CHAPTER III
RESEARCH METHODOLOGY

1. Materials

Quercetin dehydrate (98% HPLC purity), high-purity egg L-α-phosphatidylcholine, Type XVI-E (EPC), cholesterol (CHOL) and polyethylene glycol 400 (PEG) were purchased from Sigma (Barcelona, Spain). Other reagents used were analytical grade such as chloroform and ethanol (BDH Laboratory Supplies, Poole, England), glutathione reduced form (GSH), superoxide dismutase (SOD), xanthine oxidase (XOD), cytochrome c, EDTA, thiobarbituric acid (TBA), glutathione reductase (GSSGR) were analytical grade and purchased from Sigma Chemical Co., all other chemicals were at least reagent grade and used as received. Water was double distilled.

2. Preparation of Liposomes

Associate Professor Aroonsri Prempri, Departent of Pharmaceutical Sciences and Technology, Faculty of Pharmaceutical Sciences, Khon Kaen University will kindly provide liposomes. In brief, the unilamellar liposomes were prepared via a lipid thin film formation and extrusion (Priprem et al., 1999; Guo et al., 2003; Liang et al., 2005; Zu et al., 2006). The lipid thin film had been prepared as a mixture of egg phosphatidylcholine (EPC) and cholesterol (chol) at a varied ratio of EPC: chol ranging from 1:1 to 9:1. In brief, quercetin liposomes were prepared from a 0.025 M solution of EPC: chol (1:1) in 2 ml chloroform, which was evaporated at 60 °C for 30 min to form the lipid thin film. Quercetin aqueous solution was then dropped to the lipid film and sonicated to form a liquid dispersion, which was subsequently extruded through 0.2 μm and 0.1 μm polycarbonate membrane (PC membrane, Whatman, U.S.A.), thus forming quercetin liposomes. The particle size distribution of quercetin liposomes was determined by laser diffraction (Mastersizer 2000, Malvern Instruments, U.K.). The shape of quercetin liposomes were observed by the scanning electron microscopy (SEM) (Leo 1450 PV, England). The surface charge of particles was determined by using Zetasizer (nano series, Malvern instruments, U.K.).

The determination of quercetin entrapment was performed by indirect method. The QCL were ultracentrifuged (Spectrafuge, Labnet International, U.S.A.) at a speed of 10,000 rpm. The supernatant was collected for HPLC analysis to quantify the un-entrapped quercetin. The entrapment efficiency was calculated by using this following equation (Nii and Ishii, 2005):

$$\%\mathrm{EE} = \left(\frac{C_{tol} - C_{free}}{C_{tol}} \right) x \ 100$$

Where EE is the entrapment efficiency, C_{tol} is the total quantity of quercetin used to prepare the quercetin liposomes, and C_{free} is the quantity of quercetin remaining in the supernatant. The product of encapsulation will reveal spherical negative charge particle with size approximate of 200 nm. The entrapment efficiencies of the quercetin liposomes were in a range of 61% to 68%, which was satisfactory for the purpose of this study. Overall lipid content of the quercetin liposomes was more than 98%, which assures that the lipid mixture used in the preparation could actually form the vesicles.

3. Animals Treatments

Young adult male Wistar rats, 16 weeks old weight approximately 180-200 gms (National Animal Center, Salaya, Nakorn Pathom) were housed in group of 5 per cage in standard metal cages at 22 ± 2°C on 12:12 h light - dark cycle with food and water available at all time. The animals were kept in the animal care facility of the Faculty of Medicine, Khon Kaen University in the compliment of institutional and European Community (EEC directive of 1986; 86/609/EEC) guideline. In addition, they were acclimatized for 7 days to the new environment before use.

3.1 Oral quercetin

A suspension of quercetin in polyethylene glycol (PEG) was freshly prepared for oral administration. A syringe was used to draw the suspension of quercetin, which was then fed to the animals for the test. The dosages were 100, 200 and 300 mg/kg BW of the animals.

3.2 Intra nasal quercetin liposomes

Freshly prepared quercetin liposomes, containing 0.5 mg of quercetin in 20 µl (dose = 20 µg) was drawn into a micropipette, and then administered directly into the right nasal cavity of each animal. All substance administrations in this study were performed once daily between 8.00-9.00 a.m.

4. Experimental Protocol

This study was divided into 6 separated parts. The first part was designed to determine the neuropharmacological activities of quercetin with oral conventional delivery system. The second part was designed to determine the neuropharmacological activities of quercetin encapsulated liposomes via nasal administration. The third part was set up to determine the neuroprotective effect of quercetin with conventional delivery system in both healthy and cognitive deficit conditions of Alzheimer's disease model. The next part was set up to determine the neuroprotective effect of quercetin-encapsulated liposomes via nasal administration in both healthy and cognitive deficit conditions of Alzheimer's disease model. The fifth part was set up to determine the possible mechanisms underlying the cognitive enhancement and the neuroprotective effect of quercetin with oral conventional system against Alzheimer's disease. The last part of this study was set up to determine the possible mechanisms underlying the cognitive enhancement and the neuroprotective effect of quercetin encapsulated liposomes via nasal administration against Alzheimer's disease.

