CHAPTER III
MATERIALS AND METHODS

Materials

1. Experimental Animals

Pregnant Wistar albino rats of gestation day 14 were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. All pregnant rats were housed in plastic cages (one pregnant rat per one plastic cage) with wood shavings as bedding in the animal room at Faculty of Pharmaceutical Sciences, Chulalongkorn University under controlled environmental conditions (room temperature 25 ± 1 °C with 12-hr light/dark cycle, humidity of approximately 60%) They were freely fed with commercial rodent chow (C.P. rat feed, Pokaphand Animal Fed Company, Limited, Bangkok, Thailand) and allowed tap water *ad libitum*. The brains from embryonic day 18 rat fetuses were used in this study.

The protocols in this experiment were ethically approved by the Ethics Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University Bangkok, Thailand on 28 April 2004 (No 134/2004).

2. Chemicals

$A\beta_{25-35}$, quercitrin , cyclodextrin-encapsulated 17β-estradiol, trypsin, DNase I, 0.25% trypsin, Dulbecco's Modified Eagle's Medium (DMEM), DMEM nutrient mixture F-12 HAM, Dulbecco's Phosphate Buffered Saline (DPBS) without $CaCl_2$, Hank's Balance Salt Solution, HEPES buffer, putrescine, sodium bicarbonate,, sodium selenite, human transferrin, bovine insulin, penicillin G, streptomycin sulfate, poly-D-lysine hydrobromide (MW 15,000-30,000), 0.04% trypan blue, thiobarbituric acid, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB), DL-dithiothreitol (DTT), Ethylenediamine tetraacetic acid (EDTA), ethylene glycol-bis (2-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid (EGTA), glutathione (reduced form), glutathione reductase, β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), 1,1,3,3-tetraethoxypropane (TEP), manitol, phosphotungstic acid, sodium chloride, sodium dodecyl sulfate, sodium hydroxide, sodium phosphate (dibasic anhydrous), sodium phosphate (dibasic heptahydrate), sodium phosphate (monobasic anhydrous), sucrose, sulfosalicylic acid,

glycine, glycerol, Tween 20, methanol, ammonium persulfate, acrylamide, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenltetrazolium bromide) and *In vitro* Toxicology Assay Kit (Lactate Dehydrogenase Based) were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Equine serum and fetal bovine serum (FBS) were purchased from Hyclone (USA).

Dimethyl sulfoxide (DMSO) and sulfuric acid were purchased from Lab-Scan, Ltd. (Ireland).

D-Glucose, potassium chloride, and sodium hydrogencarbonate were purchased from Asia Pacific Specialty Chemicals, Ltd. (Australia).

Pyruvic acid sodium salt was purchased from Fluka Chemical (Japan).

N, N'-Methylendiacrylamide, hydrochloric acid, diethyl ether, ethanol, Tris-(hydroxymethyl) aminomethane, and skimmed milk powder were purchased from Merck (Darmstadt, Germany).

Estrogen receptor antagonist (ICI 182780) was purchased from Tocris Cookson, Inc. (Ellisville, MO, USA).

Superoxide dismutase assay kit and glutathione peroxidase assay kit were purchased from Cayman Chemical Company (USA).

MEK1 inhibitor (PD 98059), PI3K inhibitor (LY 294002), and the ApoTarget™ Caspase-3 colorimetric protease assay was purchased from Biosource International, Inc. (USA).

Anti-Bcl2 mouse mAb, anti-Bax mouse mAb, goat anti-mouse IgG (H&L) horseradish peroxidase (HRP), and cytochrome c release apoptosis assay kit were purchased from Calbiochem (San Diego, CA, USA).

Super signal West Pico chemilluminescent substrate was purchased from Pierce Biotechnology, Inc. (USA).

Page Ruler™ Prestained Protein Ladder was purchased from Fermentus Life Sciences (USA).

Transblot papers were purchased from Bio-Rad Laboratories, Inc. (Richmon, USA).

BioTrace polyvinylidine difluoride (PVDF) transfer membranes were purchased from Pall Gelman Laboratory (USA).

Film X-ray blue, developer, replenisher, fixer, and replenisher were purchased from Eastman Kodax Company (Rochester, NY).

Carbon dioxide gas was purchased from Thai Industrial Gases Public Company, Ltd. (Thailand).

All other chemicals and solvents used throughout this study were commercially available reagents or analytical grade reagents.

3. Instruments

Instruments employed in this study include adjustable pipettes: 10-100 µl, 200-1000 µl, 1-5 ml (Labsystems, Finland), bunsen burner, carbon dioxide incubator (Forma Scientific, USA), cell culture dishes: diameter 35 and 100 mm (Nunc, Denmark), 24- and 48-well cell culture plates, 96-well cell culture plates (Nunc, Denmark), conical tubes: 5 and 50 ml (Nunc, Denmark), disposable glass pasteur pipettes: 230 mm (Volac), pipette tips: 1-200 µl, 200-1,000 µl, and 1-5 ml (Labsystems, Findland), needles: 18G (Nipro), latex free syringes: 10 ml (Becton Dickinson), Syringe filter holders (13 mm) (Satorius), sterile Millex-GV (0.22 µm filter unit) (Millipore, USA), Sterivex-GS (0.22 µm filter unit with filling bell) (Millipore, USA), hemocytometer (Depth 0.100 mm) (Improved Neubauer), pH meter (Beckman Instruments, USA), Vortex mixer (Clay adams, USA), water bath (Thelco, USA), centrifuge (Kokusan, Japan), refrigerated centrifuge (Hettich zentrifugen), inverted microscope: Axiovert 135 (Zeiss, Germany), laminar air flow hood (Hepaco, USA), Vibra cell (Sonics, USA). microplate reader (Anthos Labtec HT2 version 1.21E, Australia), Multilabel microplate reader (Perkin Elmer VICTOR3 Wallac 1420, Germany), spectrofluorometer (Jasco Model FS 777), Mini-PROTEAN® 3 Electrophoresis Cell and Mini Trans-Blot® electrophoretic Transfer cell (Bio-Rad, USA).

