CHAPTER III

MATERIALS & METHODS

MATERIALS

1. Animals, Chemicals and Reagents

1.1 Animals

Male Swiss albino mice, from the National Laboratory Animal Center at Salaya campus, Mahidol University, Nakornpathom, weighing 25-35 g at the beginning of experiments, were used throughout the study. Animals were housed in plastic cages in a room with controlled temperature at 25 ± 2 °C. The mice had free access to food and water except during the task performance under a 12-h light/dark cycle (light on at 7.00 a.m.). Laboratory animal care was followed according to the institutional guidelines of the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

1.2 Chemicals and Reagents

- 90% Asiaticoside (Changzhou Natural Products Development, China) with the following specifications.

Test Item	Standard	Assay Result
Color	White powder	White powder
TLC	Conforms	Conforms
Loss on drying	≤ 5%	1.39%
Heavy metals	< 10 ppm	< 10 ppm
Sulphated ash	< 0.5%	0.36%
Assays (HPLC)	Asiaticoside 90%	Asiaticoside 90.03%
Granule size	98% pass 100 mesh	98% Pass 100 mesh
Total bacteria count	Less than 1000/gm	121/gm
Yeast/moulds	Less than 100/gm	Negative
E. coli	Negative	Negative
Pseudomonas aeruginosa	Negative	Negative
Staphylococcus aureus	Negative	Negative

- Bovine serum albumin was used as a standard protein for calculation of the protein concentration.
- Double distilled water was used as a solvent of chemical powders to develop the working solution.
- Aβ fragment 25-35 (Sigma) was used as a toxic protein to induce neuronal toxicity. It was dissolved in double distill water at 1 mg/ml and aliquots were stored at -20 °C until used.
- Acacia was used to suspend asiaticoside.
- Steriled normal saline solution (NSS) was used to wash the cerebral cortex.
- Pentobarbital sodium (Nembutal®) was used to anesthetize animals before injecting Aβ.
- Monobasic sodium phosphate was used to prepare a phosphate buffer.
- Monobasic potassium phosphate anhydrous was used to prepare a phosphate buffer.
- Dibasic sodium phosphate andydrous was used to prepare a phosphate buffer.

2. Equipments

- Erlenmayer flasks were used for the solvent preparation.
- Glass cylinders were used to measure the gross quantity of water and liquid media in preparing procedures.
- Glass tubes were used throughout the experiments in the preparation of the standard solutions, chemical tests, etc.
- Pipettes used in the experiments
    1. Glass pipettes were used to transfer liquid chemicals and solvents.
    2. Micropipettes were used to deliver small volumes of solvents, liquid chemicals and samples in analytical assays.
- Stainless steel spoons were used to transfer solid chemicals in the weighing processes.
- Eppendoph plastic tubes were used to store the liquid chemicals and samples.
- Test tube racks were used to hold and carry a large number of tubes.
- Microsyringes were used for intracerebroventricular injection of Aβ to mice.

- 1-ml disposable syringes were used for intraperitoneal injection of general anesthetic to mice.
- Feeding needle was used for intragastric intubation of asiaticoside to mice.

3. Instruments

- Autoclave was used to sterilize equipments, solvents and other materials.
- Refrigerators were used to keep or preserve reagents and chemicals during the experiments.
- Ultra-low temperature freezer was used to store the samples of brain homogenate.
- Incubator was used to control appropriate environmental temperature for aggregating Aβ.
- Water bath was used to control the appropriate reaction temperature in chemical assays.
- Hot air ovens were used to sterilize glass equipments in the experiments.
- Electronic balance was used for weighing solid chemicals and powders.
- UV Spectrophotometer was used to measure the optical density of samples in chemical assays.
- Vortex mixer was used to mix solutions or suspensions to homogeneity.
- Centrifuge was used to precipitate the samples in chemical assays.
- Bio-Rad microplate reader model 3550 was used to detect the optical density of samples in chemical microassays in a microtiter plate.
- Surgical equipments were used for the operation of mice and dissection of their brains.
- Potter-Elvehjem homogenizer was used to prepare brain homogenate.

METHODS

1. Intracerebroventricular (i.c.v) Injection of β-Amyloid Peptide

The $A\beta_{25-35}$ (Sigma) was dissolved in sterile double distilled water (vehicle) at a concentration of 1 mg/ml, and stored at -20 °C. The $A\beta_{25-35}$ (1 mg/ml) was aggregated, or 'aged' by incubation in sterile distilled water at 37 °C for 4 days, as described by by Maurice et al. (1996). The sterile double distilled water was injected into control mice.

