Cognitive enhancement and neuroprotective effects of *Bacopa monnieri* in Alzheimer's disease model

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ABSTRACT

Ethnopharmacological relevance: Bacopa monnieri (L.) Wettst., a plant belonging to the family Scrophulariaceae, has been used in the traditional system of Ayurvedic medicine to improve intelligence and memory for a long time. Therefore, the potential of this plant to protect against Alzheimer's disease has been raised but less supported document is available.
Aim of the study: To determine the effect of alcoholic extract of *Bacopa monnieri* on cognitive function and neurodegeneration in animal model of Alzheimer's disease induced by ethylcholine aziridinium ion (AF64A).
Materials and methods: Male Wistar rats were orally given the alcoholic extract of *Bacopa monnieri* at doses of 20, 40 and 80 mg/kg BW via feeding needle for a period of 2 weeks before and 1 week after the intracerebroventricular administration of AF64A bilaterally. Rats were tested for spatial memory using Morris water maze test and the density of neurons and cholinergic neurons was determined using histological techniques 7 days after AF64A administration.
Results: Bacopa monnieri extract improved the escape latency time ($p < .01$) in Morris water maze test. Moreover, the reduction of neurons and cholinergic neuron densities were also mitigated.
Conclusion: These findings suggest that *Bacopa monnieri* is a potential cognitive enhancer and neuroprotectant against Alzheimer's disease.

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1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative disorder, has been recognized as a common and challenging dementia that primarily affects the elderly population (Francis et al., 1999; Desgranges et al., 1998). At present, the etiology of Alzheimer's disease is still not clearly known. However, growing evidence had suggested that several factors especially the excess free radicals have played an important role in the neurodegeneration in AD (Guidi et al., 2006). It was reported that the brains of patients with AD showed the elevation of lipid peroxidation products such as 4-hydroxynonenal (HNE) or 2-propenal (acrolein). Moreover, the lipid peroxidation in the CSF and plasma of AD patients were also increased (Arlt et al., 2002). Recent studies have demonstrated that various compounds possessing antioxidant activity attenuated the oxidative stress induced by amyloid β-protein (Aβ), inhibited the

formation and extension of β-amyloid fibrils and decreased the plaque burden (Ono et al., 2003, 2006; Choi et al., 2001). Besides the free radicals homeostasis, the cholinergic deficit was also proposed to play a crucial role in memory impairment, an important feature in this condition (Lahiri et al., 2004; White and Ruske, 2002). Therefore, the cholinergic hypofunction induced by a cholinotoxin, AF64A, has been developed as an animal model of dementia in AD (Hanin, 1996).

Several studies reported that many free radical scavengers including α-tocopherol and ascorbic acid could slow the progression of the disease and could reduce the risk for AD (Zandi et al., 2004). Moreover, relatively long-term antioxidant treatment also delayed the onset of disease (Emilien et al., 2000; Kontush and Schekatolina, 2004). Based on the information mentioned earlier, the research to develop the substance enhancing cholinergic function from antioxidant has been focused.

Bacopa monnieri (L.) Wettst., commonly known as Brahmi, is a creeping bitter tasted plant found in a damp and marshy area (Chopra et al., 1956). It belongs to the family Scrophulariaceae, commonly used in a traditional system of Ayurvedic medicine as nerve tonic, diuretic, cardiotonic and therapeutic agents against epilepsy, insomnia, asthma and rheumatism. Numerous stud-

ies also showed that this plant extract possessed anxiolytic, anti-depression (Bhattacharya and Ghosal, 1998) and antioxidant activity (Bhattacharya et al., 2000). Based on its reputation for nerve tonic and its antioxidant activity, we hypothesized that this plant extract could mitigate the memory impairment and neurodegeneration in animal model of Alzheimer's disease.

