CHAPTER VI
EFFECT OF NOVEL FOOD SUPPLEMENT "CP1" ON MOTOR AND MEMORY DEFICIT IN ANIMAL MODEL OF PARKINSON'S DISEASE

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease. It has been reported that Parkinson's disease affects motor and non-motor symptoms leading to the poor quality of life of both the patients and caregivers and the increased socio-economic burden (Dowding *et al.*, 2006; Vossius *et al.*, 2011). Although the understanding s about the pathogenesis of neurodegeneration of both dopaminergic and cholinergic neurons and the impairments of motor and non-motor symptoms are still unclearly known, a pile of evidence has demonstrated that oxidative stress plays a crucial role on the mentioned processes (Hwang, 2013; Nakabeppu *et al.*, 2007; Sriraksa *et al.*, 2011). Despite the importance of PD, the current therapeutic efficacy is still limited and the adverse effects are commonly found (Rascol *et al.*, 2003). Therefore, the searching of the novel therapeutic strategy to improve motor and cognitive deficits in PD has gained attention.

Abundant of medicinal plants have been long term used in traditional folklore with safety and reputed for longevity promotion, neuroprotection and memory enhancer. Most of the medicinal recipes in traditional folklore have been used as polyherbalrecipes more than single plant based on the belief that the polyherbal recipes can provide more benefit due to the interaction effect of ingredients and the multi-target approach (Jayakumar, 2010). However, less scientific evidence concerning the beneficial effects of the either single plant or polyherbal recipe for treating PD was available (Li *et al.*, 2013).

Numerous lines of evidence demonstrated that *Cyperusrotundus*, a plant in a family of Cyperaceae and *Zingiberofficinale*, a plant in a family of Zingiberaceae, possess neuroprotective effect, antioxidant activity, acetylcholinesterase and monoamine oxidase -B inhibitory (Bashir *et al.*, 2012; Saenghong *et al.*, 2011; Soumaya *et al.*, 2014; Wattanathorn *et al.*, 2010). Based on these reputations and the

critical roles of oxidative stress, cholinergic and dopaminergic systems on the pathophysiology of both motor and non-motor symptoms of PD (Barnham *et al.*, 2004), the beneficial effects of both plants to improve neurodegeneration in PDare 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 enhance motor and memory deficits in Parkinson's disease. To test this hypothesis, this study aimed to determine the antioxidant effect, acetylcholinesterase and monoamine oxidase-B inhibitory of CP1. In addition, the in vivo study was also carried out to determine the neuroprotective effect and the beneficial effect on both motor and non-motor symptoms in animal model of Parkinson's disease induced by 6-OHDA.

2. Materials and Methods

2.1 Plant collection and extract preparation

Aerial part of *C.rotundus* and rhizome of *Z.officinale* were harvested from KhonKaen province, Thailand during September-November 2012. They were authenticated by Associate Professor PaneeSirisa-ard, from Faculty of Pharmacy, Chiang Mai University, Thailand. The plant materials were prepared as 95% ethanolic extract. A ratio of ethanolic 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 6-1. The combined extract was kept at -20°C in a dark bottle until used.

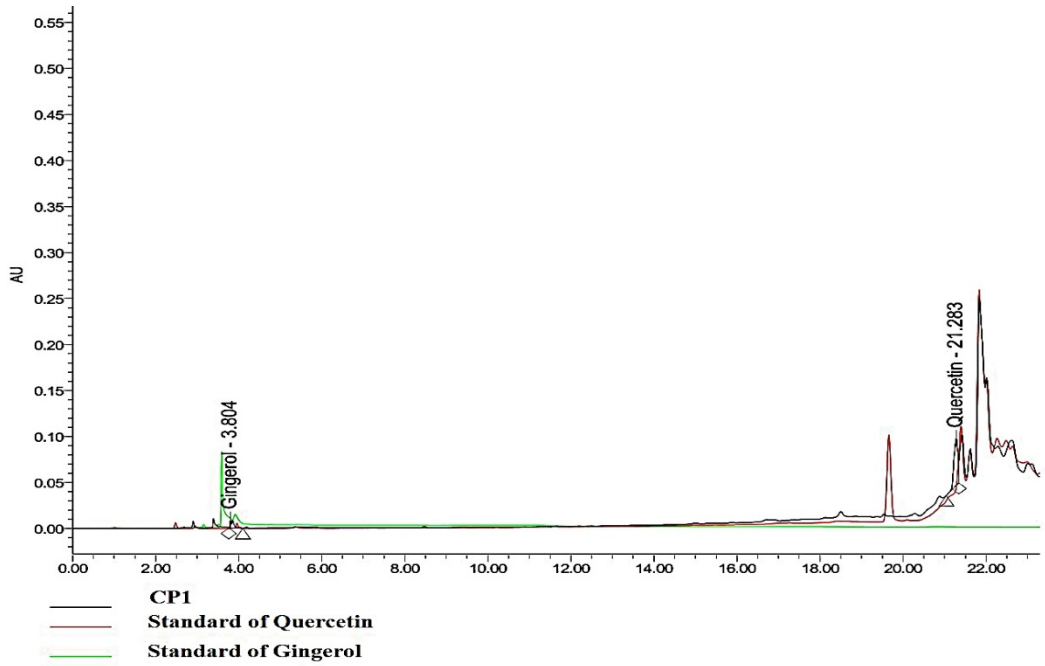

Figure 6-1 High performance liquid chromatography (HPLC) chromatogram of the combined extract of *Zingiber officinale* and *Cyperus rotundus*

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 the 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

2.3 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 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 593nm 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 mMTris–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 = $1 - (A_{sample}/A_{control}) \times 100$, where $A_{sample}$is the absorbance of the sample extracts and $A_{control}$ is the absorbance of the blank (50% aqueous methanol in buffer).

Besides in vitro assay of AChE mentioned earlier, we also determined AChE activity in hippocampal and striatal homogenate. In brief, hippocampus and striatum were 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 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. In addition to in vitro assay of MAO-B, we also determined MAO-B activity in hippocampal and striatal homogenate. The rats were divided into various groups as described in the experimental protocol (6.2.8). At the end of experiment, all rats were sacrificed. The hippocampus and striatum were isolated and prepared as a homogenate to determine the activities of MAO-B. The activities of MAO-B were determined by using the colorimetric method (Holt *et al.*, 1997).

2.6 Animals

Young adult male Wistar rats, 8 weeks old,were used as experimental animals. They were obtained from National Laboratory Animal Center, Salaya, NakornPathom. The weights of the animals on the first day of experiment are 180-220 grams. They were housed 6 per cage and maintained in 12: 12 light: dark cycle and given access to food 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).

2.7 Drugs and Chemicals

6-Hydroxydopamine hydrochloride (6-OHDA) was purchased from Sigma–Aldrich Co., USA. Sodium pentobarbital was obtained from Jagsonpal Pharmaceuticals LTD, Haryana, India. All other chemical substances were analytical grade and purchased from Sigma Chemical Company, St. Louis, MO.

