CHAPTER III
RESEARCH METHODOLOGY

3.1 Chemicals

Acetylthiocholine, ethoproprazine, glycine, sodium sulfide, silver nitrate, sodium thiosufate, cresyl violet, ethidium bromide 17β-estradiol and JumpStartTM Red HT RT-PCR Kit, catalase, superoxide dismutase, glutathione peroxidase, nitroblue tetrazolium (NBT), xanthine and xanthine oxidase, glutathione (reduced form), glutathione reductase, bovine serum albumin DL-Dithiothreitol (DDT), sodium azide (NaN_3), beta- nicotinamide adenine dinucleotide phosphate (β-NADPH) were obtained from Sigma-Aldrich Co. (USA). Hydrogen peroxide (H_2O_2) ethylenediaminetetra-acetic acid di-sodium salt (EDTA), sodium tartrate, copper(II) sulfate ($CuSO_4$), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), ferrous sulfate ($FeSO_4$), potassium thiocyanate (KSCN), sulfuric acid (H_2SO_4), dipotassium phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) were obtained from Asia Pacific specialty Chemicals Limited (Australia). Agarose, primer and ladder used in RT-PCR studies were obtained from Theera Trading CO., LTD (Thailand). Folins Reagent was from Merck (Germany). Trizol reagent was from Invitrogen (USA). Hexane (HPLC grade), dichloromethane (HPLC grade), ethyl acetate (HPLC grade) were obtained from Fisher Scientific (UK). Olive oil was obtained from the local super market (Sabroso®, Spain). 1,7–diphenyl-5-hydroxy-(1E,3E)-1,3-heptadiene (DHH), a pure compound isolated from the *C. comosa* hexane extract, was kindly provided by Prof. Apichart Suksamrarn from Ramkhamhaeng University, Thailand.

3.2 Animals and the surgeries

3.2.1 The animals were purchased from the National Animal Center, Mahidol University, Bangkok. All experiments were conducted under the National Guideline of Animal Care which was approved by the Ethic Committee of Khon Kaen University (Approved No. 0514.1.12/30). The animals were kept at 25±2 °C, relative humidity of 50-70%. Commercial food pellet for mice (C.P Ltd; Code: 082) were

provided to the animal. All efforts were made to minimize the pain and suffering of used animals.

3.2.2 For the ovariectomy in this study, rats were anesthetized with ether. Ovaries were removed via a dorsolateral incision, and ovarian blood vessels were tied off with a sterile suture and skin was then secured with wound clips. For the shamed ovariectomized surgery, a dorsolateral incision was made and the integrity of the ovaries was check before suturing the wounds.

3.3 Plant extracts and solution preparation for administration

3.3.1 The *Curcuma comosa* hexane extract (CHE) used in the short-term behaviors test and the following ER mRNA studies was kindly provided by Prof. Apichart Suksamrarn from Ramkhamhaeng University, Thailand.

3.3.2 In the other studies, the CHE was prepared in our laboratory. The method was the same as the CHE prepared in section 3.3.1. The dry piece of *C. comosa* rhizome, which was purchased from Nakornpathorn province, Thailand, was crushed into crude powder, and then was extracted with n-hexane by using a Soxhlet apparatus until the outlet hexane was colorless. Then the hexane fraction was evaporated to get a brown-yellow oily extract.

3.3.3 The CHE was diluted in olive oil at the concentration of 250 mg/ml for the low dose group and 500 mg/ml for the high dose group. For positive control, 17 beta-estradiol was also diluted in olive oil at the concentration of 10 μg/ml. The olive oil was used as a vehicle (1 ml/kg body weight).

3.4 Short-term effect of estradiol and CHE on the spatial reference memory of the ovariectomized (OVX) rats

3.4.1 Morris water maze (MWM) apparatus and test procedures

A circular stainless pool, diameter 150 cm, height 60 cm, was located in a separated room. Water at room temperature (25±2°C) was filled to the depth of 30 cm and a clear plastic circular platform (diameter: 20 cm) was located 2 cm below the surface of the water and in the center of one of the quadrants (Figure 3). White talcum powder was sprinkled on the water surface to make it opaque. Various objects or

geometric images such as circles, squares, triangles with different colors were attached on the wall of the pool in order to let the rats use these visual cues as a means of navigating in the maze. In each trial, a rat was placed into the water from the opposite site of the platform. At each day 4 trials were given to the rats. For each trial the rats were allowed to swim 60 seconds in order to find the hidden platform. When successful, the rat is allowed 15 seconds to stay on the platform. If unsuccessful within 60 seconds, the rat is given a score of 60 seconds and then physically placed on the platform and also allowed 15 seconds on the platform. A 3 to 5 minutes interval was allowed between each trial. The time of finding the platform was recorded to compare the spatial memory between groups.

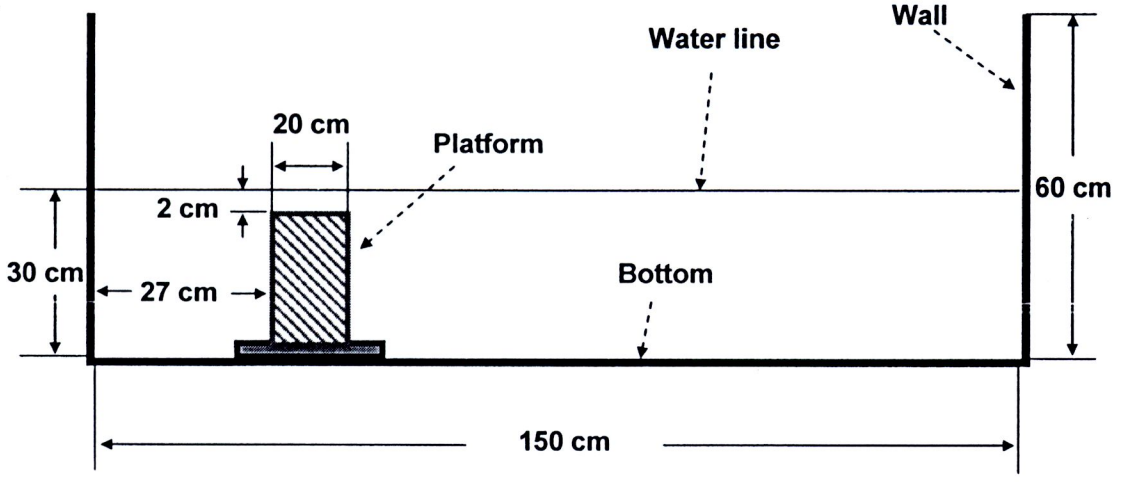

Figure 3 Apparatus (side view) of Morris water maze test in short-term behaviors study.