4.1 Neuropharmacological activities of quercetin with oral conventional delivery system

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

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

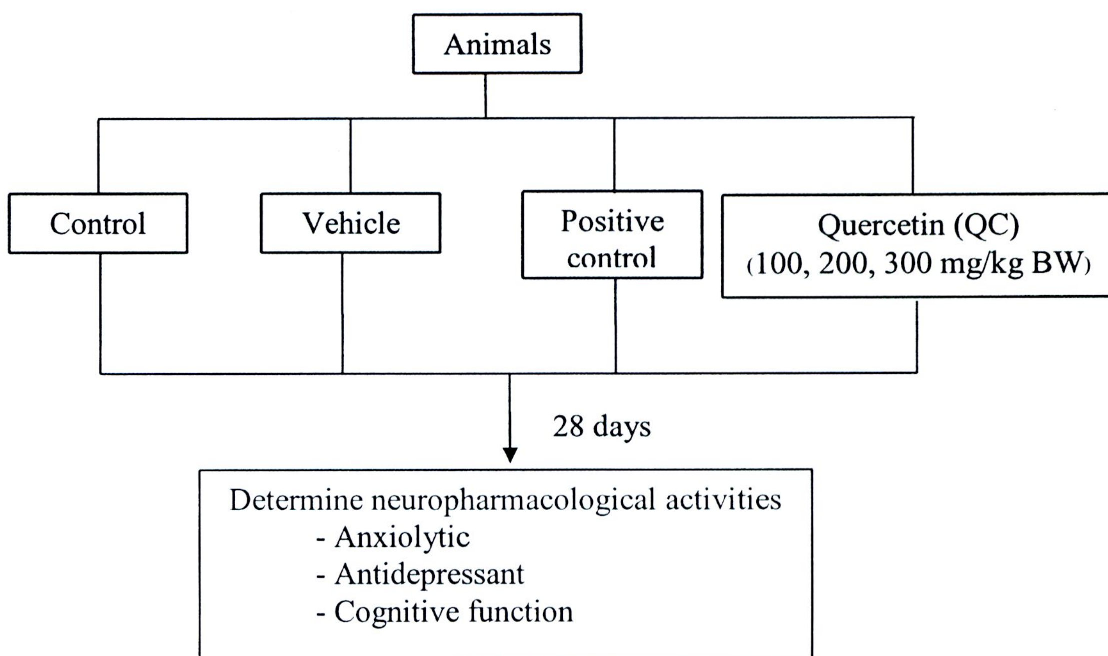
Group II: Vehicle treated group, which received polyethyleneglycol (PEG) once daily via oral route for 28 days.

Group III - VI: Positive control group: Base on previous finding in order to test the anxiolytic, antidepression and cognitive enhancing effects, the animal in positive control groups were treated with standard drug for various conditions as following; diazepam (2 mg/kg BW), fluoxetine (20 mg/kg BW), aricept or donepezil

hydrochloride (1mg/kg BW) and Vit.C 100 mg/kg BW were used as positive control for the anxiolytic, anti-depression, cognitive enhancing effect and exogenous antioxidant respectively.

Group VII – IX: Rats were orally administered of quercetin at various doses ranging from 100, 200 and 300 mg/kg BW once daily for 28 days.

Each rats in group II – IX were received the same volume of substance suspension. The animals were determined anxiolytic, anti-depression and cognitive enhancing effects within 30 minutes after the last administration of substance because the pilot data have demonstrated that this time window after treatment was the suitable time window to test the neuropharmacological profile of most agents as mentioned above. The anxiolytic effect in this study was determined using elevated plus maze test, anti depressant effect using force swimming test, whereas the spatial and non-spatial memory were determined using Morris water maze test and object recognition test respectively. All tests were performed every week in order to observe the effect time window of treatment and the dose response of quercetin.



