CHAPTER III
DEVELOPMENT OF THE NOVEL HERBAL RECIPE FOR TREATING ALZHEIMER'S DISEASE AND PARKINSON'S DISEASE

1. Introduction

It has been well established that vegetables provide numerous health benefits including anti-aging effect. The contributing factors are associated with the presence of provitamin, vitamin and polyphenolics (Craggs and Kalaria, 2011). Numerous vegetables such as *Anethumgraveolens* Linn, *Anacardiumoccidentale* Linn, *Moringaoleifera* Lam, *Zingiberofficinale* Roscoe and *Cyperusrotundus* Linn can be consumed both as food and as herb. These plants possess many health benefit including anti-aging effect. Therefore, the potential to protect against Alzheimer's disease and Parkinson's disease of the plants just mentioned have gained attention. Currently, the health concern is increased and the current lifestylesof the people prefer the ready to be used product. Therefore, the vegetable based functional food which targets at the anti-aging process is required.

According to the traditional folklore, numerous polyherbal recipes have been developed based on the belief that the ingredients containing in the polyherbal recipe can produce better benefit due to the synergistic interaction of the ingredients (Jayakumar, 2010). Based on this concept, the development of novel vegetables based food recipe as a polyherbal recipe has been considered. Since no scientific evidence concerning the potential of the selected vegetables mentioned earlier and the novel herbal recipe is available. This study was set up to clarify this issue. In addition, the acute toxicity of the novel herbal recipe is also investigated.

2. Materials and Methods

2.1 Sample preparation

Leaves of *Moringaoleifera* Lam and *Anacardiumoccidentale* Linn, aerial part of*Anethumgraveolens* Linn and *Cyperusrotundus* Linn and rhizome of *Zingiberofficinale* Roscoewere harvested from KhonKaen province, Thailand during

September-November 2012. The selected parts were air-dried, powdered and extracted by maceration in hydroethanolicsolution (50:50) (100 g in 1 L of solvent) for 72 hr. The mixtures were filtered through Whatman No.1 filter paper and the filtrates were concentrated using a rotary evaporator and then freeze dried. The lyophilysates were preserved at -20 ºC for further use. Then, the extracts were determined total phenolic compounds using Folin–Ciocalteau assay while the antioxidant activity was determined using DPPH and FRAP activities. In addition, AChEI and MAOI were also determined using colorimethod.

2.2 Determination of the total phenolic compounds

Total phenolic compounds contents were determined according to the method described previously by Quettier-Deleu *et al*. (2000) and Prior (2005) with minor modification. In brief, an aliquot of 0.1 ml of plant extract was added to 1.9 ml of deionized water and 1.0 ml of Folin-Ciocalteu phenol reagent (Sigma). After 8 min, 5.0 ml of 20% $Na_2CO_3$was added and the mixture was heated in a boiling water bath for 1 min comparatively to gallic acid standard. Absorbance was measured at 765 nm with a UV-spectrophotometer (Pharmacia LKB-Biochrom4060) after cooling in darkness and the results were expressed in mg of gallic acid/100 g vegetable extract.

2.3 Determination of flavonoids content

Total flavonoids were estimated according to the aluminum chloride method. Briefly, 0.5 ml of each sample and 300 µl of $NaNO_2$ (1:20 w/v) were pipette into a test tube. The contents were vortexed for 10 s and left at room temperature for 5 min. The mixture were then added 300 µl of $AlCl_3$ (1:10 w/v), 2 ml of 1 M NaOH, and 1.9 ml of distilled water. After 10 s of vortexing, the absorbance for each sample was measured at 510 nm. Rutin or quercitin wereused to prepare the standard calibration curve. The flavonoids contents were expressed in mg of rutin equivalent (RE) or g of quercetin equivalents (QE) /g of extract (Djeridane A.*et al*, 2006).

2.4 Determination of antioxidant activity

Radical scavenging activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical of the plant extract 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 was 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

2.5 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 mL$C_2H_4O_2$), pH 3.6, 2.5 mL of 10 m Mtripyridyltriazine (TPTZ) solution in 40 mMHCl, 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 the incubation at 37°C for 10 min. The results were expressed as µM Ascorbic acid/100 g fresh weight.

2.6 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 50 µl of 50 mMTris–HCl, pH 8.0, containing 0.1% bovine serum albumin (BSA), and 25 µl of the tested phytochemicals were added. The absorbance was measured at 405 nm after a 5-minute incubation at room temperature. Then a 25 µl 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 (%) = $1 - (A_{sample}/A_{control}) \times 100$, where A_{sample} is the absorbance of the sample extracts and $A_{control}$ is the absorbance of the blank (50% aqueous methanol in buffer).

