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Mulberry Fruit Mitigates Alcohol Neurotoxicity and Memory Impairment Induced by Chronic Alcohol Intake

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Abstract: Problem statement: To date, the therapeutic strategy efficacy against memory impairment induced by alcohol intoxication is still limited. The novel therapeutic strategy which is easy to approach, less toxic and less cost is required. Based on the role of oxidative stress in memory impairment induced by alcohol, the neuroprotective effect of substance possessing antioxidant has gained much attention. Therefore, we aimed to determine the effect of *Morus alba* fruits, substance possessing antioxidant, on spatial memory and brain damage in hippocampus. **Approach:** Male Wistar rats were induced alcoholism by increasing the alcohol concentration in drinking water gradually increased to 30% within 15-week period. Then, the alcoholic rats were orally given mulberry fruits powder at doses of 2, 10 and 50 mg kg^{-1} BW at a period of 14 days. The memory was assessed using Morris water maze after single administration and every 7 days until the end of the experimental period and at the end of experiment, hippocampus was isolated and determined the neuron density. In addition, the evaluation of Acetylcholinesterase (AChE) activity and Malondialdehyde (MDA) level were also performed. **Results:** Our results showed that all doses of mulberry fruits enhanced spatial memory and neurons density in hippocampus. The suppression of both AChE activity and MDA level were also observed. These results suggested that the neuroprotection of mulberry fruits might occur partly via the decreased oxidative stress damage while the cognitive enhancing effect might occur partly via the increased hippocampal neuron density and the suppression of AChE activity. **Conclusion:** Mulberry fruits can protect against brain damage and memory impairment induced by alcoholism. Therefore, mulberry fruits may be served as natural resource for developing food supplement against alcoholism. However, further researches about possible active ingredient and pharmacokinetic are required before moving forward to clinical trial study.

Key words: Mulberry fruits, Acetylcholinesterase (AChE), Malondialdehyde (MDA), Cyanidin-3-Glucoside Equivalents (CGE), induce impairments, excessive alcohol consumption

INTRODUCTION

Alcohol is recognized as one of the most commonly abused drugs worldwide. Alcohol has been used as psychoactive drug, but chronic and excessive alcohol consumption can produce various alcohol-related problem including social, economic and public health problem with profound impacts on brain functions and behaviors.

Chronic ethanol exposures have been shown to induce impairments in a variety of tasks requiring spatial cognition (Arendt *et al*., 1989; Matthews and Morrow, 2000). The memory impairment induced by chronic alcohol consumption was reported to be associated with the decreased cholinergic system (Arendt *et al*., 1989; Nordberg *et al*., 1982). Moreover, it was that chronic alcoholism produced the neurodegeneration and reduction of hippocampal

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volume (Agartz *et al*., 1999). The mechanism of alcohol-induced brain damage is complex. However, oxidative stress has been reported to be one factor contributing the important role on the neurodegeneration induced by alcohol (Esterbauer *et al*., 1991). It was reported that ethanol consumption increased oxidative stress both via the increase free radical formation and the decreased antioxidant enzyme activities (Das and Vasudevan, 2007). Based on the crucial role of oxidative stress mentioned earlier, the neuroprotective effect of substances possessing antioxidant has gained much attention.

Mulberry (*Morus alba* L.) or Mohn in Thai, a plant in family of Moraceae, is widely cultivated in the North and North East of Thailand. Mulberry fruit is used not only as fruit but also as medicine. According to the traditional folklore, mulberry fruit is used to protect against liver and kidney damage, strengthen the joints, improve eyesight and have anti-aging effects (Li and Luo, 2003). It is also used for the treatment of sore throat, fever, hypertension and anemia (Ma, 2002; Gong and Zhu, 2008). Recent findings have shown that mulberry fruits can protect against brain damage in various conditions including Parkinson's disease (Kim *et al*., 2010) and cerebral ischemia (Kang *et al*., 2006). Moreover, mulberry fruit extract has been reported to decrease β-amyloid protein leading to the reduction of learning and memory impairment in Senescence-Accelerated Mice (SAMP) (Shih *et al*., 2010). Although, several researches have already demonstrated the neuroprotective of mulberry fruits, less scientific evidence about the effect of mulberry fruits on hippocampus, the area contributing important role on learning and memory, in memory impairment induced by chronic alcohol consumption is available. Therefore, this present study aimed to determine the effect of mulberry fruits on spatial memory and on brain damage in hippocampus induced by chronic alcohol consumption. In addition, the alteration of AChE and oxidative damage marker were also further investigated.

