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Mulberry Fruits Protects Against Age-Related Cognitive Decline

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Abstract: Problem statement: The phenolic compounds and anthocyanin contents, the antioxidant and Acetylcholinesterase Inhibitor (AChI) activities of mulberry fruits were determined. **Approach:** Analysis of phenolic compounds and anthocyanin were performed using Folin Ciocalteu reagent and pH-differential method respectively. The antioxidant activity was determined using DPPH and FRAP assays while AChI activity was determined using Ellman method. **Results:** The mulberry fruit powder contained high amount of phenolic compounds and anthocyanin, It also exhibited strong antioxidant and AChEI activities. Based on the beneficial effect of polyphenol compounds and anthocyanin on brain, we also further evaluated the neuroprotective and cognitive enhancing effects of mulberry fruits in animal model of age-related cognitive decline. Surprisingly mulberry fruits could mitigate brain damage and memory impairment in age-related cognitive decline. The possible mechanism might be associated with its antioxidant and AChEI activities. **Conclusion:** In conclusion, Mulberry fruits are the potential neuroprotectant and cognitive enhancer. However, further investigations are required.

Key words: Mulberry fruit, hippocampal damage, memory impairment, Senescence-Accelerated Mice (SAMP), Gallic Acid Equivalents (GAE), Fresh Weight (FW)

INTRODUCTION

At present, the average age increasing at an unprecedented rate. Demographic projections suggest that the number of people aged over 65 years will nearly triple by 2050, accounting for 14% of the world (Lutz *et al*., 2008). Normal aging is reported to be associated with a slow decline in brain functions such as sensory and motor performance and at times, this decline is accompanied by progressive memory loss, dementia and cognitive dysfunctions, ultimately resulting in limited functionality. It has been reported that the cognitive impairment in both aged human and rodents is correlated with the accumulation of oxidative damage to lipids, proteins, nucleic acids (Butterfield *et al*., 2006; Forster *et al*., 1996; Murali and Panneerselvam, 2007) and the vulnerability of various neurotransmitters to oxidative stress (Govoni *et al*., 2010; Mokrasch and Teschke, 1984; Pradham, 1880). Moreover, it is also reported to be related to the hypofunction of cholinergic system and manifested by the decreased Acetylcholinesterase (AChE), one of the major markers of cholinergic function in various cerebral areas (Papandreou *et al*., 2006).

Recently, numerous researches clearly revealed that various antioxidant supplements and phytochemical components provided the beneficial for preserving brain functions and forestalling the age-related deficits (Shukitt-Hale *et al*., 2008). Thus, this raised the possibility that the substances possessing antioxidant activity should be able to protect against the brain area, contributing the important role on learning and memory resulting the improved memory impairment.

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Mulberry or Morus alba belongs to the family *Moraceae*. It has been widely planted in both the Northeast and North of Thailand. Mulberry fruit is widely regarded as a nutritious food. It can be eaten freshly or widely used in the production of wine, fruit juice, jam and canned food (Ning *et al*., 2005). Mulberry fruit is not only used 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 (Shizhen, 2008). It is also used for the treatment of sore throat, fever, hypertension and anemia (Ma, 2002; Gong and Zhu, 2008). In addition, recent findings showed that mulberry fruits could 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 also decreased β-amyloid protein and improved learning impairment in Senescence-Accelerated Mice (SAMP) (Shih *et al*., 2010). Although, several researches had already demonstrated the neuroprotective of mulberry fruits, less evidence about the effect of mulberry fruits on hippocampus, the area contributing important role on learning and memory, in the age-related cognitive impairment is available. Thus, this study was set up to elucidate this issue in animal model of age-related cognitive impairment induced by a cholinotoxin, AF64A.

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 degree 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.

Chemicals: Donepezil hydrochloride (Aricept 10 mg tablet^{-1}) (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.

Determination of phenolic compound: Total TF content of the plant extracts were determined according to the well-established colorimetric assay (Meda *et al*., 2005). Gallic acid was used as a standard and the results were expressed as milligram Gallic Acid Equivalents (GAE) per gram of dry matter. Finally, the data were converted to mg GAE/g fresh matter of fruit. Total phenolic compound was assessed both at 1 month of preparation and 1 year after preparation.

