CHAPTER V
EFFECT OF NOVEL FOOD SUPPLEMENT "CP1" ON MEMORY DEFICIT, NEURODEGENERATION AND CHOLINERGIC DYSFUNCTION IN ANIMAL MODEL OF ALZHEIMER'S DISEASE

1. Introduction

Dementia, a condition of memory and intellectual impairment, is increasing its importance in accompany with the increased older population. It has been reported that the total number of people with dementia worldwide in 2010 is estimated at 35.6 million and is projected to nearly double every 20 years, to 65.7 million in 2030 and 115.4 million in 2050. This condition produces a great impact on healthcare budget and social care (Organization, 2013). Therefore, it has gained much attention.

Dementia especially the age-related dementia is associated with many factors including forebrain and hippocampal atrophy (Lye *et al.*, 2004; Rusinek *et al.*, 2003), acetylcholine (ACh) reduction (Ikarashi *et al.*, 2004), cholinergic hypofunction (Bergmann *et al.*, 1997; Schliebs and Arendt, 2006), and degeneration of basal forebrain cholinergic neurons, neurotrophic signaling reduction (Bergmann *et al.*, 1997) and excess oxidative stress (Mecocci *et al.*, 1997). Based on the crucial role of hypocholinergic function on dementia mentioned earlier, the current anti-dementia drugs are targeting at the enhanced cholinergic function. However, the current therapeutic efficacy is still limited and the adverse effects are commonly found (Rungsanpanya *et al.*, 2012). Therefore, the protection is required.

Medicinal plants are long-term used for longevity promotion, neuroprotection and memory enhancer in traditional folklore. Both *Cyperus rotundus*, a plant in a family of Cyperaceae, and *Zingiber officinale*, a plant in a family of Zingiberaceae, are reputed for both longevity promotion. Scientific data have demonstrated that *C.rotundus* and *Z.officinale* possess antioxidant, acetylcholinesterase inhibitory (AChEI), neuroprotective and memory enhancing effects (Bashir *et al.*, 2012; Hemanth Kumar *et al.*, 2013; Rabiei *et al.*, 2013; Saenghong *et al.*, 2011; Sharma and Gupta, 2007; Wattanathorn *et al.*, 2010). Based on the crucial role of hypocholinergic

function and oxidative stress on dementia, the beneficial effect of both plants on dementia is focused. In order to optimize the benefit of the plant extracts, the positive modulation effect on the interaction of both plants has gained attention. It was hypothesized that the combination extract of *C.rotundus and Z.officinale* (CP1) could protect against age-related dementia. To test this hypothesis, we aimed to determine the antioxidant effect and AChEI of CP1. In addition, the in vivo study was also carried out to determine the neuroprotective effect against age-related dementia in animal model induced by a cholinotoxin, AF64A.

2. Materials and Methods

2.1 Plant collection and extract preparation

Aerial part of *C.rotundus* and rhizome of *Z.officinale* were harvested from Khon Kaen province, Thailand during September – November 2012. They were authenticated by Associate Professor Panee Sirisa-ard, from Faculty of Pharmacy, Chiang Mai University, Thailand. The plant materials were prepared as 95% alcoholic extract. A ratio of alcoholic extract of aerial part of *C.rotundus* and rhizome of *Z.officinale* which provided the highest potential to enhance memory (1:5) was prepared. The percent yield of the *C.rotundus* and *Z.officinale* extracts were 7.41% and 10.48% respectively. The combined extract contained gingerol and quercetin at concentration of 138.94 and 87.83 mg/g of the combined extract respectively as shown in figure 1. The combined extract was kept at -20°C in a dark bottle until used.

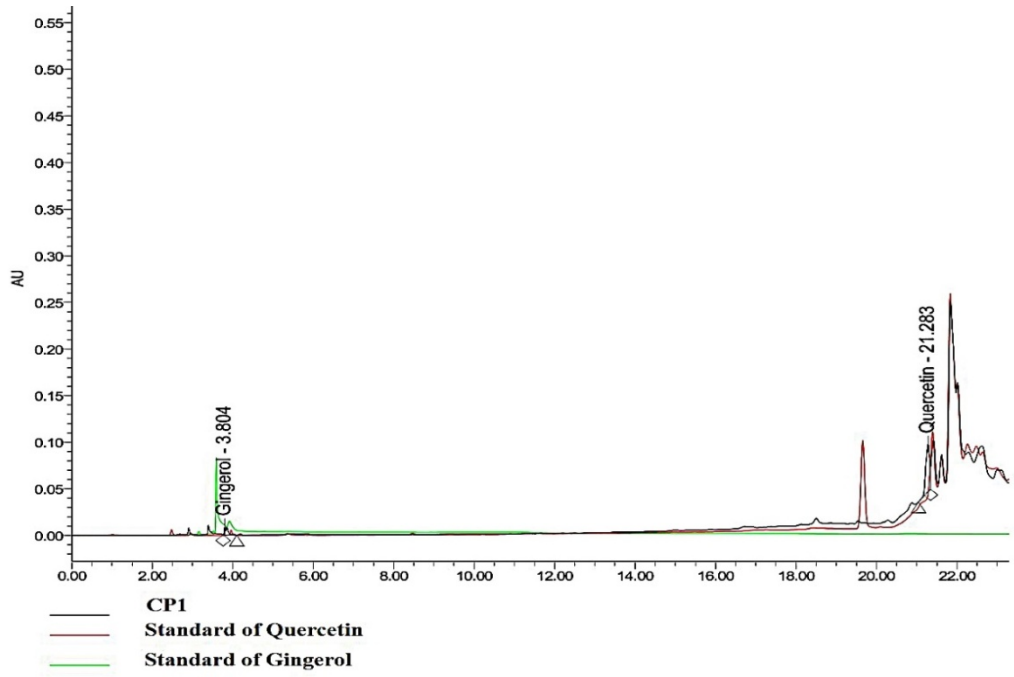

Figure 5-1 High performance liquid chromatography (HPLC) chromatogram of the combined extract of *Zingiber officinale* and *Cyperus rotundus*