MATERIALS AND METHODS

Preparation of mulberry fruits freeze-dried powder: All mulberry fruits (*Morus alba* var Chiangmai) used in this study is prepared and provided by The Institute of Queen Sirikit Seri Culture, Thailand. Mulberry fruits were collected from the Queen sirikit seri culture center udon thani. All berries were picked at the commercially ripen stage and selected according to uniformity color. Then, the fruits were dried at 70%celcius for 4 days and grounded to powder. In addition, the mulberry fruit powders were also kept in dark air tight bottle at -20°C. The mulberry fruit powder used in this study contained phenolic compounds at concentration of 519±0.004 mg GAE/g fresh matter of fruit. It also contained anthocyanin at concentration of 634.56±23.62 mg of Cyanidin-3-Glucoside Equivalents (CGE) per 100 g of fresh weight.

Animal: Adult male Wistar rats, 8 weeks old, were used as experiment animals. They were obtained from National Animal Center, Salaya, Nakornpatom. The weights of the animals on the first day of experiment were 180-220 g. All animals were given access to food and water ad libitum. They were randomly housed 5% and maintained in 12:12 light: dark cycle and given access to food and water ad libitum. The experiments were performed to minimize animals suffering and the experiment protocols were approved by the Institutional Animal Care and Unit Committee Khon Kaen University,Thailand.

Experimental protocol: All rats were induced alcoholism by means of a semi-voluntary intermittent daily intake of different concentrations of alcohol. In brief, rats were exposed to alcohol in drinking water. The alcohol concentration in drinking water was gradually increased from 5-20% from the first to fourth week (5% per week). Then the alcohol concentration was raised to 30% from the fifth to thirtieth. The animals were assessed tremor by stationary dowel test and aggression as indicators for alcoholism (Gotz *et al*., 2001). Then the alcoholic rat were divided into 6 groups as following; (1) Vehicle plus ethanol (2) Vitamin C 250 mg Kg^{-1} BW plus ethanol (3)-5) Mulberry fruits at doses of 2,10 and 50 mg Kg^{-1} BW respectively plus ethanol.

Since the effect of alcohol on oxidative stress and cognitive impairment were focused, the positive control treated groups used in this study were treated with vitamin C (well established antioxidant) and Aricept (a standard drug treated dementia) at doses of 250 and 1 mg kg^{-1} BW respectively. In addition, the animals in group 4-6 were treated with various doses of mulberry fruits ranging from 2, 10 and 50 mg kg^{-1} BW. The animals were assessed the effect of mulberry fruits on cognitive function using Morris water maze test after single intervention, 7 and 14 days of intervention. In order to determine the effect of mulberry fruits on brain damage, all rats were sacrificed and removed the brain for the determination of brain damage after 2 weeks of treatment.

Determination of spatial memory: The spatial memory was determined using Morris water maze test. The water maze was a circular pool with 160 cm in

diameter, 60 cm in height filled with water (approximately 42 cm deep, temperature of 23-2 4°C), filled up with tap water for 40 cm deep and the water surface was covered with nontoxic milk powder. The pool was divided into 4 quadrants and the removable escape platform was placed in the center on one quadrant below the water level. For animals, the location of the platform was invisible and it remained there throughout the training. The animals must memorize the environmental cues to locate the platform. Each animal was placed in the water in the starting quadrant and allowed to swim until it found and climbed onto the platform. The time for animal to reach the hidden platform was recorded as escape latency. Then, 24 hr later, the animals were exposed to the same test except that the hidden platform was removed. The time which the animals spent in the quadrant previously located the platform was recorded as the retention time.

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.

Morphological analysis: Five coronal sections of each rat in each group were studied quantitatively. Neuronal counts in hippocampus were performed by eye using a 40× 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

Determination of malondialdehyde level and acetylcholinesterase activity: Hippocampus was isolated and 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) while the activity of AChE was determined using.

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 were accepted as significance.

