American Journal of Applied Sciences, 2012, 9 (11), 1789-1795
ISSN 1546-9239

doi:10.3844/ajassp.2012.1789.1795 Published Online 9 (11) 2012 (http://www.thescipub.com/ajas.toc)

Mulberry Fruits Extract Mitigate Vascular Dementia

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Received 2012-03-05, Revised 2012-08-09; Accepted 2012-08-31

ABSTRACT

To date, Vascular Dementia (VaD) is the second most commonly found dementia following Alzheimer's disease. Though VaD is still increasing its importance, the therapeutic efficacy is still not in satisfaction level. Therefore, the searching for an acceptable cheap and safe neuroprotective agent is in a great demand. Based on the neuroprotective and cognitive enhancing effects in alcohol neurotoxicity of mulberry fruits, we hypothesized that mulberry fruits might attenuate memory impairment and brain damage in vascular dementia. Therefore, this study was set up to determine the effects and possible mechanism of actions of mulberry fruits**.** Male Wistar rats had been orally given mulberry fruits at doses of 2,10 and 50 mg.kg^{-1} BW for 7 days before and 21 days after the occlusion of right Middle Cerebral Artery (Rt. MCAO). Rats were evaluated spatial memory using Morris water maze every 7 days after Rt.MCAO throughout 21-day experimental period, then they were sacrificed for determined the density of cholinergic neuron in hippocampus and determine acetyl cholinesterase (AChE) enzyme, a key enzyme indicating Acetylcholine (ACh) turnover in hippocampus and assessment of brain infarction volume The results show that the mulberry fruits extract significantly improved memory performance in Morris water maze test, number of cholinergic neurons and decrease the level of acetyl cholinesterase activity. The mulberry fruit extract is the potential neuroprotective agent and cognitive enhancing fruits. However, further researches are required identify the possible active ingredient and precise underlying mechanism.

Keywords: Mulberry Fruit, Vascular Dementia, Stroke

1. INTRODUCTION

Vascular dementia (VaD) is the in elderly population (Malouf and Birks, 2004). It is caused by chronic reduced blood flow to the brain. The most common cause appears to be stroke or series of strokes. It has been reported that 79.5% of vascular dementia patient had a history of stroke (Lindsay *et al*., 1997). When the blood supply carrying oxygen and nutrients to the brain is interrupted by a blocked or diseased vascular system, the neurodegeneration occur in the affected brain area and the gives rise to a progressive decline in memory and cognitive functioning. It was found that level of oxidative stress markers such as Malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OHdG) (Shi *et al*., 2012) were elevated while the antioxidant systems such as the level of vitamin E (Ryglewicz *et al*., 2002) were decreased in VaD patients (Gustaw-Rothenberg *et al*., 2010). In addition, the cholinergic system was also reported to involve in cognitive impairment in VaD (Roman, 2005; Wang *et al*., 2009).

Current approaches to dementia-related neurodegenerative diseases still highly rely on relieving symptoms. Moreover, medications against this condition are very expensive. Therefore, there is a great demand of

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an acceptable cheap and safe neuroprotective agent. Based on the crucial role of oxidative stress and cholinergic system on VaD mentioned earlier, the beneficial effect of substance possessing antioxidant and acetylcholinesterase inhibitory effects have been considered.

Morus alba or mulberry belongs to the *Moraceae* family. 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. Mulberry fruit has been long term used as medicine. According to Chinese Materia Medica, mulberry fruit has been used as blood tonic to nourish the yin and blood and as anti-aging (Li and Luo, 2003). It is also used for treating weakness, fatigue, anemia and premature graying of hair. Recently, several lines of evidence have demonstrated that mulberry fruits can protect against brain damage in various conditions including Parkinson's disease (Kim *et al*., 2010), cerebral ischemia (Kang *et al*., 2006) and alcohol neurotoxicity. In addition, the memory impairment and the elevated acetyl cholinesterase induced by alcohol toxicity are also reversed. Based on the cognitive enhancing effect and neurotective effect against alcohol neurotoxicity of mulberry fruits mentioned earlier, we hypothesized that mulberry fruits might provide beneficial effects to attenuate memory impairment and brain damage in vascular dementia. Therefore, this study was set up to determine the effects and possible mechanism of actions of mulberry fruits.