2.2 Determination of antioxidant activity

Radical scavenging activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical of the combined extract of *Z.officinale* and *C.rotundus* (CP1) was determined spectrophotometrically (De Ancos *et al.*, 2002). The principle of the assay is based on the color change of the DPPH solution from purple to yellow when the radical is quenched by the antioxidant. In brief, 2.96 ml of a 0.1 mM solution of DPPH in methanol was incubated with 40 µl of various concentrations of extract (1.0, 2.0, 5.0, 10.0, 20.0, 25.0 mg/ml) at room temperature for 30 min. The decrease in DPPH radicals was evaluated by measurement of optical density at 515 nm. The stable free radical scavenging capacity is presented as the percentage of inhibition of DPPH radicals, calculated according to the following equation:

% inhibition of DPPH = (Abs control-Abs sample/Abs control) X 100

2.3 Determination of antioxidant activity by Ferric Reducing Antioxidant Power (FRAP)

Ferric reducing antioxidant power assay was evaluated according to the procedure previously described (Benzie and Strain, 1996) with some modifications.

Briefly, the working FRAP reagent was mixed with 25 mL of 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 m$LC_2H_4O_2$), pH 3.6, 2.5 mL of 10 mM tripyridyltriazine (TPTZ) solution in 40 mM HCl, and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ solution. Then, 1.8 mL of the FRAP solution was mixed with the plant extract (10µL) in 1 mL distilled water. The absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37°C for 10 min. The results were expressed as µM Ascorbic acid/100 g fresh weight.

2.4 Determination of acetylcholinesterase (AChE) inhibition

AChE inhibitory activity was measured by using Ellman's colorimetric method (Ellman *et al.*, 1961). Briefly, in the 96 well plates, 25µl of 15 mM ATCI, 75 µl of 3 mM DTNB and 50µl of 50 mM Tris–HCl, pH 8.0, containing 0.1% bovine serum albumin (BSA), and 25µl of the tested phytochemicals were added. The absorbance was measured at 405 nm after a 5-minute incubation at room temperature. Then a 25µl of 0.22 $U.ml^{-1}$ of AChE was added, incubated for 5 minutes at room temperature and the absorbance was measured at 412 nm. Acetylcholinesterase (5–1,000 µM) was used as a reference standard. The percentage inhibition was calculated using the following equation:

Inhibition (%) = $1-(A_{sample}/A_{control})\times100$, where A_{sample} is the absorbance of the sample extracts and $A_{control}$ is the absorbance of the blank (50% aqueous methanol in buffer).

Besides in vitro assay of AChE mentioned earlier, AChE activity in hippocampal homogenate was also determined in vivo. In brief, hippocampus was isolated and homogenized in ice cold 0.1 M phosphate buffer saline (pH 8.0). The homogenate was centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was used as the source of enzyme in AChE assay. AChE activity in hippocampus was evaluated based on Ellman's method with slight modifications (Isomae *et al.*, 2002).

2.5 Animals

Eight-week-old male Wistar rats weighing 180-220 g were used as experimental animals. They were derived from National Laboratory Animal Center, Salaya, Nakorn Pathom. They were housed 6 per cage and maintained in 12: 12 light: dark cycle and given standard pellets diet and water ad libutum. The experiments were performed to minimize animal suffering and the experimental protocols were approved by the Institutional Animal Care and Use Committee Khon Kaen University, Thailand (AEKKU 41/2554).

2.6 Surgical procedures

Sodium pentobarbital (Jagsonpal Pharmaceuticals LTD, Haryana, India) at dose of 60 mg/kg BW was administered to the animals via intraperitoneal route to induce anesthesia. The memory deficit was induced by the bilateral intracerebroventricular (i.c.v.) injection of AF64A (2 nmol/2 µl). The burr holes were performed through skull according to the following stereotaxic coordinates; posterior 0.8 mm, lateral±1.5 mm, and ventral (from dura) 3.6 mm. AF64A was perfused via a 30-gauge needle which was inserted through the burr holes and the perfusion rate was 1.0 µl/min. After being left at the injection site for 5 minutes, the needle was slowly withdrawn. The animals were allowed to recover from anesthesia and then placed in their cages.

2.7 AF64A administration

The preparation of AF64A was performed according to the method of Hanin (Hanin, 1996). In brief, an aqueous solution of acetylethylcholine mustard HCl (Sigma–Aldrich Co., USA) was adjusted to pH 11.3 with NaOH and stirred for 30 minutes. Then pH of the solution was adjusted to pH 7.4 with the gradual addition of HCl and stirred for 60 minutes at room temperature. The amount of AF64A was then adjusted to 2 nmol/2 µl. Artificial cerebrospinal fluid (ACSF) or vehicle of AF64A was distilled water which was prepared in the same manner as the AF64A.

2.8 Experimental protocol

All rats were randomly assigned to 7 groups as follows:

Group I Vehicle+ACSF; rats were orally given propylene glycol which served as vehicle to suspend the combination extract of CP1 once daily for 14 days after the administration of ACSF

Group II Vehicle+AF64A; rats were orally treated with propylene glycol once daily at a period of 14 days after the administration of AF64A

Group III Donepezil+AF64A; the animals were orally treated with Donepezil (Aricept) (1 mg/Kg BW) once daily for a period of 14 days after the administration of AF64A

Group IV Vitamin C+AF64A; the animals were orally treated with Vitamin C (250 mg/kg) once daily for a period of 14 days after the administration of AF64A

Group V-VII CP1 + AF64A; rats were treated with CP1 at doses of 100, 200 and 300 $mg.kg^{-1}$ BW for a period of 14 days after the administration of AF64A

Rats in all groups were orally given the assigned substances at a period of 14 days after the bilateral administration of AF64A via intracerebroventricular route. The memory assessment was performed every 7 days throughout a 14-day study period whereas the determinations of malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and acetylcholinesterase (AChE) in hippocampus were performed at the end of study. Moreover, the density of survival neurons various subregions of hippocampus including CA1, CA2, CA3 and dentate gyrus were also determined.