RESULTS

Effect of mulberry fruits on cognitive deficit induced by chronic ethanol intake: We have found that Vitamin C which used as positive control in this study significantly attenuated the increase in escape latency induced by alcohol administration after single, 7 and 14 days after mulberry administration ($p<0.001$, 01 and 0.001 respectively, compared with vehicle treated group) as shown in Fig. 1. However, Vitamin C treatment failed to attenuate the decrease in retention time induced by alcohol consumption as shown in Fig. 2. After single administration, mulberry fruits at all dosage range used in this study significantly decreased escape latency in alcoholic rat ($p<0.001$, 0.001, .05 respectively; compared with vehicle treated group). At 7days after administration, the significant reduction of escape latencies were still observed ($p<001.01$ and 0.05 respectively; compared with alcohol plus vehicle treated group). These changes were still observed when the treatment was prolonged further to 14 days ($p<0.001$ all; compared with alcohol plus vehicle treated group). The alterations of retention time were also assessed and results were shown in Fig. 2. Unfortunately, no significant change was observed.

The neuroprotective effect of mulberry fruits in alcoholic rats: The neuroprotective effect against neurotoxicity induced by alcohol was also investigated and the results were shown in Fig. 3. The current results showed that Vitamin C, the well known antioxidant, significantly increased the density of neurons in CA1, CA2 and dentate gyrus of the alcoholic rat ($p<0.01$, 0.05 and 0.001 respectively; compared with vehicle treated group). Mulberry fruits at dose of 2 mg kg^{-1} BW significantly increased the neurons density in CA1, CA2, CA3 and dentate gyrus ($p<0.01$, 0.05, 0.05 and 0.001 respectively; compared with vehicle treated group) while the mulberry fruits at higher doses (10 and 50 mg kg^{-1} BW) could increase the neuron density only in CA1 and dentate gyrus ($p<0.001$ all; compared with vehicle treated group).

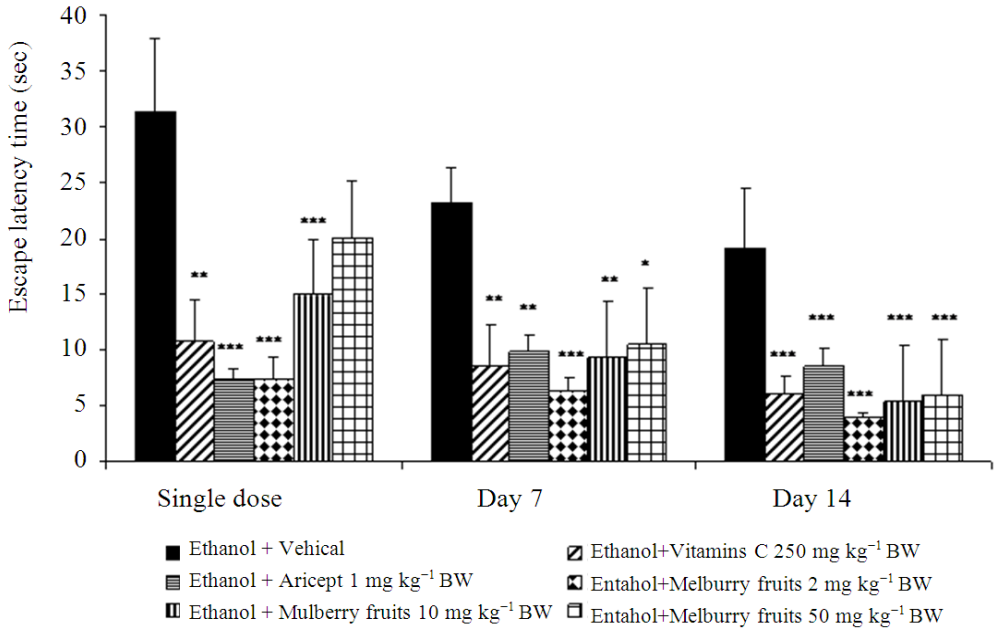

Fig. 1: Effect of Vitamin C, Aricept and various doses of mulberry fruits on escape latency time in Morris water maze test. Values given are the mean ± S.E.M. (n = 6)*p<0.05 as compared with vehicle plus ethanol.**p<0.01 as compared with vehicle plus ethanol. *** p< 0.001 as compared with vehicle plus ethanol