4.2 Neuropharmacological activities of quercetin encapsulated liposomes via nasal administration

The animals were randomly divided into 4 groups (n=8 animals/group) as described following;

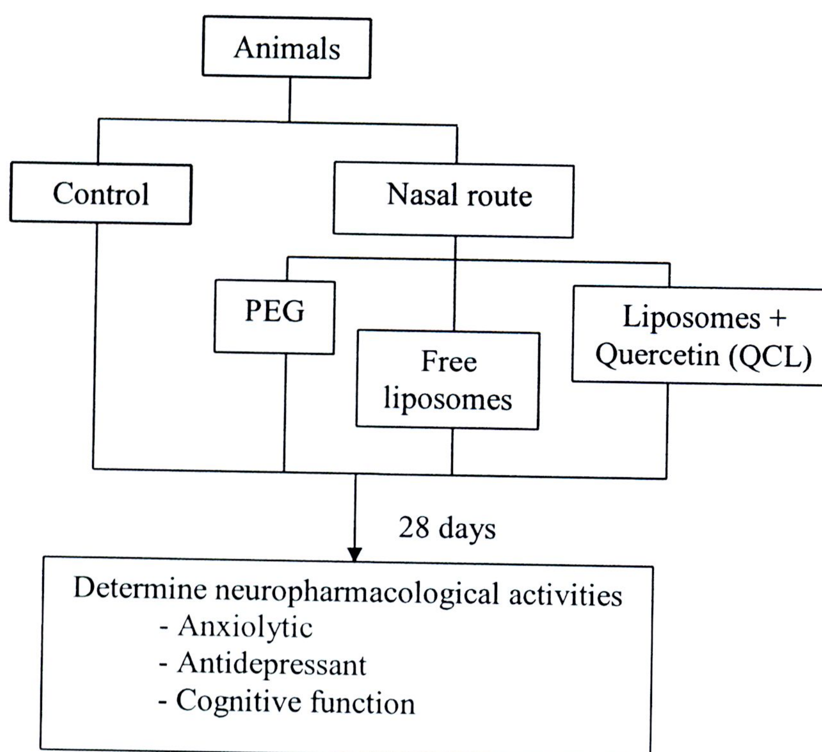
Group I: Naïve intact control group, which received no treatment.

Group II: Rats were treated with free liposomes via nasal administration for 4 weeks.

Group III: Vehicle treated group, which received polyethyleneglycol (PEG) once daily via nasal administration for 28 days.

Group IV: Rats were treated with quercetin liposomes via nasal administration for 28 days.

Rats were treated and determined the anxiolytic, anti-depressant and cognitive enhancing effect according to the time schedule as mentioned in 4.1.



4.3 Neuroprotective effect of quercetin with oral conventional delivery system in both healthy and cognitive deficit conditions of Alzheimer's disease model

This part was divided into 2 separated parts. The first part was set up to determine the neuroprotective effect of quercetin with oral conventional delivery system in healthy condition. The next part was set up to determine the neuroprotective effect of quercetin with oral conventional delivery system in Alzheimer's disease model.

4.3.1 Neuroprotective effect of quercetin with oral conventional delivery system in healthy condition

The animals were randomly divided into various groups as described following;

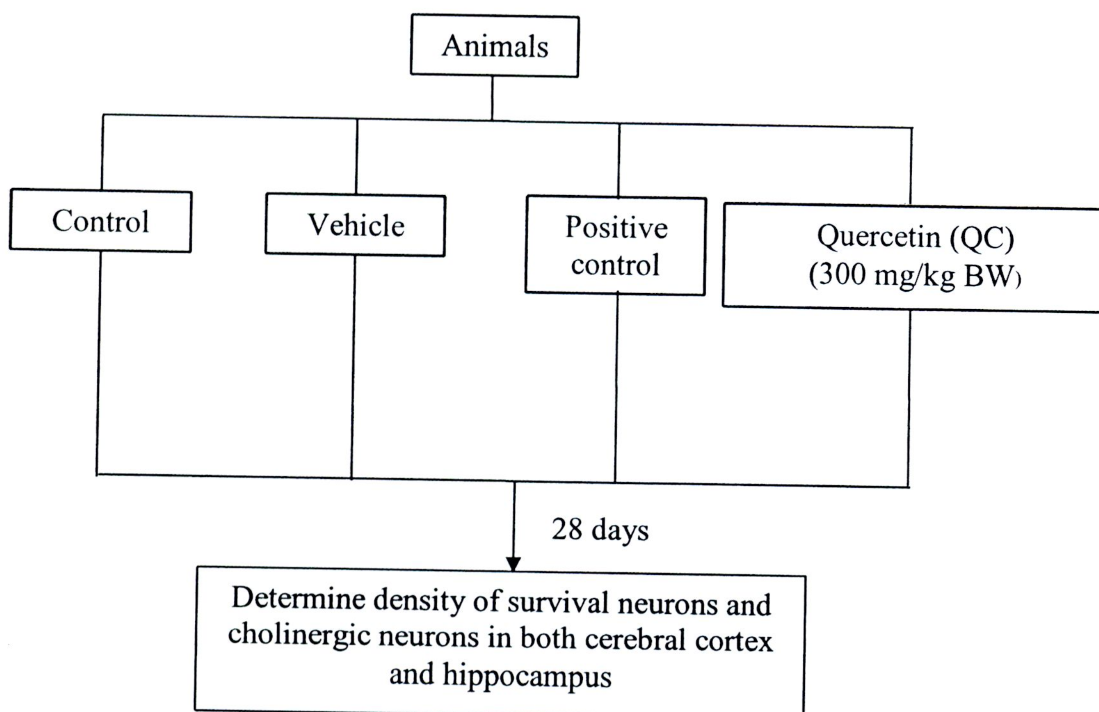
Group I: Naïve intact rats.