2.9 Determination of spatial memory

Spatial memory was evaluated using Morris water maze test. Rats were subjected to the water metal pool (170 cm in diameter × 58 cm height) which filled with a tap water (25 ºC, 40 cm deep). This pool comprised of 4 quadrants including northeast, southeast, southwest, and northwest. The water surface was covered with non-toxic milk. The removable platform was immersed below the water level at the center of one quadrant. All rats were trained to memorize the location of the invisible platform by forming the association of their location and the location of platform using the external cue. The time which animals spent to climb on the hidden platform was recorded as escape latency or acquisition time. In order to determine the capability of the animals to retrieve and retain information, the platform was removed 24 hr later and the rats were re-exposed to the same condition except that the platform was removed. The time which each animal spent in the region that previously contained the platform was recorded as retention time (McCord and Fridovich, 1969).

2.10 Determination of density of survival neuron in the hippocampus

2.10.1 Histological study

Following an anesthesia with sodium pentobarbital (60 mg/kg BW), the brain fixation was carried out by transcardial perfusion with a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. After the perfusion, the brains were removed and stored overnight in a fixative solution which was used in the perfusion, infiltrated with 30% sucrose solution and kept at 4° C. The specimens were frozen rapidly and the coronal sections at 10 μM thick were prepared using cryostat. All sections were rinsed in the phosphate buffer and picked up on slides coated with 0.01 % aqueous solution of a high molecular weight poly L-lysine.

2.10.2 Morphological analysis

Five coronal sections of each rat in each group were studied quantitatively. The evaluation of neuron density in hippocampus was performed under light microscope at 40x magnification. 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 was calculated and expressed as density of neurons per 255 μm^2.

2.11 Determination of oxidative stress markers

Rats were perfused with cold saline solution to get rid of the blood from the brain tissue. Then, hippocampus was isolated and prepared as hippocampal homogenate and the determination of the oxidative stress markers were performed. Malondialdehyde (MDA) level was indirectly estimated by determining the accumulation of thiobarbituric acid reactive substances (TBARS) (Ohkawa *et al.*, 1979). In order to determine the activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), hippocampus of each rat was weighed and homogenized with a buffer consisting of 10 mM sucrose, 10 mM Tris–HCl and 0.1 mM EDTA (pH 7.4). Then, a hippocampal homogenate was centrifuged at 3000 g at 4 °C for 15 min. The supernatant was separated and used for bioassays. The activity of SOD was determined using a xanthine/xanthine oxidase system as the source of superoxide radical production and subsequent measurement of cytochrome *c* as a scavenger of the radicals. Optical density was measured using a spectrometer (UV-1601, Shimadzu) at 550 nm (McCord and Fridovich, 1969). SOD activity was presented as units per milligram of protein (U mg^{-1} protein). One unit of enzyme activity was defined as the quantity of SOD required to inhibit the reduction rate of cytochrome *c* by 50%. CAT activity in the supernatant was measured by recording the reduction rate of H_2O_2absorbance at 240 nm (Goldblith and Proctor, 1950). The activity of CAT was expressed as μmol $H_2O_2.min^{-1}mg^{-1}$ protein. GSH-Px was determined using *t*-butyl hydro peroxide as a substrate. The optical density was spectrophotometrically recorded at 340 nm and expressed as U mg^{-1} protein(Eyer and Podhradský, 1986). One unit of the enzyme was defined as micromole (μmol) of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized per minute.

2.12 Western blot analysis

The hippocampus were removed and rapidly frozen at -80°C. The frozen tissues were homogenized in ice cold RIPA buffer with protease inhibitors. The dissolved proteins were collected after the centrifugation at 10,000 g for 30 min, and the supernatant was then collected. Protein concentration was determined using NANO drop Spectrophotometers. Equal amount of protein (35 µg) was separated by SDS-PAGE (10% SDS-polyacrylamide gel electrophoresis) and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). After transferring to membrane, the blots were incubated in a blocking buffer (5% skim milk in Tris-buffer saline with 0.05% Tween-20) for 1 h at room temperature, and incubated overnight either with phospho-ERK1/2 (1:1,000, Cell SignalingCell Signaling Technology, Inc., Boston, MA, USA) or with total ERK1/2 (1:1,000, Cell Signaling Cell Signaling Technology, Inc., Boston, MA, USA). After the incubation, the membrane was subjected to several washing steps. HRP-linked secondary antibody (1:2,000) was incubated with the membrane for 1 hr at room temperatureand signals were visualized by chemiluminescence using a ECL kit (Pierce, ThermoScientific).Images were evaluated by ImageQuant LAS 4000, GE Healthcare. Band densities were quantified with ImageQuant TL (IQTL) software, GE healthcare (Gong *et al.*, 2011).

2.13 Statistical analysis

Data were expressed as means ± S.E.M. and analyzed statistically by one-way ANOVA, followed by Post-hoc (LSD) test. The results were considered statistically significant at P-value < 0.05.