Total anthocyanin content: Anthocyanin quantification was performed by the pH-differential method (Wrolstad *et al*., 2001). Calculation of the anthocyanins concentration was based on a cyanidin-3-glucoside molar extinction coefficient 26,900 and a molecular mass of 449.2 g mol^{-1}. Results were expressed as miligrams (mg) of Cyanidin-3-Glucoside Equivalents (CGE) per 100 g of Fresh Weight (FW). Anthocyanin content was determined both at 1 month of prepparation and 1 year after preparation.

Antioxidant capacity:

DPPH radical scavenging assay: Radical scavenging capacity was determined according to the method outlined by Scapagnini *et al*. (2010). A calibration curve was prepared, using Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) and the results were expressed as mmol Trolox equivalents per 100 g of fresh weight(mmol TEAC/100 g FW).

Ferric reducing/antioxidant power assay (FRAP assay): The FRAP assay was used to estimate the reducing capacity of tested substances, according to the original method of Benzie and Strain (1996). A calibration curve was prepared, using an aqueous solution of ferrous sulphate FeSO4·7H2O and the results, obtained from three replicate extractions, were expressed as mmol $FeSO_4$·7H2O per 100 g of fresh weight (mmol Fe^{2+}/100 g FW).

Determination of Acetylcholinesterase (AChE): Inhibition of AChE was evaluated according to the method Ellman *et al*. (1961) with some modification. The assay is based on the spectrophotometric measurement of the increased yellow color produced from thiocholine when it reacts with the dithiobisnitrobenzoate ion. Rivastigmine was used as a positive control and water served as a positive control. The increase in absorbance value due to the spontaneous hydrolysis of the substrate was corrected by subtracting the ratio of the reaction before adding the enzyme from the rate after the enzyme addition. Percentage inhibition by extracts and galanthamine were calculated using the equation below.

$$\text{Inhibition } (\%) = \frac{[1 - \text{Sample reaction rate}]}{\text{Blank reaction rate}}$$

Animals: *In vivo* experiments were carried out on male Wistar rats, weighing 180-200 g, that were housed four per cage under a 12 h light/dark cycle in a room with

controlled temperature (22±1°C). Food and water were available *ad libitum*. Animals were handled daily (between 9:00 and 11:00 A.M) 5 days before the experiment in order to adapt them to manipulation and minimize non-specific stress responses. Rats were randomly allocated into several experimental groups, each comprising 8 animals. All experiments followed the guidelines on ethical standard for investigation of experimental pain in animals and approved by the Animal Experimentation Ethic Committee of Khon Kaen University.

In vivo experimental protocol: All rats were randomly assigned to 5 groups of 8 animals each.

Group I Vehicle+AF64A: Rats had been treated with vehicle for 1 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.

Group II 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.

GroupIII-V Mulberry fruits+AF64A: Rats had been treated with the plant extract at various doses ranging from 2, 10 and 50 mg kg^{-1} BW for 1 week 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. In addition, all rats were received mulberry powder treatment at 1 month after the mulberry powder preparation).

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.

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^{-1}. 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^{-1} BW. Then, AF64A (2 nmol 2 μL^{-1}) 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 in place for 5 min after infusion and then slowly withdrawn.

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.

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 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.

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 (S.E.M). One-way Analysis of Variance (ANOVA), followed by Tukey post hoc test. A probability level less than 0.05 was accepted as significance.

RESULTS

The current results demonstrated that mulberry fruit (*Morus* alba var Chiangmai) contained high amount of anthocyanin, a subclass of flavonoid and phenolic compounds. Within 1 year at -20°C, the freeze dried mulberry powder exhibited the reduction of anthocyanin and phenolic compounds approximate 26.73 and 21% respectively. Within 1 month after the mulberry fruit powder preparation, the powder still showed strong antioxidant activity and the ability to suppress AChE activity. Mulberry fruit treatment could suppress AChE activity resulting in the enhanced spatial memory at the same magnitude as standard drug or Acetylcholinesterase Inhibitor (AChI) used nowadays. Moreover, mulberry fruit also decreased oxidative stress damage in hippocampus and gave rise to the increased neuron density in the mentioned area.