2.7 Determination of monoamine oxidase type B (MAO-B) inhibition

The MAO type B activity was assessed using microplate reader method (Xu *et al.*, 2005). Rats were decapitated and brains were rapidly removed and

frozen on dried ice. Then, they were homogenized in sodium phosphate buffer (0.1 M, pH 7.4) and centrifuged at 15,000×g for 20 min. Supernatant was removed and used for the estimation of MAO-B activity. The brain supernatant 150 µl was added in to the solution containing 2.5 ml Tris buffer (0.1 M, pH 7.4) and 100 µl of 0.1 M benzylamine. Then, the absorbance was measured at 340 nm for 30 min against the blank containing Tris buffer and 5-hydroxytryptamine.

2.8 Development and determination of characteristic of CP1

Aerial part of *C.rotundus* and rhizome of *Z.officinale* had been selected for the development of CP1, a novel neuroprotectant based on their antioxidant activity, AChEI and MAOBI. They were harvested from KhonKaen province, Thailand during September-November 2012 and were authenticated by Associate Professor PaneeSirisa-ard, from Faculty of Pharmacy, Chiang Mai University, Thailand. The plant materials were prepared as 95% alcoholic extract. The percent yield of the *C.rotundus* and *Z.officinale* extracts were 7.41% and 10.48% respectively. Twenty one different combinations were prepared by varying the proportions of *C.rotundus* and *Z. officinale*. Each combination was determined the total phenolic compounds, antioxidant activity and the inhibitory activities of AChE and MAOB.

2.9 Determination of acute toxicity

Healthy young adult male and female Wistar rats, weighing 200-250 g, were purchased from National Laboratory Animal Center, Salaya, Nakorn Pathom, Thailand. The animals were maintained and treated in accordance with the guideline and approval of the Ethical Committee on Animals Experiments of Khon Kaen University (AEKKU 41/2554). Lighting was controlled to supply 12 h of light and 12 h of dark for each 24-h period. They were maintained at room temperature approximately 23 °C with constant humidity and they were allowed to acclimatize to laboratory conditions for a week before starting the experiment. They were given water ad libitum throughout the study period. Acute oral toxicity test was carried out according to the Organization for Economic Co-operation and Development (OECD) guidelines (Organization for Economic Co-operation and Development (OECD), 2012).

3. Results

3.1 Total phenolic compound and flavonoid contents and antioxidant activity

Table 3-1 showed the total phenolic compound and flavonoid contents, DPPH and FRAP activities of selected medicinal plants. The results showed that among 5 types of the selected plants, *A. occidentale*showed the highest total phenolic compounds at the concentration of 494±0.007 mg of GAE/100 g of plant. *A. graveolens* contained phenolic compounds contents at concentration of 442±0.002 mg of GAE/100 g of plant while *C.rotundus, M. oleifera* and *Z. officinale* contained phenolic compounds at concentrations of 377±0.001, 236±0.003 and 177±0.003 mg of GAE/100 g of plant respectively. It was found that *M. oleifera* contained the highest concentration of quercetin at the concentration of 3.55±0.001 mg QE/100g of plant followed by *A. occidentale, A. graveolens, C. rotundus* and *Z. officinale* which showed the concentrations of quercetin at concentrations of 3.16±0.005, 2.94±0.004, 2.46±0.012 and 2.41±0.007 mg QE/100g of plant respectively.

In order to assess the antioxidant activity of selected medicinal plants, both DPPH and FRAP assays were used as evaluation tools. The results showed that the IC_{50} of FRAP of *A. occidentale,Z. officinale*, *A. graveolens*, *M. oleifera*and *C.rotundus* were 5.00, 6.25, 6.25, 5.00, and 28.96 µg/ml respectively. In addition, the IC50 values of DPPH radical scavenging activity of *C.rotundus, Z. officinale, M. oleifera, A. occidentale* and *A. graveolens*were 47.25, 63.58, 88.48, 88.97 and 98.61 µg/ml respectively. Therefore, *A. occidentale*showed the lowest IC_{50} of FRAP while *C.rotundus* showed the lowest IC_{50} of DPPH.