3. Results

3.1 Antioxidant activity and acetylcholinesterase (AChE) inhibition of CP1

In the first part of this study,the antioxidant effects of C. rotundus, Z.officinale and the combination extract of *C.rotundus* and *Z.officinale* (CP1) were determined by using DPPH and FRAP assays. In addition, the acetylcholinesterase (AChE) inhibition was also determined using Ellman's colorimetric method. The results were shown in Table 5-1. Interestingly, our data clearly demonstrated that the

combination extract of *C.rotundus* and *Z.officinale* (CP1) had lower IC50 of FRAP (1.743 mg/ml), DPPH (1.008 mg/ml) and AChEI (0.100 mg/ml) than those of *C.rotundus* or *Z. officinale* extracts.

Table 5-1 DPPH, FRAP and AChEI activities of *Zingiber officinale*, *Cyperus rotundus* and the combination extract of *Zingiber officinale*, *Cyperus rotundus* (CP1)

Tested substance	FRAP IC_{50} mg/ml	DPPH IC_{50} mg/ml	AChEI IC_{50} mg/ml
Zingiber officinale	6.724	2.086	2.422
Cyperus rotundus	8.822	1.041	0.382
CP1	1.743	1.008	0.100

3.2 Effect of CP1 on spatial memory

In this part, the memory impairment condition as observed in age-related dementia in human was induced byinducing hypocholinergic condition via the bilateral administration of AF64A, a cholinotoxin, into lateral ventricle via intracerebroventricular route. Figure 5-2 showed that vehicle+ACSF showed no significant changes of both escape latency and retention time. Our data showed that the administration of AF64A significantly enhanced escape latency (p-value<.001 all; compared to vehicle+ACSF group) but decreased retention time (p-value<.001 all; compared to vehicle+ACSF group) at 7-day and 14-day periods. Both Donepezil and Vitamin C treatments significantly mitigated the enhanced escape latency induced by AF64A (p-value < .001 all; compared to vehicle+AF64A group). Donepezil also mitigated the decreased retention time induced by AF64A both at 7 and 14 days of treatment (p-value<.05 and.001 respectively; compared to vehicle+ACSF group). Ascorbic acid could mitigate the decreased retention time only at 14 days of treatment (p-value<.001; compared to vehicle+ACSF group). Interestingly, all doses of CP1 significantly mitigated the enhanced escape latency (p-value< .001, .01 and .001 respectively; compared to vehicle+AF64A group) at 7-day period and at 14-day period (p-value< .01, .001 and .01 respectively; compared to vehicle+AF64A group). In addition, CP1 at all doses used in this study also mitigated the decreased retention

time induced by AF64A at 14 days of treatment (p-value< .001 all; compared to vehicle+AF64A group).

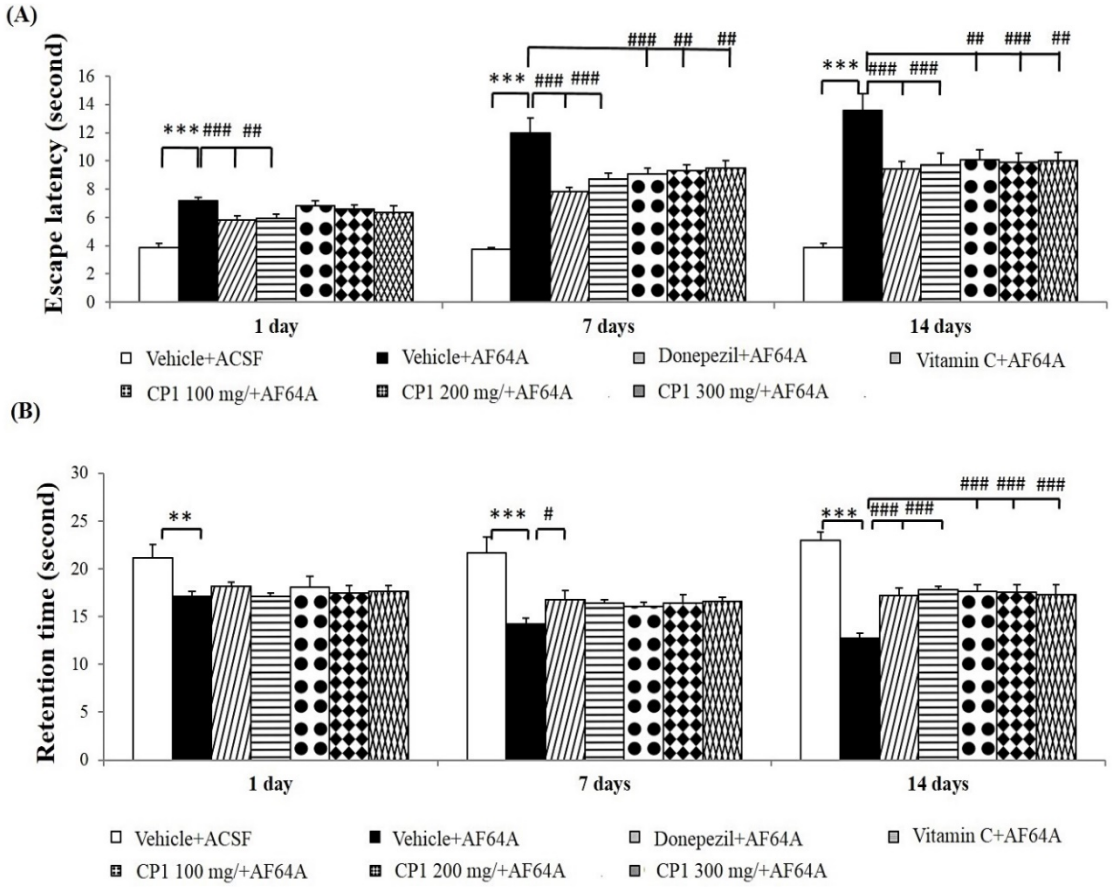

Figure 5-2 The effect of CP1, the combination extract of *C.rotundus* and *Z.officinale*, on spatial memory A) effect of CP1 on escape latency B) effect of CP1 on retention time (n=6/ group) ***p-value<.001; compared to vehicle plus ACSF group; $^{\#,\#\#,\#\#\#}$ p-value<.05, .01 and .001 respectively; compared to vehicle plus AF64A group

