ABSTRACT

Silkworm (*Bombyx mori*) pupae have long been used as food and medicine in Asian countries. It is reputed for the treatment of numerous neurological disorders related to oxidative stress including stroke. Therefore, we hypothesized that silkworm pupae could attenuate memory impairment and neurodegeneration in Alzheimer's disease. In the present study, we determined the effect of silkworm pupae on the neurodegeneration and memory impairment in animal model of Alzheimer's disease. Adult male Wistar rats, weighing 180-220 g, were orally given the silkworm pupae at doses of 60, 90 and 135 mg kg^{-1} BW 14 days before and 7 days after the bilateral administration of AF64A, a cholinotoxin, via intracerebroventricular route. The animals were determined the memory using Morris water maze test and determined the density of neurons in hippocampus. All doses of silkworm pupae used in this study significantly mitigated the memory impairment and the decreased neurons density in hippocampus. To explore the possible underlying mechanism of the cognitive enhancing effect and neuroprotective effect, the activity of acetylcholinesterase enzyme and the Malondialdehyde (MDA), the oxidative marker were determined respectively. Our results clearly demonstrated that the cognitive enhancing effect of silkworm pupae occurred at least via the increased cholinergic function while its neuroprotective effect occurred via the decrease oxidative stress. In conclusion, silkworm pupae appear to be the potential functional food to protect against Alzheimer's disease.

Keywords: Silkworm Pupae, Protects Against, Alzheimer's Disease, Bilateral Administration, Cognitive Enhancing, Memory Impairment, Clearly Demonstrated

1. INTRODUCTION

Alzheimer's disease has been considered to be the most widespread variety of dementia. The etiology of this condition is not completely understood but it has been recognized as the multiple etiologies disorder. Numerous factors have been proposed to contribute the important role on the pathophysiology of Alzheimer's Disease (AD). Recent findings demonstrated the attribution of oxidative stress in various disorders including AD (Pratico and Delanty, 2000; Markesbery, 1999). It was found that level of free radicals oxidation of lipids, proteins and DNA were elevated in postmortem brain of AD (Tuppo and Forman, 2001). Moreover, the decreased cholinergic function and degeneration of cholinergic neurons were also observed (Liston *et al*., 2004; Zarotsky *et al*., 2003; Perry *et al*., 1999). Despite the most commonly dementia found in the elderly and the increasing prevalence, the therapeutic strategy against AD is still very limited. Most of the drugs used nowadays usually produce side effects and can only slow down the progression of diseases. Therefore, the novel potential strategy is required.

Silkworm or *Bombyx mori* L. has been long term used as food and medicine in Asian countries. According to the Traditional Chinese Medicine, it is believed that silkworm could promote Chi or the body life energy while balancing the nervous system. Moreover, it has been consumed as heath food especially for cardiac and diabetic patients, bronchial asthma, primary trigeminal neuralgia, facial palsy, pain vocal nodules and polyps. In addition, it also possessed antijuvenoid (Saha *et al*., 2007), immune booster (Zhang *et al*., 2006), antioxidant (Kwon *et al*., 2006) and estrogenic effects (Yang *et al*., 2010). Previous studies demonstrated that silkworm pupae are a source of proteins and substances which are essential for the function of the nervous system such as vitamin B 1, B 2 and E (Singh and Jayasomu, 2002). Based on the role of oxidative stress in the pathophysiology of AD, its reputation to balance life energy, the antioxidant activity and its effect on the nervous system, we hypothesized that *B. mori* might be able to mitigate the memory impairment and neurodegeneration in AD. Therefore, this study was undertaken to determine the effect of *B. mori* on the memory impairment and neurodegeneration in animal model of AD.

2. MATERIALS AND METHODS

2.1 Animals

Adult male Wistar rats (180-220 g, 8 weeks old) were obtained from National Animal Center, Salaya, Nakhon Pathom and they were housed in group of 5 per cage in standard metal cages at 22 ± 2°C on 12:12 h light-dark cycle. All animals were given access to food and water ad libitum. The experiments were performed to minimize animal suffering in accordance with the internationally accepted principles for laboratory use and care of European Community (EEC directive of 1986; 86/609/EEC).The experimental protocols were approved by the Institutional Animal Care and Use Committee.