Group II: Vehicle treated group, which received polyethyleneglycol (PEG) once daily via oral administration for 28 days.

Group III-IV: Positive control group: Rats were treated with standard drug for various conditions as following; aricept or donepezil hydrochloride (1mg/kg BW) and Vit.C 100 mg/kg BW were used as positive control for cognitive enhancing effect and exogenous antioxidant respectively.

Group V: Quercetin with oral conventional delivery system. The dose of quercetin that used in this part will be the dose, which produced maximum cognitive enhancing effect: The rats in this group were orally given quercetin at dose of 300 mg/kg BW for 28 days.

After the last dose of treatment, all animals were sacrificed via transcardially perfusion with fixative. The brains were separated and determined the density of survival neurons and cholinergic neurons in both cerebral cortex and hippocampus via histological and immunohistological technique.



4.3.2 Neuroprotective effect of quercetin with oral conventional delivery system in Alzheimer's disease condition

The animals were randomly divided into various groups as described following;

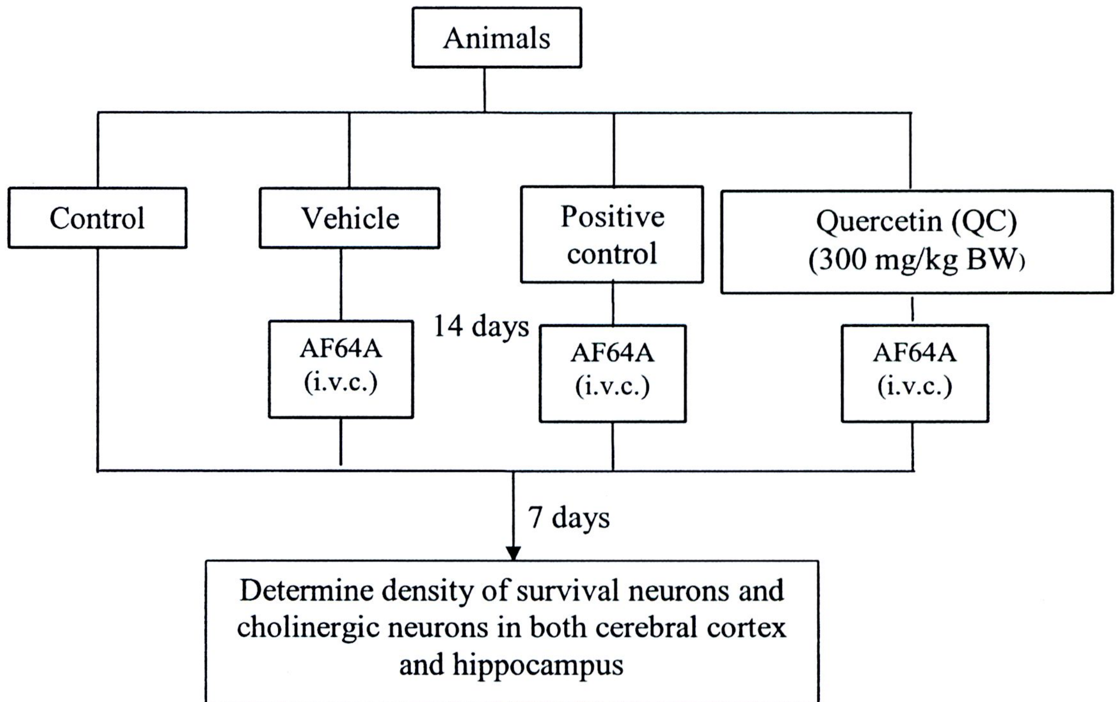
Group I: Vehicle: The rats in this group received PEG orally once daily for 21 days.

Group II: Vehicle plus AF64A: The rats in this group had received PEG orally once daily for 14 days before and 7 days after the intracerebroventricular administration of AF64A bilaterally.

Group III - IV: Positive control plus AF64A: The rats in this group had been orally given aricept or Vit.C 14 days before and 7 days after the intracerebroventricular administration of AF64A bilaterally.

Group V: Quercetin with oral conventional delivery plus AF64A. All rats in this group were orally given quercetin at dose of 300 mg/kg BW, at the same pattern as those mentioned in gr. III and IV.