3.4.2 Animal treatment

Forty eight female Wistar rats (8 weeks old) were allowed one week to be acquaint to the surrounding after obtained from the animal center. And then a 5-days MWM hidden platform training process, 4 trials per day, was conducted to generate the memory of the platform location in the swimming pool. The scores of the last 2 days of the training process were collected and used as the reference to separate the animal to 6 groups (n=8), which had the same average latency (Figure 4.1). These 6 groups were named and treated as follows. Group I (OVX+E) was ovariectomized and daily received 17-beta estradiol subcutaneously (10 μg/kg body weight). Group II

and III (OVX+C1, OVX+C2) were ovariectomized and daily received CHE orally at the doses of 250 mg/kg and 500 mg/kg body weight, respectively. Group IV (OVX+V) was ovariectomized and daily received the vehicle (olive oil), orally. Group V (Sham+V) was received shamed ovariectomy and daily received the vehicle (olive oil), orally. Group VI (Sham+C1) was received shamed ovariectomy and daily received CHE orally at the dose of 250 mg/kg body weight. Three days after the MWM training, the ovariectomized and shamed surgeries were conducted. The estradiol, CHE and vehicle were daily administered to the animal as designed began from the day next to the surgery. At the days of 1, 2 and 4 weeks after the OVX, the rats received a 1-day MWM hidden platform test which was including 4 trials at each test (Figure 4). The location of the platform was fixed at the same place through the experiment. The latency of each trial was recorded and compared between groups to evaluate the spatial reference memory.

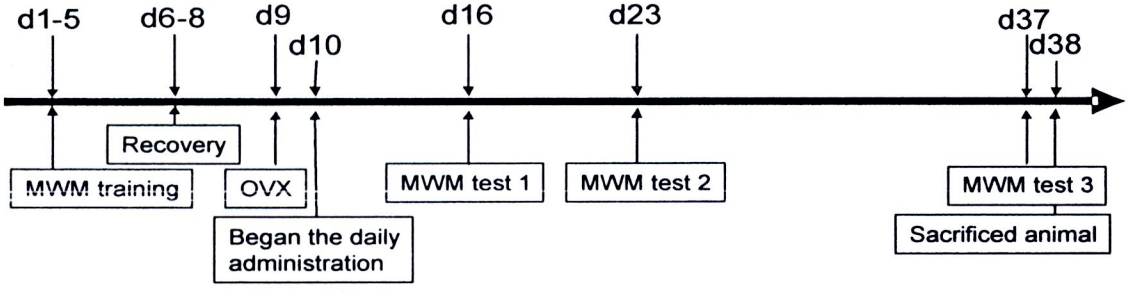

Figure 4 The time line of the experimental design of short-term behaviors study.

3.4.3 Body weight and uterus weight

The body weigh was recorded every 2 days through the experiment and the uterus weight was recorded at the autopsy.

3.5 RT-PCR quantification of the estrogen receptor mRNA in the hippocampus of the rats.

3.5.1 Sample preparation

After the behavior test (section 3.4), the animals were sacrificed. Brains were removed and the hippocampus was separated and kept in -80°C for further study.

3.5.2 RNA extraction

Total RNA from hippocampus was extracted by using Trizol reagent (Invitrogen) and following the reagent instruction described as follows.

Step 1: Around 10 mg tissue samples were put in a 1.5 ml Appendorf tube, 800 µl of Trizol reagent was added to homogenize the tissue.

Step 2: The homogenate was incubated for 5 minutes at 25°C, followed by adding 0.16 ml of chloroform. The tubes were shaken vigorously for 15 seconds and then incubated at 25°C for 2-3 minutes, followed by centrifuging the samples at 12,000 g for 15 minutes at 2-8°C.

Step 3: The aqueous phase was transferred to a fresh tube. 5-10 µg RNase-free glycogens were added and then precipitated the RNA by mixing with 0.4 ml isopropyl alcohol, followed by incubating the sample at 25°C for 10 minutes and then centrifuging at 12,000 g for 10 minutes at 2-8°C.

Step 4: The supernatant was removed and 1 ml 75% ethanol was added to the RNA pellet, and mixed by vortex followed by centrifuging at 7,500 g for 5 minutes at 2-8°C.

Step 5: The RNA pellet was briefly dried in the air for 5-10 minutes and then was dissolved in 50 µl RNase-free water and incubated for 10 minutes at 55-60°C.

To measure its concentration, RNA was diluted in Tris-buffer (pH 7.5) and determined the UV absorption at 260nm and 280nm.

3.5.3 cDNA synthesis and PCR amplification

The JumpStartTM Red HT RT-PCR Kit (Sigma) was used in these experiments. Estrogen receptor alpha and beta mRNAs were measured in this study. Housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference.

Step 1: The following reagents were added to a thin-walled 0.2 ml PCR microcentrifuge tube on ice:

Volume/reaction	Reagent
5 µl	RNase-free water
4 µl	sample (purified RNA template)
1 µl	Oligo(dT)23
Total volume: 10 µl	

After gently mixing, the tubes were briefly centrifuged to collect all components to the bottom of the tube, and then placed in thermal cycler at 70°C for 10 minutes.

Step 2: The tubes were removed from the cycler, placed on ice for 1 minute, and then the following components were added to start the reverse transcription reaction

Volume/reaction	Reagent	Final concentration
5 µl	Water, PCR reagent	
2 µl	Buffer for eAMV-RT (10X)	1X
1 µl	dNTP mix	500 µM of each dNTP
1 µl	RNase inhibitor	1 unit/µl
1 µl	eAMV-RT	1 unit/µl
Total volume: 20 µl		

The mixture was mixed well and incubated at 50°C for 50 minutes, and then the reaction was heated at 95°C for 5 minutes, followed by chilling on ice immediately

Step 3: PCR master mixture of GAPDH and ER were prepared as follows

a) Master mixture of the GAPDH

Volume/reaction	Reagent	Final concentration
4.5 ul	Water, PCR reagent	
2 µl	PCR buffer (10 X)	1 X
2 µl	$MgCl_2$ (25 mM)	2.5 mM
0.5 µl	10 mM dNTP	250 µM each dNTP
1 µl	Polymerase	0.05 unit/µl
Total volume: 10 µl		

b) Master mixture of the ER

Volume/reaction	Reagent	Final concentration
7.5 µl	Water, PCR reagent	
2 µl	PCR buffer (10 X)	1 X
2 µl	$MgCl_2$ (25 mM)	2.5 mM
0.5 µl	10 mM dNTP	250 µM each dNTP
1 µl	Polymerase	0.05 unit/µl
Total volume: 13 µl		

The master mixture was mixed well and placed on ice.

Step 4: The mixture of forward and reverse gene-specific primers were prepared (10 µM each for ER primer and 1 µM for GAPDH).

Step 5: The master mix, primer mix, and the cDNA template were added in a 0.2 ml PCR tube as follows.

PCR mixture of GAPDH

Volume/reaction	Reagent
10 µl	Master mixture
5 µl	Primer mixture
5 µl	Sample (cDNA temple)
Total volume: 20 µl	

PCR mixture of ER alpha and beta

Volume/reaction	Reagent
10 µl	Master mixture
5 µl	Primer mixture
5 µl	Sample (cDNA temple)
Total volume: 20 µl	

Step 6: The PCR amplification was started by using the program as follows.

