CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodology used in this study including chemicals, reagents, instruments, and experimental methods. The detail of each part is described below.

Chemicals and Reagents

1. Bovine serum albumin (BSA) (Lot No. 025K1031, Sigma-Aldrich, Inc., Missouri, USA)

2. BCA™ protein assay kit (Catalog No. JJ126727A, Pierce@, Rockford, USA)

3. Catalase assay kit (Lot No 110M4055, Sigma-Aldrich, Co., St Loius, MO, USA)

4. Commercial lutein (Lot No. TE090531, Kailu Brilliance Ever Biotechnology Co., Ltd, China)

5. Dimethylsulfoxide (DMSO) (A.R. grade, Lot No. 0320064, Sigma-Aldrich, Co., St Loius, MO, USA)

6. Dichlorofluorescin diacetate (DCFH-DA) (Lot No.128K4145 Sigma-Aldrich, Co., St Loius, MO, USA)

7. Dulbecco's modified Eagle's medium – low glucose (Batch No. 087k8305, Sigma-Aldrich, Co., St Loius, MO, USA)

8. EnzChek Caspase-3 Assay Kit (Lot No. 780223, Molecular Probes, Eugne, Oregon, USA)

9. Ethanol (Absolute) (A.R. grade, Lot. No. AR109G2.5L, RCI Labscan, Bangkok, Thailand)

10. Fetal bovine serum (A.R. grade, Lot No. 41Q5475K, Invitrogen Company, California, USA)

11. Glutathione peroxidase activity kit (Lot No.06141109, Enzo Life Sciences, Avenue Albert Einstein, Villeurbanne, France)

12. Hydrochloric acid (Lot No. 7647010 RIC Labscan Ltd, Bangkok, Thailand)

13. M PER, mammalian protein extraction reagent (Lot No. MA 153113 Rockford, IL, USA)

14. Sodium bicarbonate (Lot No. 41600 Riedel-de Haën®, Poland)

15. SOD assay kit (Lot No.BCBC2239, Sigma-Aldrich, Co., St Loius, USA)

16. Standard lutein (Std.lutein) (Lot No. BCBV6995V, Fluka, St Loius, USA)

17. Streptomycin/Penicillin (Lot No. 584844 Gibco, Auckland)

18. Thiobarbituric acid (TBA) (Lot No. 035K0622 Sigma-Aldrich, Germany)

19. Trichloroacetic acid (TCA) (Lot No 078K0704 Sigma-Aldrich, Germany)

20. Trolox (Lot No. D0010024, Calbiochem, Denmark)

21. Trypan blue solution (R&D grade, Lot No. 55K2342, Sigma-Aldrich, Co., St Loius, MO, USA)

22. Trypsin EDTA (A.R. grade, Lot No. 1212385, Invitrogen Company, Ontario, Canada)

Cell lines

Human retinal pigment epithelial cell line (ARPE-19) from American type culture collection (ATCC), Manassas, Virginia, USA

Instruments

1. Incubator CO_2 (Forma series II, Thermo Fisher Scientific Inc., MA, USA)
2. Autoclave (HA-300P, Hirayama Manufacturing Corporation, Saitama, Japan)
3. Laminar air flow cabinet (Heto-holten Dk 3450, Allerod, Denmark)
4. Laminar flow hood (Heal force®, HF safe 1200/c+, Shanghai, China)
5. pH meter (SevenEasy pH ^{TM} S20, Mettler-Toledo Gmbh, Schwerzenbach, Swizerland)
6. Inverted microscope (model TS100, Nikon Eclipse, Tokyo, Japan)
7. Microplate Spectrophotometer (Multimode detector DTX 880, Beckman Coulter Inc., Fullerton, USA.)
8. Ultracentrifuge (Beckman Model J2-MC, Beckman Instruments, Inc.,USA)
9. UV-chamber (Dr. Grobel, UV-Elektronik GmbH, Germany)

Methodology

1. Silk lutein extract preparation

Silk lutein extract was prepared by Suranaree University of technology. Briefly, hexane/ethanol/ethyl acetate mixture was used as a solvent for extraction. The extract was then partitioned, filtrated, and evaporated. The total lutein content in the extract was determined by HPLC. The total lutein content in the silk cocoon extract was 5.14%. The rest of content was found to be 10% fatty acids and 84.86% wax. The concentrations of silk lutein extract used in all experiments were calculated based on its lutein content. Therefore, the concentrations appeared in all results represent the concentration of lutein contains in the extract.