The aggregated form of $A\beta_{25-35}$ (9 nmol/mouse) was administered i.c.v. using a 50 µl microsyringe with a 26-gauge stainless-steel needle 3.0 mm long for all experiments. Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) and needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and ears, and perpendicular to the plane of the skull. Peptide or vehicle (3 µl) was delivered gradually within 3 sec. The administration site was confirmed by injecting Indian ink in preliminary experiments. Neither insertion of the needle nor injection of the vehicle had a significant influence on survival, and behavioral responses or cognitive functions.

2. Drug Administration

Asiaticoside was suspended in 25% acacia in water, and was administered orally 1 hour before tests for 27 consecutive days at dose of 5, 10, 25 and 50 mg/kg per day. One group consisted of 8 mice. In control groups, mice were administered with 25% acacia in water (0.15 ml/mouse). The oral administration of asiaticoside started 7 days before injection of $A\beta_{25-35}$ and the behavioral study was started 7 day after injection. On the 19^{th} day after Aβ injection, the mice were killed and cerebral cortices were used for chemical analysis.

3. Behavioral study

3.1 Locomotor activity

The measurement of locomotor activity was carried out on day 7 after Aβ injection. The apparatus consisted of a locomotor cage (23 × 35 × 20 cm), with the iron beams (3 mm) placed at 11 mm intervals on the floor of the cage. The activity was recorded by the electronic counting machine for 10 min.

3.2 Y-maze task

The Y-maze task was carried out on day 8 after Aβ injection. The Y-maze design was based on published protocols with modifications to adapt the system to mice (Yamada et al., 1996; Yan et al., 2001). The apparatus was made with black plastic. Each arm of Y-maze was 25 cm long, 14 cm high and 5 cm wide and positioned at an equal angle. The mice were placed at the end of one arm and allowed to move freely for 8 min in the maze. Arm entries were recorded manually and an arm

entry was considered to complete when the hind paws of the mouse were completely placed in the arm. Spontaneous alternation behavior was defined as the entry into all three arms on consecutive choices in overlapping triplet sets. The percent spontaneous alternation behavior was calculated as the ration of actual to possible alternation (defined as the total number of arm entries minus 2) and multiplied by 100.

3.3 Water Maze Task

The water maze was carried out on day 9 to 16 after the Aβ injection. The apparatus consisted of a circular pool (80 cm in diameter and 30 cm in height). A hidden platform (7 cm in diameter and 14 cm high) was set inside the pool that was filled with water and the surface of platform was 0.5 cm below the surface of the water. The pool was located in a contained room in which there were several cues external to the maze. These were visible from the pool and could be used by the mice for spatial orientation.

3.3.1 Reference Memory

In reference memory task, animals had to translate into memory the stable information present through all trials. This was tested on day 9 to 13 after the Aβ injection. The procedure was performed as described by Sarnyai et al. (2000). Mice were trained in a hidden-platform (spatial learning) task for 5 consecutive days. In hidden-platform training, mice were required to find the fixed location of a submerged platform in relation to external visual cues. Mice received four trials in each day; the intertrial interval was 30 min. In each day, mice were started from the four starting locations from the wall of the pool, a procedure that prevented them from associating the location of the platform with a single cue constellation. The sequence of start locations was randomized. The maximum search time allowed was 60 sec. Mice were allowed to rest on the platform for 10 sec and then were returned to their home cage. After the last trial, the platform was removed from the pool and animals were placed in a quadrant opposite to the location of the training platform and allowed to swim for 60 sec. The time spent in the quadrant where the platform had been located during training was measured.

3.3.2 Working Memory

In working memory task, animals had to translate into memory new incoming information that needed to be remembered for a specific testing day

during a short period, and that became irrelevant on the next day. This was tested on day 14 to 16 after the Aβ injection. On each day, the hidden platform position of the water maze remained constant, but changed from one day to next. The protocol for training was the same as that for reference memory.