3.3 Effect of CP1 on hippocampal neurodegeneration

Figure 5-3 showed the effect of CP1on neuron density in hippocampus. The results showed that AF64A significantly decreased neurons density in CA1, CA2, CA3 and dentate gyrus (p-value<.001 all; compared to vehicle+ACSF group). It was found that rats subjected to AF64A which received donepezil showed the significant

elevation of neuron density only in CA1 CA2, CA3 and dentate gyrus (p-value<.01, .05, .05, .01 respectively; compared to vehicle+AF64A group). In addition, Vitamin C significantly enhanced neuron density in CA2 and CA3 in rats which subjected to AF64A treatment (p-value<.01 and .01 respectively; compared to vehicle+AF64A group). Interestingly, CP1 at low concentration (100 mg/kg) significantly attenuated the reduction of neuron density in CA1 (p-value <.05; compared to vehicle+AF64A group) in rats which received AF64A. The enhanced neuron densities in CA2 and dentate gyrus were observed in rats which subjected to AF64A and received CP1 at doses of 200 and 300 mg.kg^{-1} BW respectively (p-value <.05 all; compared to vehicle+AF64A group). No significant changes were observed in CA3.

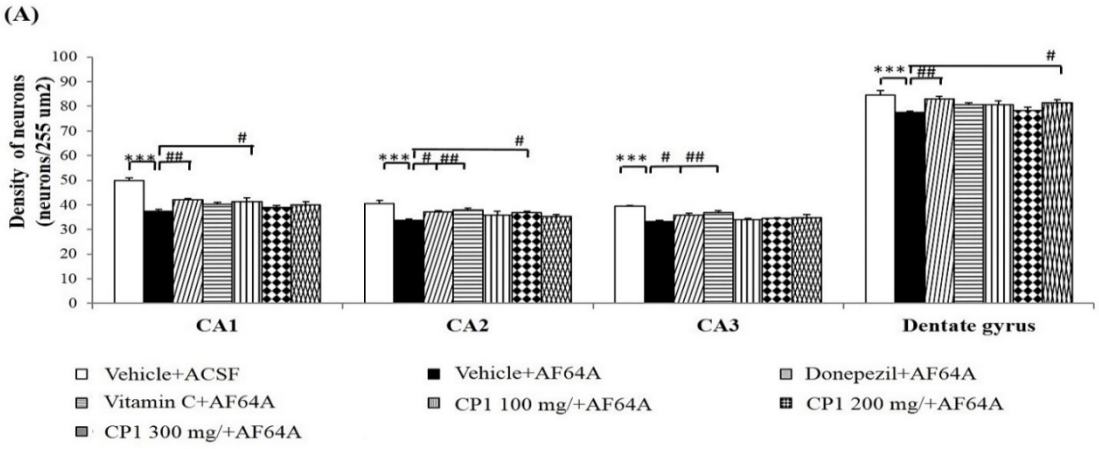

Figure 5-3 The effect of CP1 on neuron density in various subregions of hippocampus including CA1, CA2, CA3 and dentate gyrus. A) Average density of neurons in CA1, CA2, CA3 and dentate gyrus B) Photograph of neuron density in CA1, CA2, CA3 and dentate gyrus. (n=6/group) *** p-value<.001; compared to vehicle+ACSF group. #, ##, ### p-value<.05, .01 and .001 respectively; compared to vehicle+AF64A group

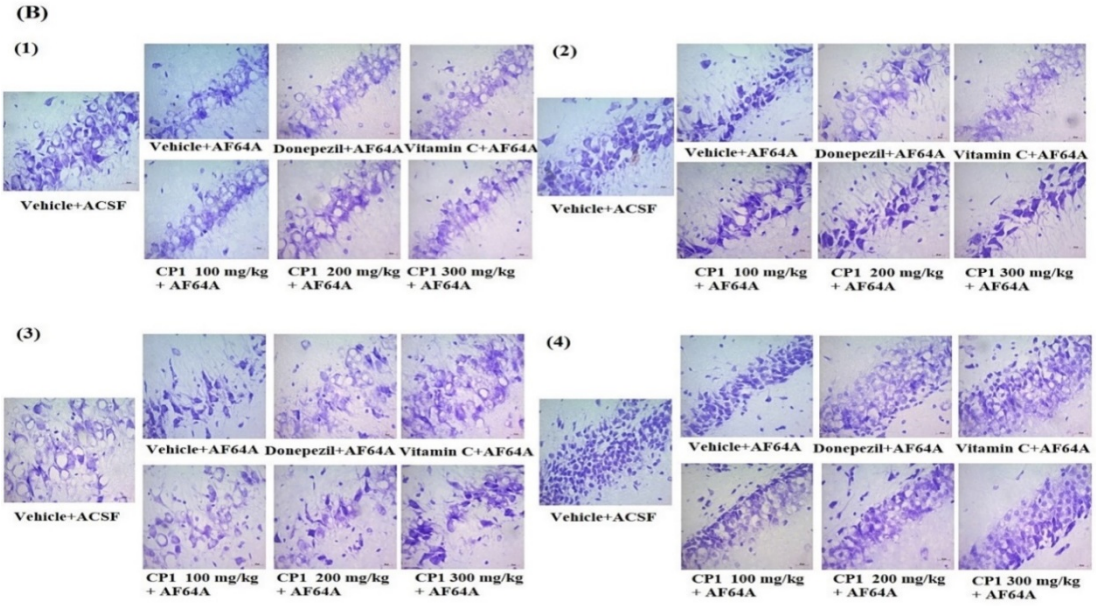

Figure 5-3 The effect of CP1 on neuron density in various subregions of hippocampus including CA1, CA2, CA3 and dentate gyrus. A) Average density of neurons in CA1, CA2, CA3 and dentate gyrus B) Photograph of neuron density in CA1, CA2, CA3 and dentate gyrus. (n=6/group) *** p-value<.001; compared to vehicle+ACSF group. #, ##, ###p-value<.05, .01 and .001 respectively; compared to vehicle+AF64A group (Cont.)