Cycling program for GAPDH

Denaturation		95°C	2 min
PCR cycling 30 X	Denaturation	95°C	20 sec
	Annealing	59°C	30 sec
	Extension	68°C	30 sec
Final extension		68°C	10 min
Keeping		4°C	

Cycling program for ER

Denaturation		95°C	5 min
PCR cycling 35 X	Denaturation	95°C	1 min
	Annealing	57°C	1 min
	Extension	72°C	1 min
Final extension		72°C	4 min
Keeping		4°C	

Step 7: Prepared a 2% agarose gel containing 0.5 μg/ml ethidium bromide dyes by adding 1 g agarose in 50 ml H_2O_2 and boiling it, followed by adding 2.5 μl of ethidium bromide (10 mg/ml) and mixed well.

Step 8: 5 μl of the RT-PCR products was added on the 2% agarose gel and separated by electrophoresis (100 V). The run time was 20-30 minutes, until the band of target gene was in the middle of the gel according to the reference ladder.

The primer sequences of ER alpha, beta and GAPDH were show in Table 1.

Table 1 Primer sequences of ER alpha, beta and GAPDH

Genes	Forward (5'-3')	Reverse (5'-3')	cDNA band
ER-alpha	GGC TGC GCA ABT GTT ACG AA	CAT TTC GGC CTT CCA AGT CAT	116 bp
ER-beta	GAG GCA GAA AGT AGC CGG AA	CGT GAG AAA GAA GCA TCA GGA	141 bp
GAPDH	GTC ATC ATC TCT GCC CCC TCT	CAG CCC CAG CGT CAA AGG TG	544 bp

3.5.4 Quantitative analysis

The separated mRNA on the gel was photographed by Gel Doc 2000 (Bio Rad). The band densities were analyzed by using Genie® software. All values were normalized with internal control (GADPH) and the data were expressed as mean±SEM (n=8). One way ANOVA was performed to analyze the variance between groups. The ER primer design and cycling program followed the previous reported method (Rose et al., 2003).

3.6 Long-term effect of estradiol and CHE on the spatial reference memory and working memory of ovariectomized rats

3.6.1 Apparatus

a) Morris Water Maze

A large circular plastic pool, diameter 200 cm, height 40 cm, with the bottom painted blue was located in a room separated by a black curtain. Water at room temperature (25±2°C) was filled to the depth of 20 cm and a clear plastic circular platform (diameter: 10 cm) was located 2 cm below the surface of the water, and 20 cm to the wall of the pool. White talcum powder was sprinkled on the water surface to make it opaque. Various objects or geometric images such as circles, squares, and triangles with different colors were hung on the wall in the testing room for the rats to use as visual cues to navigate the maze. Swimming activity of each rat was monitored via a camera mounted overhead, which relays information including

latency to find the platform, total distance traveled, time and distance spent in each quadrant (Figure 5).

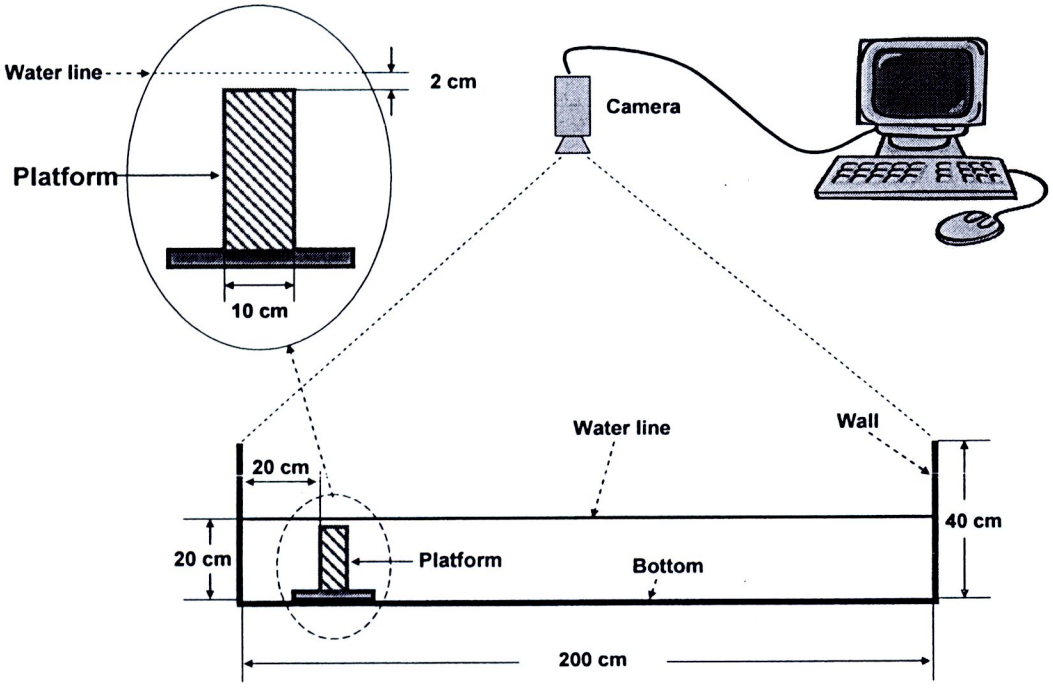

Figure 5 The apparatus (side view) of Morris water maze test in the long-term behaviors study.

b) Radial Arm Maze

An eight arm maze was used in this experiment. Clear Plexiglas was used to construct the apparatus with the bottom painted dark brown. The octagonal central arena is 29 cm across; and the arm length is 70 cm with the height 15 cm and the width 12 cm. Food dishes are located at the end of each arm. A removable slide door was set at the entry of each arm to control the rat to enter the designed arms. The maze was elevated 70 cm above the floor and located in a room separated by a black curtain and various objects or geometric images such as circles, squares, triangles with different colors were hung on the wall (Figure 6).