2.8 Experimental Protocol

All rats were randomly assigned to 7 groups of 12 animals each as following

Group I Vehicle + saline; rats were orally given propylene glycol which served as vehicle to suspend the combination extract of CP1 once daily for 14 days after the injection of salineinto right substantia nigra

Group II Vehicle + 6-OHDA; rats were orally treated with propylene glycol once daily at a period of 14 days after the injection of 6-OHDAinto right substantia nigra

Group III Vitamin C + 6-OHDA; the animals were orally treated with Vitamin C (200 $mg.kg^{-1}$) once daily for a period of 14 days after the injection of 6-OHDAinto right substantianigra

Group IV L-DOPA + 6-OHDA; the animals were orally treated with L-DOPA (5 $mg.kg^{-1}$) once daily for a period of 14 days after the injection of 6-OHDAinto right substantia nigra

Group V-VII CP1 + 6-OHDA; rats were treated with CP1 at doses of 100, 200 and 300 $mg.kg^{-1}$ for a period of 14 days after the injection of 6-OHDAinto right substantia nigra

Rats in all groups were orally given the assigned substances at a period of 14 days after the injection of 6-OHDA into right substantia nigra. The memory and motor assessment were 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), acetylcholinesterase (AChE) and monoamine oxidase-B (MAO-B) in hippocampus and striatum were performed at the end of study. Moreover, the density of survival neurons various sub-regions of hippocampus including CA1, CA2, CA3 and dentate gyrus were also determined.Immunohistochemical staining to investigate the density of dopaminergic neurons in substantia nigra was performed using the tyrosine hydroxylase (TH).

2.9 Substantianigra lesion

The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (Jagsonpal Pharmaceuticals LTD, Haryana, India) at dose of 60 $mg.kg^{-1}$ BW. Each animal was mounted on a stereotaxic stand, the skin overlying the skull was cut to expose the skull, and the coordinates for the substantia nigra parcompacta (SNpc) were accurately measured (anteroposterior -0.5 mm from bregma, mediolateral 2.1 mm from midline and dorsoventral -7.7 mm from the skull). Total 6 µg of 6-OHDA was dissolved in 2 µl 0.2% ascorbic acid saline (Ferro *etal*., 2005) and were perfused into right substantia nigra through a 30 gauge stainless needle. After the surgery, all animals were allowed to recover from anesthesia and then placed in their cages.

2.10 Determination of spatial memory

Spatial memory was evaluated via the Morris water maze test. The water maze consists of a metal pool (170 cm in diameter × 58 cm tall) filled with tap water (25 ºC, 40 cm deep). The pool was divided into 4 quadrants (Northeast, Southeast, Southwest, and Northwest). The water surface was covered with non-toxic milk. The removable platform was placed below the water level at the center of one quadrant. For each animal, the location of the invisible platform was placed at the center of one quadrant and remained there throughout training. The times which animals spent to climb on the hidden platform were recorded as escape latency. 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 released into the quadrant diagonally opposite to that which contained the platform. Time spent in the region that previously contained the platform was recorded as retention time (Brandeis *et al*., 1989).

2.11 Determination of motor function

When all rats regained consciousness after the last laser acupuncture treatment, the changes in apomorphine-induced rotation behavior (0.5 $mg.kg^{-1}$, s.c.) were assessed using a cylindrical container. The diameter of the cylindrical container used for measuring rotational activity was 40 cm. The animals were habituated in the cylindrical container for 10 min and the rotations made by the animals to either side was counted for 45 min. The net number of rotations was counted as follows: the number of contralateral rotations−the number of ipsilateral rotations with respect to the 6-OHDA injection side. This behavioral test was performed blindly (Ahmad *et al*., 2005; Yu *et al*., 2010).

2.12 Histological procedure

After the anesthesia with sodium pentobarbital (60 $mg.kg^{-1}$ BW), brains were subjected to transcardial perfusion with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3. After the perfusion, brains were removed and stored over a night in a fixative solution that used for perfusion. Then, they were infiltrated with 30% sucrose solution at 4° C. The specimens were frozen rapidly and 10 μM thick sections were cut on cryostat. The selected sections 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.

2.13 Immunohistochemical evaluation

Immunohistochemical staining to investigate dopaminergic neuron was performed using the monoclonal anti-tyrosine hydroxylase antibody produced in mouse (Sigma, St Louis, MO, 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_2in methanol. Sections were washed in running tap water and distilled water for 1 min. each, then rinsed in KPBS and KPBS-BT for 5 minutes per each process. Excess buffer was removed, then incubated for 30 min in a blocking solution composed of 5% normal goat serum in KPBS-BT. The sections were then incubated in mouse monoclonal anti-tyrosine hydroxylase antibody diluted 1: 400 in KPBS-BT at room temperature for 2 hours and then incubate at 4^oC for 48 hours. The tissue was rinsed in KPBS-BT (two washes x 7 min), incubated for 1 hours in biotinylated goat anti-mouse IgG antibody, rinsed in KPBS-BT (two washes x 7 min) and then incubated in Strep ABC Complex/HRP for 4 hours.According to the preparation for visualization step, sections were rinsed inKPBS-BT (1 min), and KPBS (two washes x 10 min). Tyrosine hydroxylaseimmunoreactivity was visualized using 0.025% 3, 3' diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2 for 48 hours. Finally, sections were rinsed in running tap water, air dried and cover-slipped using permount.

2.14 Morphological analysis

Five coronal sections of each rat in each group were studied quantitatively. Neuronal counts in hippocampus and substantia nigra were performed by eye using a 40x and 20x magnification respectively with final field 255 μm^2. 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.

2.15 Determination of oxidative stress markers

Hippocampus was isolated and prepared as hippocampal homogenate and the determination of the oxidative stress markers in hippocampus 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 mMTris–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 for the production of superoxide radical and subsequent measurement of cytochrome c as a scavenger of the radicals. Optical density was determined 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 protein (Eyer and Podhradsky, 1986). One unit of the enzyme was defined as micromole (μmol) of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized per minute.

2.16 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 effect of *C. rotundus*, *Z.officinale* and the combination extract of *C.rotundus* and *Z.officinale* (CP1) was determined 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 1. Interestingly, the current 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), AChEI (0.100 mg/ml) and MAOBI (0.100 mg/ml) than those of *C.rotundus*or *Z. officinale*extracts.