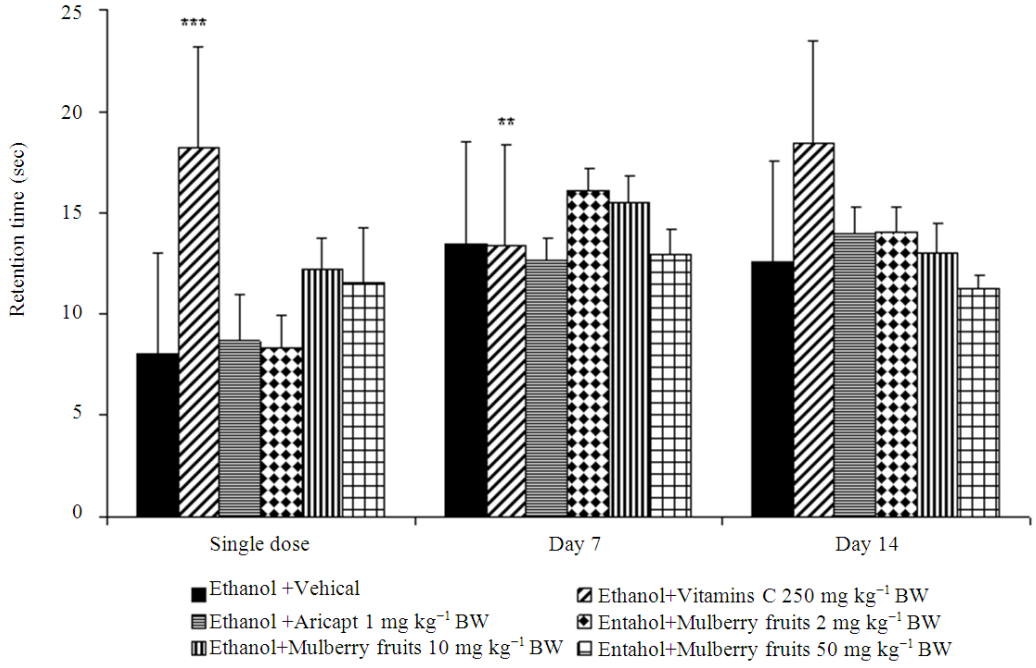

Fig. 2: Effect of Vitamin C, Aricept and various doses of mulberry fruits on retention time in Morris water maze test. Values given are the mean ± S.E.M. (n = 6)* p<0.05 as compared with vehicle plus ethanol. **p<0.01 as compared with vehicle plus ethanol. ***p<0.001 as compared with vehicle plus ethanol

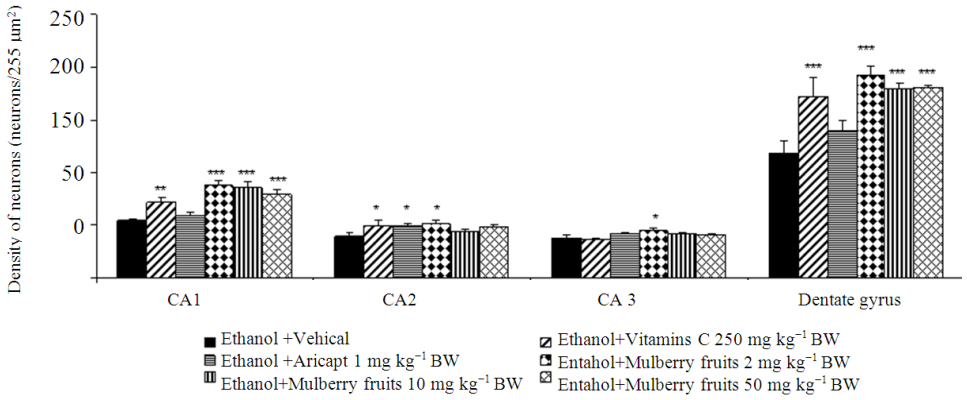

Fig. 3: Effect of Vitamin C, Aricept and various doses of mulberry fruits on neurons density in various subregions of hippocampus. Values given are the mean ± S.E.M. (n = 6) * p<0.05 as compared with vehicle plus ethanol. ** p<0.01as compared with vehicle plus ethanol. *** p<0.001 as compared with vehicle plus ethanol