Methods

1. Primary cultures of embryonic rat hippocampal neurons (Figure 5)

Dissociated cell cultures were prepared from dissected hippocampal regions of embryonic day 18 Wistar rats. After removal of the meninges, the tissue was cut into pieces and incubated for 30 min at 37 °C with 0.25% trypsin and 0.01% DNase I

in Ca^{2+}, Mg^{2+}-free Hank's balance salt solution. The incubation was terminated by the addition of heat-inactivated Equine serum and tissue fragments were centrifuged at 3,000 rpm for 10 min. The tissue pellet was gently rinsed and resuspended in DMEM supplemented with 10% FBS, 99 μg/ml sodium pyruvate, 3.7 mg/ml sodium bicarbonate, 54 μg/ml penicillin G sodium and 90 μg/ml streptomycin sulfate. The single cells were dissociated by gently passing the suspension through a flame-polished pasteur pipette 15 to 20 times. The cell suspension was filtered through two sheets of nylon net (50 μm-mesh) to remove cell lumps and was further diluted with DMEM containing 10% FBS and plated on poly-D-lysine coated plates (100 μg/ml) at the density of 6×10^5 cells/cm^2 or 1×10^6 cells/cm^2 in 48-well plates or 1.2×10^6 cells/cm^2 in 24-well plates. They were cultured at 37°C for 24 hr in a humidified 5% CO_2-95% air atmosphere and the medium was changed to DF/TIP medium containing a 1:1 (v/v) mixture of DMEM (high glucose) and Ham's nutrient mixture F-12 supplemented with 3.8 mg/ml sodium bicarbonate, 30 nM sodium selenite, 80 μg/ml human transferrin, 10 μg/ml bovine insulin, 100 μM putrescin, 54 μg/ml penicillin G sodium and 90 μg/ml streptomycin sulfate. Cultures were used in the experiments 24 hr after plating *in vitro*.

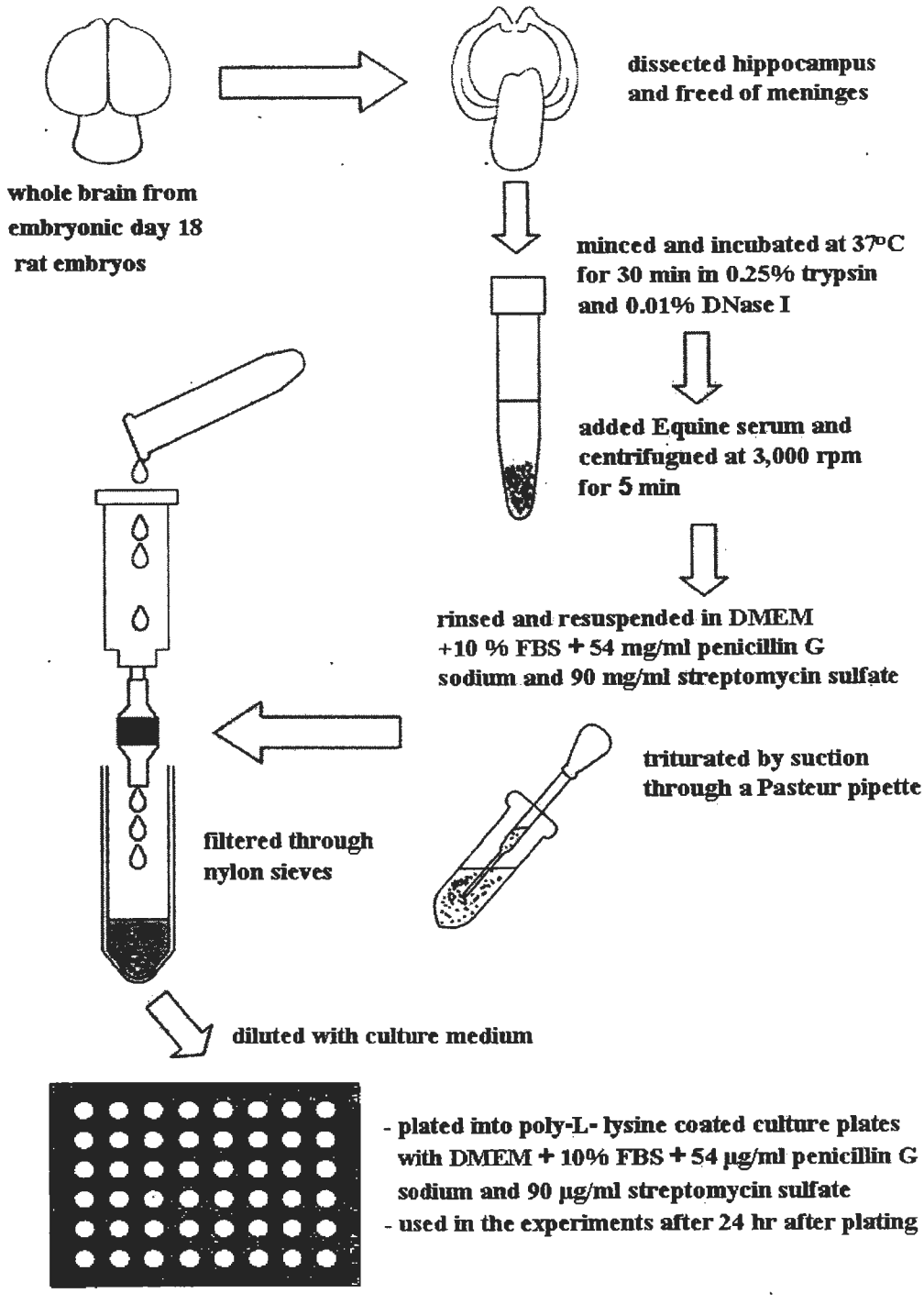

Figure 5 Preparation of rat hippocampal neuronal cultures.

