CHAPTER III
RESEARCH METHODOLOGY

1. Materials

Quercetin, zein, 6-hydroxydopamine and apomorphine have been purchased from Sigma-Aldridge Company.

L-dopa was kindly given by Associated Professor Dr. Somsak Tiamkao which produced by Roche company while vitamin C was a product of the government pharmaceutical organization.

2. Preparation of Animals

Adult male Wistar rats (8 weeks) were used as experimental animals. They were obtained from National Laboratory Animal Center, Mahidol University. The weights of the animals in the young adult were 200-250 grams, they were housed 5 per cage and maintained in 12:12 light: dark cycle and given excess to food and water *ad libitum*. This study has been reviewed and approved by the Animal Ethics Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand. The record number is AEKKU 2/2552 and the reference number is 0514.1.12.2/11.

3. Preparation of Zein Based Quercetin Nanofiber Patch

The zein based quercetin nanofiber patch was fabricated by electrospinning method. Briefly, zein was dissolved in N, N-Dimethylformamide (DMF) at a ratio of 1:2 (w/v). Slight stirring was performed approximate 30 minutes to expedite dissolution. Quercetin was added to the solution to produce the solution which contained 10 and 15% quercetin and the solution was stirred for further 60 minutes. The solution was loaded into a plastic syringe equipped with a 22-gauge needle made of stainless steel. The needle was connected to a high voltage supply (DEL High Voltage (0–100 kV), DEL Electronics Corp., USA). The solution was fed at a rate of 0.2 ml/h for 12 hours using a syringe pump (TERUMO Terufusion Syringe pump TE-

331, Japan). A piece of flat aluminum foil was placed 15 cm below the tip of the needle, and used to collect the nanofibers. The voltage for electrospinning was 13 kV. All electrospinning processes were carried out at room temperature.

4. Experimental Protocol

This study was divided into 3 separated parts.

The first part was set up to determine the neuroprotective effect against Parkinson's disease of conventional quercetin administration.

The second part was set up to determine the neuroprotective effect against Parkinson's disease of zein based quercetin nanofiber patch.

The third part was set up to compare the effectiveness of conventional quercetin and zein based quercetin nanofiber patch by comparing the density of neuron and tyrosine hydroxylase positive neuron in various brain areas and compare the effect of quercetin administered by different interventions on free radicals and antioxidant enzymes activities.

4.1 The determination the neuroprotective effect against Parkinson's disease of conventional quercetin administration.

The animals were randomly divided into 9 groups (n=8 animals/group) as described following;

Group I: Control group which received no treatment

Group II: Vehicle treated group alone, rats were received normal saline solution (NSS) which served as vehicle to suspend quercetin, once daily via oral route.

Group III: Vehicle + NSS injection, rats were received NSS at a period of 14 days before and 14 days after NSS injection into the right substantia nigra, (NSS served as vehicle for 6-OHDA).