Quantification of phenolic compounds and anthocyanin: The results were shown in Table 1. Our results showed that the total phenolic compounds of mulberry fruit powder used in this study was 519±0.004 mg GAE/g fresh matter of fruit. However, the content of phenolic compounds decreased to 410.28±.001 mg GAE/g fresh matter of fruit or approximate 21% when kept in the dark air tight bottle at -20°C.

Table 1: Total phenolic compounds and anthocyanin content of mulberry fruit powder

Materials/ duration time	Total phenolic compound mg GAE /g fresh matter of fruit	Anthocyanin mg CGE /100 of fresh weight
1month	519.33±0.004	634.56±23.62
1 year	410.28±0.001	551.06±5.90
Percentage change	21%	13.17%

Table 2: The scavenging ability of mulberry powder and Vitamin C. * p<0.05 as compared with vitamin C. ** p< 0.01 as compared with vitamin C. *** p<0.001 as compared with vitamin C

	DPPH (mmol TEAC/100g FW)		FRAP activity (mmol Fe^{2} +/100 g FW)	
Concentration (ug/mg)	vitamin C	Mulberry fruity powder	vitamin C	Mulberry fruity powder
25	36.13+0.61	17.63_0.2	2.30±0.01	5.86+0.03
50	40.75+0.2	22.83+0.61	10.90±0.1	9.7+0.130
100	44.80+0.2	32.95+2.04	31.52±0.27	18.86+0.74
250	52.60+1.63	52.95+2.25	53.24±0.20	28.76+0.24
500	63.87+0.20	64.74+1.63	77.71±1.62	59.29+0.24
1000	86.71+0.82	82.66+1.63	98.29±0.13	95.24+0.67

Since anthocyanin pigment is recognized as a subclass of flavonoid, one types of phenolic compounds commonly found in mulberry fruits and playing the crucial role on learning and memory, we also determined the total anthocyanin content of mulberry fruit powder used in this study. Our results showed that the anthocyanin content assessed at 1 month after the mulberry powder preparation was 634.56±23.62 mg of Cyanidin-3-Glucoside Equivalents (CGE) per 100 g of fresh weight. However, the content of anthocyanin decreased to 551.06±5.90 mg of Cyanidin-3-Glucoside Equivalents (CGE) per 100 g of fresh weight (13.17% reduction) within 1 year at -20°C as shown in Table 1.

Determination of antioxidant activity of mulberry fruits powder: It has been well established that the principle of antioxidant activity is the availability of electrons to neutralize free radicals. Since, antioxidant mechanisms are diverse, a variety of in vitro technique has been developed. It is suggested that the evaluation of antioxidant activity should be performed via different assays based on different mechanisms. Therefore, in this study we determined the antioxidant activity of mulberry fruit powder using DPPH and FRAP. Table 2 showed the scavenging ability of mulberry powder and Vitamin C. The results showed that at concentration of 25, 50 and 100 $\mu g\ mL^{-1}$, vitamin C significantly exhibited DPPH scavenger efficacy (p<0.03; compared to vitamin C). However, when the concentrations of both substances were increased further to 250,500 and 1000 $\mu g\ mL^{-1}$, no significant changes in DPPH scavenger efficacy between vitamin C and mulberry fruit powder were observed. Our results showed that at concentration of 25 $\mu g\ mL^{-1}$, mulberry powder exhibited higher ferric reducing antioxidant power compared to vitamin C (p<0.0002; compared to vitamin C. Therefore both DPPH and FRAP assays also

confirmed the antioxidant activity of mulberry fruit powder. Previous study reported that there was a strong relationship between polyphenol and reducing power (Sofidiya *et al*., 2006). Therefore the antioxidant activity of mulberry fruit observed in this study might be associated with its polyphenolic compound content.

In vitro assessment of AChE activity: The hypofunction of cholinergic system has been reported to play a crucial role on the cognitive impairment in both aged human and rodents (Papandreou *et al*., 2006), therefore, we also investigated the effect of mulberry fruit powder on the activity of AChE. The effect of mulberry fruit powder was shown in Fig. 1. It was found that mulberry fruit powder also demonstrated the suppression of AChE activity in a dose-dependent manner. Previous study had demonstrated that the principal role of AChE is the termination of acetylcholine (ACh), an important neurotransmitter at the cholinergic synapse (Mukherjee *et al*., 2007) resulting in the increase concentration of ACh at the synapse leading to the increased nerve impulse transmission (Rollinger *et al*., 2004). Our data showed that mulberry fruit powder exhibited the suppression of AChE at low concentration. Therefore, it should have the potential to enhance learning and memory.