2. Materials and methods

2.1. Plant materials and preparation of crude extract

Aerial parts of *Bacopa monnieri* (Scrophulariaceae) were kindly donated by Health and Herbs Company. The plants were collected from Petchaburi province, Thailand, in September 2004. It was authenticated by Associate Professor Wongsatit Chuakul, Mahidol University, and prepared as alcoholic extract by Associate Professor Kornkanok Ingkaninan, Department of Pharmaceutical Chemistry and Botany, Faculty of Pharmaceutical Science, Naresaun University. The voucher specimen (Phrompittayarat001) was kept at the PBM Herbarium, Mahidol University, Thailand. Plant material was dried at $50\,^{\circ}C$ for 12 h. The dry material was milled, obtaining crude powder and then extracted by percolation with 95% ethanol at the ratio of 1 g:7 ml. The plant materials were dried again under the same conditions and extracted exhaustively with ethanol. The alcoholic extract was dried in a rotary evaporator under reduced pressure. The percent yield obtained was 10% (Phrompittayarat et al., 2007a,b). The extract contained 5% (w/w) of total saponins, the mixture of bacoside A_3, bacopaside II, bacopasaponin X, bacopasaponin C and bacopaside I. The total saponin content was determined using HPLC as previously reported (Phrompittayarat et al., 2007a,b). The extract was stored at $-20\,^{\circ}C$ in a dark bottle until used.

2.2. Animals

Adult male Wistar rats (180–200 g, 8 weeks old) were obtained from National Animal Center, Salaya, Nakorn Pathom, and were housed in group of 5 per cage in standard metal cages at $22 \pm 2\,^{\circ}C$ on 12:12 h light–dark cycle. All animals were given access to food and water ad libitum. The experiments were performed after the approval of protocol by the Ethical Committee of the Institution and every effort was made 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).

2.3. Experimental protocol

All rats were randomly assigned to 6 groups of 8 animals each as follows: (1) Vehicle: rats were orally given propylene glycol which served as vehicle to suspend the *Bacopa monnieri* extract fed via feeding needle for a period of 2 weeks before and 1 week after the administration of artificial cerebrospinal fluid (ACSF); (2) Vehicle + AF64A: rats were treated as similar as group I except that the administration of AF84A, a cholinotoxin, was performed instead of ACSF administration; (3) Aricept + AF64A: the animals were orally given an acetylcholine esterase inhibitor, Aricept (donepezil), which is known as standard drug for dementia treatment that served as positive control in this study. Then, they were administered with AF64A as mentioned in group II (4)–(6) BM + AF64A: rats were treated with the plant extract at different doses of 20, 40 and 80 mg/kg BW (Chowdhuri et al., 2002) for 2 weeks before and 1 week after the administration of AF64A. The animals were tested for the spatial memory 1 week after AF64A administration and then they were sacrificed and the density of survival neurons in various subregions of hippocampus was determined. The plant

extract at dose of 40 mg/kg BW was selected for further study on the alteration of density of cholinergic neurons in all areas of hippocampus because this dose produced the maximum cognitive function improvement.

2.4. AF64A administration

AF64A was prepared as described previously by Hanin (1996). Briefly, 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 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. 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 and the needle was left in place for 5 min after infusion and then slowly withdrawn.

2.5. Morris water maze test

The water maze consisted of a metal pool (170 cm in diameter × 58 cm tall) filled with tap water ($25\,^{\circ}C$, 40 cm deep) divided into 4 quadrants (NE, NW, SE, and SW) by two imaginary lines crossing the center of the pool. In the center of 1st quadrant was a removable escape platform below the water level and covered with a nontoxic milk powder. For each animal, the location of invisible platform was placed at the center of one quadrant and remained there throughout training. The rats must memorize the platform location in relation to various environmental cues because there was nothing to directly show the location of the escape platform in and outside the pool. Therefore, the placement of the water tank and platform were the same in all acquisition trials. 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 found and climbed onto the platform. During training session, the rat was gently placed on the platform by experimenter when it could not reach the platform in 60 s. In either case, the subject was left on the platform for 15 s and removed from the pool. The time for animals to climb on the hidden platform was recorded as escape latency. Time spent in the region that previously contained the platform was recorded as retention time. In each trial, the animal was quickly dried with towel before being returned to the cage. All tests were carried out within 45 min after the administration of vehicle or plant extract or aricept, a cholinesterase inhibitor, which served as positive control.

2.6. Histological procedure

Following anesthesia with sodium pentobarbital (60 mg/kg 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 overnight in a fixative solution that is used for perfusion. Then, they were infiltrated with 30% sucrose solution at approximately $4\,^{\circ}C$. The specimens were frozen rapidly and 30 μM thick sections were made using 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.