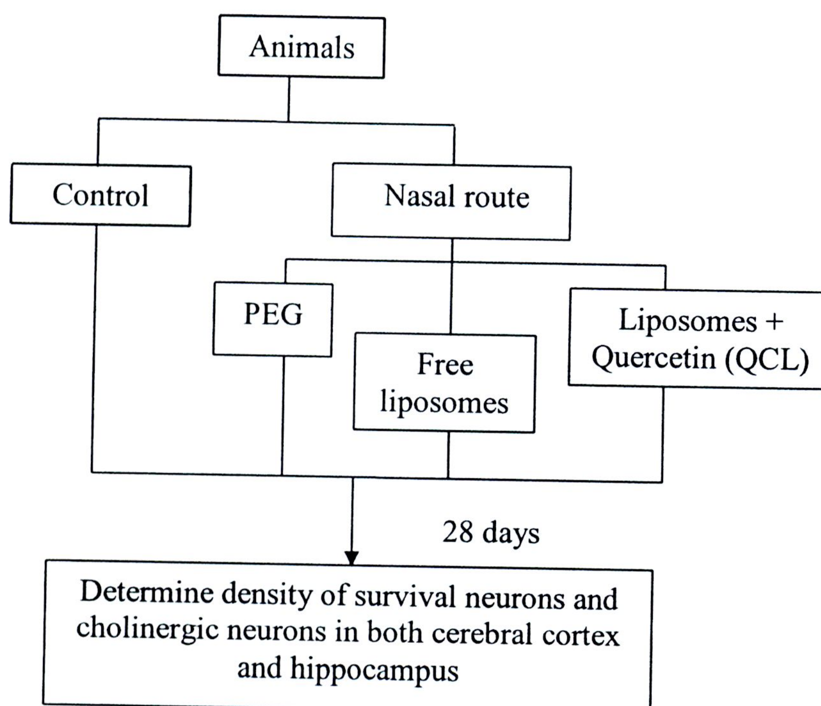
After the last dose of treatment, all animals were determined the density of survival neurons and cholinergic neurons in both cerebral cortex and hippocampus as mentioned in 4.3.1.



4.4 Neuroprotective effect of quercetin encapsulated liposomes via nasal administration in both healthy and cognitive deficit conditions of Alzheimer's disease model

4.4.1 Neuroprotective effect of quercetin encapsulated liposomes via nasal administration in healthy condition

In this part of study, rats were divided into various groups as mentioned in 4.2, after the last dose of treatment, all animals were determined the density of survival neurons and cholinergic neurons in both cerebral cortex and hippocampus as mentioned in 4.3.1.



4.4.2 Neuroprotective effect of quercetin encapsulated liposomes via nasal administration in Alzheimer's disease condition

The animals were randomly divided into various groups as described following;

Group I: Rats were treated with free liposomes via nasal administration for 21 days.

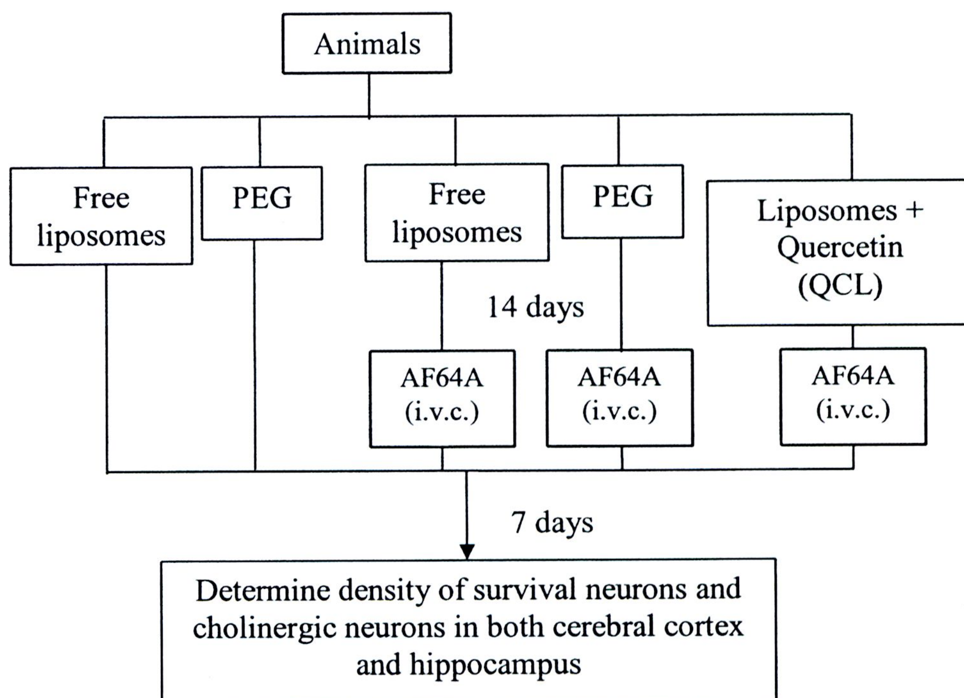
Group II: Vehicle treated group, which received polyethyleneglycol (PEG) once daily via nasal administration for 21 days.