Table 6-1 DPPH, FRAP, AChEI and MAOBI activities of *Zingiberofficinale*, *Cyperusrotundus* and the combination extract of *Zingiberofficinale*, *Cyperusrotundus* (CP1)

Tested substance	FRAP IC_{50} mg/ml	DPPH IC_{50} mg/ml	AChEI IC_{50} mg/ml	MAOBI IC_{50} mg/ml
Zingiber officinale	6.724	2.086	2.422	5.000
Cyperus rotundus	8.822	1.041	0.382	5.000
CP1	1.743	1.008	0.100	0.100

3.2 Effect of CP1 on spatial memory of 6-OHDA lesion rats

Figure 6-2 showed that the administration of 6-OHDA significantly enhanced escape latency (p-value<.001 all; compared to vehicle+saline group) but decreased retention time (p-value<.001 all; compared to vehicle+saline group) at 7-day and 14-day period. Both Vitamin C and L-DOPA treatments significantly improved the reduction of escape latency induced by 6-OHDA (p-value < .001 all; compared to vehicle+6-OHDA group) throughout the experimental period. Both mentioned substances also mitigated the decreased retention time induced by 6-OHDA at 14 days of treatment (p-value<.05, .01 respectively; compared to vehicle+6-OHDA group). Interestingly, medium and high doses of CP1 significantly mitigated the enhanced escape latency at 7-day period (p-value<.01 and .001 respectively; compared to vehicle+6-OHDA group) and at 14-day period (p-value<.001 all; compared to vehicle+6-OHDA group). Low dose of CP1 could significantly improve the reduction of escape latency only at 14 days of treatment (p-value<.001; compared to vehicle+6-OHDA group). Moreover, CP1 at all doses also mitigated the decreased retention time induced by 6-OHDA at 14 days of treatment (p-value<.05, .01, .01 respectively; compared to vehicle+6-OHDA group).

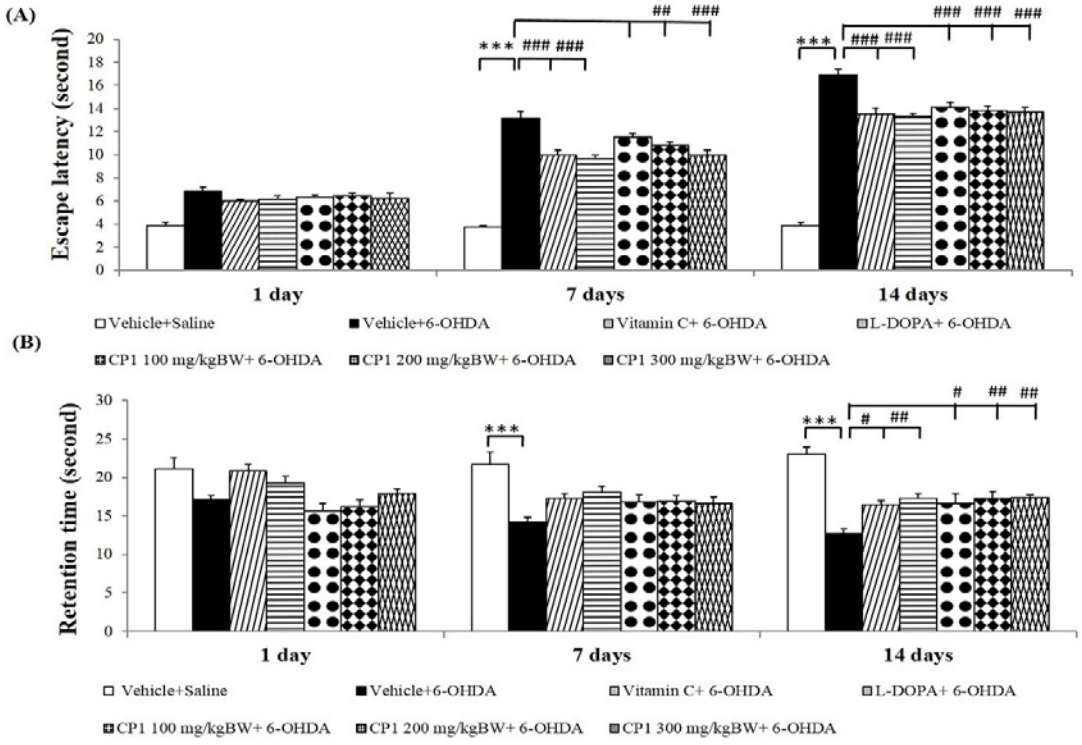

Figure 6-2 Effect of CP1 on spatial memory using the Morris water maze test in rats subjected to 6-OHDA treatment (A) escape latency (B) retention time. Values are given as mean ± S.D. (n = 6) *** p-value<.001 as compared with vehicle+saline group and # p-value<.05, ## p-value<.01 as compared with vehicle+6-OHDA group

3.3 Effect of CP1 on apomorphine-induced rotation behavior

The present data showed that the administration of 6-OHDA into right substantia nigra significantly increased rotation number at 7-day and 14-day period after the 6-OHDA administration (p-value<.001 all; compared to vehicle+ saline group). Both Vitamin C and L-DOPA treatments significantly suppressed the elevation of rotation number induced by 6-OHDA (p-value<.01 all; compared to vehicle+ 6-OHDA group) at 7-day and 14-day period after the 6-OHDA administration (p-value<.001 all; compared to vehicle+6-OHDA group). In addition, it was found that the medium and high doses of CP1 significantly suppressed the elevation of rotation number induced by 6-OHDA at only 14-day intervention period (p-value<.001 all; compared to vehicle+6-OHDA group).

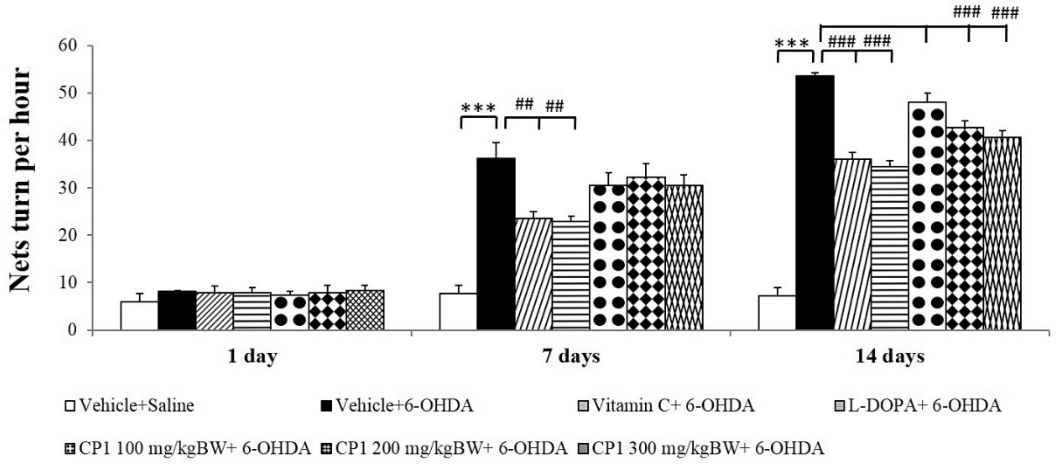

Figure 6-3 Effect of CP1 on apomorphine - induced rotations in rats subjected to the 6-OHDA. Values are given as mean ± S.D. (n = 6) *** p-value<.001 as compared with vehicle+ saline group and ## p-value<.01, ### p-value<.001 as compared with vehicle+ 6-OHDA group