Table 3-1 Total phenolic compounds and flavonoid contents, DPPH and FRAP activities of selected medicinal plants

Plant extract	Total phenolics mg GAE/100 g plant extract	Flavonoids mg QE/100 g plant extract	FRAP IC_{50} µg/ml	DPPH IC_{50} µg/ml
Moringa oleifera Lam.	236±0.003	3.55±0.001	12.47	88.48
Zingiber officinale Roscoe.	177±0.003	2.41±0.007	6.25	63.58
Anethum graveolens Linn.	442±0.002	2.46±0.012	6.25	98.61
Anacardium occidentale Linn.	494±0.007	3.16±0.005	5.00	88.97
Cyperus rotundus Linn.	377±0.001	2.94±0.004	28.96	47.25

3.2 Acetylcholinesterase inhibitory (AChEI) activity

The AChE inhibitory activity of the selected medicinal plants were shown in Figure 3-1. It was found that IC50 values of AChEI of *M. oleifera, C.rotundus, Z. officinale, A. occidentale* and *A. graveolens*were 5.00, 5.00, 9.25, 9.78 and 10.22 µg/ml respectively. Interestingly, *M.oleifera* and *C.rotundus* showed the lowest IC_{50} of AChEI and followed by *Z. officinale, A. occidentale* and *A. graveolens.*

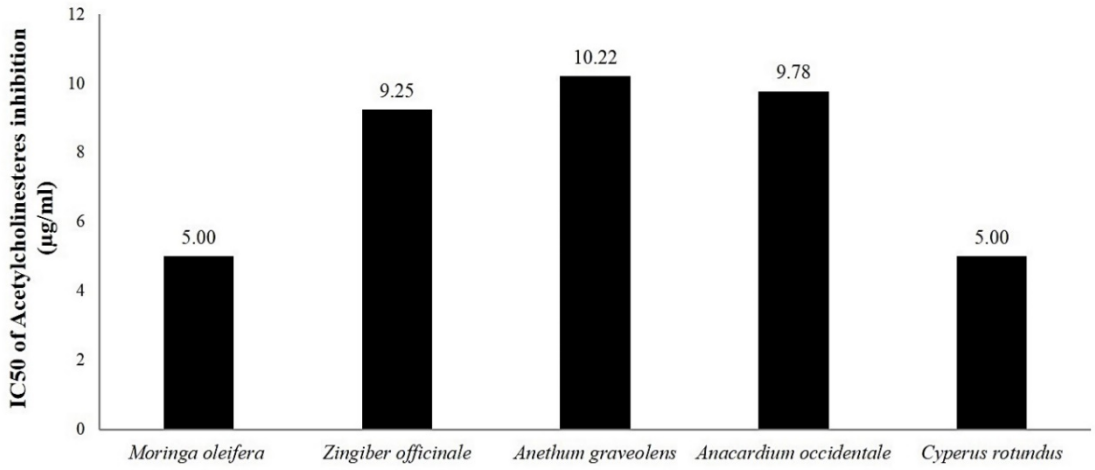

Figure 3-1 IC_{50} of acetylcholinesterase inhibition of the selected plants consisting of *M. oleifera, Z. officinale, A. graveolens, C. rotundus and A. occidentale*

3.3 Monoamine oxidase B suppression activity

In this study, the suppression ability of MAOB of *M. oleifera, Z. officinale, A. graveolens, C.rotundus* and *A. occidentale* were also evaluated. The results were shown in Table 3-2. Interestingly, all selected medicinal plants showed potent suppression activity on MAOB with the value less than 50 µg/ml.

Table 3-2 Monoamine oxidase type B (MAOB) inhibitory activity of selected medicinal plants

MAOB (% inhibition)					
Concentration (µg/ml)	*Moringa oleifera*	*Zingiber officinale*	*Anethum graveolens*	*Anacardium occidentale*	*Cyperus rotundus*
50	89.560±1.166	91.389±0.943	85.782±0.643	93.023±0.243	83.713±0.672
100	89.569±1.609	93.902±0.540	91.739±0.618	98.130±0.207	90.222±0.870
250	88.028±1.593	93.310±0.536	90.476±1.180	97.137±0.088	90.486±0.389
500	83.571±1.304	92.482±0.975	87.681±1.676	97.143±0.883	91.244±0.564
1000	91.002±0.825	91.905±0.401	88.042±1.838	94.455±0.425	92.068±0.939
IC50	<50	<50	<50	<50	<50

3.4 Antioxidant and biological activities of CP1

Table 3-3 showed the total phenolic compounds, antioxidant activity and the inhibitory activities of AChE and MAOB of various combinations of *C. rotundus and Z. officinale* (CP1).The results showed the combination of *C.rotundus* and *Z. officinale* which provided high amount of phenolic compounds were ranking in the first fifth rank as following; 1:5, 4:5, 4:3, 5:4 and 4:1. In addition, it was found that the first fifth ranks of the combinations of *C.rotundus* and *Z. officinale* which showed potent ferric reducing antioxidant power were 1:5, 4:5, 4:3, 2:3 and 3:4 while the first fifth ranks of combinations provided high ability to directly scavenge stable free radicals were 5:4, 5:3, 5:2 and 5:1 and the fifth rank was 5:1.