Group III: Free liposomes plus AF64A. All rats in this group had received free liposomes administered via nasal route once daily for 14 days before and 7 days after the intracerebroventricular administration of AF64A bilaterally.

Group IV: PEG plus AF64A: The rats in this group received PEG administered via nasal route once daily for 14 days before and 7 days after the intracerebroventricular administration of AF64A bilaterally.

Group VI: Quercetin liposomes via nasal administration plus AF64A: The rats in this group were given quercetin liposomes via nasal administration for 14 days before and 7 days after the intracerebroventricular administration of AF64A bilaterally.

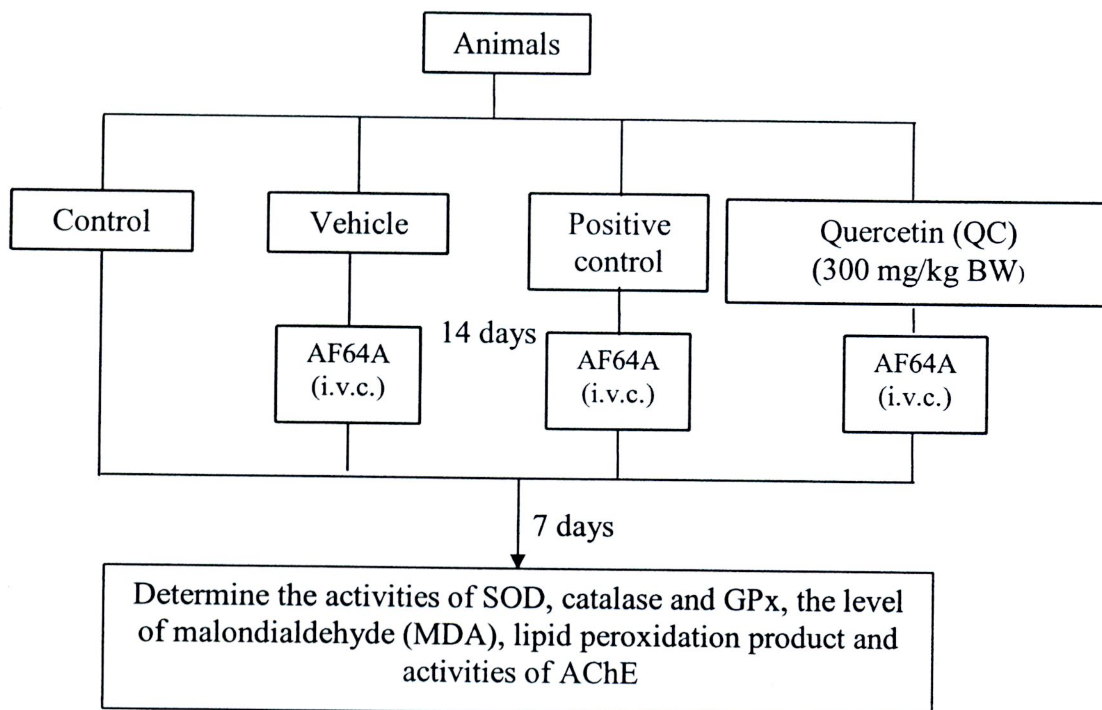
After the last dose of treatment, all animals were determined the density of survival neurons and cholinergic neurons in both cerebral cortex and hippocampus as mentioned in 4.3.1.



4.5 Possible mechanism(s) underlying the cognitive enhancement and neuroprotective effect against Alzheimer's disease of quercetin with oral conventional system

Based on previous knowledge that flavonoids could exert neuroprotection via the scavenging enzymes and improve cognitive function by inhibiting the acetylcholinesterase enzyme, therefore, this study aimed to determine the effect of quercetin with oral conventional system on the alterations of parameters mentioned above.

In this part of study, rats were divided into various groups, which received the same treatment as mentioned in 4.3.2, after sacrificed, hippocampus and cerebral cortex were isolated and determined the activities of scavenger enzymes such as superoxide dismutase, catalase and glutathione peroxidase. The level of malondialdehyde (MDA), lipid peroxidation product and acetylcholinesterase enzymes activities (AChE) were also determined in this study.



4.6 Possible mechanism(s) underlying the cognitive enhancement and neuroprotective effect against Alzheimer's disease of quercetin encapsulated liposomes via nasal administration

All rats were divided into various groups, which received the same treatment as mentioned in 4.4.2, and then the brains were removed to determine the activities of scavenger enzymes, the level of malondialdehyde (MDA) and acetylcholinesterase enzymes activities (AChE) as mentioned in 4.5.

Animals

Free liposomes | PEG | Free liposomes | PEG | Liposomes + Quercetin (QCL)

14 days

AF64A (i.v.c.) | AF64A (i.v.c.) | AF64A (i.v.c.)