2. Experimental designs for the study

2.1 Effects of $A\beta_{25-35}$ on cultured hippocampal neurons

$A\beta_{25-35}$ was dissolved in sterile distilled water at a concentration of 1 mg/ml as a stock solution. This stock solution was aliquoted and stored at -20 °C. An aliquot of the stock solution was aged at 37 °C for 3 days to aggregate the peptide before use.

Hippocampal neurons were cultured in 48-well culture plates at the density of 6×10^5 cells/cm^2 for 24 hr and exposed to various concentrations of $A\beta_{25-35}$ (1-20 μM) for 24 and 72 hr. Cultured cells received the equivalent amount of vehicle were used as control cultures. After $A\beta_{25-35}$ exposure, cell viability was analyzed using MTT reduction and LDH release assays to determine neurocytotoxic effects of $A\beta_{25-35}$ at various concentrations and durations of exposure.

2.2 Effects of quercitrin on cultured hippocampal neurons

Quercitrin was dissolved in pure DMSO and diluted with sterile distilled water as a stock solution and directly added to the culture medium to attain final concentrations as indicated in the experiment. Cyclodextrin-encapsulated 17β-estradiol was dissolved in sterile distilled water as a stock solution and directly added to the culture medium to attain final concentrations as indicated in the experiment.

Hippocampal neurons were cultured in 48-well culture plates at the density of 6×10^5 cells/cm^2 for 24 hr and treated with various concentrations of quercitrin (0.001-100 μM) for 24 and 72 hr. Neuronal cultures received the equivalent amount of vehicle were used as the controls. Neuronal cultures were also treated with 17β-estradiol at different concentrations (0.001-100 μM) for 72 hr as the positive controls. After treatment, cell viability was analyzed using MTT reduction assay to determine effects of quercitrin or 17β-estradiol at various concentrations and durations of exposure.

In a preliminary study, it was found that nearly 50% of cultured cells died after 3 days of exposure to 5 μM Aβ. In order to cover delayed effects of quercitrin and 17β-estradiol that required a time for protein synthesis, 3 days of $A\beta_{25-35}$ exposure and 3 days of quercitrin or 17β-estradiol treatment were used in subsequent experiments.

2.3 Effects of co-exposure to quercitrin on $A\beta_{25-35}$ -induced neurotoxicity in hippocampal neuronal cultures

Hippocampal neurons were cultured in 48-well culture plates at the density of 6×10^5 cells/cm^2 for 24 hr. Neuronal cells were treated with the combination of quercitrin at concentrations of 0.1, 1, 10, 50 and 100 μM and $A\beta_{25-35}$ at the concentration of 5 μM for 3 days. Cells received the equivalent amount of vehicle are used as the vehicle control. Cells were also treated with the combination of 17β-estradiol at concentrations of 0.1, 1, 10, 50 and 100 μM and $A\beta_{25-35}$ at the concentration of 5 μM for 3 days were used as the positive control. After the co-exposure, cell viability was analyzed using MTT reduction assay. Additionally, quantitative determinations of LDH, ROS, TBARS, GSH, GPx and SOD would be analyzed if co-exposure to quercitrin could protect hippocampal neurons from Aβ-induced neurotoxicity.

2.4 Effects of pre-exposure to quercitrin on $A\beta_{25-35}$-induced neurotoxicity in hippocampal neuronal cultures

Hippocampal neurons were cultured in 48-well culture plates at the density of 6×10^5 cells/cm^2 for 24 hr and pretreated with quercitrin at concentrations of 50 and 100 μM for 3 days. Thereafter, culture medium was changed and replaced with fresh medium containing $A\beta_{25-35}$ at the concentration of 5 μM for 3 days. Cells received the equivalent amount of vehicle were used as the vehicle control. Cells treated with 17β-estradiol at an effective concentrations (50 and 100 μM) were used as the positive control for quercitrin. After $A\beta_{25-35}$ exposure, cell viability was analyzed using MTT reduction assays. Quantitative determinations of LDH, ROS, TBARS, GSH, GPx and SOD would be analyzed if pre-exposure to quercitrin could protect hippocampal neurons from Aβ-induced neurotoxicity.

2.5 Effects of ER antagonist (ICI 182780) on protective effects of quercitrin against $A\beta_{25-35}$-induced neurotoxicity in hippocampal neuronal cultures

ICI 182780, an estrogen receptor antagonist, was dissolved in DMSO at a concentration of 50 μM as a stock solution and used at a final concentration of 1 μM.

Hippocampal neurons were cultured in 48-well culture plates at the density of 6×10^5 cells/cm^2 for 24 hr. Thereafter, medium was changed to DF/TIP medium containing a 1:1 (v/v) mixture of DMEM (high glucose) and Ham's nutrient mixture F-12 supplemented with 3.8 mg/ml sodium bicarbonate, 30 nM sodium selenite, 80 μg/ml human transferrin, 10 μg/ml bovine insulin, 100 μM putrescin, 54 μg/ml penicillin G sodium and 90 μg/ml streptomycin sulfate. Hippocampal neurons were treated with 1 μM ICI 182780 or vehicle for 2 hr. Then, quercitrin or 17β-estradiol at effective concentrations (50 and 100 μM) and $A\beta_{25-35}$ at the concentration of 5 μM were added to the medium for 3 days. After co-exposure, cell viability was analyzed using MTT reduction assay.