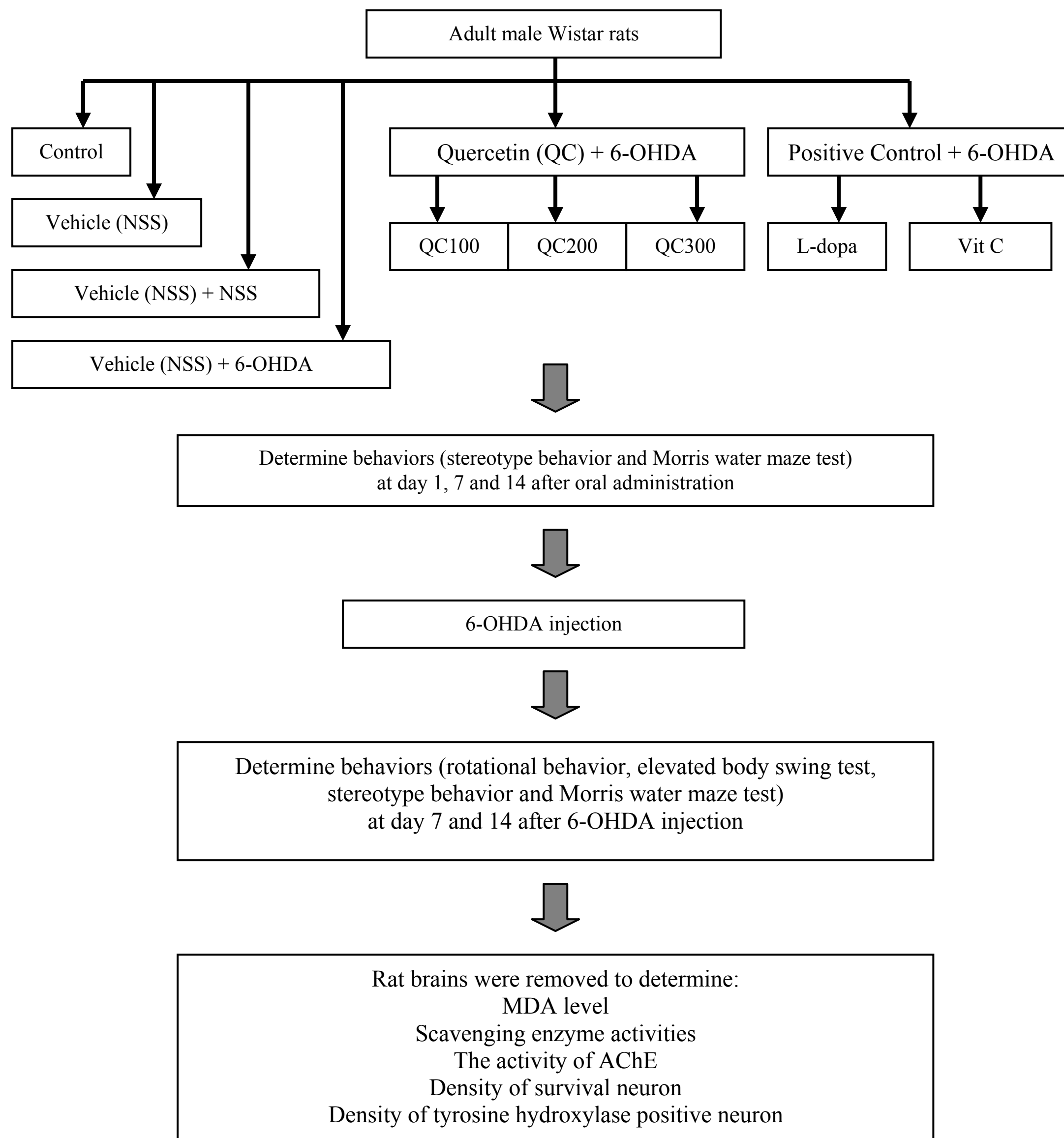
Group IV: Vehicle + 6-OHDA, rats were given NSS orally at a period of 14 days before and 14 days after 6-OHDA injection into the right substantia nigra.

Group V-VII: Quercetin treated group, rats were orally administered various doses of quercetin ranging from 100, 200 and 300 mg/kg BW once daily at a period of 14 days before and 14 days after 6-OHDA injection.

Group VIII-IX Positive control groups, rats were orally treated with L-dopa (5 mg/kg BW) (Mandel, 2000) and vitamin C (200 mg/kg BW) (Fakher *et al*, 2007) and received 6-OHDA injection just like mentioned groups above.

All rats except group I were received the same volume of administered treatment. They were determined locomotor activity, cognitive enhancing effect by using Morris water maze test. The escape latency and retention time were recorded as indices for spatial memory, according to the following time schedule; after single dose, 1 and 2 weeks of substance administration. Rats were subjected to 6-OHDA injection by using the stereotaxic apparatus and infused the toxin by the Hamilton syringe into the right substantia nigra (co-ordinates: anteroposterior -0.5 mm from bregma, mediolateral 2.1 mm from midline and dorsoventral -7.7 mm from the skull after substances treatment for 2 weeks. After the operation, the rats were allowed to recovery for 1 week. At the period of 1 week and 2 weeks after 6-OHDA injection, rats were determined the locomotor activity, Morris water maze test. In addition, rats were determined rotational behavior and elevated body swing test, which these two tests were performed in order to determine the motor changes following nigrostriatal lesion in rats.