2. Cell culture

ARPE-19 cells were cultured in DMEM/F12 growth medium with 3 mM glutamine and supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 U/ml and streptomycin 0.1 mg/ml). Cells were plated at density 1×10^7 cells/cm^2 in 175 cm^3 flask and then kept in a CO_2 incubator with 5% CO_2. Culture medium was changed every 2–3 days. All experiments were carried out within 20 passages, between passage 10 to passage 30 to ensure uniformity of cell population and reproducibility.

3. Lutein and trolox treatment

Cells were plated in 24-well plates at a density of 1×10^5 cells/cm^2 in 0.5 ml DMEM/F12 containing 10% FBS without phenol red. Twenty–four hours after plating, treatment with lutein and trolox was initiated. Lutein and trolox were prepared in 5% v/v DMSO, and 5% v/v ethanol, respectively. Final concentration of these solvents in culture medium was 0.5 % v/v which showed no effect on cell viability. ARPE-19 cells were treated with various concentrations of lutein or trolox prior to UV-B exposure.

4. UV-B irradiation

After lutein or trolox treatment, 450 µl of culture medium were removed and left 50 µl that enough to cover cells. UV-B irradiation was carried out in UV-chamber (Dr. Grobel, UV-Elektronik GmbH, Germany) using a 5 W UV-B lamp which emits most of its energy within the UV-B range (290–320 nm). Cells were irradiated in non phenol red and serum free medium with different doses of UV-B (20-

80 mJ/cm^2). After UV-B irradiation, 500 µl of the fresh medium were added and cells were further incubated for 24 h in 5 % CO_2 incubator prior to conducting specific measurement.

5. Cell viability

5.1 MTT assay

The viability of ARPE-19 cells was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. This assay is based on the ability of a mitochondrial dehydrogenase enzyme in viable cells to cleave the tetrazolium rings of the pale yellow of MTT and form dark blue formazan crystals. This formazan is impermeable to cell membranes, thus resulting in its accumulation within living cells, according to the method previous described [135] with slightly modifications. In this experiment, ARPE-19 cells were cultured into 96-wells plate at density $2.5x10^4$ cells/well. After UV-B exposure, MTT solution was added into culture medium at a final concentration of 0.5 mg/ml for 2 h. Thereafter, the purple formazan crystals was dissolved in 200 µl DMSO:ethanol (1:1) and absorbance was measured at 595 nm using a Microplate reader.

5.2 Tryphan blue assay

The trypan blue assay was also used to determine ARPE-19 cell viability. This assay is based on the principle that live cells possess intact cell membranes that exclude certain dyes, whereas dead cells can not.

After the particular treatment, cells were trysinized with 0.2 % trysin/EDTA (100 µl) for 3 minutes and suspended in serum free medium. ARPE-19 cells were then mixed with 0.4% trypan blue solution (1:1). By thise method, death cells were stained whereas viable cells were unstained [136]. The cell suspensions (10 µl) were carefully filled on the hemocytometer chamber, incubated for 30 sec at room temperate, and finally counted under the microscope.