2.6 Effects of MEK inhibitor (PD 98059) or PI3K inhibitor (LY 294002) on protective effects of quercitrin against Aβ-induced neurotoxicity in hippocampal neuronal cultures

PD 98059 and LY 294002 were dissolved in DMSO at concentrations of 20 mM and 50 mM as stock solutions and used at final concentrations of 30 μM. and 40 μM, respectively.

Hippocampal neurons were cultured in 48-well culture plates at the density of 6×10^5 cells/cm^2 for 24 hr. Thereafter, medium was changed to DF/TIP medium. Cultured neurons were treated with 1 μM ICI 182780 or vehicle for 2 hr. Then, quercitrin or 17β-estradiol at effective concentrations (50 and 100 μM) and $A\beta_{25-35}$ at the concentration of 5 μM were added to the medium for 3 days. After co-exposure, cell viability was analyzed using MTT reduction assay.

Hippocampal neurons were cultured in 48-well culture plates at the density of 6×10^5 cells/cm^2 for 24 hr. Thereafter, medium was changed to DF/TIP medium. Cultured neurons were pretreated with 30 μM PD98059 or 40 μM LY294002 or vehicle for 2 hr before further co-incubation with quercitrin or 17β-estradiol at effective concentrations (50 and 100 μM) and $A\beta_{25-35}$ at the concentration of 5 μM for 3 days. After co-exposure, cell viability was analyzed using MTT reduction assay.

2.7 Effects of quercitrin on apoptotic signaling, cell survival and cell death factors in cultured hippocampal neurons

Hippocampal neurons were cultured in 24-well culture plates at the density of 1.2×10^6 cells/cm^2 for 24 hr. Hippocampal neurons were treated with the combination of quercitrin or 17β-estradiol (at concentrations of 50 and 100 μM) and $A\beta_{25\text{-}35}$ at the concentration of 5 μM for 3 days. After co-exposure, measurements of caspase-3 activity, Bcl-2 and Bax proteins, and cytochrome c release were performed.

3. Analytical methods

Assessment of cell morphology

The morphology of cultured cells after $A\beta_{25\text{-}35}$ exposure with or without pretreatments was observed and monitors by an inverted microscope with phase-contrast optics in combination with microcomputer-assisted image capture system.

Measurement of cell viability

MTT reduction assay

Reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenltetrazolium bromide (MTT) is one of the most frequently used methods for measuring cell proliferation and cytotoxicity. MTT reduction is an index of mitochondrial viability because it requires metalbolically active mitochondria. The MTT assay based on the reduction of yellow-colored 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenltetrazolium bromide into a purple insoluble formazan product by mitochondrial dehydrogenase in living cells but not in dead cells or their lytic debris (Figure 6) (Mosman, 1983). The resulting intracellular purple formazan can be quantitatively measured by spectrophotometry after an extraction with DMSO. It is widely assumed that MTT is mostly reduced by active mitochondria in living cells. A decrease in cellular MTT reduction could be an index of cell damage.

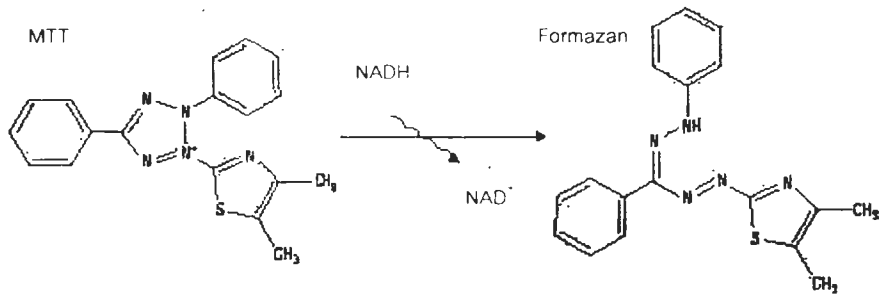

Figure 6 Molecular structure of MTT and their corresponding reaction products. (Methods for studying cell proliferation and viability in cell populations: assays that measure metabolic activity).

MTT reduction was determined by colorimetric method with modifications (Hansen et al, 1989, Ishikawa et al, 2000) of the original procedure (Mosmann, 1983). Ten µl of MTT stock solution was added to the medium in each well (a final concentration was 100 µg/ml). Cultures were incubated in a 5% CO_2 incubator at 37 °C for 1 hr and the culture medium in each well was aspirated off without disturbing the formazan precipitate. Then 200 µl of DMSO was added to each well to solubilize the formazan crystals. After formazan solubilization, 180 µl aliquots of soluble formazan were transferred to a 96-well microtiter plate. The absorbance in each well was measured using an Anthos Labtec HT2 microplate reader at a measuring wavelength of 570 nm and a reference wavelength of 655 nm. The cellular reduction of MTT which represents metabolic activity and viability was expressed as the percent absorbance of treated cells compared with the absorbance of control cells.

LDH release assay

The lactate dehydrogenase assay is a means of measuring either the number of cells via total cytoplasmic lactate dehydrogenase (LDH) or membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture medium when the plasma membrane is damaged, thus it can be used as an indicator of plasma membrane damage. The assay is based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye (Figure 7). The resulting compound is measured spectrophotometrically.