3.4 Effect of CP1 on hippocampal neurons

Figure 6-4 showed the effect of CP1on neuron density in hippocampus. The results showed that the administration of 6-OHDA into right substantia nigra induced the decreased survival neuron density in CA1, CA2 and CA3 of hippocampus (p-value<.001 all; compared to vehicle+ saline group). It was found that rats subjected to 6-OHDA which received vitamin C significantly enhanced neuron density only in CA1 (p-value<.01; compared to vehicle+6-OHDA group). L-DOPA significantly enhanced neuron density in CA1 and CA3 in rats which subjected to 6-OHDA treatment (p-value<.001 all; compared to vehicle+6-OHDA group). Interestingly, CP1 at all doses significantly mitigated the reduction of neuron density in CA1 (p-value <.05 all; compared to vehicle+6-OHDA group) and CA3 (p-value<.01 all; compared to vehicle+6-OHDA group) in rats which received 6-OHDA. No significant changes were observed in CA2 and dentate gyrus.

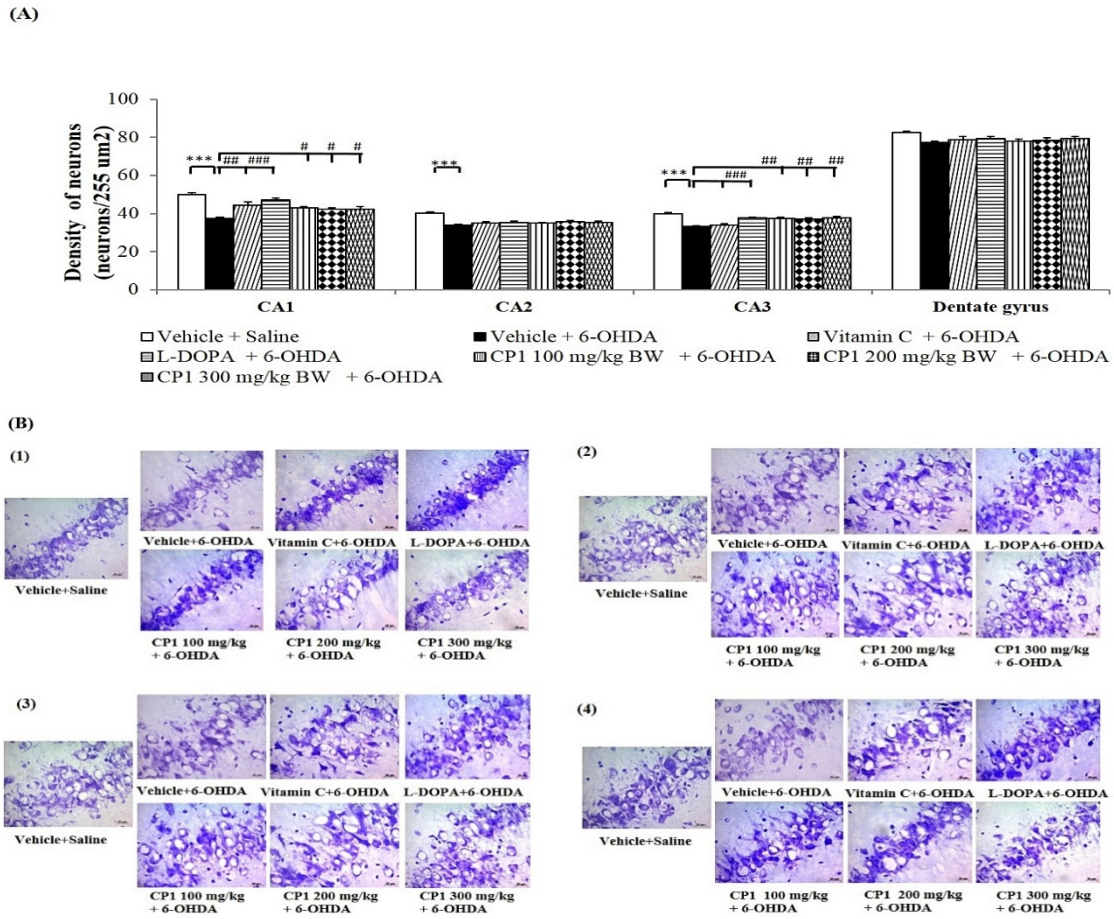

Figure 6-4 The effect of CP1 on neuron density in various subregions of hippocampus including CA1, CA2, CA3 and dentate gyrus of rats subjected to 6-OHDA. A) Average density of neurons in CA1, CA2, CA3 and dentate gyrus B) Photograph of neuron density in CA1, CA2, CA3 and dentate gyrus (n=6/group). ***p-value<.001; compared to vehicle+saline group and $^{\#\#}$ p-value<.01, $^{\#\#\#}$ p-value<.001 as compared with vehicle+6-OHDA group

3.5 Effect of CP1 on tyrosine hydroxylase positive neuron

In this part, the density of tyrosine hydroxylase positive neuron in substantia nigra was investigated by using immunohistochemistry technique. The rats subjected to the unilateral lesion of substantia nigra induced by 6-OHDA showed the reduction of dopaminergic neuron in the substantia nigra (p-value<.001; compared to vehicle+saline group). Both Vitamin C and L-DOPA treatments significantly enhanced the reduction of dopaminergic neuron induced by 6-OHDA (p-value<.001 all;

compared to vehicle+6-OHDA group). Interestingly, CP1 at doses of 200 and 300 mg/kg BW also significantly attenuated the reduction of dopaminergic neuron density (p-value<.05, .01 respectively; compared to vehicle+6-OHDA group) while no significant change was observed in low dose of CP1 group as shown in figure 6-5.