The main outcome of this study had been focused on neuroprotective effect, the alterations of neurotransmitters including acetylcholine and monoamine transmitters were evaluated. The current results showed that the combination of

C.rotundus and *Z. officinale* which provided high ability to suppress AChE with the value less than 0.1 mg/ml were 1:1, 1:2, 1:5, 2:1, 2:3, 2:5, 3:1, 3:2, 3:5, 4:1, 4:3, 4:5 and 5:1. In addition, the present findings also showed that the combinations of *C. rotundus and Z. officinale* which showed the potent MAOBI with the value less than 0.1 mg/ml were the combinations of 1:5, 2:1, 2:3, 2:5, 3:1, 3:2, 3:4, 3:5, 4:3, 4:5, 5:1, 5:2, 5:3 and 5:4.

Therefore, the current findings showed that combination of *C. rotundus and Z. officinale* which provided optimum biological activities was 1:5 and this combination was selected for further investigation in next part as a product CP1.

Table 3-3 The total phenolic compounds, antioxidant activity and the inhibitory activities of AChE and MAOB of various combinations of *C.rotundus* and *Z. officinale*

C. rotundus : Z. officinale	**Total phenolics** mg GAE/100 g plant extract	**FRAP IC_{50}** mg/ml	**DPPH IC_{50}** mg/ml	**AChEI IC_{50}** mg/ml	**MAOBI IC_{50}** mg/ml
0:1	131.371±0.003	6.724	2.086	2.422	>5
1:0	180.133±0.003	8.822	1.041	0.382	>5
1:1	173.562±0.009	3.429	1.350	<0.1	>5
1:2	216.371±0.018	3.293	1.181	<0.1	2.078
1:3	219.657±0.009	2.889	1.458	>5	0.839
1:4	212.990±0.001	4.254	1.426	0.197	1.594
1:5	258.467±0.002	1.743	1.008	<0.1	<0.1
2:1	240.514±0.003	3.026	1.169	<0.1	<0.1
2:3	233.133±0.010	1.962	1.489	<0.1	<0.1
2:5	237.229±0.002	7.873	1.345	<0.1	<0.1
3:1	205.276±0.004	3.908	3.565	<0.1	<0.1
3:2	214.371±0.002	2.711	1.787	<0.1	<0.1
3:4	236.705±0.006	1.981	1.624	0.475	<0.1
3:5	223.657±0.001	2.574	1.883	<0.1	<0.1
4:1	240.800±0.009	2.392	1.486	<0.1	0.448
4:3	244.848±0.003	1.807	1.296	<0.1	<0.1
4:5	246.705±0.005	1.746	1.027	<0.1	<0.1
5:1	235.514±0.003	2.769	0.906	<0.1	<0.1
5:2	239.324±0.001	2.273	0.854	>5	<0.1
5:3	232.705±0.008	2.836	0.840	>5	<0.1
5:4	243.800±0.003	10.232	0.805	>5	<0.1

3.5 Acute toxicity of CP1

In the acute toxicity study, it was found that CP1 up to the level of 5,000 mg/kg BW failed to exhibit the lethality and toxic symptoms. No behavioral changes and macrospic changes of histology of vital organs were observed. According

to the Organization of Economic Cooperation and Development (OECD) guidelines for acute oral toxicity, an LD50 of 2,000 mg/kg BW or above is categorized as unclassified and hence the product is found to be safe. Therefore, CP1 is safe especially for short duration application.

4. Discussion

To date, numerous lines of evidence have demonstrated that the imbalance of oxidative stress homeostasis gives rise to the enhanced oxidative stress which plays the crucial role on numerous pathophysiology including neurodegenerative disease (Gandhi and Abramov, 2012; Uttara *et al.*, 2009). In addition, it has been reported that damage and impairment induced by oxidative stress can be attenuated by antioxidants (Pandey and Rizvi, 2009; Pham-Huy *et al.*, 2008).

All data obtained from in vitro study of this study indicated that all selected medicinal plants showed high potential to be served as natural resources for developing neuroprotective product. Since the combinations of *C. rotundus and Z. officinale* are the medicinal plants and less expensive, they were selected for the development of combined herbal recipes, CP1.

Polyherbal formulation has been long-term used in Oriental Medicine. Several lines of evidence have demonstrated that polyherbal formulation provide more benefits in the management of various ailments (Parasuraman *et al.*, 2014; Srivastava *et al.*, 2012). This was also in agreement with the present results which clearly demonstrated that the combined extract of *C. rotundus and Z. officinale* at a ratio of 1:5 provided optimum benefit concerning about antioxidant, AChEI and MAOI activities. In addition, the current data showed that it was safe up to the dose of 5,000 mg/kg BW.

In conclusion, the novel herbal recipe shows the potential to protect against neurodegenerative disease especially in Alzheimer’s disease and Parkinson’s disease. However, further study is necessary to fully elucidate the possible active ingredients of the polyherbal formulation.