The neuroprotective and cognitive enhancing effects of mulberry fruit: Based on the antioxidant activity of mulberry fruit and the role of phenolic compound including anthocyanin on memory, we have also evaluated the neuroprotective effect of mulberry fruit on the hippocampal neuron density, the area contributing the important role on learning and memory. Figure 2 demonstrated the effect of various doses of mulberry fruit powder on the neuron density in various subregions of hippocampus, the area playing the crucial role on learning and memory. Donepezil treated group significantly enhanced neuron density in CA1 ($p<0.01$ and .001 respectively; compared to vehicle+AF64A), CA2 ($p<0.001$ all; compared to vehicle+AF64A), CA3 CA2 ($p<0.001$ all; compared to vehicle+AF64A) and dentate gyrus ($p<.001$ and .05 respectively; compared to vehicle+AF64A). Rats subjected to mulberry fruit powder treatment at dose of 2 mg kg^{-1} BW significantly enhanced neuron density only in CA1 and CA3 ($p<.05$ all; compared to vehicle+AF64A). The medium dose or mulberry fruit at dose of 10 mg kg BW could produce the significant increase in neuron density in CA1 ($p<0.01$; compared to vehicle+AF64A), CA3 ($p<0.01$; compared to vehicle+AF64A) and dentate gyrus ($p<0.05$; compared to vehicle+AF64A). Unfortunately, no change was observed at the higher dose.

Based on the crucial role of oxidative stress mentioned earlier, the level of MDA which previously reported to be the marker of oxidative damage was also determined in hippocampus. Figure 3 clearly revealed that both Donepezil and mulberry fruits at all doses of mulberry fruits could decrease MDA level in hippocampus ($p<0.01$ all; compared to vehicle+AF64A). Taken all data together, the neuroprotective effect of mulberry fruit powder observed in this study might occur partly via the decreased oxidative stress damage indicating by the decreased MDA level.

Since mulberry fruit powder exhibited the neuroprotective effect in hippocampus, the cognitive enhancing effect of this substance was also determined and results were demonstrated in Fig. 4 and 5. It was found that both Donepezil and mulberry fruit powder treated groups at all dosage range used in this study significantly decreased escape latency time as shown in Fig. 4 ($p<0.001$ all; compared to vehicle+AF64A). In addition, Fig. 5 showed that Donepezil and mulberry fruit treated groups at doses of 2 and 50 mg kg^{-1} BW also produced significant elevation in retention times ($p<0.05$ all; compared to vehicle+AF64A).