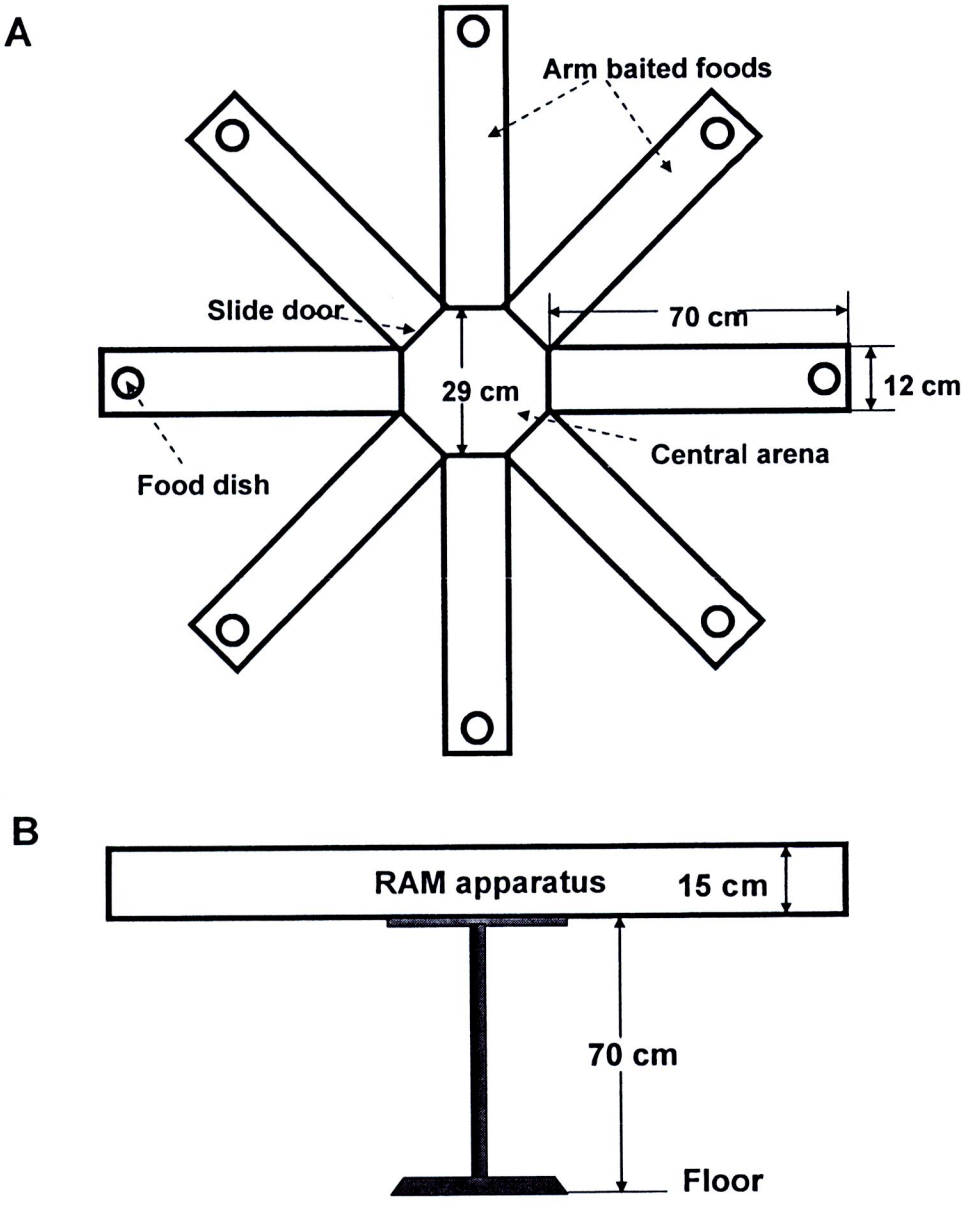

Figure 6 The planform (A) and the side view of the radial arm maze apparatus.

3.6.2 Morris water maze testing procedures

a) Hidden platform test

In the swimming pool, the platform as described above was located at 1 of the 4 quadrants designated NE, NW, SW, and SE, which were set up on the computer software (Figure 7). In each trial, a rat was placed into 1 of the 4 quadrants except for the quadrant located at the platform. At each day, 4 trials were given to the rats. For each trial the rats were allowed to swim 60 seconds in order to find the hidden platform. When successful, the rat was allowed 15 seconds to stay on the

platform. If unsuccessful within 60 seconds, the rat was given a score of 60 seconds and then physically placed on the platform and also allowed 15 seconds on the platform. A 3-5 minute interval was allowed between each trial. The time to find the platform and the distance to the platform were recorded to compare the spatial memory and learning ability between groups.

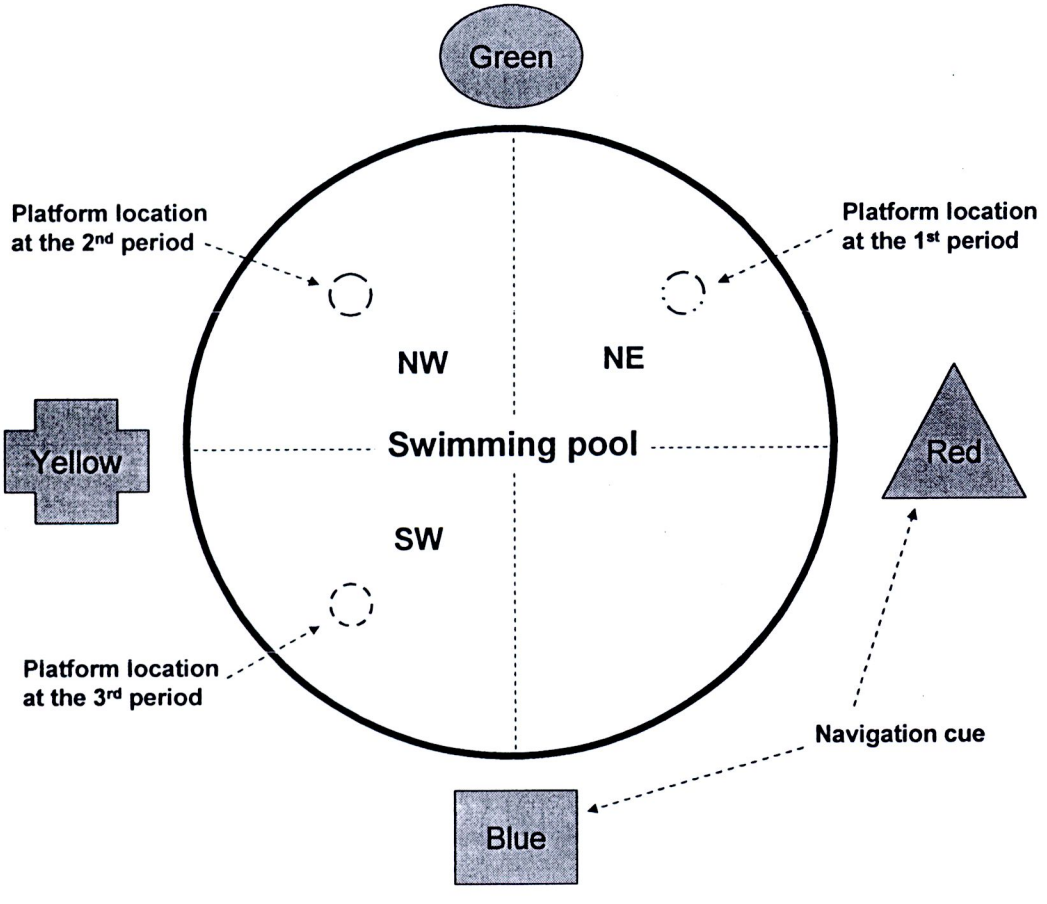

Figure 7 The platform location in the swimming pool (planform) for the Morris water maze

b) Probe test

On the probe test, 3 trials on each day were given while the platform was removed from the pool. The time that the animal spent on the quadrant with the platform removed was recorded to measure the reference spatial memory after a long term interval by measuring the time the rats traveled in each of the four quadrants.

c) Video tracking and analysis systems

All the animal behavior in the swimming pool was recorded by a camera set above the apparatus. Video document was analyzed by the Wintrack software

(version 2.4.50923), developed by Dr. David P. Wolfer, Institute of Anatomy, University of Zurich.