2. MATERIALS AND METHODS

2.1. Plant Material and Extraction

2.1.1. Animals

Adult male Wistar rats (300-350g, 8 weeks old) were obtained from National Laboratory Animal Center, Salaya, Nakorn Pathom and 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 (AEKKU 1/2552).

2.2. Drugs

Mulberry fruits were extract from The Queen Sirikit Department of Seri culture, Ministry of Agriculture and Cooperative, Thailand. Both Donepezil (Aricept®), a standard drug used for mild cognitive impairment treatment and vitamin C, an antioxidant possessing the neuroprotective and cognitive enhancing effects were used as positive controls in this study. Propylene Glycol (PG) was used as vehicle throughout the study. All administered substances were freshly prepared.

2.3. Plant Preparation

All mulberry fruits used in this study is prepared and provided by The Queen Sirikit Department of 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°C for 4 days and grounded to powder. Then, 4 kilograms of mulberry fruit powder were extracted 3 times with ethyl alcohol 5 liters per time by percolation techniques. The obtained extracts were evaporated under reduced pressure to yield 7.37% of ethanol extract.

2.4. Experimental Protocol

All rats were randomly divided into 6 groups. Each group contained 6 rats.

Group 1

Vehicle treated group. The animals in this group were treated with Propylene Glycol (PG)

Group 2-3

Positive control treated group. The positive control group was treated with the standard drugs used for treating the related the condition. Vitamin C, a well-known antioxidant, was orally administered at dose of 250 kg^{-1}BW. In the determination of cognitive function, the positive control group was treated with Donepezil (Aricept®, a cholinesterase inhibitor) at dose of 1mg kg^{-1}BW.

Group 4-6

Mulberry fruits extract treated group. The animals in group 4-6 were treated with the Mulberry fruits extract at various doses ranging from 2, 10 and 50 mg kg^{-1} BW respectively via oral route administration for a week once daily throughout the experimental period and three weeks after induce middle cerebral artery occlusion.

All animals were treated with vehicle, positive control or mulberry fruits extract at a period of 7 days before and 21 days after right Middle Cerebral Artery Occlusion (MCAO).

2.5. Surgical Procedure to Induction of Middle Cerebral Artery Occlusion (MCAO)

Focal cerebral ischemia was performed according to modified method of (Longa *et al*., 1989). In brief, rats were anesthetized by thiopental sodium at dose of 50 mg kg^{-1} BW. The right common carotid artery and the right external carotid artery were exposed through a ventral midline neck incision and were ligated proximally. A silicone coated nylon monofilament (4-0) suture (USS DGTM sutures; Tyco Healthcare group LP, Connecticut, USA) with its tip

rounded by heating near a flame was inserted through an arteriectomy in the common carotid artery just below the carotid bifurcation and then advanced into the internal carotid artery approximately 17-18 mm distal to the carotid bifurcation until a mild resistance was felt. Occlusion of the origins of the anterior cerebral artery, the middle cerebral artery and the posterior communicating artery was thereby achieved. Then, the wound was sutured, the rats were returned to their cages with free access to food and water. The incision sites were infiltrated with 10% Povidone-Iodine Solution for anti-septic postoperative care.

2.6. Assessment of Cognitive Function

Animals were tested spatial memory by the water maze test (Morris *et al*., 1982). The apparatus was a pool with 170 cm diameter filled up with tap water for 40 cm deep and the water surface was covered with nontoxic powder. The pool was divided into four 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 environment 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 or acquisition time.

2.7. Acetylcholinesterase Assay

After the animals were sacrificed, brains were isolated and kept cool in ice buckets. Then the tissues of hippocampus were homogenized in 4 volume of 1.15% KCl with glass Potter-Elvehjim homogenizer (Trounce, 1973). An AChE assay was performed using the colorimetric method (Ellman *et al*., 1961) with minor modifications. The hippocampus was homogenized in 0.1 M phosphate buffer, pH 8. The reaction mixture consist of 2.6 mL of phosphate buffer (0.1M, pH 8.0)0.4 ml aliquot of homogenate and 0.1 mL of 0.01 M dithiobisnitrobenzoic Acid (DTNB). After the addition of the substrate acetylthiocholine iodine (0.075 M) change in the absorbance was noted every 2 min for 10 min at 412 nm using a spectrophotometer. The activity was expressed as micromoles hydrolyzed per milligram protein of tissue (μmg^{-1}.protein).