After the last dose of administration, they were sacrificed and their brains were isolated in order to determine level of malondialdehyde (MDA), activities of scavenging enzymes (SOD, GPx, CAT) and the activity of acetylcholinesterase (AChE) in hippocampus and striatum. In addition, rat brains were determined the density of survival neuron (hippocampus, striatum, and substantia nigra) and determine tyrosine hydroxylase immunohistochemistry in substantia nigra.



4.2 The determination of the neuroprotective effect against Parkinson's disease of zein-based quercetin nanofiber patch.

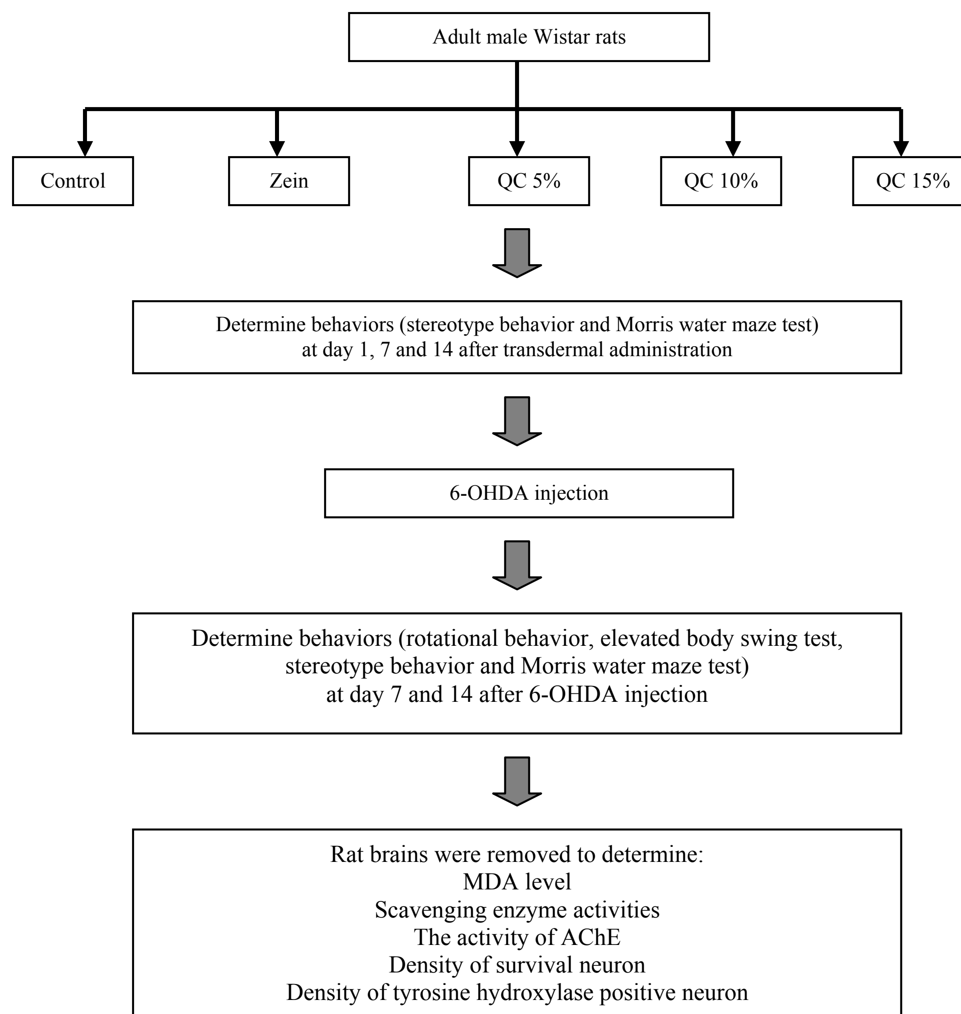
The animals were randomly divided into 5 groups (n=8 animals/group) as described following;

Group I: Naïve intact control group, which received no treatment.