3.6.3 Radial arm maze testing procedures

a) Acclimation

First, food was provided daily in measured portions, to reduce the body weight of the rats to 85% to 90% of that when of free-feeding. Water was available *ad libitum*. Then the animals were subjected to 6 days of acclimation in which they were allowed to explore the maze for a 5-10 minute period each day. Small pieces of food pellets were put in the food dishes located at the end of each arm. The slide door equipped with each arm was randomly closed to avoid the algorithmic pattern of arm selections, such as always visiting the immediately neighboring arm to the left; thus the subject would not make any errors. The animals were reinforced to know the location of the food under the food deprivation condition. Only the un-visited arms were baited.

b) All arm accessible test

Following the acclimation sessions, the animals were tested for 1 trial per day. In this procedure, an animal was placed in the center of the RAM and allowed to visit all the 8 arms freely; food pellet rewards were available upon the first visit to each arm. Re-entry into an arm previously visited within any daily trial was not rewarded and was scored as an error. After each arm had been visited once, the session ended; a default time limit was set after 5 minutes or after visiting the arms 16 times, which rarely occurred. The feces and urine were removed immediately to eliminate the effect of the odors. The all arm accessible test was performed in 3 consecutive days. The error times that the animal made at the first-8-probe and total probes to the non-visited arms were recorded to compare between the groups.

c) Delay Non-Match-to-Sample (DNMTS) test

Following acquisition of the all arms open RAM task, animals were trained to perform the task with a certain time delay imposed between the 4^{th} and 5^{th} arm choices. During the pre-delay session, animals were allowed to choose freely in all 8 baited arms until the 4^{th} accessible arm had been chosen. Upon completion of the pre-delay session, animals were returned to their home cages for a certain delay interval. Following the delay, animals were returned to the maze and allowed to

choose freely among all 8 arms. Entry into an arm visited during the pre-delay session was not rewarded and constituted an error, as those repeated entry into post-delay choices. The pre-delay and the post-delay sessions were limited to 5 minutes each. The animal that did not complete the pre-delay session in the ascribed time was not tested in a post-delay session.

The data from animals that did not make at least 4 choices in 5 minutes during the post-delay session was not used. The feces and urine were cleaned to minimize odor cues, and then the maze was rotated clockwise 45 degrees between the pre-delay and the post-delay while maintaining a consistent orientation of the arms of the maze relative to the landmarks in the test room to minimize intra-maze cues. In our experiments, the intervals between the 4^{th} and the 5^{th} probes were set at 10 minutes, 1 hour and 2 hours, each delay interval was tested for 2 or 3 consecutive days. The 10 minutes and 1 hour interval were tested at the 1^{st} period of RAM test, while the 1 hour and 2 hours delay were included in the 2^{nd} period. All the RAM tests were performed at 19:00~23:00. The error times the animals made at the first-4-probe and total probes to the non-visited arms were recorded to compare between the groups.

3.6.4 Animals and treatment

After housing at 25±2 °C under a 12/12 light-dark cycle and with accessing to food and water *ad libitum* for 1 week, the rats were randomly separated into 5 groups (n=10). Group I (OVX+E) was ovariectomized and daily received 17-beta estradiol subcutaneously (10ug/kg body weight). Group II, III (OVX+C1 and OVX+C2) were ovariectomized and daily received CHE orally at the doses of 250mg/kg and 500 mg/kg body weight, respectively. Group IV (OVX+V) was ovariectomized and daily received vehicle orally. Group V (Sham+V) received shamed ovariectomies and daily received vehicle orally. The treatment was begun on the next day following ovariectomy and the feeding time at each day was 3 hours before the behaviors test. Thirty days after the ovariectomy, the animals were given the first period MWM hidden platform test (denoted as M-H1) for 5 days, 4 trials per day. The platform was located at the NE quadrant; the released point of the four trials was at the SW, NW, SE and then SW quadrant. After the first period of the MWM hidden platform test, food supply was controlled so that the animal body weight decreased to 85-90% of their normal level. Then a 13-day RAM probe training and

test (denoted as RAM1) was given to each group in order to test the working memory, which included 6 days of acclimation, 3 days of all arms accessible test, 2 days of 10-minute delay non-match to sample (DNMTS) test and 2 days of 1 hour DNMTS test. A free food supply was given to the animals after the RAM test. Thirty days after the hidden platform test (M-H1), the 2-day MWM probe test (M-P1) was given to the animal while the platform was removed in order to test the reference memory after long term intervals. Immediately following the M-P1, the platform was moved to the NW quadrant and the animal received a second period hidden platform test (M-H2) for 5 days. Twenty days later, a 2 day MWM probe test (M-P2) was given to the animal while the platform was removed. Right after that the platform was moved to SW quadrant and the animal received the 3rd hidden platform test (M-H3) for 5 days. Then 20 days later, a 2 day MWM probe test (M-P3) was given to the animal while the platform was removed. Between the 3rd MWM hidden platform and probe test, the second RAM test (RAM2) was conducted, which included 3 days of an all arm accessible test, 3 days of a 1 hour DNMTS test and 3 days of a 2 hours DNMTS test (Figure 8).

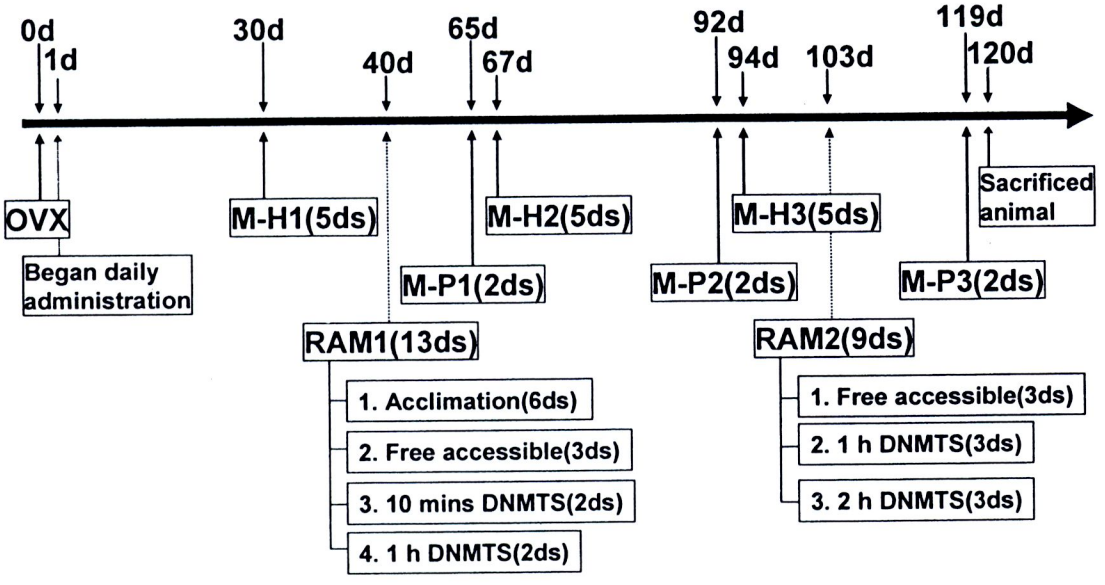

Figure 8 The experiment timeline of long-term behaviors test. M-H1, 2 and 3 denoted the MWM hidden platform test and M-P1, 2 and 3 denoted the MWM probe test at 1st, 2nd, and 3rd, period, respectively. RAM1 and RAM2 denoted the 1st and 2nd period of radial arm maze test.