7 days

Determine the activities of SOD, catalase and GPx, the level of malondialdehyde (MDA), lipid peroxidation product and activities of AChE

5. Determination of Neuropharmacological Activity

5.1 Spontaneous motor behaviors

All animals were assessed spontaneous motor behaviors including grooming, licking and rearing. In this study, the number of grooming and rearing were recorded in open field within 5 minutes.

5.2 Anxiolytic activity

The anxiolytic effect was assessed using the elevated plus maze, which consisted of open arms (50×10 cm) and two enclosed arms (50 ×10 cm) with 40 cm high walls, extending from a central platform (10×10 cm). The arms were connected with a central square, 10 × 10 cm, to give the apparatus a plus sign appearance. The maze was raised to a height of 50 cm above floor. The maze floor and walls were constructed from dark opaque wood. Each rat was placed on the center of the platform facing an enclosed arm. Animals were tested individually and only once a day for 5 minutes. The number of opened arm entries and time spent in the opened arms were recorded as anxiolytic indices (Lister et al., 1987).

5.3 Anti-depressant effect

The force swimming test is an animal model commonly used for both the screening of antidepressant drugs and the analysis of the neurobiological bases of depression. In this model, rats were forced to swim and eventually adopt a floating posture identified as immobility behavior, which is considered as an index of 'behavioral despair'. In the Force swimming test, antidepressants induce a decrease in immobility (Borsini et al., 1988). The rats were forced to swim in a glass aquarium (22 cm in diameter, 40 cm in height) containing 20 cm high fresh water at 25° C. The total duration of immobility was measured during the 5-minutes test. Upon removal from the water, rats were towel-dried and finally returned to their home cage.

5.4 Spatial memory

The Morris water maze test is one of the most important paradigms used for testing spatial memory, which is thought to be dependent on the proper functioning of hippocampus (Morris, 1984). The Morris water maze consisted of a circular water tank (170 cm in diameter × 58 cm tall) filled with tap water (25° C, 40 cm deep) divided into 4 quadrants. In the center of 1 quadrant was a removable escape platform below the water level and covered with a nontoxic milk powder. The pool was divided into 4 quadrants (NE, NW, SE, and SW) by two imaginary lines crossing the center of the pool. For each animal, the location of invisible platform was placed at the center of one quadrant and remained there throughout training. The rats must memorize the platform location in relation to various environmental cues because there was nothing directly showed the location of the escape platform in and outside the pool. Therefore, the placement of the water tank and platform were the same in all acquisition trials. Each rat was gently placed in the water facing the wall of the pool from one of the four starting points (N, E, S or W) along the perimeter of the pool, and the animal was allowed to swim until it found and climbed onto the platform. During training session, the rat was gently placed on the platform by experimenter when it could not reach the platform in 60 S. In either case, the subject was left on the platform for 15 s and removed from the pool. The time for animals to climb on the hidden platform was recorded as escape latency or acquisition time. On the next day, the rats were also exposed to the test again except that the platform was removed and the retention time or the time that the animal spent to swim around the previous location of platform before removing the platform was recorded.

5.5 Non -spatial memory

Non – spatial memory was assessed using the object recognition test. The apparatus were test in open box made of plexiglass (88 X 88 X 60 h cm). The objects used were in two different shapes: the cubes were from wood and the cylinders were glass. In addition, these objects had no genuine significance for rats and had never been associated with reinforcement.

Object recognition sessions consisted of two 3-min trials: During the 'sample' trial (T1), two identical samples (objects) were placed in two opposite corners of the apparatus 10 cm from the sidewall. A rat was placed in the middle of the apparatus and was left to explore these two identical objects. After T1, the rat was put back in its home cage and a recognition trial (T2), each separated by an inter-trial interval of 1/2 h, 6 h and 24 h. These delays were chosen based on previous studies of our group and other laboratories, which reported that even though performance because less consistent with long inter-trial delays. During T2, a new object differed from the familiar object as either texture or as shape had been replaced one of the samples presented in T1; thence, the rats were re-exposed to two objects. The total time each subject interacted with each of the objects was recorded. Exploration was defined as follows: directing the front paws or the nose. Non-directed contacts were not counted; such behaviors include backing into the object or brushing against the object with the side or tail (Ennaceur and Delacour., 1988).

6. Histological and Immunohistochemical Studies

6.1 Tissue preparation

The brain of the animals were perfused transcardially with fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3 and they were postfixed in the same fixative overnight at 4°C. Then they were immersed sequentially for 48-72 h each in a cryoprotectant containing 30% sucrose. Serial sections of tissues containing striatum were cut frozen on a sliding microtome at 25 μm thick. Sections were stored in phosphate buffer and they were picked up no 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.

6.2 Cresyl violet staining for Nissl substance

Adjacent series of sections containing hippocampus from all groups were stained with 0.5% cresyl violet to aid in neuronal density determination.