6. Intracellular reactive oxygen spices (ROS) measurements

The intracellular ROS in ARPE-19 cells was measured by a fluorescent probe, 2, 7-dichlorofluorescin diacetate (DCFH DA). DCFH-DA dyes can be diffused through the cell membrane and hydrolyzed by intracellular esterases to non fluorescent dichlorofluorescin (DCFH). In the presence of ROS, DCFH is rapidly oxidized to highly fluorescent dichlorofluorescein [137]. This assay was modified according to

previous report [138]. It is a reliable method for the measurement of intracellular ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), and hydroperoxide (ROOH).

After lutein or trolox treatment, cell cultures were replaced with medium containing 50 μM DCFH-DA. After 30 minute incubation with DCFH-DA, medium were removed and washed with PBS to remove the excess amount of dye. After that cell were covered with 50 μl of culture medium for UV-B irradiation. After UV-B exposure, 500 μl medium was added and cells were further incubated in CO_2 incubator. The fluorescence intensity was monitored at 0.5, 1, and 24 h by spectrofluorometer at excitation wavelength 485 nm and emission wavelength 530 nm.

7. Lipid peroxidation determination

Lipid peroxidation was investigated by thiobarbituric acid reactive substances (TBARS) assay using modification method from previous study [139]. This assay measures lipid hydroperoxides and aldehydes, such as malondialdehyde (MDA) in cells and/or culture media. MDA can bind to thiobarbituric acid (TBA) in a 1:2 ratio to form a fluorescent adduct.

After treatment, 500 μl of TBARs reagent containing 0.4% TBA, 1.4% TCA, and 8 % HCl (1:2:1) was directly added into ARPE-19 cells without removing culture medium. The mixture was then heated at 90 °C for 60 min, cooled, and read by fluorometry at 535 nm excitation, and 595 nm emission. Concentrations of TBA products were determined from a standard curve constructed using malondialdehyde (MDA). Lipid peroxidation values were expressed as nanomole of MDA per 10000 cells.

8. Glutathione peroxidase (GPx) activity determination

The glutathione peroxidase activity in ARPE-19 cells was determined by a commercial enzymatic assay kit. The reagent mixture contains, t-butyl hydroperoxide (tBOOH) used as the peroxide substrate, glutathione reductase (GSSG-R), and NADPH (reduced-nicotinamide adenine dinucleotide phosphate). In the presence of GSSG-R and NADPH, oxidized glutathione (GSSG) is immediately converted to the reduced glutathione (GSH) by GPx with a concomitant oxidation of NADPH to $NADP^+$. The decreased rate of NADPH in the absorbance at 340 nm is directly proportional to the GPx activity in the sample [140].

For GPx activity determination, ARPE-19 cells were lysed with mammalian protein extraction reagent. After centrifugation, supernatant was collected. Cell lysated (20 µl) was mixed with 160 µl the reaction mixture contains the GR, reconstituted GSH, and NADPH. To initiate the reaction, 20 µl of curmene hydrogenperoxide was quickly added. The decrease in NADPH absorbance at 340 nm was immediately measured for 5 min.

9. Superoxide dismutase (SOD) activity determination

The total superoxide dismutase activity in ARPE-19 cells was determined using a commercial assay kit according to the manufacturer's instruction. This activity was assessed by measuring the dismutation of superoxide radicals generated by the conversion of xanthine to uric acid and hydrogen peroxide by xanthine oxidase (XOD), and then superoxide radical converts WST to WST-diformazan. SOD activity was determined employing xanthine and xanthine oxidase (XO) to generate O_2. which reacts with (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-di sulfophenyl)-2H-tetrazolium) (WST) to form a yellow dye. The SOD activity in the test sample was measured by the degree of inhibition of this reaction [141].

After treatment, cells were lyzed in 50 µl of mammalian protein extraction reagent and the aqueous fraction was separated by centrifugation. Then, in a 96 wells microplate, 20 µl of the sample was mixed with 180 µl reaction mixture containing xantine substrate, WST and XO. The absorbance was recorded at 340 nm for 30 min in a microplate reader. The activity was expressed as the difference in absorbance of reaction per mg of protein. Protein concentration was determined using the BCA protein assay kit (Pierce®) according to the manufacturer's instructions.