3.6.5 OVX and estradiol efficiency inspection

The body weight of each animal was recorded every 3 days throughout the experiment and the uterus weight was collected at the postmortem check to inspect the efficiency of the OVX and the estradiol effect.

3.7 Brain morphology study

3.7.1 Sample preparation

After the long term behaviors test (Section 3.6), 5 animals in each group were sacrificed by injecting overdose thiopental sodium (120mg/kg BW). Under the deep anesthesia, 150 ml ice cold normal saline and 150 ml 10% formalin PBS buffer were perfused through the cardiac route to clean the blood and fix the tissue. The brains then were removed from the skull right after the perfusion and kept in the 10% formalin-30% sucrose solution for 24 hours. Before mounting the brain on the cryostat, a needle was punched longitudedly through the brain to make a landmark for the later 3D reconstruction (Figure 9). The knife for section the brain was adjusted to

be vertical to the needle followed by fixing the relative position of the sample and the knife. The needle was then removed after the brain was frozen, then the brain were sectioned coronally in the cryostat at the thickness of 40 um. The sample and the chamber temperatures were set at -16 and -18 °C, respectively. Two serials sections (2 of every 3 sections) were collected for AChE and Nissl staining, respectivelly.

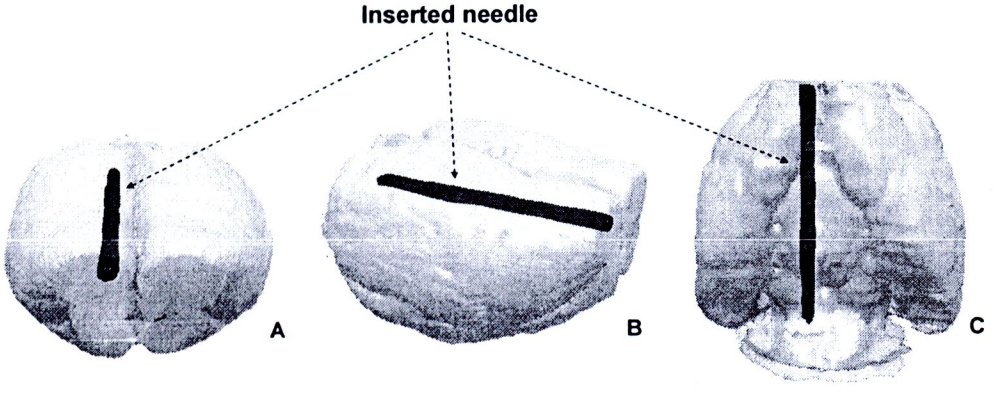

Figure 9 The position of an inserted needle for alignment in the 3D reconstruction. The anterior (A), lateral (B) and ventral (C) position were provided.

3.7.2 AChE staining

a) Solution preparation (Appendix A)

b) Staining process

Brain sections which had been mounted on the slides were rinsed and re-hydrated in water and then were incubated in acetylthiocholine solution for 10-15 hours at 37 °C with slight vibration. Then the slides were rinsed in the ice cold water for 6 times following by incubating in sodium sulfide solution at room temperature for 20 minutes. Then the slides were rinsed in ice cold water for 6 times following by incubating in silver nitrate solution for 1-2 minutes until the section got staining. Then the slides were rinsed in sodium thiosufide solution and tab water to washed out unspecific staining and the reacted chemicals following by dehydrating in gradient alcohol and xylene, and finally coverslipped with Permount.

3.7.3 Nissl staining

a) Solution preparation (Appendix A)

b) Staining process

Slides were rinsed and re-hydrated in water, then were emerged in 0.5% cresyl violet for 15-30 min, following by differentiating in water for 3-5 min and then dehydrated through 70% alcohol, 95% alcohol, 100% alcohol, and 100% alcohol. Then they were put in xylene and coverslipped.

3.7.4 Three-dimensional reconstruction of the brain

The AChE staining sections of each brain were captured by the camera system in the orders from frontal brain to the hindbrain and saved in a digital image format. The landmark, a hole that from a pre-punched needle and the midline of the brain, was used for locating the section while taking the pictures (Figure 3.3). These images were imported to 3D Doctor (Able Software Corp) with the calibrated factor. The area of interest, such as cortex, hippocampus, amygdala, thalamus and the 3rd ventricle were segmented in each slides used the software tools following by running the reconstruction process. Two times of surface smoothing process were run to smoothed the reconstructed hippocampus before reading the volume.

3.7.5 Volume comparison of the hippocampus

The hippocampus from each animal was reconstructed and the volumes were measured by using the *Calculate Volumes* function. Then the hippocampal volumes between groups were compared by running the ANOVA analysis to evaluate the effect of the OVX and the designed treatments on the hippocampus morphology.

3.7.6 Neuron density study

The Nissl staining sections were used for neuron density study. The specific area of CA1 and CA3 area of each slide were captured by the camera under the light microscope at 40 time magnification. All the cells with the size bigger than the glia cells in the counting region were count as neurons. The neuron density was compared between groups to find the difference due to the OVX and the designed treatments.

3.8 The effect of the CHE on the antioxidative enzyme activities of the rat brain against the ethanol-induced oxidative stress.

3.8.1 Experimental design

Forty-nine female Wistar rats (8 weeks old) were kept in an environment maintained at 25±2°C, relative humidity of 50~70% and a 12-h light/dark cycle, five per cage. Food and tap water were provided *ad libitum* and the animals were allowed one week to be familiar the environment. The rats were divided into 7 groups, which were daily intraperitoneally administered as follows: control group (normal saline 2g/kg body weight); vehicle group (olive oil 2g/kg body weight); ethanol group (20% ethanol 2g/kg body weight); C1 group(CHE 100mg/kg body weight); C2 group (CHE 250mg/kg bodyweight); EtOH+C1 group (CHE 100mg/kg plus 20% ethanol 2g/kg body weight) and EtOH+C2 group (CHE 250mg/kg plus 20% ethanol 2g/kg body weight) for 14 days.

3.8.2 Brain tissue collection and sample preparation

At the fifteenth day, the rats were sacrificed by cervical dislocation. The brain was removed immediately from the skull and washed in the ice-cold buffer and then separated the tissues on the ice-cold stage. Hypothalamus, pituitary, hippocampus, cortex, and cerebellum were respectively collected. These tissues were weighed and then wrapped in an aluminum foil and kept in a dry ice box, then transferred to a freezer of -80°C.