2.2. Preparation of *Bombyx mori* Powder

The male silk *Bombyx mori* pupae state 5 were authenticated by Wiroje Kaewrueng, the Queen Sirikit Institute of Sericulture, Ministry of Agricture and Cooperative, Thailand. The voucher specimen was also kept there. The *B.mori* pupae were isolated from their cocoon, dried at 60°C at a period of 4 days. After being dried, the weight of B mori pupae was decreased to 20% of wet weight. The dried *B.mori* pupae were grounded to powder, then separately packed (100 grams/pack) and kept in the cool dry place until use. The suspension of *B.mori* pupae was freshly prepared to a desired concentration by using Propylene Glycol (PG) as vehicle. In order to provide the feeding convenient, *B.mori* powder was suspended in a suspending agent of PG.

2.3. Drugs and Chemicals

Donepezil hydrochloride (Aricept 10 mg/tablet) (Pfizer pharmaceuticals Inc.) was used as standard drugs in this study. They were dissolved in propylene glycol and administered via oral route. All chemical substances used in this study were analytical grade.

2.4. Experimental Protocol

All rats were randomly assigned to 6 groups of 8 animals each.

2.5. Group I

Vehicle+ACSF Rats had been treated with propylene glycol which served as vehicle to suspend the *B.mori* for 2 weeks before and 1 week after the administration of Artificial Cerebrospinal Fluid (ACSF), a vehicle of AF64A.

2.6. Group II

Vehicle+AF64A Rats had been treated with vehicle for 2 weeks before and 1 week after the administration of AF64A, a cholinotoxin, in order to induce a cholinergic deficit as found in Alzheimer's disease.

2.7. Group III

Donepezil+AF64A: Animals were treated with donepezil, a cholinesterase inhibitor which used as standard drug for dementia treatment that served as positive control in this study, as same as that mentioned in group II.

2.8. Group IV

VI *B.mori*+AF64A: Rats had been treated with the plant extract at various doses ranging from 60, 90 and 135 mg kg^{-1} BW for 2 weeks before and 1 week after the administration of AF64A respectively (The doses used in this study were selected based on our preliminary data on the cognitive enhancing effect).

The animals were determined the spatial memory 1 week after AF64A administration. Then, they were sacrificed and determined the density of survival neurons and in various subregions of hippocampus.

2.9. AF64A Administration

AF64A was prepared as an aqueous solution of acetylethylcholine mustard HCl (Sigma, St. Louis, MO) was adjusted to pH 11.3 with NaOH. After stirring for 30 min at room temperature, the pH was lowered to 7.4 with the gradual addition of HCl and stirred for 60 min. The amount of AF64A was then adjusted either to 2 nmol/2 µL. The vehicle of AF64A was distilled water prepared in the same manner as the AF64A and recognized as ACSF.

In order to administer AF64A bilaterally via intracerebroventricular (i.c.v.) route, the animals were anesthetized with the intraperitoneal injection of sodium pentobarbital at dose of 60 mg kg BW^{-1}. Then, AF64A (2 nmol/2 μL) was infused bilaterally via intracerebroventricular (i.c.v.) route with a 30-gauge needle inserted through a burr hole drilled into the skull into both the right and left lateral ventricles. Stereotaxic coordinates were (from the bregma): posterior 0.8 mm, lateral ± 1.5 mm and ventral (from dura) 3.6 mm. The rate of infusion was 1.0 μL min^{-1} and the needle was left inplace for 5 min after infusion and then slowly withdrawn.

2.10. Morris Water Maze Test

The Morris water maze test is one of the most important paradigms used for testing spatial navigation task, which is thought to be dependent on the proper functioning of the hippocampus. The testing apparatus for all task used in this study was a stainless steel circular pool that 147 cm in diameter and 47 cm in dept. The interior of the pool was flat and the pool was placed on the steady floor. The pool was filled with water to a depth of 12 cm. The water were maintained at 23±1°C and darkened by nontoxic powder.

The pool was divided into four quadrants (NE, NW, SE and SW) by two imaginary lines crossing the center of the pool. For each animal, the invisible platform was placed in the center of one of the quadrants and was remained there for a training period of 4 days. Each rat was gently placed in the water facing the wall of the pool from one of the four starting points (N, E, S or W) along the perimeter of the pool and the animal was allowed to swim until it climbed onto the platform. When an animal could not reach the platform in 60 s, it was gently placed on the platform by the experimenter. In either case, the animal was left on the platform for 10 s and removed from the pool. Then, it was quickly dried with a towel before being returned to the home cage. The behavior of the experimental animal such as latency to finding the platform, total distance traveled, time spent in the target quadrant of the pool were recorded.