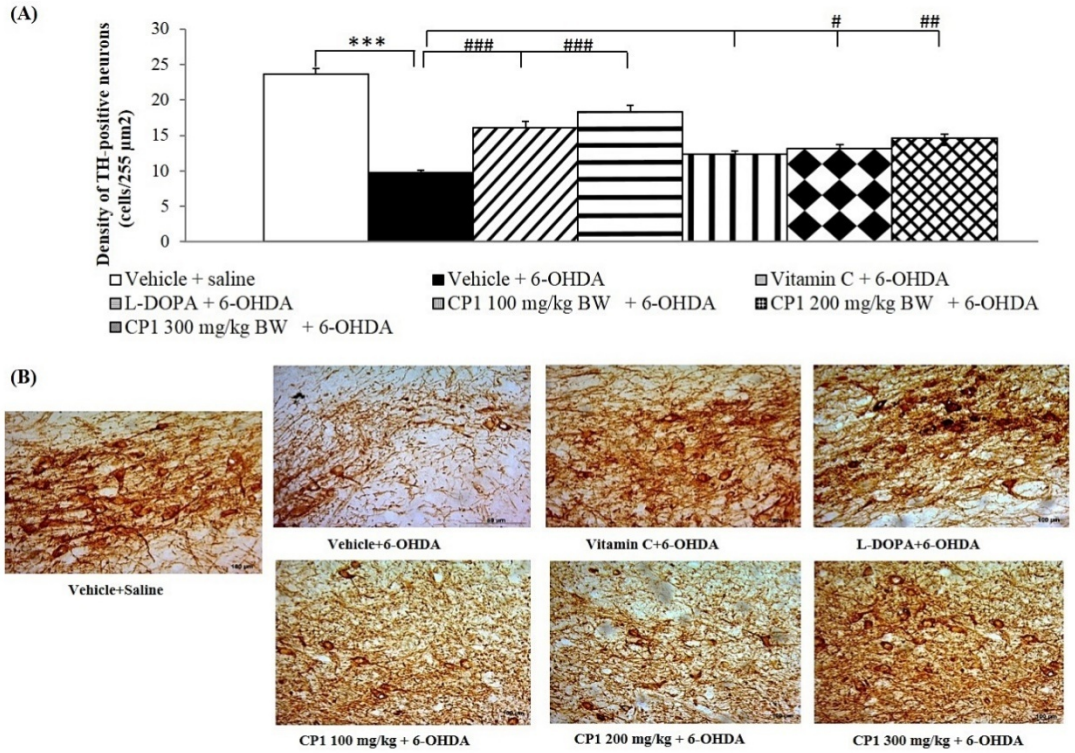

Figure 6-5 The effect of CP1 on tyrosine hydroxylase positive neurons in rats subjected to 6-OHDA treatment. Values are given as mean ± S.D. (n = 6) *** p-value<.001 as compared with vehicle+saline group and $^{\#}$ p-value<.05, $^{\#\#}$ p-value<.01, $^{\#\#\#}$ p-value<.001 as compared with vehicle+6-OHDA group

3.6 Effect of CP1 on AChE and MAO-B activities

Since acetylcholine and monoamine oxidase B had been reported to play the crucial roles on cholinergic and dopaminergic function, this study also focused on the effect of CP1 on the activities of AChE and MAO-B. The results were shown in figure 6-6. Rats which exposed to 6-OHDA showed the elevation of AChE in hippocampus and striatum (p-value<.001 and .05 respectively; compared to vehicle+saline). However, the elevated AChE in hippocampus was reversed by

L-DOPA, Vitamin C and all doses of CP1 (p-value<.001 all; compared to vehicle+ 6-OHDA group). No significant changes of AChE induced by 6-OHDA in striatum were observed in rats treated with L-DOPA, Vitamin C, and all doses of CP1. Figure 6-7 showed the effect of CP1 on MAO-B activity in hippocampus and striatum. Rats which subjected to the unilateral lesion of substantia nigra induced by 6-OHDA demonstrated the significant reduction of MAO-B in the striatum (p-value<.01; compared to vehicle+ saline group). In addition, it was found that L-DOPA treatment significantly mitigated the elevation of MAO-B activity in striatum (p-value<.05; compared to vehicle+6-OHDA group). Interestingly, the high dose of CP1 significantly mitigated the elevation of MAO-B activity in striatum (p-value<.05; compared to vehicle+6-OHDA group). However, no significant change was observed in hippocampus.

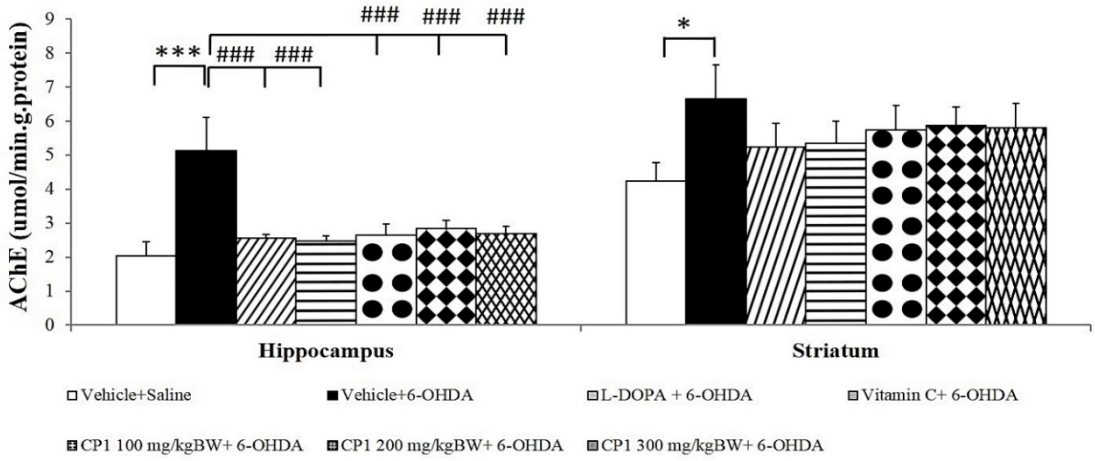

Figure 6-6 Effect of CP1 on the activity of acetylcholinesterase (AChE) in the hippocampus and striatum. Values are given as mean ± S.D. (n = 6) ** p-value<.01, *** p-value<.001 as compared with vehicle+ saline group and ### p-value<.001 as compared with vehicle+6-OHDA group

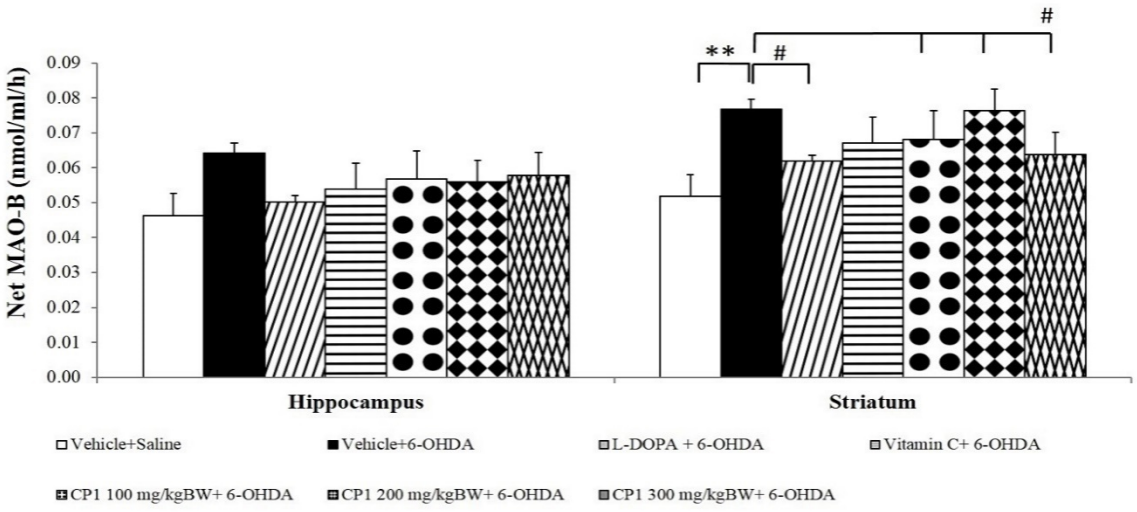

Figure 6-7 Effect of CP1 on the activity of monoamine oxidase-B (MAO-B) in the hippocampus and striatum of rats subjected to 6-OHDA rats. Values are given as mean ± S.D. (n = 6) ** p-value<.01 as compared with vehicle+ saline group and #p-value<.05 as compared with vehicle+6-OHDA group