Group II: Vehicle treated group which received zein nanofiber via transdermal patch.

Group III-V: Rats were treated with transdermal zein based quercetin nanofiber patch at various concentrations (5%, 10% and 15% respectively).

In this part rats were received quercetin via transdermal route. They received the same protocol of behavioral determination and the same schedule of quercetin treatment and 6-OHDA injection as oral part. In addition, all biochemical assays, histology and immunohischemistry were performed as same as the conventional part.



4.3 Comparison of the effectiveness of conventional quercetin and zein based quercetin nanofiber.

In order to compare the effectiveness of conventional quercetin and transdermal quercetin administration, after the last dose of administration the rats were sacrificed by brain perfusion and their brains were removed in order to determine following parameters;

First: the density of the density of survival neuron (hippocampus, striatum and substantia nigra) and tyrosine hydroxylase positive neuron in substantia nigra.

Second: the level of malondialdehyde (MDA, the activities of scavenging enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in hippocampus and striatum.

Third: the activity of acetylcholinesterase (AChE) in hippocampus and striatum.

5. The Administration of 6-OHDA

6-OHDA were prepared as described previously by Ferro and co-workers (Ferro *et al.*, 2005). Briefly, rats were anesthetized with 40 mg/kg sodium thiopental (i.p.), each animal were mounted on a stereotaxic stand, the skin overlying the skull were cut to expose the skull, and the burr hole was performed. The 6 μg 6-OHDA in 2 μl 0.2% ascorbic acid saline was perfused into SNpc through a 30 gauge stainless needle according to the following co-ordinates; anteroposterior -0.5 mm from bregma, mediolateral 2.1 mm from midline and dorsoventral -7.7 mm from the skull (Paxinos and Watson, 1982). After surgery, animals were allowed to recover from anesthesia and then placed in their cage.

6. Behavioral Studies

Behavioral tests of all animals were conducted every 7 days by blinded observer. The animals were assessed the changes in neurobehaviors including locomotor activity, elevated body swing test, rotational behavior and Morris water maze test.

6.1 Elevated body swing test

The swing test is a simple and easy behavioral test that only requires handling the animal by its tail and recording the direction of swings made by the animal for 1 min. The rat was held approximately 1 inch from the base of its tail. It was then elevated to an inch above the surface on which it has been resting. The rat was held in the vertical axis, a swing was recorded whenever the animal moved its head out of the vertical axis to either side. Before attempting another swing, the animal must return to the vertical position for the next swing to be continued. In cases

when the animal swings and redoubles its effects to move toward one side without returning to the vertical position, only one swing was counted. The total number of swings made to each side was divided by the overall total number of swings made to both sides to get percentages of left and right swings. The criterion for biased swing behavior were set at 70% or higher (Borlongan and Sanberg, 1995).

6.2 Rotational behavior

Prior to determine rotational behavior, rats were injected with apomorphine (0.5 mg/kg BW, s.c). They were then measured behavior by placing animals in a cylindrical container, 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 (Ahmad *et al*., 2005).