2.11. Histological Procedure

Following anesthesia with sodium pentobarbital (60 mg kg^{-1} BW), fixation of the brain was carried out by transcardial perfusion with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3. The brains were removed after perfusion and stored over a night in a fixative solution that used for perfusion. Then, they were infiltrated with 30% sucrose solution for approximately 4°C. The specimens were frozen rapidly and 30 μM thick sections were cut on cryostat. They were rinsed in the phosphate buffer and picked up on slides coated with 0.01% of aqueous solution of a high molecular weight poly L-lysine.

2.12. Choline Acetyltransferase and Immunohistochemistry

A series of rat brain sections containing hippocampus from each groups were reacted in parallel experiments using a mouse monoclonal antibody detected against Choline Acetyltransferase (ChAT) (Chemicon Internation, Inc., CA, USA) and a modification of a previously described protocol employing the DAKO Strept ABC Complex/HRP duet kit. In brief, rat brain sections were eliminated endogenous peroxidase activity by 0.5% H_2O_2 in methanol. Rat brain sections were washed in running tap water and distilled water for 1 min each, then rinsed in KPBS and KPBS-BT for 5 min per each process. Excess was removed, then, the rat brain sections were incubated for 30 min in a blocking solution composed of 5% normal horse serum in KPBS-BT. The rat brain sections were then incubated in mouse primary antibody against ChAT diluted 1 : 100 in KPBS-BT at room temperature for 2 h and then incubated at 4°C for 48 h. The tissue was rinsed in KPBS-BT (two washes ×7 min), incubated for 4 h in biotinylated goat antimouse IgG antibody, rinsed in KPBS-BT (two washes ×7 min) and then incubated in Strep ABCComplex/HRP for 4 h. In preparation for visualization step, rat brain sections were rinsed in KPBS-BT (1 min) and KPBS (two washes ×10 min). ChAT immunoreactivity was visualized using 0.025% 3, 3' diaminobenzidine (DAB, Sigma) and 0.01% H2O2. Finally, rat brain sections were rinsed in running tap water, air dried and cover-slipped using permount.

2.13. Morphological Analysis

Five coronal rat brain sections in each group were studied quantitatively. Neuronal counts in hippocampus were performed by eye using a 40x magnification with final field 255 μm^2 according to the following stereotaxic coordinates: AP -4.8 mm, lateral ± 2.4-6 mm, depth 3-8 mm. The observer was blind to the treatment at the time of analysis. Viable stained neurons were identified on the basis of a stained soma with at least two visible processes. Counts were made in five adjacent fields and the mean number extrapolated to give total number of neurons per 255 μm^2. All data are represented as number of neurons per 255 μm^2.

2.14. Acetylcholinesterase and Malondialdehyde Assays

The rats were divided into various groups as previously described in 2.3. After the last dose of administration, all rats were sacrificed. Hippocampus was isolated, prepared as hippocampal homogenate and the determination of the Malondialdehyde (MDA) level and acetylcholinesterase activity in hippocampus were performed. Malondialdehyde was indirectly estimated by determining the accumulation of Thiobarbituric Acid Reactive Substances (TBARS) in the hippocampal

homogenate whereas acetylcholinesterase activity was performed using the colorimethod.

2.15. Statistical Analysis

Data are presented as mean ± Standard Error of Mean (SEM). One-way Analysis of Variance (ANOVA), followed by Tukey post hoc test. A probability level less than 0.05 was accepted as significance.

3. RESULTS

3.1. The Effect of *B.mori* on Spatial Memory

The effect of *B.mori* on spatial memory was determined using the validated test, Morris water maze test. The results were shown in **Fig. 1**. The results clearly revealed that AF64A significantly increased escape latency time (p-value < 0.001, compared with vehicle+ACSF). Donepezil and all doses of *B.mori* could reverse the elevation of escape latency induced by AF64A (p-value < 0.001 all, compared with vehicle+AF 64A). The retention time was evaluated 24 h after the assessment of escape latency. **Figure 2** showed that AF64A significantly decreased retention time (p-value < 0.01, compared to vehicle+ACSF). Again, these changes were reversed by donepezil (p-value < 0.01, compared to vehicle+AF64A) and all doses of *B.mori* (p-value < 0.001 all, compared to vehicle+AF64A).