3.4 Multiple-trial passive avoidance task

Multiple-trial passive avoidance task was carried out on day 17 and 18 after the Aβ injection as described by Yamada et al. (1996). The apparatus consisted of two compartments (15×25 cm and 15 cm in height), one light and one dark, both equipped with a grid floor. A guillotine door separated the two compartments. During the training trial, each mouse was placed in the light compartment, when animal entered to dark compartment, the door was closed and an inescapable foot shock (0.3 mA, 5 sec) was delivered through the grid floor. The mouse was removed after receiving the footshock and was placed back into the light compartment by the experimenter. The door was again opened 30 sec later to start the next trial. Training continued in this manner until the mouse stayed in the light compartment for 120 sec on a single trial. In the testing trial, given 24 h after the training trial, the mouse was again placed in the light compartment and the time until it entered the dark compartment was measured as step-through latency. When the mouse did not enter for at least 300 sec, a score of 300 sec was assigned.

4. Chemical Analysis

4.1 Sample Preparation

4.1.1 Reagents

1) 0.9% w/v NaCl
2) phosphate buffer, pH 7.4

   One liter of phosphate buffer, pH 7.4, consisted of 1.78 g of KH_2PO_4, 9.55 g of Na_2HPO_4. The solution was adjusted to pH 7.4 with NaOH or HCl.

4.1.2 Brain Homogenate

1) The mice were sacrified by cervical dislocation and brains were quickly removed from the skull.
2) Cerebral cortices were weighed and then washed in ice-cold saline.

3) Tissues were chilled in ice-cold phosphate buffer, pH 7.4, and homogenized at 1g tissue per 9 ml buffer (10% w/v) at 0 °C.
4) The homogenates were centrifuged at 700 g for 10 min at 4 °C.
5) The supernatant fluid was recovered and aliquots were finally frozen at -80 °C until assay.

4.2 Protein Assay

The protein concentration of sample was determined by the method of Bradford (1976). The protein determination method involves the binding of dye to protein.

4.2.1 Reagents

1) Phosphate buffer, pH 7.4, as homogenizing buffer.
2) Bradford reagent (sigma).
3) 2 mg/ml standard BSA in distilled water was frozen -80 °C until use.

4.2.2 Procedure

1) Prepared fresh standard BSA at concentrations 0.05-1.4 mg/ml in microcentrifuge tubes:

Working standard (mg/ml)	1.40	1.20	1.00	0.60	0.40	0.20	0.00
BSA 2 mg/ml (µl)	140	120	100	60	40	20	0
H_2O (µl)	60	80	100	140	160	180	200

2) Pipetted 10 µl of sample or working standard and transferred to 96-well plate in duplicate.
3) Added 190 µl of Bradford reagent.
4) The absorbance at 595 nm was measured by the microplate reader after 5 min and before 1 h.

4.2.3 Calculations

1) The average absorbance of each standard was plotted against its amount of protein. The best fit regression line was drawn through the points. The amount of protein in each unknown sample was obtained by comparing its absorbance against the standard curve.

2) Protein concentrations were expressed as milligram protein per gram of brain weight.

4.3 Thiobarbituric Acid Reactive Substances (TBARS)

The TBA test is a colorimetric technique, which measures the absorbance of the pink compound formed between TBA and TBARS. The formation was determined according to the method of Ohkawa et al. (1979).

4.3.1 Reagents

1) Phosphate buffer, pH 7.4, as homogenizing buffer.
2) 8.1% w/v SDS in distilled water.
   4.05 g of SDS was dissolved in 50 ml of warm distilled water until the solution was clear.
3) 20% w/v acetic acid in distilled water.
   10 g of acetic acid was dissolved in 450 ml of distilled water and the solution was adjusted pH to 3.5 with NaOH or HCl.
4) 0.8% w/v TBA in distilled water was prepared freshly.
   2.4 g of TBA was dissolved in 300 ml of warm distilled water.
5) 4 M TEP (1M TEP = 1M MDA) from sigma.

4.3.2 Procedure

1) Prepared fresh standard TEP at concentration 1-20 μM in the tubes:
   1.1) Prepared 40 mM TEP

4M TEP	10	μl
Add buffer to	1	ml

   1.2) Prepared 100 μM TEP

40mM TEP	10	μl
Add buffer to	4	ml

Working standard (μM)	0.00	2.50	5.00	10.00	15.00	20.00
100 μM TEP (μl)	0	13	25	50	75	100
Buffer (μl)	500	488	475	450	425	400

2) Pipette 100 μl of sample or working standard and transferred to tubes in duplicate.