2.8. Immunohistochemical Staining of Cholineacetyltransferase (ChAT)

A series of sections containing hippocampus were reacted in a mouse monoclonal antibody directed 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, the sections were eliminated endogenous peroxidase activity by 0.5% H_2O_2 in methanol. 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 buffer was removed and then incubated for 30 min in a blocking solution composed of 5% normal goat serum in KPBS-BT. Then, the sections were incubated in mouse primary antibody against ChAT diluted 1: 100 in KPBS-BT at room temperature for 2 h and incubate at 4°C for 48 h. The tissue was rinsed in KPBS-BT (2 washes x 7 min), incubated for 1 h in biotinylated goat antimouse IgG antibody, rinsed in KPBS-BT (2 washes x 7 min) and then incubated in Strept ABC Complex/HRP for 4 h. The sections were rinsed in KPBS-BT (1 min) and KPBS (2 washes x 10 min). ChAT immunoreactivity was visualized using 0.025% 3, 3' diaminobenzedine (DAB, Sigma) and 0.01% H_2O_2. For 24 h. Finally, sections were rinsed in running tap water, air dried and cover-slipped using permount.

2.9. 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.

2.10. Statistical Analysis

Data were presented as mean±Standard Error of Mean (SEM). The analysis was performed using one-way Analysis of Variance (ANOVA), followed by LSD test. All statistical results were considered significant at p-value<0.05.

3. RESULTS

3.1. Effect of mulberry Fruits Extract in Animal Model of Stroke in Morris Water Maze Test

Cognitive deficit is commonly clinical sign in vascular dementia. In this study examined the effect of mulberry fruit extract on spatial memory both healthy condition and stroke condition, after induce middle cerebral artery occlusion. Data were recorded escape latency and retention time. In healthy condition, the results showed significant changes in escape latency and retention times in rats which received either vitamin C or Aricept®. Moreover, mulberry fruit significantly decreased escape latency time in dose 50 mg kg^{-1}BW in single dose administration and increased retention time in mulberry fruit at dose of2, 10 mg kg^{-1}BW on single dose (p-value < 0.05, all; compared with vehicle treated groups) (**Fig. 1-2**).

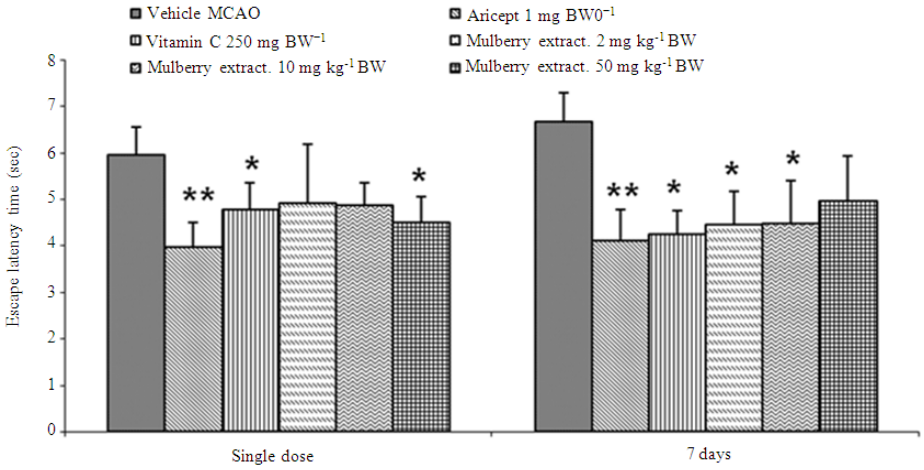

Fig. 1. The effect of mulberry fruit extract on escape latency time in healthy condition. Rats were treated with vehicle, Aricept®, vitamin C or the mulberry fruit extract at various doses ranging from 2, 10 and 50 mg kg^{-1}-1BW via oral route for 7 days, then