3.7 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 and striatum were also evaluated. The results were shown in figure 6-8 to 6-11. It was found that 6-OHDA injection into right substantia nigra significantly increased MDA level but decreased the activities of CAT and GSH-Px in hippocampus (p-value<.001, .05 and .01 respectively; compared to vehicle+ saline group). However, the elevation of MDA level in hippocampus was mitigated by L-DOPA, Vitamin C and CP1 at doses of 100 and 200 mg/kg BW (p-value<.01, .01, .05 and .05 respectively; compared to vehicle+ 6-OHDA group). No significant changes of CAT, SOD and GSH-Px in hippocampus were observed in rats subjected to 6-OHDA which treated with L-DOPA, Vitamin C, and all doses of CP1. In addition, rats which exposed to 6-OHDA showed the elevation of MDA level but decreased the activities of CAT, SOD and GSH-Px in striatum (p-value<.001, .001, .01 and .01 respectively; compared to vehicle+ saline group). L-DOPA treatment significantly mitigated the elevation of MDA level and the reduction of CAT activity in striatum (p-value<.05 all; compared to vehicle+ 6-OHDA

group). Interestingly, medium dose of CP1 significantly mitigated the reduction of CAT activity in striatum (p-value<.05; compared to vehicle+ 6-OHDA group).

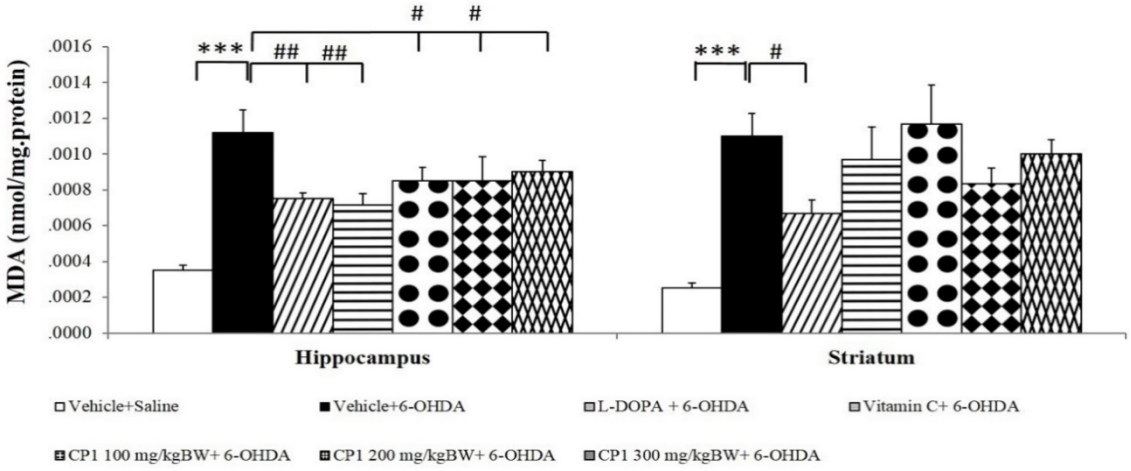

Figure 6-8 Effect of CP1 on the malondialdehyde (MDA) level in the hippocampus and striatum of rats subjected to 6-OHDA rats. Values are given as mean ± S.D. (n = 6) *** p-value<.001 as compared with vehicle+saline group and [#] p-value < .05, [##] p-value < .01 as compared with vehicle+6-OHDA group

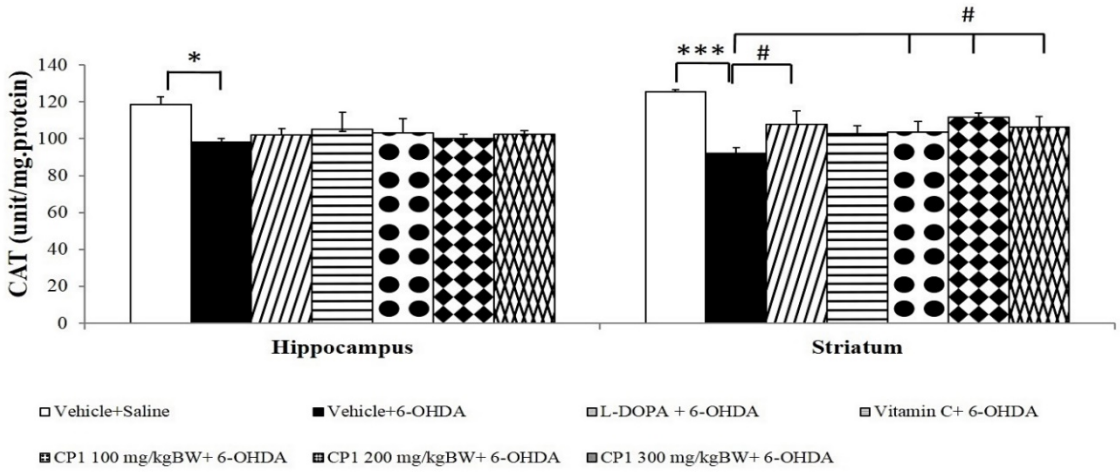

Figure 6-9 Effect of CP1 on the activity of catalase (CAT) in the hippocampus and striatum of rats subjected to 6-OHDA rats. Values are given as mean ± S.D. (n=6). * p-value<.05, *** p-value<.001 as compared with vehicle+ saline and [#] p-value < .05 as compared with vehicle+6-OHDA group