3) Added 200 µl of 8.1% SDS, 1.5 ml of 20% acetic acid, and 1.5 ml of 0.8% TBA respectively and then mixed together.
4) After that, adjusted the volume with distilled water to 4 ml.
5) Boiling at 95 °C for 60 min
6) After cooling, added distilled water to 5 ml.
7) Extracted with 5 ml of n-butanol.
8) The reaction solution was centrifuged at 3,000 rpm for 10 min.
9) The absorbance of organic layer was measured at 532 nm with a spectrophotometer.

4.3.3 Calculations

1) The average absorbance of each standard was plotted against its amount of TEP. The best fit regression line was drawn through the points. The amount of MDA in each unknown sample was obtained by comparing its absorbance against the standard curve.
2) MDA concentrations were expressed as nanomoles per milligram protein

4.4 Total glutathione measurement

Total GSH measurement was based on the method of Griffith's (1980) and Tietze's (1969). GSH can be measured by an enzymatic recycling procedure. The sulfhydryl group of GSH reacts with DTNB (Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). NADPH reduces the disulfide that is produced, in the presence of glutathione reductase. The rate of formation of TNB is measure at 415 nm.

4.4.1 Reagents

1) 0.1 M Sodium phosphate buffer with 0.005 M EDTA, pH 7.5, was used to prepare solutions of 0.3 mM NADPH, 6 mM DTNB, 50 units/ml GSH reductase and 5% 5-SSA.
   - 0.2 M Monobasic sodium phosphate stock solution

Monobasic sodium phosphate monohydrate	1.10	g
Distilled water	40	ml

   - 0.2 M Dibasic sodium phosphate stock solution

Dibasic sodium phosphate anhydrous	2.84	g
Distilled water	100	ml

Combine

0.2 M Monobasic sodium phosphate	16	ml
0.2 M Dibasic sodium phosphate	84	ml
EDTA disodium salt dihydrate	0.3722	g
Distilled water	50	ml
Adjust with HCl and NaOH to pH 7.5		
Add distilled water to	200	ml

2) 5% w/v 5-SSA.

5 g of 5-SSA was dissolved in 100 ml of 0.1 M Sodium phosphate buffer with 0.005 M EDTA, pH 7.5.

3) 0.3 mM NADPH.

0.0085 g of NADPH was dissolved in 34 ml of 0.1 M Sodium phosphate buffer with 0.005 M EDTA, pH 7.5.

4) 6 mM DTNB.

0.01427 g of DTNB was dissolved in 6 ml of 0.1 M Sodium phosphate buffer with 0.005 M EDTA, pH 7.5.

5) 25 units/ml GSH reductase.

19 µl of GSH reductase (500 units/0.27 ml) was dissolved in 1.4 ml of 0.1 M Sodium phosphate buffer with 0.005 M EDTA, pH 7.5

4.4.2 Procedure

1) Prepared fresh standard GSH in microcentrifuged tubes:

- Prepared 0.5 mM GSH.

GSH	0.0015 g
Add Buffer to	10.00 ml

Working standard (µM)	0	30	60	90	120	150	200
0.5 mM GSH (µl)	0	30	60	90	120	150	200
Buffer (µl)	500	470	440	410	380	350	300

2) Added 1 vol. of 5% 5-SSA to standard GSH and sample for precipitate protein.

3) Centrifuged at 10,000 rpm for 10 min at 4 °C.

4) Pipetted 40 µl of supernatant of sample or working standard and transferred to 96-well plate in duplicate.
5) Added 140 µl of 0.3 mM NADPH.
6) Then added 5 µl of GSH reductase.
7) Incubated the mixture at 25 °C for 20-30 min.
8) Added 20 µl of 6 mM DTNB and mixed together.
9) The absorbance at 415 nm was measured by microplate reader every 15 sec for 3 min.

4.4.3 Calculations

1) The average absorbance of each standard was plotted against its amount of GSH. The best fit regression line was drawn through the points. The amount of GSH in each unknown sample was obtained by comparing its absorbance against the standard curve.
2) GSH concentrations were expressed as nanomoles per milligram protein.

5. Statistical analysis

The results were expressed as mean ± SEM of experimental data obtained from 8 mice. Differences among means were tested by one-way analysis of variance (ANOVA) followed by Scheffe's test for multi-group comparison. $P < 0.05$ was considered to be statistically different.