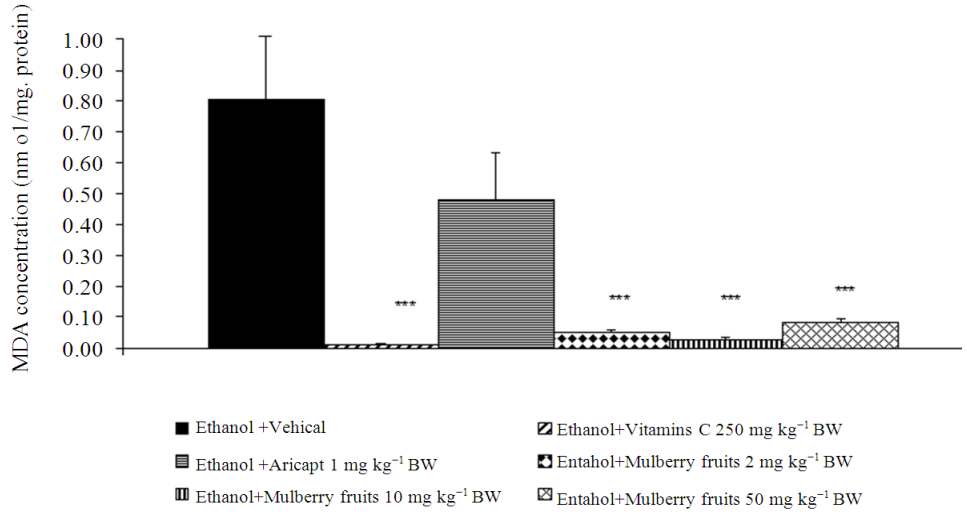

Fig. 4: Effect of Vitamin C, Aricept and various doses of mulberry fruits on the level of Malondialdehyde (MDA), a product of lipid peroxidation in hippocampus. Values given are the mean ± S.E.M. (n = 6)* p<0.05 as compared with vehicle plus ethanol. ** p<0.01 as compared with vehicle plus ethanol. ***p<0.001 as compared with vehicle plus ethanol

Effect of mulberry fruits on Malondialdehyde (MDA) level: Based on the previous information about the crucial role of oxidative stress on the neurodegeneration and the neuroprotective effect of mulberry fruits, the effect of mulberry fruits on oxidative stress damage marker, the Malondialdehyde (MDA) was determined. It was found that after 2 weeks of treatment, Vitamin C significantly decreased MDA level in hippocampus as shown in Fig. 4. Mulberry fruits at all doses used in this study also significantly decreased MDA level in the mentioned area (p<05 all; compared with vehicle treated group).

Effect of mulberry fruits on Acetylcholinesterase (AChE) activity: Based on the previous information that cholinergic system played the crucial role on the memory impairment induced by chronic alcohol consumption, we also determined the activity of AChE activity in hippocampus. Figure 5 showed that Vitamin C significantly suppressed AChE activity (p<0.05; compared to vehicle treated group). In addition, mulberry fruits at dose of 2, 10 and 50 mg kg^{-1} BW also suppressed AChE activity (p<05.01 and 0.05 respectively; compared to vehicle treated group).

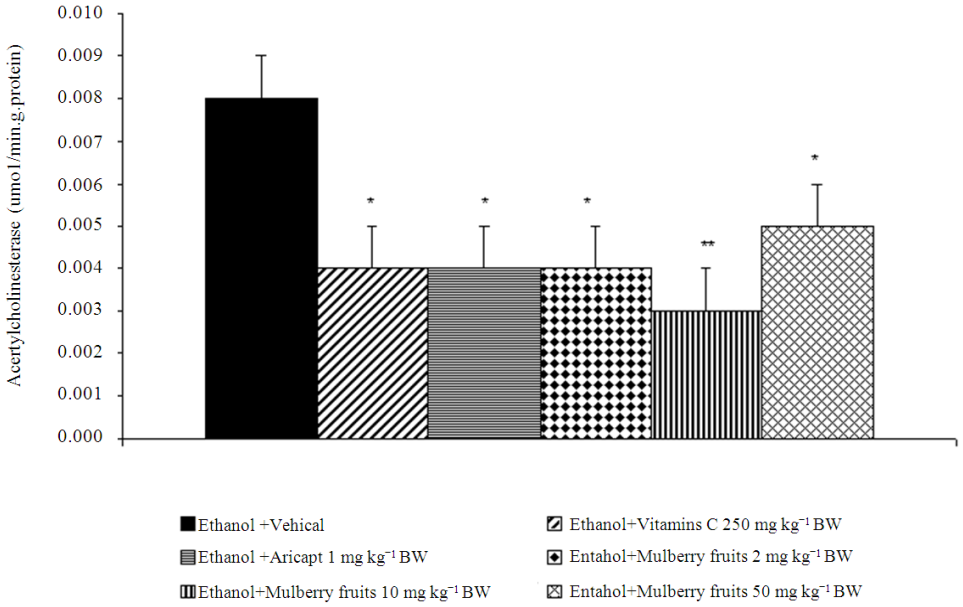

Fig. 5: Effect of Vitamin C, Aricept and various doses of mulberry fruits on the level of Acetylcholinesterase (AChE) in hippocampus. Values given are the mean ± S.E.M. (n = 6)* $p<0.05$ as compared with vehicle plus ethanol. **$p<0.01$ as compared with vehicle plus ethanol.*** $p<0.001$ as compared with vehicle plus ethanol