2.7. Nissl staining

The duplicate coronal sections of brains were stained with 0.75% cresyl violet, dehydrated through graded alcohols (70, 95, 100% 2×), placed in xylene and cover-slipped using DPX mountant.

2.8. Choline acetyltransferase and immunohistochemistry

A series of section containing hippocampus from each groups were reacted in parallel experiments using a mouse mono-clonal antibody detected against choline acetyltransferase (ChAT) (Chemicon International, 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 was removed, and then incubated for 30 min in a blocking solution composed of 5% normal horse serum in KPBS-BT. The 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, 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% H_2O_2. 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 400× 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. Statistic analysis

All data were expressed as mean ± S.E.M. value. The significant differences among various groups were compared by ANOVA and followed by Duncan's test. The statistical difference was regarded at $p < 0.05$.

3. Results

3.1. Effect of Bacopa monnieri on spatial memory

Fig. 1A and B showed that AF64A administration significantly increased the escape latency but decreased retention time ($p < .001$ and .05, respectively). Aricept and the plant extract treatment at dosage used in this study significantly decreased the escape latency ($p < .01$). However, no significant change in retention time
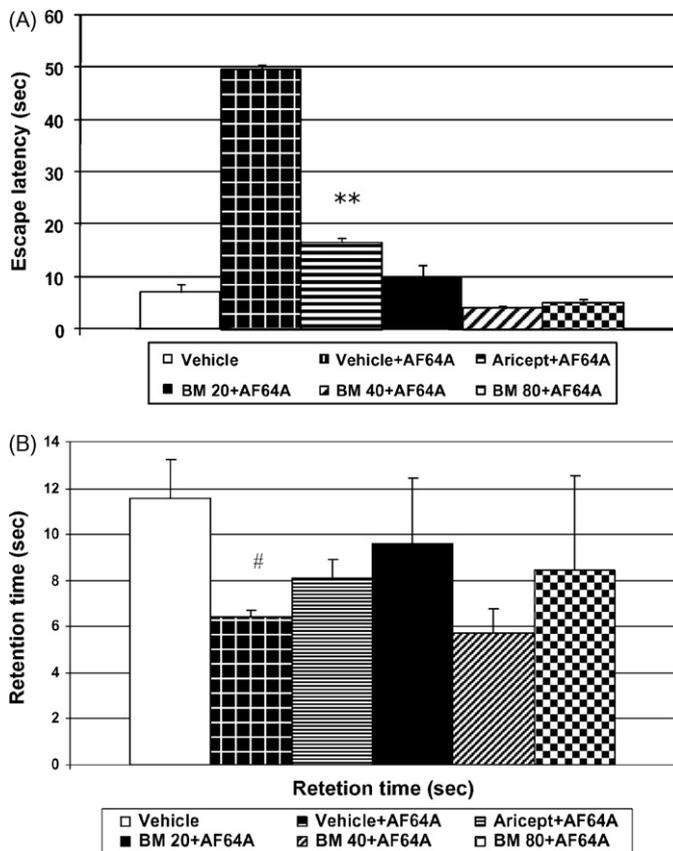

Fig. 1. (A) The effect of *Bacopa monnieri* extract on the escape latency in Morris water maze test. Rats had been orally treated with vehicle or aricept or *Bacopa monnieri* extract at various doses ranging from 20, 40 and 80 mg/kg BW for 2 weeks before and 1 week after the AF64A administration via intracerebroventricular route bilaterally, then they were determined the escape latency or the time to reach the platform in Morris water maze test. Data are presented as mean ± S.E.M. $n = 8$/group. ###$p < 0.001$ compared with vehicle treated group. *$p < 0.01$ compared with vehicle + AF64A treated group. (B) The effect of *Bacopa monnieri* extract on the retention time in Morris water maze test. Rats were treated with vehicle or aricept or *Bacopa monnieri* extract at various doses ranging from 20, 40 and 80 mg/kg BW via intragastric route for 2 weeks before and 1 week after the AF64A administration via intracerebroventricular route bilaterally, then they were determined the retention time in Morris water maze test. Data are presented as mean ± S.E.M. $n = 8$/group. #$p < 0.05$ compared with vehicle treated group.

was observed. Our study failed to show the dose-dependent manner.