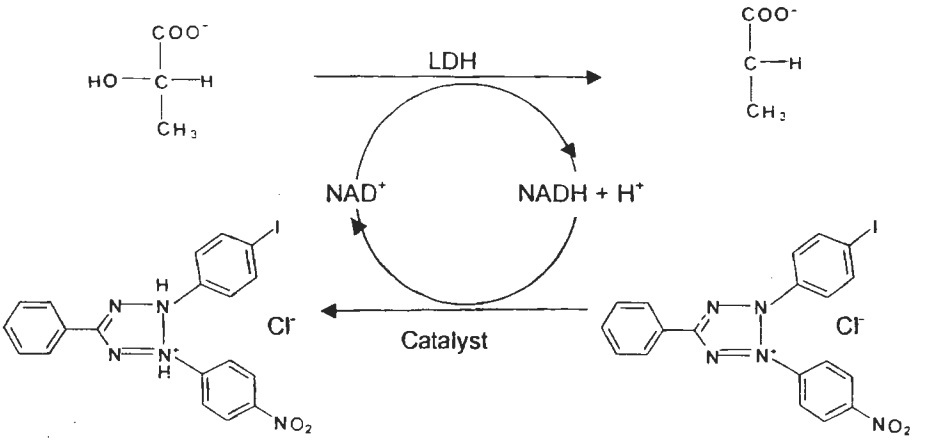

Figure 7 The reactions in lactate dehydrogenase (LDH) assay. (Cytotoxicity detection kit (LDH) : instruction manual version 5, 1999).

LDH activity was determined by using an *In vitro* Toxicology Assay Kit Lactate dehydrogenase based (Sigma, USA) with a procedure according to the manufacturer's instructions. In brief, medium LDH was assayed by pipetting 100 µl aliquot of culture medium from each well into a 96-well microtiter plate. Cellular LDH in cultures was measured by carefully removed the remaining culture medium from the adherent cells and solubilized cells with 500 µl of 0.5% Triton X-100 in PBS, Then, 100 µl aliquots were pipetted into a 96-well microtiter plate. The reaction was initiated by adding 50 µl of assay mixture into each well. Thereafter, the plate was covered with aluminum foil and left at room temperature for 30 min. Then, the reaction was terminated by an addition of 50 µl of 0.5 N HCl into each well. The spectrophotometric absorbance in each well was determined with a measuring wavelength of 490 nm and a reference wavelength of 690 nm. The LDH release was expressed as the percentage of total LDH activity by the following formula and comparative LDH release in test conditions was presented as the percentage of that in control conditions.

$$\% \text{ LDH release} = \frac{\text{LDH activity in medium} \times 100}{\text{LDH activity in medium + LDH activity in cells}}$$

Measurement of lipid peroxidation:

Thiobarbituric acid reactive substance (TBARS) assay

The TBARS assay is the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress (Yagi et al., 1998). This assay measures the amount of malondialdehyde (MDA), an end product of polyunsaturated fatty acid oxygenation. It is widely used as a screening assay to quantify the extent of lipid peroxidation *in vitro* (Holly and Cheeseman, cited in Ljybucid et al., 1996). One molecule of MDA can react with two molecules of thiobarbituric acid (TBA) to generate the production of pink pigment which can be determined by spectrofluorometric method (Halliwell and Gutteridge, 1989; Esterbauer and Cheeseman, 1990; Sattler et al., 1998) (Figure 8)

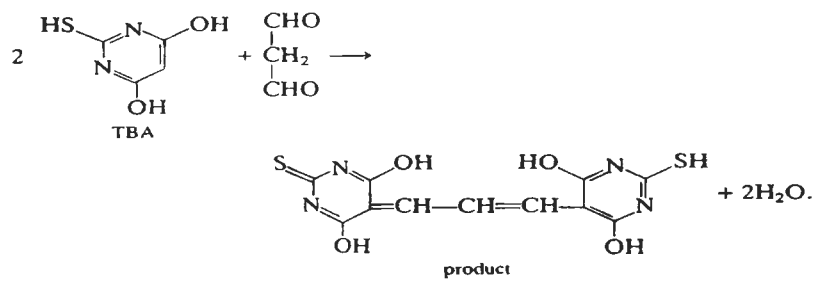

Figure 8 The reaction of thiobarbituric acid (TBA) and malondialdehyde (MDA) (Halliwell and Gutteridge, 1989).

TBARS was measured using a technique that modified from Ohkawa et al. (1979) and Storch et al. (2000). Briefly, cells were washed twice with PBS and lysed with 160 µl of 2% sodium dodecyl sulfate for 30 min. Cell lysates collected from two culture wells of cultured hippocampal neurons were pooled and transferred to a conical tube. The lysates were adjusted with 2% sodium dodecyl sulfate to 1 ml and added serially with 50 µl of 4% butylated hydroxytoluene in ethanol, 1 ml of 10% phosphotungstic acids in 0.5 M sulfuric acid and 1.5 ml of 0.7% TBA. The reaction mixtures were boiled at 95 °C for 60 min. After cooling by tap water, the mixtures were extracted with n-butanol and the precipitate was removed by centrifugation at 3,500 rpm for 10 min. The fluorescence of n-butanol layer (supernatant) was

measured at 515 nm excitation and 553 nm emission wavelengths using Jasco Model FS 777 spectrofluorometer. Fluorescence measurement was expressed in term of the percentage of control. In this experiment, 1,1,3,3-tetraethoxypropane (TEP) was used as standard. Lipid peroxidation was expressed in term of MDA equivalence (1M TEP = 1M MDA).