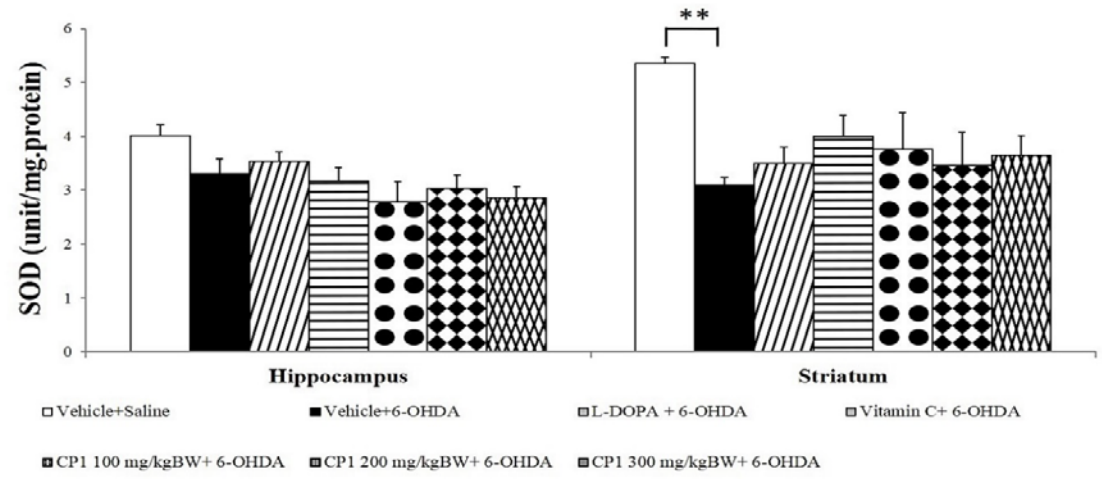

Figure 6-10 Effect of CP1 on the activity of superoxide dismutase (SOD) in the hippocampus and striatum of rats subjected to 6-OHDA rats. Values are given as mean±S.D. (n=6). *p-value<.05, *** p-value<.001 as compared with vehicle+ saline

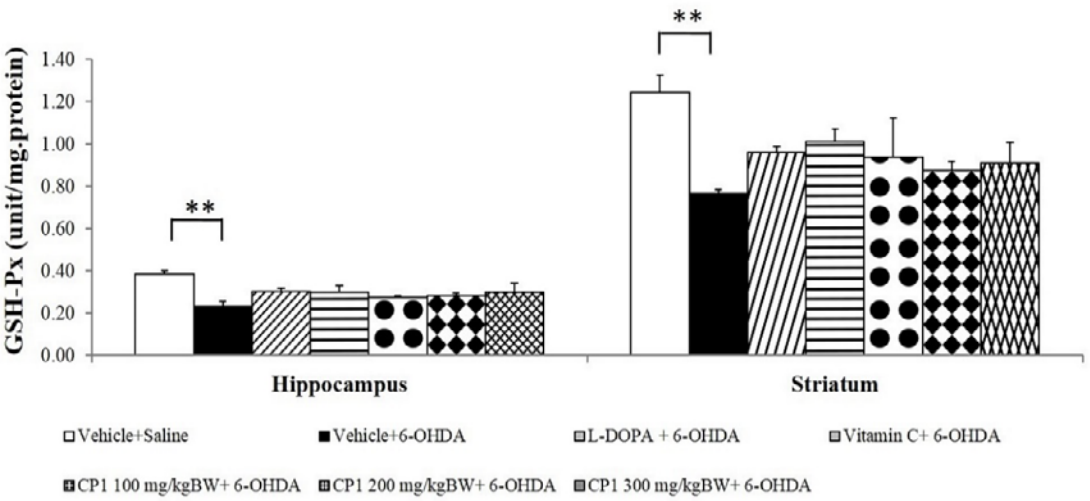

Figure 6-11 Effect of CP1 on the activity of glutathione peroxidase (GSH-Px) in the hippocampus and striatum of rats subjected to 6-OHDA rats. Values are given as mean±S.D. (n=6). **p-value<.01 as compared with vehicle+ saline

4. Discussion

In this study, it had been clearly demonstrated that the combination of *C.rotundus* and *Z.officinale* (CP1) at a ratio used in this study showed higher antioxidant activities, AChEI and MAOI effects than *Z.officinale* or *C.rotundus* alone.It also attenuated the degeneration of neurons, and cholinergic neurons in hippocampus. In addition, the degeneration of dopaminergic neurons in substantia nigra was also observed. Moreover, the improved both motor and non-motor impairments were also observed.It has been demonstrated that 6-OHDA induces nigrostriatal damage and the impairments of both motor and non-motor symptoms such as cognitive deficit similar to that observed in patients with PD (Chaturvedi *et al.*, 2006; De Leonibus *et al*., 2007 ;Hefco *et al*., 2003; Mura and Feldon, 2003; Tadaiesky *et al*., 2008). Therefore it has been used as a validated model of PD. The current results also confirm these changes. It was demonstrated that the administration of 6-OHDA into substantia nigra produced the motor and memory deficit, the elevation of MAO-B, AChE and oxidative stress in hippocampus and striatum. Based on the connection between substantia nigra and the limbic system including hippocampus (Sriraksa *et al*., 2011), the administration of 6-OHDA into substantia nigra could induce the dopaminergic damage not only in substantia nigra but also in striatum and hippocampus which in turn induced the functional disturbances of the affected areas (Pennartz *et al*., 2011;Sriraksa *et al*., 2011).

Accumulative lines of evidence have shown that spatial memory impairment is associated with the neurodegeneration in hippocampus (Oswald and Good, 2000; Devi *et al*., 2003) and the neurodegeneration which occurs in this area is under the influence of oxidative stress status in the mentioned area (Smith *et al*., 1991; Lyras *et al.*, 1997; Mecocci *et al*., 1997; Harman, 1998). It has been shown that all doses of CP1 used in this study enhance spatial memory and neuron density in hippocampus. In addition, the cholinergic function and oxidative stress status in hippocampus also increase in CP1 treated rats. Based on the crucial role of oxidative stress status on the neurodegeneration and the role of neurodegeneration on spatial memory impairment mentioned earlier, it has been suggested that CP1 improve oxidative stress status reflected by the decreased MDA level giving rise to the increased neuron density in both CA1 and CA3, the areas contributing the role on encoding and retrieval, (Ji and

Maren, 2008) which in turn enhance spatial memory. Although CP1 treated rats at all doses used in this study decreased MDA level, no changes of SOD,CAT and GSH-Px activity in hippocampus were observed in rats which received CP1. Therefore, the reduction of MDA level might also involve other factors rather than the increase of enzymatic scavenging enzymes such as non-enzymatic antioxidant system (Nikam *et al.*, 2009) and the decreased oxidative stress formation (Uttara *et al*., 2009). Rats which received CP1 at high dose also showed the increased neurons density in CA1 and CA3 while no improved oxidative stress status in hippocampus was observed. Therefore, other factors such as the decreased apoptosis might also involve (Tatton *et al*., 2003). In addition, it has been reported that the suppression of AChE which in turn increased cholinergic function in hippocampus also plays the pivotal role on the increased learning and memory impairment in Parkinson-liked condition induced by 6-OHDA (Sriraksa *et al.,* 2012). Therefore, the alteration of AChE in hippocampus of rats treated with CP1 was also investigated. The current data also revealed that all doses of CP1 also suppressed AChE in hippocampus. Therefore, the suppression of AChE in hippocampus might also play a role on the improved spatial memory impairment in 6-OHDA lesion rats in this study.