3.4 Effect of CP1 on oxidative stress markers

The effects of CP1 on oxidative stress markers including the level of MDA and the activities of SOD, CAT and GSH-Px in hippocampus were also evaluated. The results were shown in Table 5-2. It was demonstrated that AF64A injection significantly increased MDA level (p-value<.001 compared to vehicle+ ACSF group) but decreased the activities of SOD, CAT and GSH-Px (p-value< .001 all; compared to vehicle+ACSF). The elevation of MDA level in hippocampus induced by AF64A was mitigated by Donepezil, Vitamin C and all doses of CP1 (p-value<.01, .05, .05, .01 and .05 respectively; compared to vehicle+AF64A group). It was also found that the increased CAT activity was observed in rats which subjected to AF64A and received Donepezil, Vitamin C and all doses of CP1 (p-value<.01, .01, .05, .01,. 01; compared to vehicle+AF64A group). All treatments mentioned earlier failed to modulate the reduction of GSH-Px induced by AF64A.

Table 5-2 Effect of CP1 on the oxidative stress markers such as malondialdehyde level and the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) in hippocampus. (n=6/group) *, ***p-value<.05 and.001 respectively; compared to vehicle+ACSF group. $^{\#,}$ $^{\#\#,}$ $^{\#\#\#}$p-value<.05, .01 and .001 respectively; compared to vehcle+AF64A group)

Group	MDA (μ/mg·protein)	SOD (μ/mg·protein)	GSH-Px (μ/mg·protein)	CAT (μ/mg·protein)
Vehicle + ACSF	0.0008±0.0002###	3.3230±0.1195###	0.0829±0.0155	109.2071±1.5467###
Vehicle + AF64A	0.0021±0.0005***	2.1633±0.1244***	0.0518±0.0163	84.6617±2.5905***
Donepezil + AF64A	0.0011±0.0001##	2.9833±0.3891##	0.0743±0.0185	106.4200±5.6561##
Vitamin C + AF64A	0.0011±0.0001#	3.1532±0.1500	0.0870±0.0095	106.8167±3.6787##
CP1 100 mg/kg BW + AF64A	0.0013±0.0002#	2.9210±0.2465##	0.0705±0.0235	101.3417±6.1163#
CP1 200 mg/kg BW + AF64A	0.0011±0.0001##	2.4226±0.0834***	0.0624±0.0096	105.8271±3.6703##
CP1 300 mg/kg BW + AF64A	0.0015±0.0001* #	2.3510±0.0531***	0.0612±0.0088	102.3483±5.4788##

3.5 Effect of CP1 on acetylcholinesterase (AChE) activity

The effect of CP1 on cholinergic function was evaluated indirectly by using the activity of AChE as indirect indicator reflecting the available acetylcholine in hippocampus. The results were shown in figure 5-4. Rats which exposed to AF64A showed the elevation of AChE (p-value<.001; compared to vehicle+ACSF). However, this change was reversed by Donepezil, Vitamin C and all doses of CP1 (p-value<.05, .05, .05, .01 and .01 respectively; compared to vehicle+AF64A group)

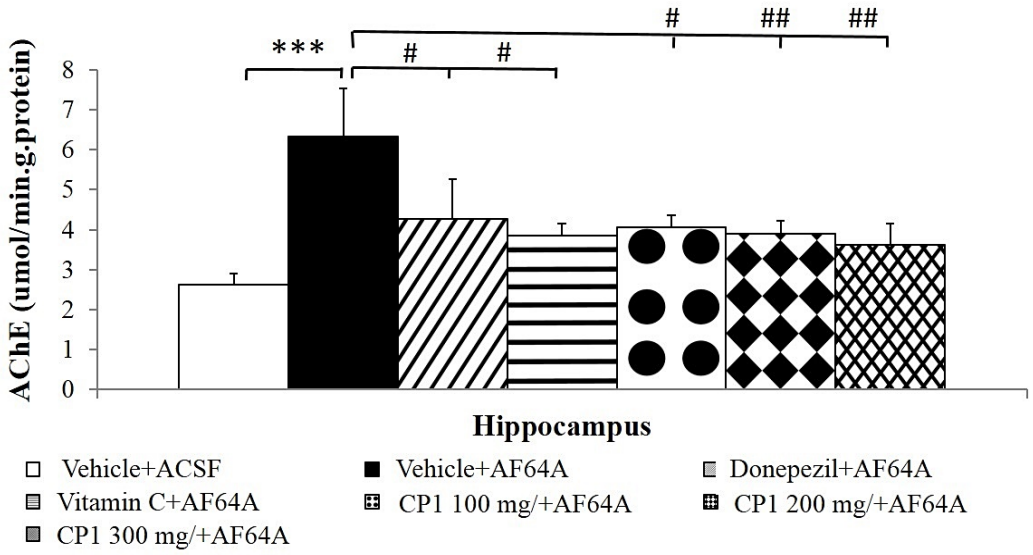

Figure 5-4 Effect of CP1 on an acetylcholinesterase (AChE) enzyme activity in hippocampus. (n=6/group) ***p-value<.001; compared to vehicle+ACSF group. #, ##p-value<.05 and .01 respectively; compared to vehicle+AF64A group

3.6 Effect of CP1 on ERK1/2 activation

Since ERK cascade plays an important role on synaptic plasticity, long-term potentiation and cell survival, the effect of CP1 on ERK1/2 in the hippocampus was also assessed. The results were shown in figure 5-5. It was found that AF64A injection significantly decreased density of the pERK1/2 bands (p-value<.001; compared to vehicle+ACSF). Interestingly, the enhanced expression of pERK1/2 was observed in AF64A treated rats which received Donepezil, medium and high doses of CP1 (p-value<.001, .05 and .01 respectively; compared to vehicle+AF64A group). No significant change was observed in AF64A treated rats which received either Vitamin C or low doses of CP1.