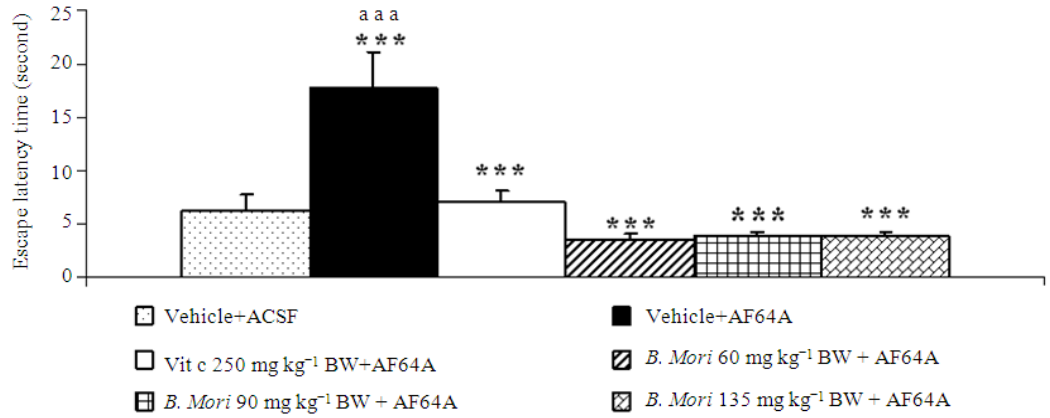

Fig. 1. The effect of *B.mori* on spatial memory. (Escape latency time**)**

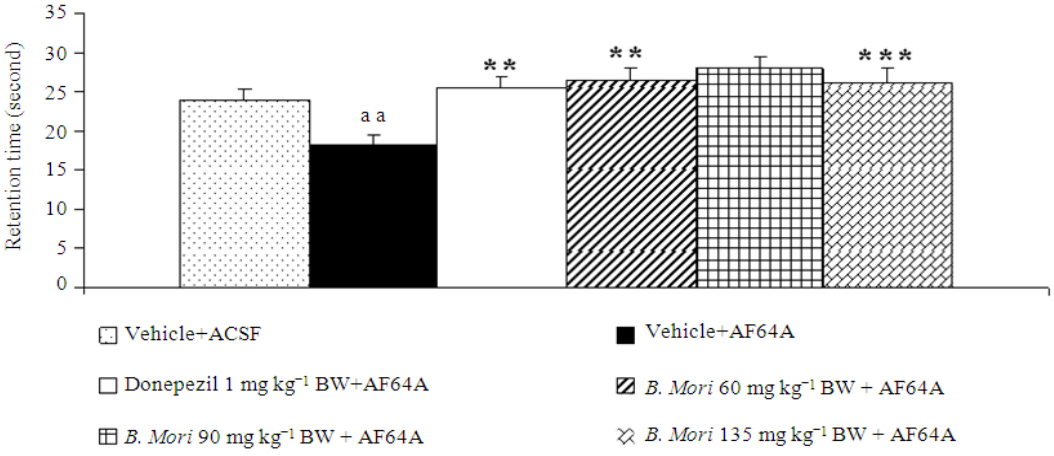

Fig. 2. The effect of *B.mori* on spatial memory. **(**Retention time**)**

3.2. The Effect of *B.mori* on Cholinergic Neurons Density

Since the spatial memory has been reported to involve the function of cholinergic system and hippocampus, we also determined the effect of *B.mori* on cholinergic neurons density in hippocampus. **Figure 3** showed that AF64A significantly decreased the cholinergic neuron density in CA1, CA2, CA3 and dentate gyrus (p-value <.001 all, compared with vehicle+AF 64A). Donepezil significantly reversed the cholinergic neuron density in CA1, CA2 and dentate gyrus (p-value < 0.05, 0.01 and 0.01 respectively, compared with vehicle+AF64A). It was found that rats subjected to *B.mori* treatment at dose of 90 mg kg^{-1} BW significantly increased cholinergic neuron density in CA1, CA2, CA3 and dentate gyrus (p-value < 0.01, 0.01, 0.05 and 0.05 respectively, compared with vehcle+AF64A) while the high dose treatment could enhance the cholinergic neuron density in CA1, CA2 and dentate gyrus (p-value < 0.05, 0.05 and 0.001 respectively, compared with vehicle+AF64A).