6.3 Morris water maze test

The Morris water maze test is one of the most important paradigms used for testing spatial memory. This test is believed to be associated with the proper functioning of hippocampus. Animals were tested in a spatial version of Morris water maze was tested as described of a circular water task (120 diameter, 50 cm height) that were partially filled with water (25 °C). Powder was used to render the water opaque. The training started by acclimatization the rat to the task environment with 4 days of free-swimming in the pool with no platform. Each session lasted for 2 min. The pool was divided virtually into four equal quadrants, labeled N-S-E-W. A platform (10 cm diameter) was placed in one of the four maze quadrants (the target quadrant) and submerged 1.5 cm below the water surface. The platform remained in the same quadrant during the entire experiment. The rats were required to find the platform using only distal spatial cues available in the testing room. The cues were maintained constant throughout the test. The rats received four consecutive daily training trials in the following 5 days, with each trial having a ceiling time of 60 seconds and a trial interval of approximately 30 seconds. The rat have to swim until it climbs onto the platform submerge underneath the water. After climbing onto the platform, the rat will remain there for 30 seconds before the commencement of the next trial. The escape platform was kept in the same position relative to the distal cues. If the rat fails to reach the escapes platform within the maximally allowed time of 60 seconds, it was gently placed on the platform and allowed to remain there for

the same amount of time. The time to reach the platform (latency in seconds) was measured (Morris, 1981).

6.3.1 Probe trial

A probe trial was performed wherein the extent of memory consolidation was assessed. The time spent in the target quadrant indicates the degree of memory consolidation that has taken place after learning. In the probe trial, the rat was placed into the pool as the training trial, except that the hidden platform was removed from the pool. The time of crossing the former platform quadrant and the total time of crossing all quadrants was recorded for 1 minute.

7. Tissue Preparation

The brains of the animal were perfused transcardially with fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, weighed the brains after removed and then they were postfixed in the same fixative overnight at 4°C following by immersed sequentially for 48-72 h each in a cryoprotectant containing 30% sucrose. Serial sections of tissue were cut frozen on a sliding microtome at 30 μm thick. Sections were stored in phosphate buffer and they were picked up on slides coated with a 0.01% aqueous solution of a high molecular weight poly-L-lysine. The serial cut sections were either stored at 4°C or processed immediately (APPENDIX A).

8. Cresyl Violet Staining for Nissl Substance

Adjacent series of sections throughout striatum, substantia nigra and hippocampus from all groups were stained with 0.5% cresyl violet to aid in neuronal density determination (APPENDIX C).

9. Tyrosine Hydroxylase Immunohistochemistry Technique

Tyrosine hydroxylase immnohistochemistry was performed according to the method of Ahmad and co-workers (Ahmad *et al.*, 2005). The animals were deeply anesthetized 50 mg/kg sodium thiopental (i.p.) and perfused transcardially through ascending aorta with 100.0 ml of 0.1 M phosphate buffer saline (PBS) at pH 7.5 followed by 300.0 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Brains

were immediately removed out and further immersed in the same fixative for an additional 24 h at 4°C. Furthermore, the tissues were preserved in 10%, 20%, and 30% sucrose solution (in phosphate buffer) until they sank. The tissues were then kept in final sucrose solution until sectioning. The fixed tissues were embedded in OCT compound (polyvinylglycol, polyvinyl alcohol, and water) and frozen at -20°C. Coronal sections of 30 µm thickness were cut on a freezing cryostat (Leica), collected in PBS, and stored at 4°C. The sections were then transferred to gelatin-coated slides and immersed in wash buffer (sodium phosphate 100 mM, sodium chloride 0.5 M, Triton X-100, sodium azide) at pH 7.4 for 20 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and 10% methanol in PBS and incubated for half and hour at room temperature. Thereafter, the slides were washed with PBS, and the sections were overlaid with 20 µl of anti-tyrosine hydroxylase antibodies (2% in PBS) and incubated for 2 h in a humid chamber at room temperature. The slides were washed again with 20 µl solution of biotinylated anti-mose IgG (2% in PBS) for 3 h at 4°C in the humid chamber. Then slides were exposed to streptavidin-peroxidase and the labeled sites were visualized with a solution of diaminobenzidine and hydrogen peroxide. Finally the sections were dehydrate, cover-slipped, and viewed under microscope, and photomicrographs were taken (APPENDIX D).