Before the enzyme analysis, the brain tissues were homogenized with 0.1M phosphate buffer (pH 7.4) by a hand homogenizer with 10 times buffer volume for the hippocampus, pituitary, and hypothalamus weights, and 3 times buffer volume for the cortex and cerebellum weights. The homogenates were centrifuged at 10000g, 4°C for 10 minutes. The supernatants were taken into aliquots and kept at -20°C until further analysis.

3.8.3 Protein assay

a) Solution preparation for reaction (Appendix A)

b) Analysis protocol

Step1: 100 µl of Lowry A and B solution, 10 ml of Lowry C solution were mixed just before the assay, and then 200 µl of this solution were pipetted to each well of a 96-well microplate.

Step 2: the standard curve was made by adding 0, 10, 20, 40 and 50 µl of bovine serum albumin (1 mg/ml) to the standard wells of the microplate, mixed well.

Step 3: 10 µl of hippocampus, hypothalamus and pituitary samples and 3 µl of cortex and cerebellum samples were pipetted to the sample wells of the microplate, mixed well.

Step 4: the sample solution were incubated for 10 minutes at 25°C.

Step 5: 25 µl of 1 N Folins reagent were pipetted into the wells containing the samples by a multi-channel pipette. The mixture was mixed well and then incubated for 20 minutes at 25°C.

Step 6: The absorbance (ABS) was detected at 650 nm by micro plate reader. The protein concentration of each sample was calculated by comparing the ABS with that of the standard curve.

3.8.4 Catalase (CAT) activity assay

Hydrogen peroxide was catalyzed by catalase in the sample in the *Kinetic Plates*. At the intervals of 8 minutes, 20 µl of solutions in the *Kinetic Plate* was removed to the *Color-Reaction Plate*, where the non-reacted hydrogen peroxide oxidized the Fe^{2+} to Fe^{3+}, followed by reacting with SCN^- to generate a red color $Fe(SCN)_3$, which was sensitive at 490 nm.

a) Reaction formulation

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

$$2FeSO_4 + H_2O_2 + H_2SO_4 \longrightarrow Fe_2(SO_4)_3 + 2H_2O$$

$$Fe_2(SO_4)_3 + 6KSCN \longrightarrow \underset{\Delta 490nm}{2Fe(SCN)_3} + 3K_2SO_4$$

b) Solution preparation *for reaction* (Appendix A)

c) Analyses *protocol*

Step 1: 160 µl of PBS (0.1 M, pH 7.4) were pipetted to the each well of the *Kinetic Plate* (a 96 wells microplate).

Step 2: 40 ml of H_2SO_4 solution (0.3 M) and 8 ml of $FeSO_4$ solution (10 mM) were mixed and then 230 µl of this mixture were pipetted to each wells of the *Color-Reaction Plate* (another 96 wells microplate, 2 *Color-Reaction Plate* matched 1 *Kinetic Plate*)

Step 3: a standard curve was prepared by adding 4, 8, 12, 16 and 20 µl of H_2O_2 (60 mM) to the standard wells of the *Kinetic Plate*.

Step 4: 10 µl of hippocampus, hypothalamus and pituitary samples and 3 µl of cortex and cerebellum samples were pipetted to the sample wells of the *Kinetic Plate* and mixed well.

Step 5: 20 µl of the H_2O_2 solution (60 mM) were pipetted to the sample wells of the *Kinetic Plate* by using a multi-channel pipette to initial the reaction. The temperature was 25°C.

Step 6: At the 2nd minute and the 10th minutes after the reaction started, 20 µl of the reaction mixture was pipetted from the *Kinetic Plate* to the related wells of the *Color-Reaction Plate*. The reaction was ceased by the H_2SO_4 in the *Color-Reaction Plate*

Step 7: 20 µl of KSCN solution (1.25 M) was added to the *Color-Reaction Plate* by a multi-channel pipette to generate the color (incubated 1 or 2 minutes for the reaction).

Step 8: The ABS was detected at 492 nm by a micro plate reader

The final concentration of each chemical in the plate was: 0.1 M PBS, 6 mM H_2O_2 in the *Kinetic Plate*, 0.21M H_2SO_4, 1.42 mM $FeSO_4$ and 0.092 M KSCN in the *Color-Reaction Plate*.

One unit of catalase activity was defined as a conversion of 1.0 µmole of H_2O_2 to H_2O per min at pH 7.4 at 25°C. The unit/mg protein was used for analysis the CAT activity in the brain between groups.

3.8.5 Superoxide dismutase (SOD) activity assay

In this reaction, xanthine and xanthine oxidase was used to generate the surperoxide free radical, which was then converted to oxygen catalyzed by SOD. The non-reacted surperoxide free radical was reacted with nitroblue tetrazolium (NBT) to form a blue-color NBT formazan, which was sensitive at 560 nm.

a) Reaction formulation

$$Xanthine + O_2 + H_2O \xrightarrow{XOD} Uric\ acid + O_2^{\bullet -}$$

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

$$O_2^{\bullet -} + NBT \longrightarrow \underset{\Delta 560nm}{NBT\ formazan} + O_2$$

b) Solution *preparation for reaction* (Appendix A)

c) Analyses protocol

Step 1: A mixture containing 6 ml of PBS solution (0.05 M), 3 ml of EDTA (0.5 mM), 6 ml of NBT (0.25 mM), 6 ml of Xathine (0.25 mM) (total 21 ml, for 2 plates) was freshly prepared. Then 175 µl of this mixture were added to each well of a 96-well microplate.

Step 2: 10 µl of hippocampus, hypothalamus and pituitary samples and 3 µl of cortex and cerebellum samples were pipetted to the sample wells of the microplate and mixed well.

Step 3: 70 µl of the freshly prepared xanthine oxidase solution were pipetted to each well by a multi-channel pipette to initial the reaction at 25°C.

Step 4: at the 1st minute and the 16th minute after the reaction started, the ABS was detected at 562 nm by a microplate reader.

The final concentration of the chemicals in the reaction was: 0.05M PBS; 0.05 mM EDTA; 0.05 mM NBT; 0.05 mM Xanthine; 0.045 unit Xanthine oxidase.

One unit of SOD activity was defined as the ability of inhibiting 50% of the reaction speed comparing to the uninhibited status at pH7.4, 25°C. The unit/mg protein was used for analysis the CAT activity in the brain between groups.

3.8.6 Glutathione peroxidase (GPx) activity assay

In this reaction, GPx catalyzed the glutathione reduced form (GSH) to glutathione oxidized form (GSSG) and water under the present of hydrogen peroxide. The GSSG reacted with the β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH) to genate the β-nicotinamide adenine dinucleotide phosphate oxidized form (β-NADP). The ABS of β-NADPH was detected at 340 nm.

a) Reaction *formulation*

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

$$GSSG + \beta - \underset{\Delta 340nm}{NADPH} \xrightarrow{GR} \beta - NADP + 2GSH$$

b) Solution preparation *for reaction* (Appendix A)

c) Analyses *protocol*

Step 1: A mixture containing 46 ml of the cocktail solution, 0.5 ml of the GR solution, 0.25 ml of the GSH solution was prepared before running the reaction. Then 200 µl of this mixture were pipetted to the sample wells of a 96 wells microplate.