3.2. Neuroprotective effect of Bacopa monnieri

The effect of *Bacopa monnieri* extract on the neurons density in various subregions of hippocampus was shown in Fig. 2A. AF64A administration significantly decreased the neuron density in CA2, CA3 and dentate gyrus ($p < .05$ all). Aricept which used as positive control in this study could attenuate the decrease of neurons density only in CA3 ($p < .05$). It was found that low dose of *Bacopa monnieri* extract could mitigate the reduction of neurons density induced by AF64A in all areas of hippocampus while the medium dose could produce significant changes only in CA1, CA2 and CA3 ($p < .05$ all). The highest dose could produce significant change of this parameter only in CA1 ($p < .05$).

Based on the information about cognitive enhancing effect of this extract mentioned earlier, the *Bacopa monnieri* extract at dose of 40 mg/kg BW was selected for further study on the changes of cholinergic neuron densities in hippocampus, the area play-

ing an important role on spatial memory. Data were shown in Fig. 2B. AF64A produced significant reduction in cholinergic neuron densities in all areas of hippocampus. Aricept could mitigate the decrease of cholinergic neuron densities in all areas of hippocampus investigated in this study but *Bacopa monnieri* extract at dose of 40 mg/kg BW could significantly attenuate the decrease of cholinergic neuron densities only in CA1 and CA2 ($p < .05$ all).
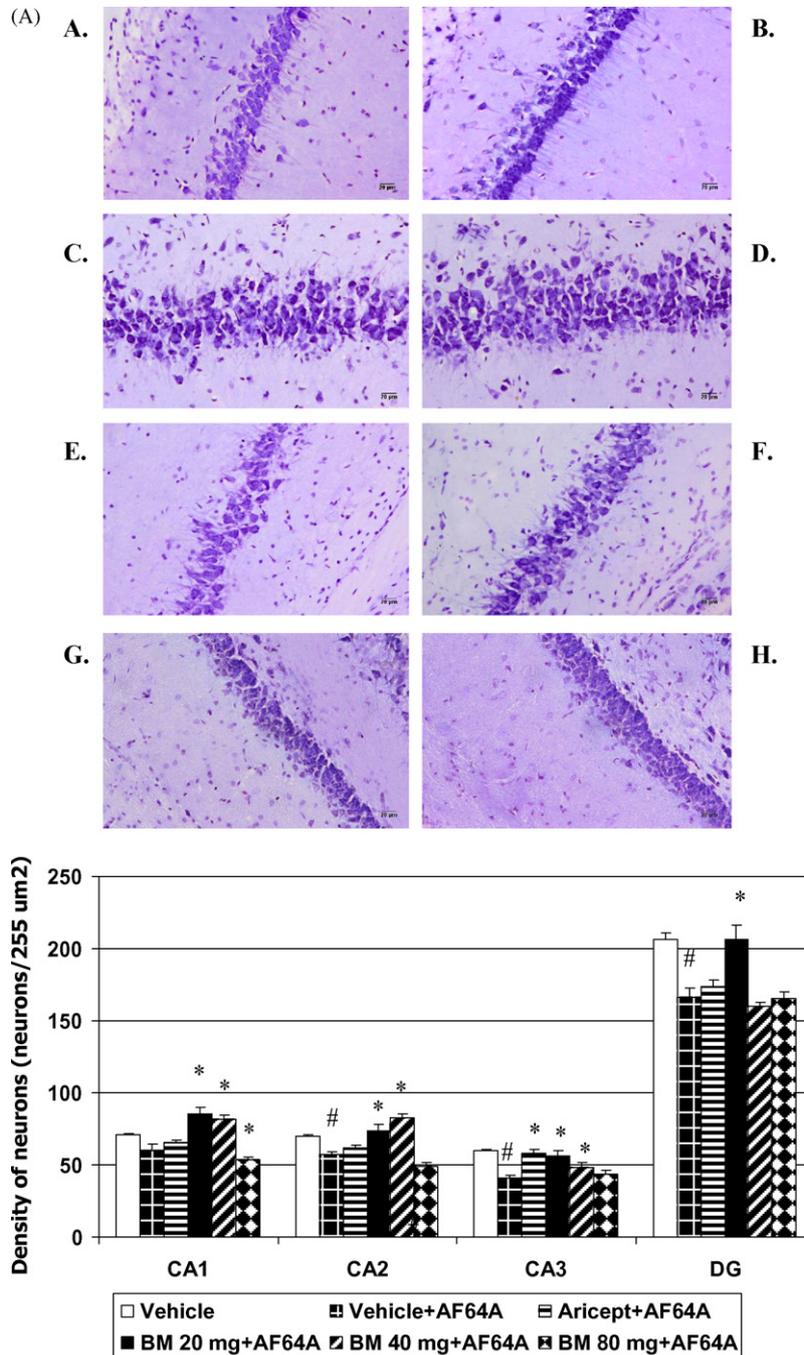