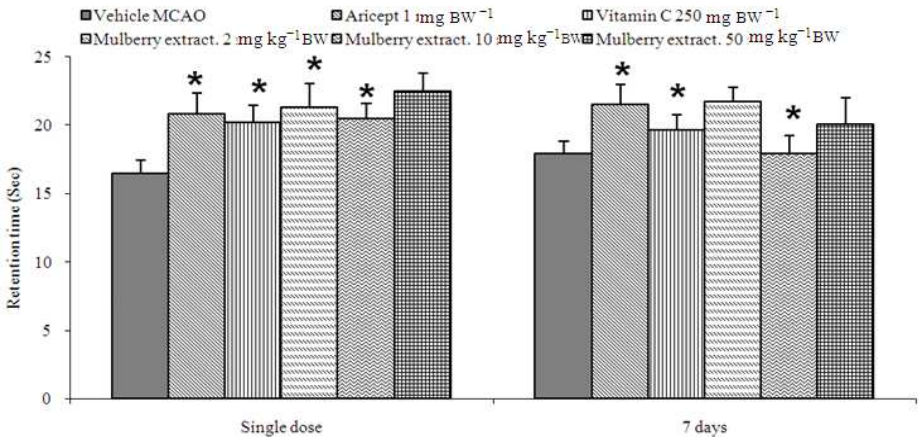

Fig. 2. The effect of mulberry fruit extract on retention time in healthy condition. Rats were treated with vehicle, Aricept®, vitamin C or the mulberry fruit extract at various doses ranging from 2, 10 and 50 mg kg^{-1}-1BW via oral route for 7 days, then they

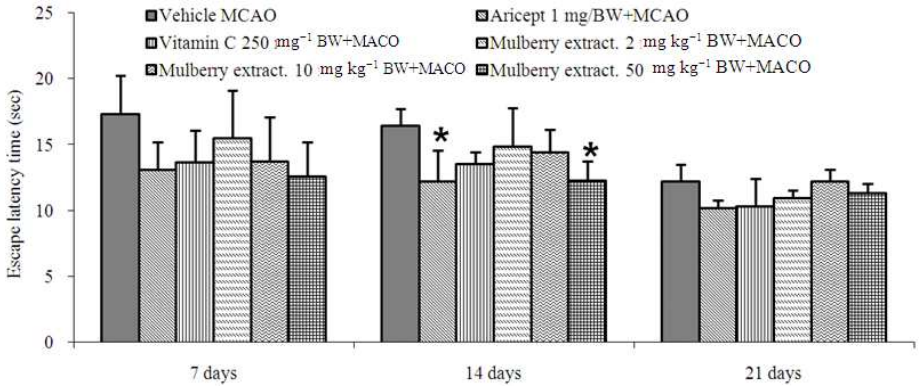

Fig. 3. The effect of mulberry fruit extract on escape latency time in stroke condition. Rats were treated with vehicle, Aricept®, vitamin C or the mulberry fruit extract at various doses ranging from 2, 10 and 50 mg kg^{-1}-1BW via oral route for 21 days, then

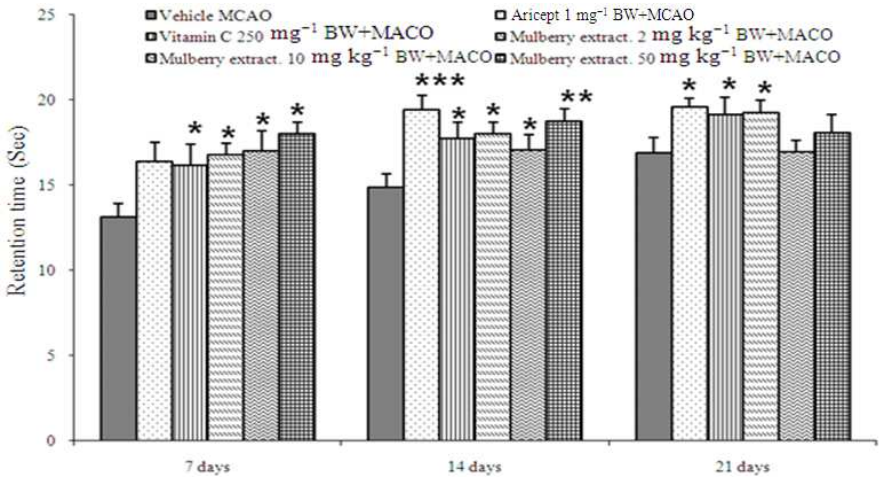

Fig. 4. The effect of mulberry fruit extract on retention time in stroke condition. Rats were treated with vehicle, Aricept®, vitamin C or the mulberry fruit extract at various doses ranging from 2, 10 and 50 mg kg^{-1}-1BW via oral route for 21 days, then they