10. Catalase activity determination

The catalase (CAT) activity in ARPE-19 cells was measured by assay kit (Sigma-Aldrich). This assay is based on the measurement of the hydrogen peroxide substrate remaining after the action of catalase. Catalase converted hydrogen peroxide to water and oxygen. The amount of hydrogen peroxide remaining after reaction was determined by a colorimetric method. A substituted phenol (3,5-dichloro-2 hydroxy benzenesulfonic acid), couples oxidatively to 4-aminoantipyrine in the presence of hydrogen peroxide and horseradish peroxidase (HRP) to give a red quinoneimine dye (N-(4-antipyryl)-3-chloro-5-sulfonatep- benzoquinone-monoimine)[142].

After treatment, cells were lysed with mammalian protein extraction reagent and cytosolic fraction was collected by centrifugation. Cell lysated 10 µl was mixed with 90 µl of substrate solution containing 200 mM H_2O_2 and incubated for 5 min. After that the reaction was stoped by adding 100 µl of the stop solution and 20 µl of the reaction mixture was transfered to another microplate. Finally, the reaction mixture was mixed with 80 µl of color reagent which containing the chromogen solvent and peroxidase enzyme an incubated for 30 min. The absorbance was measured at 520 nm.

11. Caspase 3 activity determination

The caspase 3 activity of ARPE-19 cells was measured by commercial assay kit. Casepase 3 substrate is a bisamide derivative of rhodamine 110 (R110) containing DEVD peptides covalently linked to each of R110's amino groups, thereby suppressing the dye's visible absorption and its fluorescence. Upon caspase 3 cleavages, the nonfluorescent bisamide substrate is converted in a two-step process [143]. The fluorescent monoamide and then to the even more fluorescent R110 were detected at excitation 496 nm and emission 520 nm.

After treatment, cells were lyzed in 50 µl of mammalian protein extraction reagent and the aqueous fraction was be separated by centrifugation at 12,000×*g* for 15min at 4 °C. In a 96 black well microplate, 25 µl of the sample was mixed with 25 µl the caspase-3 substrate, (Z-DEVD–R110) that was prepared as a stock solution of 10 mM in DMSO and stored at −20°C. Prior to the assay, the caspase-3 substrate was mixed in an assay buffer reagent containing 20 mM HEPES, pH 7.4, with 2 mM EDTA, 0.5% CHAPS, and 5 mM DTT, yielding a final concentration of 250 µM Z-DEVD-R110 in the solution stock. Cell lysate (25 µl) and of substrate (25 µl) were mixed to a 96 black well plate and fluorescence of the cleavage product was measured at 37°C for 30 min in a microplate spectrofluorometer, at Ex 485 nm and Em 535 nm.

12. HPLC analysis

To determine the concentration of lutein(s), HPLC was used for this purpose. HPLC system (Shimadzu, Kyoto, Japan) is consisted of a LC20-AT pump connected to a SIL-10ADVP auto-injector, a SPD-6AV system controller, SPD-20A UV-visible detector. Lutein was separated using a Vertisep bio C30 column (5 µm,

4.6x100 mm) and a guard column. Sample was dissolved in mobile phase containing 2-propanol-dichloromethane-methanol at a ratio of 20:10:70 (v/v) [144]. The injection volume is 50 μl. The flow rate was 1.0 ml/min and the column was kept at a constant temperature (25°C). Retention times of lutein was 3.5 min. Lutein was quantified from its peak area by comparison with a standard reference curve established with different amounts of the respective standard lutein (from 0.01 to 5.0 μg/ml) in 2-propanol-dichloromethane-methanol at a ratio of 20:10:70 (v/v) at 446 nm.

Data statistic analysis

Results are presented as means±SEM from at least three independent experiments. Group comparisons were made by one-way ANOVA. Significant differences were achieved when p value equal or less than 0.05.