Fig. 2. (A) The effect of *Bacopa monnieri* extract on the density of survival neurons in various subregions of hippocampus in Alzheimer's disease model induced by AF64A. Rats had been orally administered vehicle or aricept or *Bacopa monnieri* at various doses ranging from 20, 40 and 80 mg/kg BW for 2 weeks before and 1 week after the bilateral administration of AF64A via intracerebroventricular route, then they were sacrificed and the brains were cut as coronal sections at 25 μm thick. The sections were stained with cresyl violet. The density of survival of neurons in hippocampus was determined under light microscope at 40× magnification using Image proplus program. (A) CA1 of Vehicle plus AF64A, (B) CA1 of BM 40 mg/kg BW plus AF64A, (C) CA2 of Vehicle plus AF64A, (D) CA2 of BM 40 mg/kg BW plus AF64A, (E) CA3 of Vehicle plus AF64A, (F) CA3 of BM 40 mg/kg BW plus AF64A, (G) dentate gyrus of Vehicle plus AF64A, (H) dentate gyrus of BM 40 mg/kg BW plus AF64A. Data are presented as mean ± S.E.M. $n = 8$/group. (DG: dentate gyrus). $^{\#}p < 0.05$ compared with vehicle treated group. $^{*}p < 0.05$ compared with vehicle + AF64A treated group. (B) The effect of *Bacopa monnieri* extract on the cholinergic neurons density in various subregions of hippocampus in Alzheimer's disease model induced by AF64A. Rats had been orally administered vehicle or aricept or *Bacopa monnieri* at dose of 40 mg/kg BW for 2 weeks before and 1 week after the bilateral administration of AF64A via intracerebroventricular route, then they were sacrificed and the brains were cut as coronal sections at 25 μm thick. The sections were stained with monoclonal antibody against choline acetyltransferase enzyme (ChAT). The density of cholinergic neurons in hippocampus was determined under light microscope at 40× magnification using Image proplus program. (A) CA1 of Vehicle plus AF64A, (B) CA1 of BM 40 mg/kg BW plus AF64A. Data are presented as mean ± S.E.M. $n = 8$/group. (DG: dentate gyrus). $^{\#}p < 0.05$ compared with vehicle treated group. $^{*}p < 0.05$ compared with vehicle + AF64A treated group.
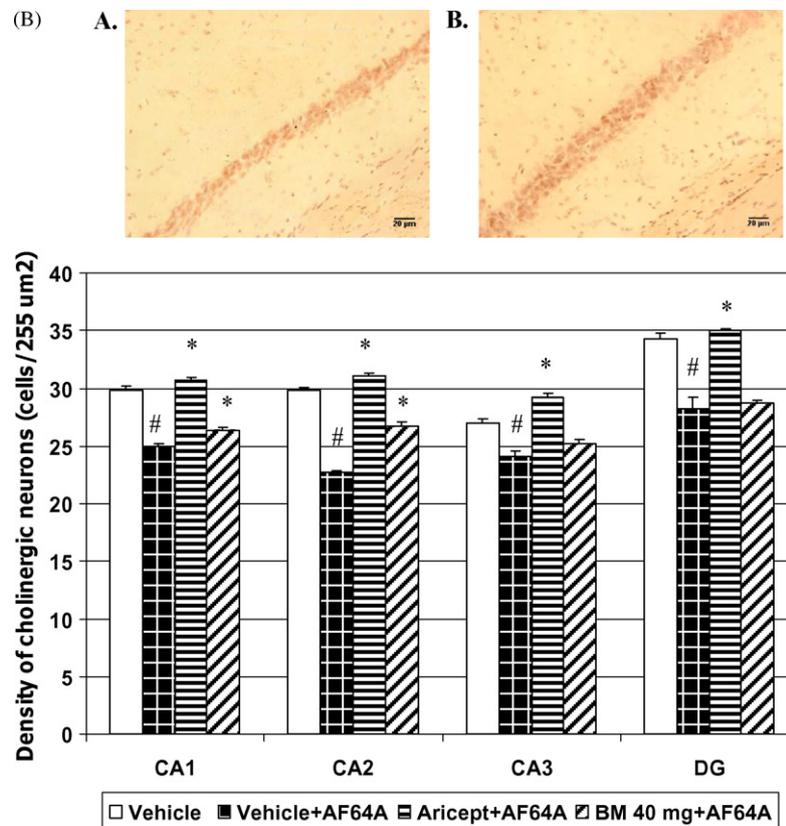
(B)