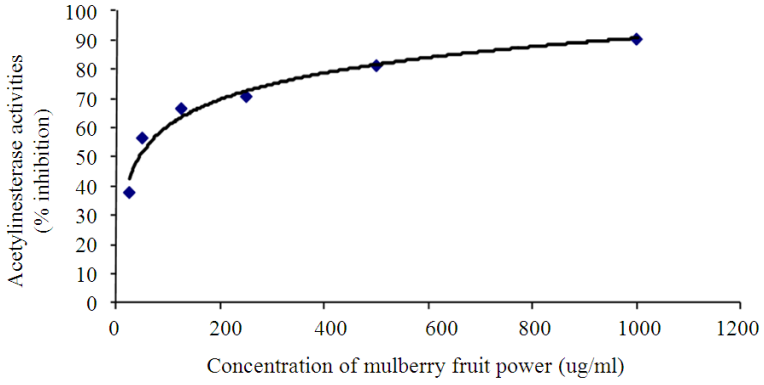

Fig. 1: The effect of mulberry fruit powder on the activity of AChE

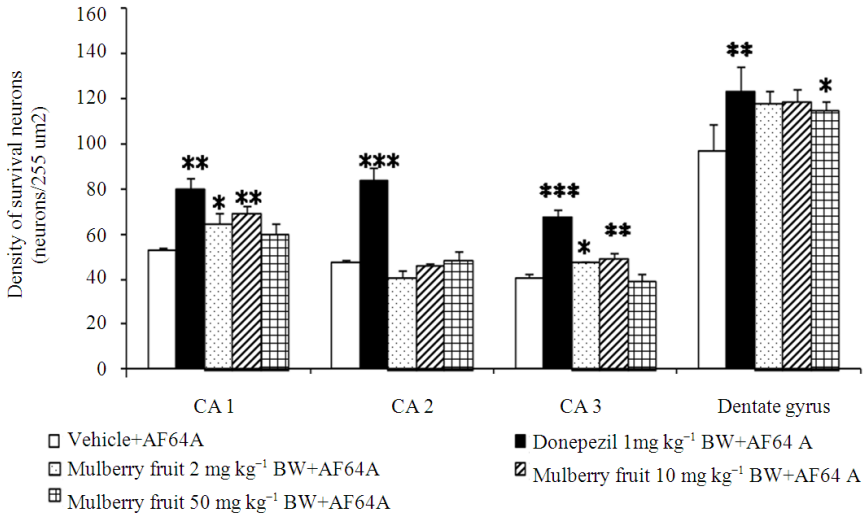

Fig. 2: The effect of mulberry fruit powder on the neuron density in various subregions of hippocampus. * p<0.05 as compared with vehicle plus AF64A. ** p<0.01 as compared with vehicle plus AF64A. *** p<0.01 as compared with vehicle plus AF64A

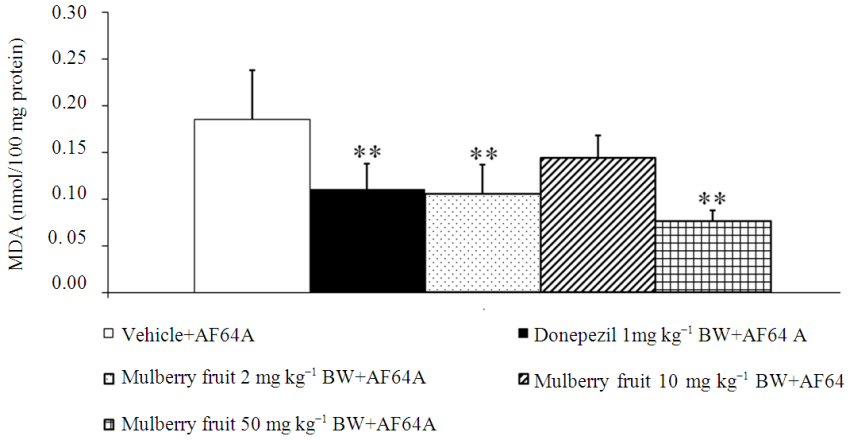

Fig. 3: The effect of mulberry fruit powder on the malondialdehyde (MDA) level in hippocampus.** *p<0.*01 as compared with vehicle plus AF64A

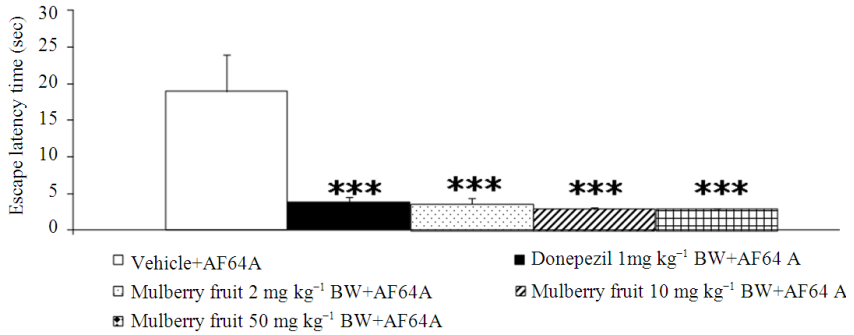

Fig. 4: The effect of mulberry fruit powder on the escape latency time in Morris water maze test. *** *P*-value < .01 as compared with vehicle plus AF64A

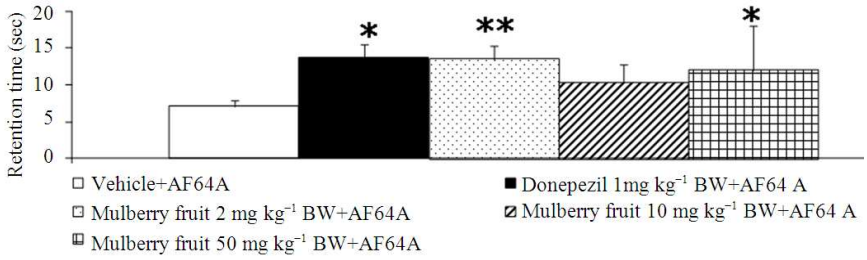

Fig. 5: The effect of mulberry fruit powder on the retention time in Morris water maze test. * p< 0.05 as compared with vehicle plus AF64A. ** p< 0.01 as compared with vehicle plus AF64A