CP1 at doses of 200 and 300 $mg.kg^{-1}$BW also improved motor symptom and dopaminergic neurons density in substantia nigraof 6-OHDA lesion rat, an animal model of Parkinson's disease. It has been reported that the improved dopaminergic function (Fox *et al*., 2011) play an important role on the improved motor behaviors disorder such as rotational behavior. Therefore, the improved rotational behaviors in CP1 treated rats might be due to the increased dopaminergic neurons in substantia nigra, an important area involving the pathogenesis of Parkinson's diseases. On the basis of previous finding which demonstrated the role of apoptotic pathway in the degeneration of dopaminergic neurons in substantia nigra (Tatton *et al*, 2003), it has been suggested that the enhanced dopaminergic neurons density in substantia nigra in 6-OHDA lesion rats treated with CP1 at medium and high doses may be due to the decreased apoptosis in substantia nigra. However, this required further investigation. Since oxidative stress and monoamine oxidase type B (MAO-B) suppression have been reported to be the targets for therapeutic strategy against Parkinson's disease (Ebadi *et al*, 1996), the alterations of oxidative stress markers and the activity of

MAO-B in striatum, a key area in regulating the basal ganglia function was also investigated. However, only the rats which received CP1 at high dose showed the suppression of MAO-B activity in striatum. Although rats which received medium dose of CP1 showed the enhanced CAT activity in striatum, no significant change of MDA level was observed. Therefore, the suppression of MAO-B in striatum induced by high dose of CP1 might increase dopaminergic function which in turn improved the function of basal ganglia and improved motor symptom in animal model of Parkinson's disease. The improved oxidative stress was less likely to play a role in the improved motor symptom induced by CP1 both at medium and at high doses. No dose dependent manner of CP1 was observed in this study because the relationships between the concentration of CP1 and the observed parameters were not the simple linear relationships.

Based on the high content of gingerol in CP1 and the anti-apoptotic effect (Wang *et al*, 2014) of gingerol and the anti-parkinson effect of quercetin, a constituent content in CP1, it has been suggested that the improved both motor and non-motor symptoms of Parkinson's disease induced by CP1 may be associated with gingerol and quercetin. The interaction effect of both substances are also possibly to contribute the role on the anti-parkinson effect of CP1. However, further investigations are very much essential.

In conclusion, CP1 is the potential candidate to improve both motor and non-motor symptoms such as memory impairment in Parkinson's disease. The memory enhancing effect might occur via the suppression of AChE whichinturn increased cholinergic function in hippocampus and the decreased oxidative stress status which in turn increased neuron density in CA1 and CA3 in hippocampus leading to the improved memory impairment. The motor improvement induced by CP1 might occur via the enhanced dopaminergic neurons density in substantia nigra which in turn improved dopaminergic function and motor disorder. In addition to the the enhanced density of dopaminergic neurons in substantia nigra, the improved dopaminergic function in striatum induced by CP1 especially at high dose also occurred via the suppression of MAO-B in striatum. Therefore CP1 might be served as the potential phytotherapy against Parkinson's disease or as an adjuvant therapy against Parkinson's disease. However, further researches concerning the active ingredient, subchronic toxicity and the interaction between CP1 and other medicines are essential.

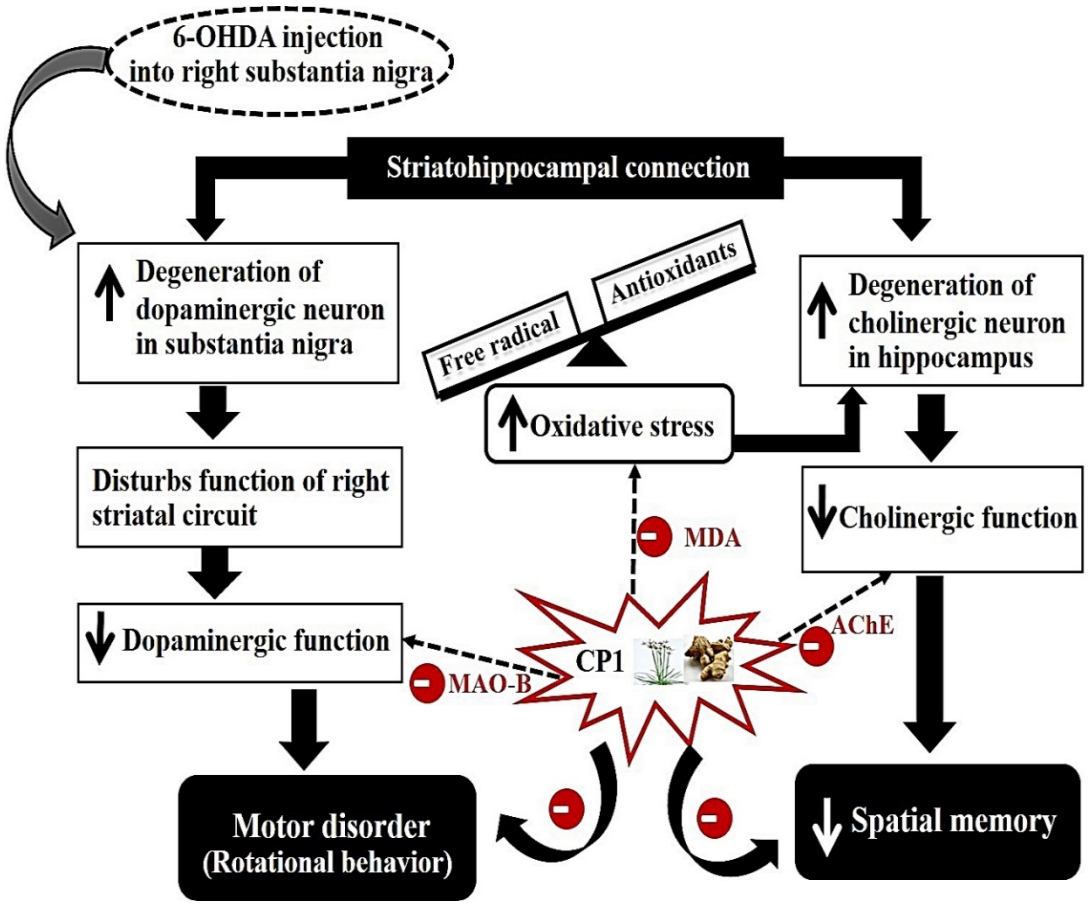

Figure 6-12 Schematic diagram concerning the possible mechanisms to improve memory and motor disorders of CP1 in hemiparkinsonian rats induced by the unilateral injection of 6-OHDA into right substantia nigra