Fig. 2. (Continued).

4. Discussion and conclusion

The present study demonstrated that *Bacopa monnieri* extract could mitigate the memory impairment and the degeneration of neurons in hippocampus in animal model of Alzheimer's disease induced by AF64A. Our data showed no tight correlation between the effect of cognitive enhancing effect and the neuroprotective effect of *Bacopa monnieri* extract. This suggested that other mechanisms might also play roles on cognitive enhancing effect. Numerous factors were reported to improve the memory impairment including the cerebral blood flow (Tong et al., 2009), the brain oxidative stress status (Nicolakakis et al., 2008), the balance function of various neurotransmitters including acetylcholine, serotonin, catecholamine (Reis et al., 2009), GABA (Kant et al., 1996) and glutamate (Saraf et al., 2009). Recent findings showed that the alcoholic extract of this plant could improve acetylcholine and cerebral blood flow (Kishore and Singh, 2005). This might be responsible in part for the explanation of the similar magnitude of cognitive enhancement between *Bacopa monnieri* and Aricept even though Aricept could produce neuroprotective effect in hippocampus area more than *Bacopa monnieri*.

It had been reported that the alcoholic extract of *Bacopa monnieri* comprised of various types of saponin including bacopasaponin A, B, C, D, pseudojujubogenin, bacopaside I, II, II, IV and V. Besides the substance mentioned above, the extract also contained other ingredients such as brahmine, herpestine and monnierin (Kapoor et al., 2009). Therefore, the increasing doses of the extract might increase all ingredients concentration and result in the masking effect of active ingredient. This might attribute to the lack of dose-dependent response in this study.

We also found that various subregions of hippocampus showed different vulnerability to the substances either AF64A or the plant extract. The difference in this vulnerability might be associated with the difference in distribution of various types of neuronal system such as cholinergic neurons, granule cells, the different distribution of various factors contributing important roles on the survival of neurons and cholinergic neurons including the ability to form free radicals, the distribution of antioxidant system, the distribution of calcium binding proteins and the difference in the trophic factor particularly nerve growth factor (NGF) and brain derived growth factor (BDNF) distribution. However, the precise underlying mechanisms still required further investigation.

Although the precise underlying mechanism of the *Bacopa monnieri* extract to protect neurons and cholinergic neurons against toxicity induced by AF64A was beyond the scope of this study. Based on previous findings that this plant extract could directly inhibit the superoxide anion formation (Russo and Borrelli, 2005) and could increase the hippocampal superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities, we suggested that the neuroprotective and cholinoprotective effects of the plant extract might be partly due to its antioxidant (Bhattacharya et al., 2000). However, this still required further investigations. In conclusion, the present data supported the efficacy of *Bacopa monnieri* according to the traditional system and provided supported document about the neuroprotective effect against the cholinergic degeneration and cognitive enhancing effect of *Bacopa monnieri* in Alzheimer's disease model. Therefore, this plant is a valuable candidate for cognitive enhancer and neuroprotective agent in Alzheimer's disease. However, further researches are needed to identify the active constituent of the extract responsible for its nootropic activity and the possible underlying mechanism.

Acknowledgements

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