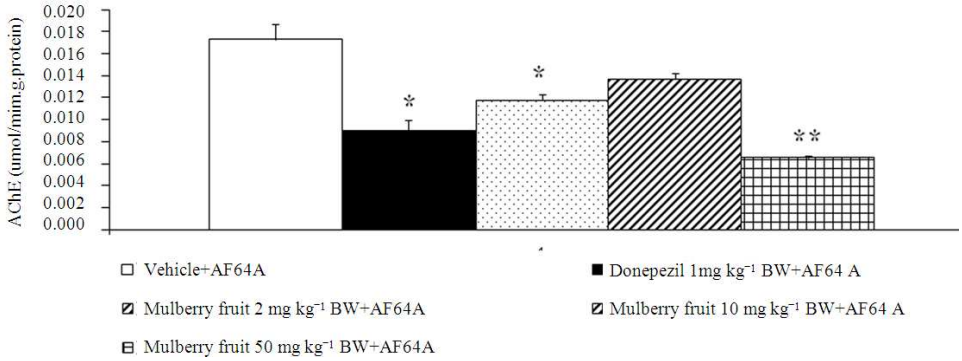

Fig. 6: The effect of mulberry fruit powder on the Acetylcholinesterase (AChE) activity in hippocampus. * p<0.05 as compared with vehicle plus AF64A. ** p<0.01 as compared with vehicle plus AF64A

Although our in vitro study had demonstrated that mulberry fruit powder exhibited the AChE suppression effect, the difference environment between in vitro and in vivo might also influence on the response to mulberry fruit powder leading to the difference results. Therefore, in order to confirm that the suppression of AChE activity might play a crucial role on the cognitive enhancing effect of mulberry fruit, we also evaluated the activity of AChE in hippocampal homogenate at the end of the experimental period. The results were shown in Fig. 6. It was found that Donepezil produced a significant decrease in AChE activity in hippocampus. Rats subjected to mulberry fruit treatment at dose of 50 mg kg^{-1} BW also significantly suppress AChE ($p<0.01$; compared to vehicle+AF64A) while no significant changes were observed in other doses treatment. These results suggested that the cognitive enhancing effect might occur partly via the suppression of AChE. In addition, our data also suggested that besides the suppression of AChE activity, other factors such as the enhanced neuron density in hippocampus, the area contributing the important role on learning and memory, might also contribute their roles on the cognitive enhancing effect of mulberry fruit. However, the precise underlying mechanism is still required further investigation.

DISCUSSION

In this study, our results showed that AF64A treated rats induced the degeneration of neurons density in all subregions of hippocampus Previous work has demonstrated that hippocampus is regarded as a brain region essential for intact cognitive abilities and appears to be particularly vulnerable to the oxidative stress during aging (Fukui *et al*., 2002; Candelario-Jalil *et al*., 2001). The reduction of neuron number and the functional impairment in the mentioned area were reported to associate with oxidative damage. Increasing oxidative damage to proteins, lipids and nucleotides may contribute to neuron dysfunction in normal and pathological aging in humans (Ames *et al*., 1993; Harman, 1992). Recent study suggested that lipid peroxidation might be the potential therapeutic target in age-related cognitive decline (Montine *et al*., 2002).

Mulberry fruit powder exhibited the suppression of AChE activity in vitro and in hippocampus. In accompany with these changes, the memory impairment was also improved. In addition, Mulberry fruit powder 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 age-related cognitive decline (Stewart and Heales, 2003).

The reduction of MDA level observed in this study 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 age-related cognitive decline. Therefore, the cognitive enhancing effect of Mulberry fruit powder in this study might occur partly via the inhibition of AChE.

CONCLUSION

Therefore, mulberry fruit is the potential cognitive enhancer and neuroprotectant against age-related cognitive decline. However, further investigation about the possible active ingredient is still essential.

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