Measurement of glutathione

The total glutathione, comprising reduced glutathione (GSH) and glutathione disulfide (GSSG), contents of biological samples is conveniently determined with an enzymatic recycling assay based on glutathione reductase (Tietze, 1969; Xu and Thornalley, 2001). The sum of reduced and oxidized forms of glutathione can be determined using a kinetic assay in which catalytic amounts of GSH or GSSG and glutathione reductase bring about the continuous reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) by NADPH according to the following reactions (Figure 9).

$$2GSH + DTNB \xrightarrow{\text{nonenzyme}} GSSG + 2TNB$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSH reductase}} 2\,GSH + NADP^+$$

$$NADPH + H^+ + DTNB \xrightarrow[\text{GSH reductase}]{\text{GSH /GSSG}} 2\,TNB + NADP^+$$

Figure 9 The reaction of GSH, GSSG and GSH reductase (Akerboom and Sies, 1981).

DTNB (5,5'-Dithiobis (2- nitrobenzoic acid)), known as Ellman's Reagent, was developed for detection of thiol compounds. The glutathione recycling system by DTNB and glutathione reductase created a highly sensitive glutathione detection method. DTNB and glutathione (GSH) react to generate 2-nitro-5-thiobenzoic acid and glutathione disulfide (GSSG). Since 2-nitro-5-thiobenzoic acid is a yellow colored product, GSH concentration in a sample solution can be determined by the

measurement at 450 nm absorbance (Tietz, 1969; Baker, 1990). GSH is generated from GSSG by glutathione reductase and react with DTNB again to produce 2-nitrobenzoic acid. Therefore, this recycling reaction improved the sensitivity of total glutathione detection.

In this study, total glutathione (GSH) was determined by using enzymatic cycling method that modified from Tietz (1969) and Baker et al. (1990). In brief, after removing the medium, cells were washed twice with 500 µl of cooled PBS (2 ml) on ice and 150 µl of 1% (w/v) sulfosalicylic acid was added. Then, cells were immediately scraped and two wells of cultured hippocampal neurons were pooled and transfered to 1.5 ml Eppendorf tube. The extracted cells were centrifuged at 13,000 g for 5 min at 4 °C and 20 µl of the supernatant was transferred to a 96-well microtiter plate and then volume was adjusted to 100 µl with 0.1 M sodium phosphate buffer containing 1 mM EDTA (pH 7.5). Then, 100 µl of reaction mixture (25 µl of 0.15 mM 5-5'-dithiobis-(2-nitrobenzoic acid), 25 µl of 0.2 mM NADPH and 50 µl of 1U GSH reductase in 0.1 M sodium phosphate buffer, pH 7.5) was added to each well. The formation of 2-nitro-5 benzoic acid in each well was monitored at the wavelength of 405 nm every 10 seconds for 2 min using an Anthos Labtec HT2 microplate reader. The slope of initial rate of reaction was used for calculating GSH content from a standard curve obtained by plotting known amount of GSH. The cellular GSH content was expressed in term of the percentage of control.

Measurement of superoxide dismutase

Superoxide dismutase (SOD) is antioxidant enzyme that catalyses the dismutation of highly reactive superoxide anion to molecular oxygen and the less reactive species, hydrogen peroxide, and thus forms a crucial part of the cellular antioxidant defense mechanism (Fridovich, 1995; Sandalio et al., 1997; Teixeira et al., 1998). Peroxide can be destroyed by catalase or glutathione peroxidase reaction

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

SOD is widely distributed in both plants and animals. It occurs in high concentrations in brain, liver, heart, erythrocytes and kidney. In humans, there are

three forms of SOD: cytosolic Cu/Zu-SOD, mitochondrial Mn-SOD and extracellular SOD (Sandstrom et al., 1994).

The SOD activity is measured by monitoring the inhibition of cytochrome c reduction by xanthine/xanthine oxidase reaction as described by McCord and Fridovich (1969). Hippocampal neurons were cultured at the density of 1×10^6 cells/cm^2 in 48-well culture plates. After 24 hr of culturing, they were treated with the combination of quercitrin or 17β-estradiol at concentrations of 50 and 100 μM and $A\beta_{25-35}$ at the concentration of 5 μM for 3 days.

Sample preparation for SOD

SOD was detected using the Cayman Chemical SOD assay kit. Sample preparation was performed as follows. Briefly, after treatment, treated cells were washed twice with ice-cold PBS and harvested in cold 20 mM HEPES buffer, pH 7.2 containing 1 mM ethylene glycol-bis (2-aminoethyl ether)-N,N,N′,N′,-tetraacetic acid (EGTA), 210 mM manitol and 70 mM sucrose using a cell scraper. Cell lysates obtained from pooled two culture wells were transferred to a 1.5 ml microcentrifuge tube and centrifuged at 1,500 g for 5 min at 4 °C. The supernatant was transferred to the fresh tube for assay and kept on ice. If not assay on the same day, the samples were frozen at -80 °C and the assay was done within 1 month.

SOD assay procedure

The SOD assay was performed according to the manufacturer's protocol as the followings. Two hundred μl of the diluted radical detector was added to a 96-well plate and 10 μl of sample or standard SOD was added to each well. The reactions were initiated by adding 20 μl of the xanthine oxidase to all the wells. Subsequently, the 96-well plate was carefully shaked for a few seconds to mix, covered with the plate cover and incubated on shaker for 20 min at room temperature. The optical density was measured at 450 nm using a Perkin-Elmer VICTOR3 Wallac 1420 microplate reader. Determination of the reaction rate was begun by dividing standard blank absorbance by itself and all other absorbances of standards and samples to yield the linearized rate (LR). Then, the linearized SOD standard rate as a function of final SOD activity (U/ml) was plotted. SOD activity was calculated using the equation obtained from the linear regression of the standard curve substituting the linearized rate (LR) for each sample. One unit of SOD activity is defined as the amount of enzyme need to exhibit 50% dismutation of superoxide radical. SOD activity was

standardized using the cytochrome c and xanthine oxidase coupled assay. SOD activities were expressed as units of activity per milligram of protein. The protein concentration was determined according to Bradford (1976).

$$\text{SOD (U/ml)} = \frac{\text{sample LR-y-intercept}}{\text{Slope}} \times \frac{\text{total volume of reaction}}{\text{volume of sample}}$$

Measurement of glutathione peroxidase

Glutathione peroxidase (GPx) catalyses the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and protects cells from oxidative damages. In fact, glutathione metabolism is one of the most essential antioxidant defense mechanisms (Paglia and Valentine, 1967). GPx activity was indirectly measured by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, was recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ was accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity was rate limiting, the rate of decrease in the A_{340} was directly proportional to the GPx activity in the sample (Paglia and Valentine, 1967).