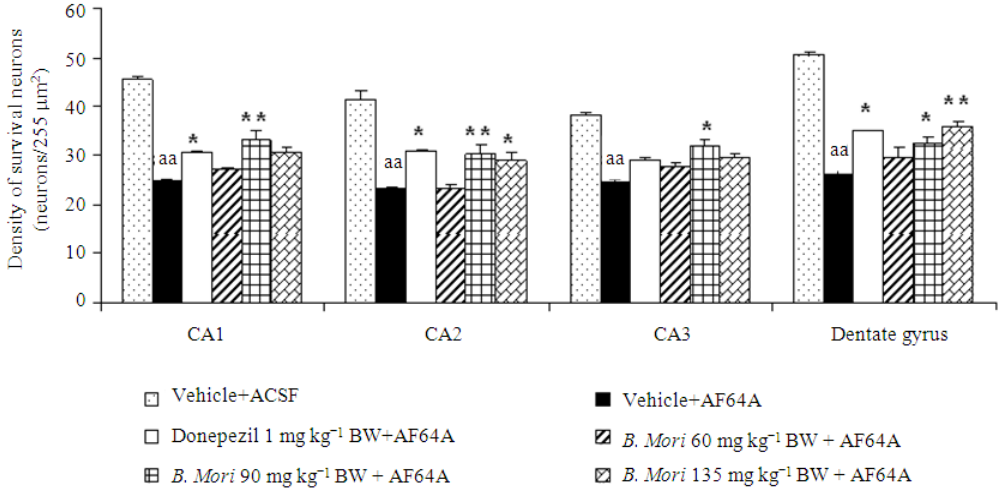

Fig. 3. The effect of *B.mori* on cholinergic neurons density

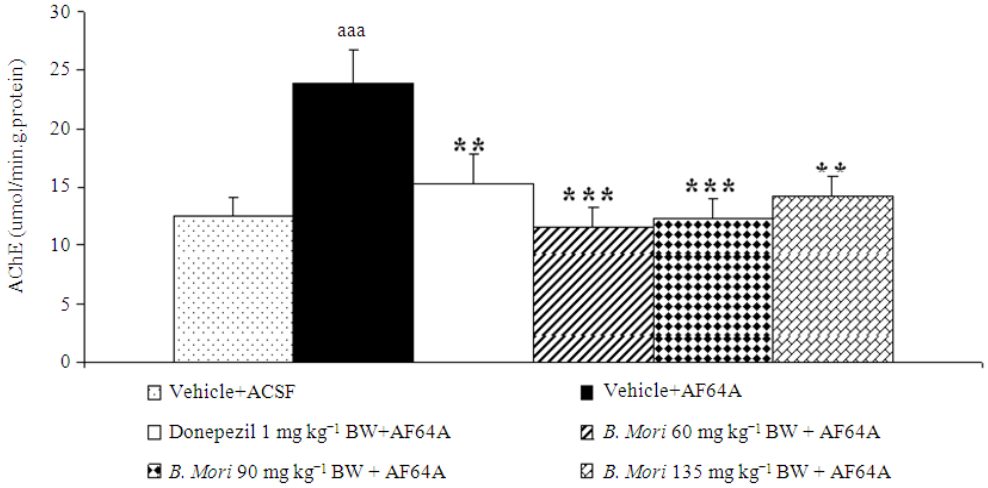

Fig. 4. Effect of *B.mori* on acetylcholinesterase activity

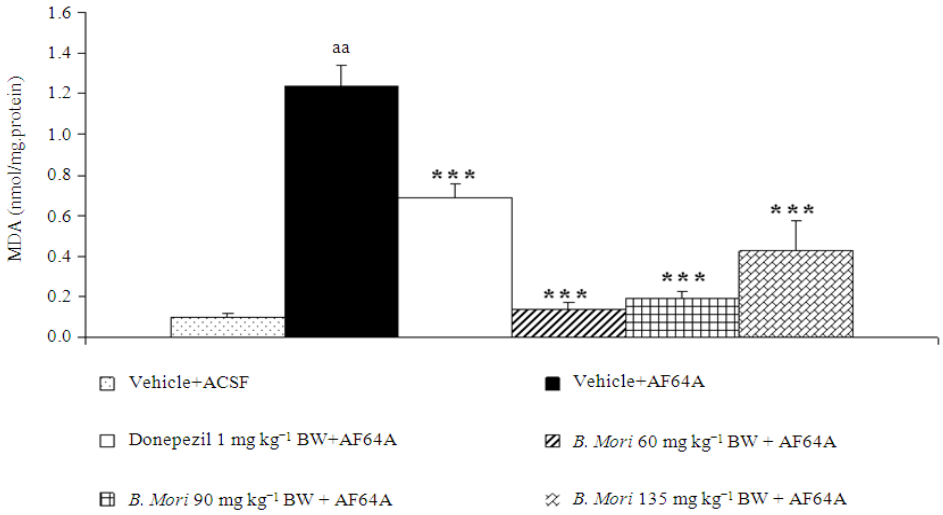

Fig. 5. Effect of *B.mori* on malondialdehyde level in hippocampus

3.3. Effect of *B.mori* on Acetylcholinesterase Activity and Malondialdehyde Level in Hippocampus

Based on the previous information that Acetylcholinesterase (AChE) indirectly indicated the level of Acetylcholine (ACh), we also determined the activity of AChE in hippocampus. In **Fig. 4**, it was found that rats subjected to AF64A significantly increased AChE activity in hippocampus (p-value < 0.001, compared with vehicle+ACSF). Donepezil treated groups significantly reversed the elevation of AChE induced by AF64A (p-value < 0.01. compared with vehicle+AF64A). Surprisingly, *B.mori* treated groups at all dosage range used in this study could reverse the elevation of AChE activity induced by AF64A much more than that observed in donepezil treated group (p-value < 0.001 all, compared with vehicle+AF64A).

Due to the crucial role of oxidative damage on neurodegeneration, we also determined the effect of *B.mori* on the level of Malondialdehyde (MDA) level in hippocampus. The results were shown in **Fig. 5**. It was demonstrated that AF64A produced a significant elevation of MDA in hippocampus (p-value < 0.001, compared with vehicle+ACSF). All doses of *B.mori* also reversed this elevation (p-value < 0.001 all, compared with vehicle+AF64A).

4. DISCUSSION

In this study, our results showed that AF 64A treated rats induced the degeneration of cholinergic neurons density in all subregions of hippocampus as those observed in AD. Dietary supplementation with *B.mori* pupae powder could enhance the cholinergic function by decreasing both the cholinergic neurons density and AChE activity in hippocampus. In accompany with these changes, the memory impairment was also improved. In addition, *B.mori* treated group also showed the markedly reduction of MDA level in hippocampus.