10. Preparation of Tissue Homogenates

After the last administration of substances, rats were anesthetized with 50 mg/kg sodium thiopental (i.p.), and then were perfused transcardially with ice-cold 0.9% NaCl to remove the free radical scavenging and free radical generating source in the brain. The striatum and hippocampus were carefully excised, and the tissues stored at -80 °C then tissues were homogenized in 4 volume of 1.15% KCL with glass potter-Elvehjim homogenizer (APPENDIX E).

11. Enzymatic Assay of Superoxide Dismutase (SOD)

SOD was assayed utilizing the technique of McCord and Fridovich (1969) based on as the inhibition of the rate of reduction of cytochrome *c* by the superoxide radical, which was observed at 550 nm. In a 1 ml system mixture, the final concentrations were 50 mM potassium phosphate, 0.1 mM ethylenediaminetetraacetic

acid (EDTA), 0.01 mM cytochrome *c*, 0.05 mM xanthine, 0.005 unit xanthine oxidase and 1 unit superoxide dismutase solution or sample. The superoxide dismutase solution was used as a standard enzyme activity. The standard curve was plotted as percentage inhibition against the SOD activity. One unit activity was defined as the amount of enzyme necessary to inhibit the rate of reduction of cytochrome *c* by 50 % in the coupled system using xanthine-xanthine oxidase at pH 7.8 at 25°C. The data was presented in units of SOD activity per mg protein. (APPENDIX H).

12. Enzymatic Assay of Glutathione Peroxidase (GPx)

The glutathione peroxidase activity was determined by the method of Wendel (1980). Oxidized glutathione, produced upon reduction of hydrogen peroxide by glutathione peroxidase, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A340nm is directly proportional to the glutathione peroxidase activity. In the final 1 ml of the system mixture contained 48 mM sodium phosphate, 0.38 mM EDTA, 0.12 mN β-NADPH, 0.95 mM sodium azide, 3.2 units of glutathione reductase, 1 mM glutathione (GSH), 0.02 mM DL-dithiothreitol, 0.0007% H_2O_2, and the standard enzyme glutathione peroxidase solution or a homogenate brain sample. The glutathione peroxidase solution was used as a standard enzyme activity. The standard curve was plotted as the rate of A340 nm per minute against the GPx activity. One unit activity was defined as the amount of enzyme necessary to catalase the oxidation by H_2O_2 of 1 μmole of GSH to GSSG per minute at pH 7 at 25°C. The data was reported in units of GPx per mg of protein (APPENDIX I).

13. Enzymatic Assay of Catalase (CAT)

CAT was assayed using colorimetric method as described by Goldblith and Progtor (1950). The enzyme samples or the standard enzyme solution was allowed to react with hydrogen peroxide for one minute. The reaction was then stopped by a sulfuric acid solution. A potassium permanganate was next added to the mixture and allowed to react with the excess peroxide, which was not decomposed by catalase. After the addition of permanganate, the excess permanganate from the reaction with

peroxide was determined the absorbance at 515 nm. The standard curve was plotted as the A515nm against the catalase activity. The data was reported in units of catalase per mg protein (APPENDIX J).

14. Estimation of Malondialdehyde (MDA)

Tissue homogenate was determined oxidative damage marker by thiobarbituric acid reaction according to the method of Ohkawa et al. (1979) and determine protein concentration by the colorimetric method of Lowry et al. (1951) (APPENDIX G).

15. Enzymatic Assay of Acetylcholinesterase (AChE)

The determination of AChE in brain homogenate was performed according to a method developed by Ellman et al. 1961. This method employed acetylthiocholine iodide (ATChI) as a synthetic substrate for AChE. ATChI was broken down to thiocholine and acetate by AChE and thiocholine was reacted with dithiobisnitrobenzoate (DTNB) to produce a yellow color. The quantity of yellow color which developed over time was used as an index of the activity of AChE and can be measured using a spectrophotometer (APPENDIX K).

16. Statistical Analysis

All data were expressed as mean $\pm$ S.E.M. value. The differences among various groups will be compared by ANOVA. The statistical difference were regarded at p-value<0.05.