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPx}} \text{R-O-H} + \text{GSSG} + H_2O$$

$$\text{GSSG} + \text{NADPH} + H^+ \xrightarrow{\text{GR}} 2\text{GSH} + NADP^+$$

Hippocampal neurons were cultured at the density of 1×10^6 cells/cm^2 in 48-well culture plates. After 24 hr of culturing, they were treated with the combination of quercitrin or 17β-estradiol at concentrations of 50 and 100 μM and $A\beta_{25\text{-}35}$ at the concentration of 5 μM for 3days. GPx activity is measured according to the method described by Paglia and Valentine (1967) using cumene hydroperoxide.

Sample preparation for GPx

GPx was detected using the Cayman Chemical GPx assay kit. Sample preparation was performed as follows. Briefly, after treatment, treated cells were washed twice with ice-cold PBS and harvested in cold buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM Ethylenediamine tetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) using a cell scraper. Cell lysates from pooled two culture wells were transferred to a 1.5 ml microcentrifuge tube and centrifuged at 10,000 g for 15 min at

4 °C. The supernatant was transferred to the fresh tube for assay and kept on ice. If not assay on the same day, the samples were frozen at -80 °C and the assay was done within 1 month.

GPx assay procedure

The GPx assay was performed according to the manufacturer's protocol as the followings. For non-enzymatic wells as background, 120 µl of assay buffer and 50 µl of co-substrate mixture were added to 2 wells of a 96-well plate. For positive control or sample wells (bovine erythrocyte GPx), 100 µl of assay buffer, 50 µl of co-substrate mixture and 20 µl of diluted bovine erythrocyte GPx (control) or samples were added to a 96-well plate. The reactions were initiated by adding 20 µl of cumen hydroperoxide to all wells. Subsequently, the 96-well plate was carefully shaken for a few seconds to mix. The optical absorbances were read every minute at 355 nm using a Perkin-Elmer VICTOR3 Wallac 1420 microplate reader. The change in absorbance ΔA_{355}/min was monitored and the rate of ΔA_{355}/min for background or non-enzymatic wells was calculated and subtract this rate from that of the sample wells. The reaction rate could be determined using the NADPH extinction coefficient of 0.00373 μM^{-1}. One unit was defined as the amount of enzyme that catalyzed the oxidation of 1.0 nmol of NADPH to $NADP^+$/min at 25 °C. GPx activity was expressed relatively to the amount of protein (in milligrams) in cell extracts determined by Bradford protein assay (Bradford, 1976). GPx activity was calculated from the following formula.

$$\text{GPx Activity} = \frac{\Delta A_{355}/\text{min}}{0.00373\ \mu M^{-1}} \times \frac{\text{total volume of reaction (ml)}}{\text{volume of sample (ml)}}$$

Measurement of reactive oxygen species

The accumulation of intracellular ROS in hippocampal cells was determined by analyzing 2′,7′-dichlorofluorescein (DCF) fluorescence as described previously (Bastianetto et al., 2000b). The nonfluorescent probe 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was used to assess levels of net intracellular generation of ROS. DCFH-DA, a peroxide/redox-sensitive fluorescent probe, is nonpolar and diffusible into the cell. Intracellular esterases cleave the diacetate ester group and untrap the polar, nonfluorescent DCFH within the cell. During oxidative burst, the cell-permeable DCFH-DA is readily converted into DCFH, which is able to interact

with intracellular free radicals and peroxide to generate the highly fluorescent compound DCF. Measurement of intracellular ROS levels was performed as the followings.

Briefly, a stock solution of DCFH-DA at the concentration of 20 mM was prepared in DMSO and stored at -20 °C in the dark. Hippocampal neurons at the density of 1×10^6 cells/cm^2 in 48-well plates were treated with the combination of quercitrin or 17β-estradiol at concentrations of 50 and 100 μM and $A\beta_{25-35}$ at the concentration of 5 μM for 6, 12, 18 and 24 hr. After treatment, neuronal cells were washed twice with cooled PBS on ice and loaded with 50 μM of DCFH-DA at 37 °C in dark for 45 min. Neuronal cells that did not receive DCFH-DA were used as blank. Cells incubated with 100 μM of hydrogen peroxide were used as a positive control. After loading of the dye, cells were washed 3 times with cooled PBS on ice and subsequently scraped into PBS and collected in microcentrifuge tubes. The cell suspension was pelleted by centrifugation at 5,000 g for 3 min at 4 °C and then the cell pellet was resuspended in cooled PBS. The fluorescent intensity of the cell pellet was quantified with a Perkin-Elmer VICTOR3 Wallac 1420 microplate reader using excitation and emission wavelengths of 485 and 535 nm, respectively. The fluorescence of the cell population is proportional to the levels of intracellular ROS generated. Intracellular ROS levels were expressed in term of the percentage of control.