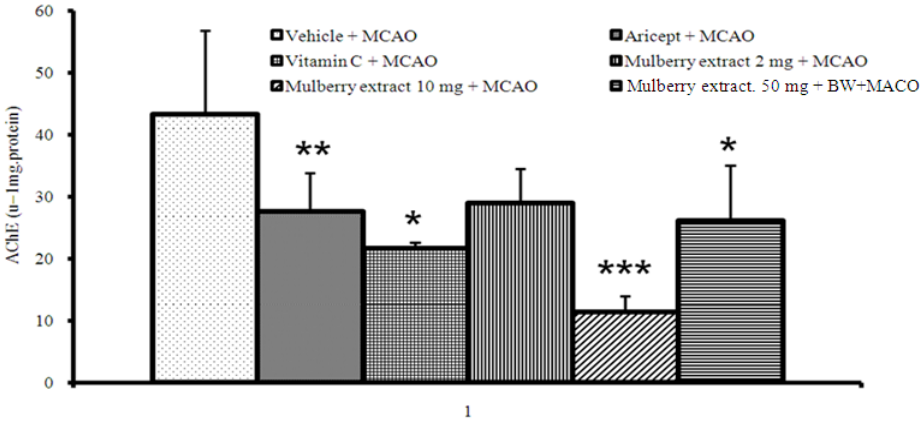

Fig. 5. The effect of mulberry fruit extract on the alteration of acetylcholinesterase enzyme activity in hippocampus. Rats were treated with vehicle, Aricept®, vitamin C and various doses of mulberry fruit extract via oral route for 7 days, then they w

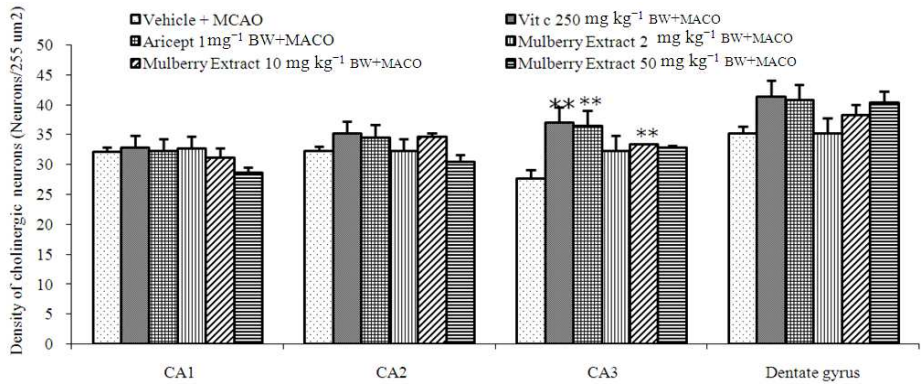

Fig. 6. The effect of mulberry fruit extract on alteration of density of cholinergic neuron in various subregion of hippocampus. Data are presented as mean ± SEM (n=6/group) * p-value<0.05 compared with vehicle plus MCAO group

Table 1. Effect of Aricept®, vitamin C and various doses of mulberry fruit extract on motor function using neurological score. Values given are the mean ± SEM (n = 6)

		Post surgery		
Group/day	Pre surgery	7 days	14 days	21 days
Vehicle + MCAO	5.00±0.00	2.87±0.23	3.50±0.20	3.75±0.21
Aricept® + MCAO	5.00±0.00	3.5±0.22	4.25±0.18**	4.75±0.15***
Vitamin C + MCAO	5.00±0.00	4.5±0.92***	4.62±0.34***	4.87±0.27***
Mulberry extract 2 mg kg^{-1}BW + MCAO	5.00±0.00	3.87±0.21*	4.25±0.17***	4.75±0.15***
Mulberry extract 10 mg kg^{-1}BW + MCAO	5.00±0.00	4.12±0.20***	4.62±0.17***	4.87±0.14***
Mulberry extract 50mg kg^{-1}BW + MCAO	5.00±0.00	3.00±0.23	4.62±0.15***	4.75±0.15***

*, **, ***p-value <0.05; .01 and .001 respectively ; compared with vehicle plus MCAO

In stroke condition, Vitamin C, Aricept® and mulberry fruit extract extract at dose 50 mg kg^{-1}BW significantly decreased the escape latency time in Morris water maze test after MCAO 7 days after MCAO when compared to the vehicle plus MCAO group. Moreover, the significant effect of mulberry fruit extract at dose 2, 10, 50 mg kg^{-1} BW was observed in retention time of Morris water maze test after 7 and 14 days of post MCAO but no significant was observed in 21 days post MCAO (**Fig. 3-4**). Vitamin C treated rats significantly increased neurological score throughout the experimental period (p-value<.001 all; compared with vehicle plus MCAO; compared with vehicle plus MCAO). All doses of mulberry fruit extract significantly improved neurological score throughout the experimental period except the high dose of mulberry fruit extract which started to show the significant improvement at 14 days of treatment until the end of experimental period. (p-value<0.001 all; compared with vehicle plus MCAO) (**Table 1**).