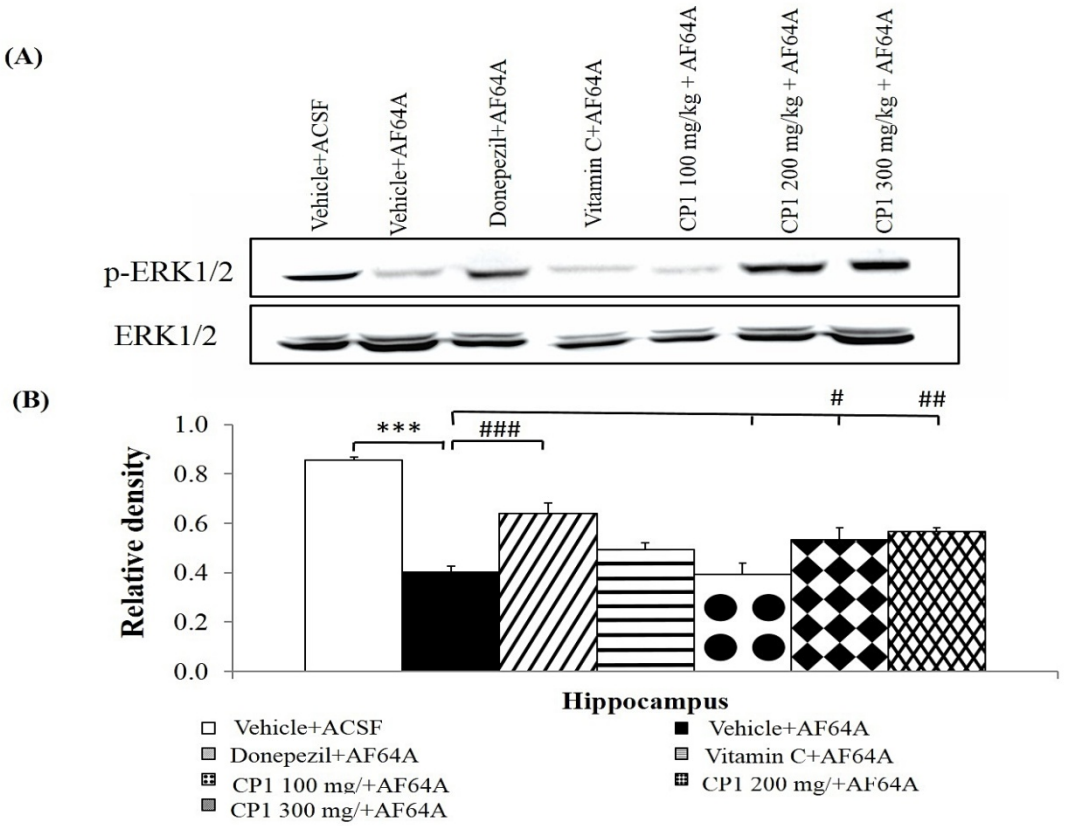

Figure 5-5 Effect of CP1 on the level of ERK1/2 and pERK1/2 in hippocampus. (n=6/group). ***p-value<.001; compared to vehicle+ACSF group. $^{\#, \#\#, \#\#\#}$p-value<.05, .01 and .001 respectively; compared to vehicle+AF64A group

4. Discussion

Medicinal plants have been long term used for treating various ailments either as single plant or as polyherbal recipes. However, the polyherbal recipes have been very much widely used than the single plant based on the concept that the synergistic effect of the plants can provide more beneficial effects (Jayakumar, 2010). However, less scientific evidence is available. In this study, we have clearly demonstrated that CP1, the combination extract of *C.rotundus* and *Z.officinale* showed the lower IC_{50} of both antioxidant effect via DPPH and AChEI effect. Therefore, our results confirmed hypothesis that the interaction of both medicinal plants mentioned earlier could provide more benefit. This was also in agreement with the other study which demonstrated the beneficial effect of the combined extract (Sungkamanee *et al.*, 2014; Thiraphatthanavong *et al.*, 2014; Thukham-mee and Wattanathorn, 2012).