DISCUSSION

The current study demonstrated that mulberry fruits powder could mitigate memory impairment in animal model of alcoholism. In addition, mulberry fruits also increased neuron density in hippocampus in accompanied with the suppression of AChE activity and MDA level.

Brain has been reported to be susceptible to lipid peroxidation, since it consumes a large amount of oxygen and is rich in polyunsaturated fatty acids, which are especially prone to reactive oxygen injury. Oxidative stress appears to be one key factor inducing the pathogenesis of alcohol related brain damage (Gotz *et al*., 2001; Montoliu *et al*., 1994; Sun *et al*., 1997). Previous study had demonstrated that chronic ethanol-induced increases in CYP2E1 and other oxidases resulting in the increased lipid peroxidation and reactive oxygen radicals in the brain (Montoliu *et al*., 1994; Crew, 1999).

Recent studies have shown that oxidative related damage including brain damage and memory impairment can be prevented and attenuated by polyphenolic compounds which exhibited potent antioxidant activity (Rodrigo *et al*., 2011; Kim *et al*., 2010; Spencer, 2010; Sun *et al*., 2002). In addition, mulberry fruit extract have been previously reported to exhibit the neuroprotective effect via its antioxidant activity leading to the decreased oxidative stress and finally decreased neurodegeneration (Kim *et al*., 2010). These lines of evidence are in agreement with our finding that the mulberry fruits powder used in this study contained abundant of polyphenol and anthocyanin and it could decrease oxidative stress damage indicating by the reduction of MDA level in the hippocampus. Therefore, the neuroprotective effect of mulberry fruits observed in this study might occur partly via its antioxidant effect which in turn gave rise to the decreased oxidative damage. However, other effect related to the neuroprotection induced by mulberry fruits such as anti-apoptotic effect was also reported (Kim *et al*., 2010). Therefore, the neuroprotection of mulberry fruits related to anti-apoptotic effect still could not be excluded. The possible active ingredient might be associated with the content of polyphenol compounds including anthocyanin in the mulberry fruits powder.

Previous studies had clearly demonstrated that the memory impairment was related to the hippocampal damage (Goodrich-Hunsaker and Hopkins, 2010; Graham *et al*., 2006). Therefore, it might be possible that the mulberry fruits decreased oxidative damage in hippocampus resulting in the increased neuron density in hippocampus leading to the improved spatial memory impairment induced by alcoholism. In addition, it was reported that Acetylcholine (ACh)

modulated the encoding and retrieval of spatial memory in hippocampus (Rogers and Kesner, 2003). Accumulating lines of evidence had suggested that drugs or natural products exhibiting Acetylcholinesterase inhibitor (AChEI) could enhance the available ACh resulting in the improved memory impairment in various conditions (Chonpathompikunlert *et al*., 2010; Wattanathorn *et al*., 2011; Yuede *et al*., 2007). Our results also showed that mulberry fruits powder suppressed AChE activity in hippocampus. Therefore, these findings suggested that the cognitive enhancing effect of mulberry fruits might occur partly via the AChE suppression.

The present results failed to show the dose dependent effect of both neuroprotection and cognitive enhancing effects induced by mulberry fruits because mulberry fruits contained numerous ingredients. Therefore, the increasing doses of mulberry fruits might also increase the concentration of other ingredients which in turn can mask the effect of active ingredient. In addition, it was also possible that the relationships between mulberry fruits and the interested parameters which included both memory and neuron density in hippocampus were not the simple relationship.

CONCLUSION

Mulberry fruits can provide the beneficial effect to protect against brain damage induced by alcoholism and can be served as the valuable functional food. However, further researches about possible active ingredients, precise underlying mechanism and chronic toxicity are still essential before move forward to clinical trial study to confirm the beneficial effect of mulberry fruit as functional food to protect against brain damage and memory impairment in alcoholism.

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