It is widely accepted that oxidative stress is implicated in the pathogenesis of neurodegeneration in numerous conditions including Alzheimer's disease (Stewart and Heales, 2003). Previous studies showed that both the degeneration of cholinergic neurons and the decreased ACh were also observed in the brains of patients attack by AD (DeLaGarza, 2003). In addition, it was reported that the level of antioxidant in plasma of AD patients were decreased in accompany with the cognitive decline. The reduction of antioxidant enzymes in AD patients gave rise to the inadequate antioxidant enzymatic activities to counteract the increased free radicals. The administration of dietary rich in antioxidant could decrease the risk of AD (Engelhart *et al*., 2002). Recently, antioxidant has been proposed to be the potential therapeutic against AD (Behl and Moosmann, 2002; Thukham-Mee *et al*., 2012). Administration of substances possessing antioxidant activity could increase the activity of Superoxide Dismutase (SOD) but decreased MDA level resulting in the attenuation of memory impairment in animal model of Alzheimer's disease (Li *et al*., 2010; Luo and Huang, 2006). This was in agreement with our results which demonstrated that *B.mori*, a substance possessing antioxidant activity, could decrease MDA level in hippocampus. The

reduction of MDA level indicated the improvement of oxidative stress status which in turn resulted in the increased cholinergic neurons density leading to the attenuation of memory impairment.

Previous study also clearly demonstrated that acetylcholinesterase inhibitor could also improve memory impairment in Alzheimer's disease (DeLaGarza, 2003). Therefore, the cognitive enhancing effect of *B.mori* in this study might occur partly via the inhibition of AChE.

5. CONCLUSION

Although the precise underlying mechanism and the details of the active substances in *B.mori* remained to be discovered, this local food and herbal medicine according to the folklore might offer a novel approach for the prevention of Alzheimer's disease.

6. ACKNOWLEDGEMENT

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