The current results also demonstrated that CP1 significantly increased spatial memory, enhanced cholinergic function and decreased oxidative stress in hippocampus. In addition, CP1 also significantly enhanced densities of neuron in CA1, CA2 and dentate gyrus and increased pERK1/2 level in the area just mentioned. It has been reported that ERK1/2, a subclass of mitogen-activated protein (MAP) kinases, plays a pivotal role on neurodegeneration via mitochondria apoptotic mechanism (Lu *et al.*, 2011; Lu and Xu, 2006). The neurodegeneration in hippocampus, an important area of learning and memory, is associated with the memory deficit (Arlt, 2013; Daulatzai, 2013). Therefore, the memory enhancing effect of CP1 may occur partly via the decreased oxidative stress by enhancing the antioxidant enzyme activities in hippocampus which turn induced the increased pERK1/2 (Lee *et al.*, 2003) giving rise to an increased neuron density in CA1, CA2 and dentate gyrus leading to the improved encoding, retrieval and consolidation processes resulting in the enhanced spatial memory (Riedel *et al.*, 2003). Although the decreased oxidative stress could increase the phosphorylation of ERK1/2 resulting anti-apoptotic effect leading to the enhanced neuron density in hippocampus, no closely relationship between the increased pERK1/2 and the decreased oxidative stress was observed especially at the low concentration of CP1. Since the decreased oxidative stress in AF64A–induced memory deficit rats can increase neuronal density in hippocampus and can improve memory impairment, (Sutalangka *et al.*, 2013; Wattanathorn *et al.*, 2014), we did suggest that the antioxidant effect of CP1 might decrease oxidative stress status in the hippocampus which in turn decreased neurodegeneration induced by the attack of free radicals resulting in increased neuron density in this area. In addition, the activation of ERK1/2 giving rises to the phosphorylation of ERK1/2 which in turn plays the important role on the function of acetylcholine via nicotinic receptor (Bitner *et al.*, 2007). Therefore, it is also possible that CP1 at all doses used in this study may suppress AChE leading to the increased available acetylcholine (ACh) which in turn may bind to nicotinic receptor resulting in the activation and phosphorylation of ERK1/2 and finally leading to the improved spatial memory.

Our results also showed the differential vulnerability to CP1. It was found that CA3 regions showed less vulnerability among various sub-regions assessed in this study. The possible explanation may be due to the difference in distribution of signal

molecule and growth factor which plays the important role on cell survival (Alonso *et al.*, 2004).

It has been demonstrated that gingerol (Alonso *et al.*, 2004) and quercetin (Pangpookiew *et al.*, 2012) could protect against oxidative stress-related neurodegeneration. Therefore, they might be responsible for the neuroprotective effect of CP1 in this study. In addition to the direct effect of both substances mentioned earlier, the interaction effect of various ingredients including the interaction of both ingredients might also contribute the role. However, this required further investigations.

In conclusion, CP1, the combination extract of *C.rotundus* and *Z.officinale*, is the potential supplement to improve neurodegeneration and memory impairment. The possible mechanism may possibly occur via the improved oxidative stress status which in turn increased pERK1/2 in hippocampus leading to the improved memory impairment. In addition, CP1 can also suppress AChE activity in hippocampus giving rise to the increased available ACh and the function of ACh via nicotinic receptor resulting in the enhanced memory performance. However, further researches are necessary to investigate the precise active ingredients, sub-chronic toxicity and the interaction with drugs which commonly used in the elderly patient to assure the safety consumption.

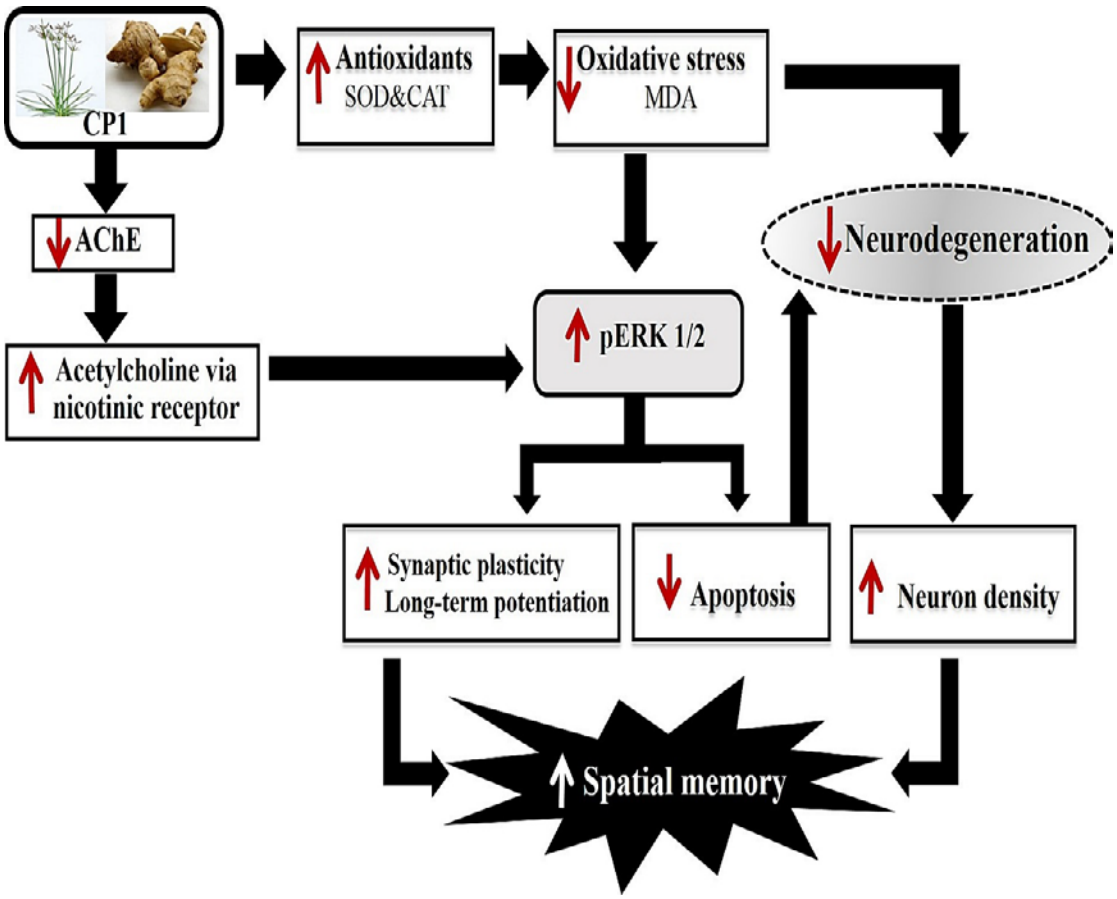

Figure 5-6 Schematic diagram shows the possible underlying mechanism of CP1