3.2. Effect of Mulberry Fruit Extract on Acetylcholineasterse Activity

The cholinergic system plays the crucial role in spatial memory, the effect of mulberry fruit extract on the activity of AChE, an indirect indicator of cholinergic function was evaluated. It was found that the mulberry fruit extract at 10 and 50 mg^{-1}kg BW ,vitamin C and Aricept® significantly decreased the AChE activity in the hippocampus homogenate (p-value < 0.05 compared to vehicle+MCAO) (**Fig. 3**). However, the low doses (2 mg kg^{-1}BW respectively) of mulberry fruit extract failed to show significant alterations on this enzyme activity (**Fig. 5**).

3.3. Effect of Mulberry Fruit Extract on Cholinergic Neuron Density

The alteration of density of Cholineacetyl Transferees (ChAT) positive stained neurons in various sub-regions of hippocampus after induction of MCAO, The medium dose of mulberry fruit extract (10 mg kg^{-1}BW) significantly enhanced cholinergic neurons density in CA3 ($P < 0.01$, compared to vehicle plus MCAO) (**Fig. 6**).

4. DISCUSSION

In this study, we have demonstrated the neuroprotective effect of Mulberry fruits extract via oral route administration in the vascular dementia model rats. Our data showed that mulberry fruits decreased the escape latency time and increased retention time of Morris water maze test. The neurological score was increased. The number of cholinergic neurons was increases in hippocampus CA3 and decreased the level of acetyl cholinesterase activity when compare with vehicle treated groups.

The cognitive enhancing effect observed in this study associated with the inhibition of acetylcholinesterase in hippocampus, an area which played a crucial role in learning and memory. Acetyl cholinesterase inhibitors use as drug in mild cognitive impairment and behavioral symptoms of Alzheimer's disease with cerebrovascular disease and vascular dementia. For Donepezil (Aricept®) is a drug that moderate the acetylcholine response by inhibiting acetylcholinesterase enzyme. It has been suggested that could produce a beneficial cascade of neurotransmitters in the brains of dementia patients (Maelicke, 2000).

Mulberries (*Morus alba* Linn.) are anthocyanins rich fruits, which are a traditional Chinese medicine. The major anthocyanins identified in the fruit extract are cyanidin 3-glucoside and cyanidin 3-rutinoside (Suh *et al*., 2003). Recently, mulberry fruit has been reported to possess medicinal benefit, such as potent anti-oxidant activity (Chen *et al*., 2005; Kang *et al*., 2006), anti-inflammation (Shin *et al*., 2006), anti-thrombotic (Allen and Bayraktutan, 2009). Moreover, mulberry exhibit inhibitory effect on the migration and invasion of a human lung cancer cell line (Chen *et al*., 2005). Therefore, exploring the diverse bioactivities of this extract can be exploited pharmacologically resulting to candidate for decreasing the aliments associated with stroke. It had been shown that the memory impairment was involved with the neuronal damage in brain (Mori *et al*., 1997). Therefore, the neuron density and cholinergic neurons density were also investigated in hippocampus. Mulberry fruit extract could significantly increase neurons density in CA3 hippocampus while no significant changes in CA1, CA2 and dentate gyrus.

Based on this finding and the crucial role of cholinergic system on learning and memory, the cognitive enhancing effect particularly the spatial memory of mulberry fruit extract might be related to the increase cholinergic function in hippocampus. So, the exploring of this extract can be exploited pharmacologically resulting to candidate for decreasing the aliments associated with stroke.

5. CONCLUSION

In summary, we conclude that, this study provides experimental evidence for the potential effect of mulberry fruits extract to enhance cognitive effect in model of vascular dementia. But further studies are still required.

6. ACKNOWLEDGEMENT

This study was supported by the Integrative Complementary Alternative Medicine Research Group, Faculty of Medicine, Thailand and The Queen Sirikitti Department of Sericulture, Ministry of Agriculture and cooperative, Bangkok, Thailand.

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