6.3 Immunohistochemical study of choline acetyltransferase(ChAT) enzyme

A series of sections containing hippocampus from various groups were reacted in parallel experiments using a mouse monoclonal antibody directed against choline acetyltransferase (ChAT) (Chemicon Internation, Inc., CA, USA) and a modification of a previously described protocol employing the DAKO Strept ABC Complex/HRP duet kit. In brief, the sections were eliminated endogenous peroxidase activity by 0.5% H_2O_2 in methanol. Sections were washed in running tap water and distilled water for 1 min. each, then rinsed in KPBS and KPBS-BT for 5 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 primary antibody against ChAT diluted 1 : 100 in KPBS-BT at room temperature for 2 hours and then incubate at 4°C 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. In preparation for visualization step, sections were rinsed in KPBS-BT (1 min), and KPBS (two washes x 10 min). ChAT immunoreactivity 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.

6.4 Morphological analysis

All sections of cerebral cortex and hippocampus were evaluated with the aid of Olympus light microscope model BH-2. For the determination of neuronal density, five representative sections of cerebral cortex and hippocampus were selected for analysis. The observer was blind to the treatment at time of analysis. The density of neurons, and ChAT immunoreactived neurons were determined under 40x magnification.

7. Lipid peroxidation and Enzymes Assay

7.1 Preparation of tissue homogenate

After the last administration of substances, rats will be anesthetized with pentobarbital (50 mg/kg) and then perfused transcardially with ice-cold 0.9% NaCl. The cerebral cortex, hippocampus will be carefully excised, and the tissues stored at −80 °C. then the tissues will be homogenized in 4 volume of 1.15% KCL with glass Potter-Elvehjim homogenizer. (APPENDIX B)

7.2 Determination of Acetylcholinesterase

The activity of AChE in both cerebral cortex and hippocampus were measured according to a method developed by Thompson et al. (1993). This method employed acetylthiocholine iodide (ATChI) as a synthetic substrate for AChE. ATChI was broke down to thiocholine and acetate by AChE and thiocholine was then reacted with dithiobisnitrobenzoate (DTNB) to produce a yellow color. The quantity of yellow color, which developed over time, was a measured and expressed as the activity of AChE using a spectrophotometer (spec. 20). (APPENDIX H)

7.3 Determination of lipid peroxidation

Tissue homogenate was determined lipid peroxide levels by malondialdehide (MDA) (APPENDIX F) according to the method of Ohkawa et al. (1979) and determined protein concentration by the colorimetric method of Lowry et al. (1951).

7.4 Determination of scavenging enzymes activities

The superoxide dismutase activity SOD (EC 1.15.1.1) was measured at 505 nm and 37 °C and calculated using inhibition percentage of formazon formation. One EU was defined as the enzyme inhibition percentage of formazon formation 1 mol/min at 25 °C and optimal pH (pH 7.8) (McCord and Fridovich, 1969). The catalase activity was measured at 515 nm and determined by method of Goldblith (Goldblith et al., 1950), wheares glutathione peroxidase activity (GPx) (EC 1.11.1.9) was determined by method of Wendel (Wendel A, 1980), t-butyl hydroperoxide was used. The GSSG in the medium was reduced to GSH by GPx and NADPH. The activity of GPx was assayed at 37°C and 340 nm by calculating the difference in absorbance values during the oxidation of NADPH. One EU was defined as the enzyme oxidation 1 lmol of NADPH per minute at 25 °C and optimal pH (pH 8.0).

8. Preparation and Administration of AF64A

AF64A was prepared as described previously by Fisher et al.(1982). Briefly, an aqueous solution of acetylethylcholine mustard HCL (Sigma, St. Louis, MO) was adjusted to pH 11.3 with NaOH. After stirring for 30 minutes at room temperature, the pH was lowered to 7.4 with gradual addition of HCl and stirred for 60 minutes. The amount of AF64A was then adjusted to 2 nmol/2μl. The vehicle of AF64A was distilled water prepared in the same manner as the AF64A and recognized as ACSF.

Rats were anestesized with 60 mg/kg BW of sodium pentobarbital via intraperitoneal injection. Then they were administered either ACSF or AF64A.

AF64A (2 nmol/2 μl) or ACSF was infused bilaterally via intracerebroventricular (i.c.v.) route with a 30-gauge needle inserted through a burr hole drilled into the skull into both the right and left lateral ventricles. Stereotaxic coordinates were (from the bregma): posterior 0.8 mm, lateral ± 1.5 mm, and ventral (from dura) 3.6 mm. The rate of infusion was 1.0 μl/min and the needle was left in place for 5 min after infusion and then slowly withdrawn.

9. Statistical Analysis

All data were expressed as mean ± S.E.M value. The difference among various groups were compared by ANOVA followed by LSD. The statistical difference was regarded at p-value < (0.05).