Measurement of caspase-3 activity

Caspase-3 is a member of the interleukin-1B converting enzyme (ICE) family of cysteine proteases. Caspase-3 has been shown to play an imperative role in the apoptotic cascade. The overexpression of caspase-3 can result in apoptosis. Likewise, the inhibition of caspase-3 or other caspases can prevent cells from entering the apoptotic pathway. The caspase-3 colorimetric protease assay provides a simple and convenient means for quantitating caspases that recognize the amino acid sequence, DEVD. The substrate, DEVD-*p*NA, is composed of the chromophore, *p*-nitroanilide (pNA) and a synthetic tetrapeptide, DEVD (Asp-Glu-Val-Asp), which is the upstream amino acid sequence of the caspase-3 cleavage site in poly(ADP ribose) polymerase. Upon cleavage of the substrate by caspase-3, light absorbance of free

pNA can be quantified using a spectrophotometer or a microplate reader at 400 or 405 nm.

The caspase-3 activity is measured by the ApoTarget™ caspase-3 protease assay (Biosource, USA). Briefly, cells were collected and resuspended in 50 µl of chilled cell lysis buffer and then incubated on ice for 10 min. After incubation, cells were centrifuged at 10,000 g for 1 min at 4 °C. The supernatant (cytosol extract) was transferred to a fresh tube and put on ice. Protein concentration was measured by Bradford protein assay (Bradford, 1976). Each cytosol extract was diluted to a concentration of 50-200 µg protein per µl cell lysis buffer (1-4 mg/ml). Fifty µl of cell lysates was transferred to a 96-well microtiter plate. Then, 50 µl of 2× reaction buffer containing 10 mM DTT and 5 µl of 4 mM DEVD-pNA substrate for caspase-3 (200 µM final concentration) were added to each sample. A 96-well microtiter plate was incubated at 37 °C in the dark for 2 hr (the samples are kept in the dark during incubation). The absorbance in each well was measured using a Perkin-Elmer VICTOR3 Wallac 1420 microplate reader at the wavelength of 405 nm. The enzyme activity is calculated as µmol of pNA released per min per milligram of protein. Fold-increase in caspase-3 activity was determined by direct comparison of enzyme activities in treated cells with that of uninduced control.

Bcl-2 and Bax protein

Hippocampal neurons were cultured at the density of 1.2×10^6 cells/cm^2 in 24-well culture plates. After 24 hr of culturing, they were treated with the combination of quercitrin or 17β-estradiol at the concentrations of 50 and 100 µM and $A\beta_{25\text{-}35}$ at the concentration of 5 µM for 3 days.

Sample preparation of Bcl-2 and Bax protein

After treatment, treated cells were washed twice with ice-cold PBS; lysed in lysis buffer containing 50 mM Tris-HCL (pH 8.0), 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 1% NP-40, 0.02% NaN_3 with protease inhibitor (1 mM phenylmethane-sulfonyl fluoride, 2 µg/ml leupeptin and 2 µg/ml aprotinin). Then, cells were harvested using a cell scraper. Each cell lysate from pooled two culture wells was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was collected to detect Bcl-2 and Bax protein.

Cytochrome c release

Preparation of cytosolic extracts

Cytochrome c release was detected using cytochrome c release apoptosis assay kit (Calbiochem, USA). Cytosolic extracts were prepared according to the manufacturer's protocol. After treatment, treated cells were washed twice with ice-cold PBS and cells were harvested in PBS using a cell scraper. Each cell lysate from pooled four culture wells was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 600 g for 5 min at 4 °C. The supernatant was removed and cells were resuspended with 120 µl of 1× cytosol extraction buffer mix containing DTT and protease inhibitor cocktail and then incubated on ice for 10 min. Cell pellets were homogenized using Vibra cell (Sonics) at amplitude of 20 twice (1 second each). Homogenized cells were centrifuged at 700 g for 10 min at 4 °C and the supernatant was collected as cytosolic fraction.

Protein content was determined by Bradford method using bovine serum albumin as a protein standard. Lysate samples containing 30 µg of protein were mixed with loading dye (225 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 9% 2-mercaptoethanol and 0.009% bromphenol blue) (2:1), boiled at 95 °C for 5 min and stored at -20 °C until use.

Western blot and immunoblot analysis

Thirty micrograms of protein samples were electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under constant voltage of 150 V for 45 min. After electrophoresis, protein bands in the gel were electrotransferred to polyvinylidene difluoride (PVDF) membrane using Mini Trans-Blot electrophoretic Transfer cell (Bio-Rad), a semi-dry electrophoretic transfer cell under constant voltage of 90 V for 60 min. The membranes were blocked with 5% skimmed milk in Tris buffered saline-tween 20 (TBST) for 60 min at room temperature. The membranes were probed with specific primary antibodies. Primary antibody was diluted (1:100 for anti Bcl-2 mouse mAb and anti-Bax mouse mAb, 1:200 for cytochrome c antibody and 1:500 for anti-Actin) in blocking solution and then added to the blot for 2 hr at room temperature. After washing 4 times for 7 min each with TBST, the membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody, goat anti-mouse immunoglobulin

(IgG) for one hour at room temperature. Subsequently, the membranes were washed as described above and incubated with an enhanced chemiluminescence detection reagent, Supersignal® chemiluminescent substrate (West Pico, Pierce) for 5 min. The immunoblots were exposed to X-ray films (Eastman Kodak). Actin bands were monitored on the same blot to verify the consistency of protein loading. Films were scanned and the band intensity of each treatment was compared to that of control.

Data Presentation and Data Analysis

All data were expressed as mean ± SEM of 6 to 8 samples within the same sister culture from a representative experiment. The difference between mean values of two sample groups was analyzed by the independent sample Student *t*-test. One-way analysis of variance (ANOVA) was used for testing the differences of the mean values among groups and if significant, pairwise comparison between groups was analyzed by Scheffe post hoc testing. *P*-values less than 0.05 were considered to be significant.