Step 2: A standard curve solution was prepared by pipetting 0, 50, 100, 150, 200 µl of the **mixture** described in Step 1 to the standard wells and then PBS (0.05M) was added to make the total volume up to 200 µl, so the 5 concentration of the β-NADPH in the standard cascade was 0, 0.03, 0.06, 0.09, 0.12 mM.

Step 3: 10 µl of hippocampus, hypothalamus and pituitary samples and 3 µl of cortex and cerebellum samples were pipetted to the sample wells of the microplate and mixed well.

Step 4: 20 µl of H_2O_2 (0.007%) was pipetted to initiate the reaction by a multi-channel pipette at 25°C.

Step 5: At the 1^{st} $_{minute}$ and the 5^{th} minute after the reaction was initiated, the ABS was detected at 340 nm by a miroplate reader.

The final $_{concentration}$ of the solution is: 50 mM of PBS, 0.4 mM of EDTA, 0.12 mM β-NADPH, 1 unit of GR, 1 mM GSH, 1 mM NaN_3, 0.02 mM DDT, 0.0007% H_2O_2.

One unit of catalase activity defined as converting 1.0 µmole of β-NADPH to β-NADP per min at pH 7.0 at 25°C. The unit/mg protein was used for analysis the CAT activity in the brain between groups.

d) Calculation of the GPx activity

The ABS value in each microplate well was converted to the β-NADPH concentration (mM).

Equation to calculate the GPx units

$$Units_{GPx} = \frac{(C_{end} - C_{start}) \times V_{total}}{T_{reaction}}$$

C_{end}: the β-NADPH concentration at the end of the reaction

C_{start}: the β-NADPH concentration at the beginning of the reaction

V_{total}: total volume of the reaction mixture

$T_{reaction}$: the reaction time

3.9 The pharmacokinetics of CHE

3.9.1 Apparatus and high pressure liquid chromatography (HPLC) conditions

The purpose to develop the mobile phase system is to maximally separate the compound from the crude extract.

The analysis system was shown as follows.

Equipment:	Agilent LC 1200 HPLC
Mobile phase:	dichloromethane: n-hexane (6:4)
Column:	Hypersil Silica (Thermo®), 250×4 (mm), particle size: 5 um.
Temperature:	25°C
Flow rate:	1.5 ml/min
Detection:	UV (302 nm and 250 nm)
Inject volume:	20 µl
Run time:	23 min

The peaks in the chromatogram were name following the order that they flowed out from the column as peak 1 to peak 13. The compounds related to the peaks were temporarily named as compound 1 to compound 13, since the structure or the name of the compound was unknown yet.

3.9.2 HPLC method Validation

a) Determination of the optimal wavelength

CHE (1 mg/ml in mobile phase) was injected to the HPLC and detected at different wavelength as follows; 210, 230, 250, 270, 290, 302, 310, 330, 350, 370 nm. The peak patterns were compared to select the optimized wavelength for further analysis.

b) Linearity

Linearity study was performed by analysis of CHE diluted by the mobile phase to gradient concentrations as follows: 0.0001, 0.001, 0.005, 0.05 and 0.5 mg/ml. Calibration curves of each compound were established using peak area versus the relative concentration. Slopes, intercepts and correlation coefficients (r^2) were obtained by linear regression analysis.

c) Precision and stability

One CHE sample was injected to HPLC in triplicate and the peak area detected at 302 nm was recorded. The precision was reported by calculating the percentage of coefficient of variation (%C.V), which expressed as (S.D/mean) × 100. Three CHE samples were prepared and analyzed in duplicate with 24 hours interval. The peak areas of each compound were compared between the intervals to evaluate the stability of the CHE in the analysis process.

d) Specificity

Specificity of the method was evaluated by analyzing the CHE-free tissue samples (blood, brain, liver, kidney, ovary and uterus) and compared to the peak patterns of CHE (1 mg/ml in mobile phase) and olive oil (45 mg/ml in mobile phase)

e) Recovery

0.1 mg CHE (10 µl, 10 mg/ml in ethyl acetate) was added to 0.2 ml blood which was obtained from a normal female rat, followed by vortex for 1 minute. 0.6 ml ethyl acetate (3 times to the blood volume) was then added to extract the CHE by strong vortex for 1 minute and then ultra-sonication for 10 minutes followed by centrifugation (10,000 g) for 10 minutes. The supernatant was removed to the evaporated dish, and then allow the evaporation of the ethyl acetate at room temperature. The extract process was repeated 3 times. The residue in the evaporated dish was diluted with the mobile phase and made the volume up to 1 ml. Each sample was prepared in triplicate as described above. The samples were analyzed by HPLC and the peak areas were recorded to compare with those of the 0.1 mg/ml CHE diluted in mobile phase

3.9.3 Sample preparation

a) Intravenous (I.V) administration group

Thirty five adult female Wistar rats (180-220g) were separated into 7 groups (n=5). Each group received intravenous injection of CHE at a dose of 125 mg/kg body weight via the lateral tail vein. At the time point of 0, 0.5, 1, 2, 4, 8, 12 hours after the injection, 5 animals were sacrificed by cervical dislocation. Blood was drawn immediately from the heart followed by a perfusion of 200 ml ice cold normal saline into the rat. Then the brain, liver, kidney, ovary, and uterus were removed,

weighted and homogenized. The blood and the tissue homogenates were extracted and prepared for HPLC analysis according to the procedure described in the method validation.

b) Oral administration group

Forty female Wistar rats (180-220g) were separated into 8 groups (n=5). Each group received CHE orally at a dose of 125 mg/kg body weight. At each time point (0, 0.5, 1, 2, 4, 8, 12, 24 hours) after the administration, 5 animals were sacrificed by cervical dislocation. The samples were prepared for HPLC analysis as described in the section 3.9.3 (a).

c) Standard reference

A 1.0 mg/ml CHE and a 0.1 mg/ml 1,7 –diphenyl-5-hydroxy-(1E, 3E)-1,3-heptadiene (DHH, a compound purified from CHE) dissolved in mobile phase were used as reference with every HPLC analysis of each group.

3.9.4 Bioavailability of CHE

In the HPLC chromatograms, any peaks existed in the blood samples were integrated and the peak areas were used to generate a concentration-time curve for each compound. Bioavailability was calculated by comparing the area under the curve (AUC) of oral feeding groups to the I.V groups.

3.9.5 Organ distribution of CHE

In the HPLC chromatograms, any peaks existed in the liver, kidney, brain, ovary and uterus samples were integrated and the areas were used to generate a concentration-time curve for each compound in each organ. For each organ, the distribution and clearance time of each compound was compared.

3.10 Statistics

All data was expressed as mean ± Standard error of the mean (SEM). Statistical analysis of the experimental data was carried out using SPSS (Version 11.5). The significance of differences among groups was analyzed by using one-way ANOVA. The criterion for statistical significance was $P<0.05$.