CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodology of this research including chemical, reagents and instruments. The detail of each topic is described below.

Materials

Chemicals and reagents

1. Curcuminoids powder (The government pharmaceutical organization (GPO), Bangkok, Thailand)
2. Ethanol (RCI Labscan Ltd, Bangkok, Thailand)
3. Sodium bicarbonate (Riedel-de Haën®, Poland)
4. Dimethylsulphoxide (RIC Labscan Ltd, Bangkok, Thailand)
5. Dimethylsulphoxide (Sigma-Aldrich, Germany)
6. Xylene (RIC Labscan Ltd, Bangkok, Thailand)
7. Paraplast Plus (McCormick Scientific, St. Louis, Missouri, USA)
8. Hematoxylin (Merk, Darmstadt, Germany)
9. Eosin (Merk, Darmstadt, Germany)
10. 2-Thiobarbituric acid (TBA) (Sigma-Aldrich, Germany)
11. Trichloroacetic acid (TCA) (Sigma-Aldrich, Germany)
12. 37% Hydrochloric acid (RIC Labscan Ltd , Bangkok, Thailand)
13. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Amresco®,Solon, USA)
14. 4, 5-Diaminofluorescein diacetate (DAF-2DA)(Calbiochem® Darmstadt, Germany)
15. Aspartate aminotransferase assay kit (AST kit) (Human GmbH, Wiesbade, Germany)
16. Alanine aminotrasferase assay kit (ALT kit) (Human GmbH, Wiesbaden, Germany)

17. Lactate dehydrogenase assay kit (LDH kit) (Human GmbH, Wiesbaden,Germany)
18. Alkaline phosphatase assay kit (ALP kit)(Human GmbH, Wiesbaden, Germany)
19. Trypsin-EDTA 0.25% (Invitrogen ^{TM}, GibcoR, Ontario, Canada)
20. Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham, DMEM/F12 (Sigma-AldrichR, St. Louis, Missouri, USA)
21. Fetal bovine serum (Invitrogen^{TM}, Sao Paulo, Brazil)
22. Trypan blue solution (Sigma- Aldrich Laboratory, Missouri, USA)
23. Glycin (Research organics, INC, USA)
24. Carboxymethylcellulose (Sigma-AldrichR, St., Louis,Missouri, USA)
25. D-glucose (Riedel-de Haën®, Germany)
26. BCA™ Protein assay kit (Thermo scientific, Rockford, Illinois, USA)
27. Micro BCA™ Protein assay kit (Thermo scientific, Rockford, Illinois, USA)
28. Triton X-100 (Fisher Scientific, Leicestershire LE11 5RG, UK)
29. Permount (Lot No. 1813446, Fisher Scientific, New Jersey, USA)

Cell lines

Human hepatocellular carcinoma cell line (HepG2, American type culture collection (ATCC), Manassas, Virginia, USA)

Animals

Male Sprague Dawley rats (180-220 g) were obtained from national laboratory animal center, Mahidol University, Nakornpathom, Thailand.

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 ™ S20, Mettler-Toledo Gmbh, Schwerzenbach, Swizerland)
6. Microplate reader (Beckman Coulter, Multimode Detector Model DTX 880, Vienna , Austria)
7. Inverted microscope (model TS100, Nikon Eclipse, Tokyo, Japan)
8. Microplate Spectrophotometer (Multimode detector DTX 880, BeckmanCoulter Inc., Fullerton, USA.)
9. Leica TP1020 tissue processor (Lieca TP1020, Leica Microsystems, Nussloch, Germany)
10. Leica EG1160 paraffin embedding station (Leica EG1160)
11. Leica RM2235 Manual rotary microtome (Leica RM2235)
12. Ultra Centrifuge (Beckman Model J2-MC, Beckman Instruments, Inc., California, USA)

Animal models

The rats were housed in the animal room with 12 hours light-dark cycle, and controlled temperature (25 ± 2 °C). All rats were rested 7 days before experiments. The rats were fed with regular diet and water *ad libitum*. Animal handling and experiments reported herein were approved by the Ethical Committee for the Use of Animal, Naresuan University. Rats were divided into 7 groups of six rats in each group. The control groups, normal rats without ethanol. Glucose group, rats were received glucose as the isocaloric equivalence. The alcohol-induced toxicity rats were induced by daily gastric intubations for 60 days. The first and second week, rats were received 4 and 5 g/kg /day ethanol respectively and then after that 6 g/kg /day ethanol was fed into rats throughout 60 days. The ethanol-induced toxicity, rats were divided into 7 groups for treatment of various concentrations of curcuminoids, silymarin and carboxymethyl cellulose (CMC). Duration of curcuminoids treatment into the rats were 45 days.

The rats were randomly divided in to 7 groups as followed:

1. Control group : normal rats

2. CMC group: alcohol-induced toxicity rats with CMC as the vehicle control.

3. Glucose: rats received glucose as the isocaloric equivalence.

4. Silymarin: alcohol-induced toxicity rats with 100 mg/kg/day silymarin.

5. 250 mg/kg/day curcuminoids : alcohol-induced toxicity rats fed with curcuminoids at dose 250 mg/kg/day

6. 500 mg/kg/day curcuminoids : alcohol-induced toxicity rats fed with curcuminoids at dose 500 mg/kg/day

7. 750 mg/kg/day curcuminoids : alcohol-induced toxicity rats fed with curcuminoids at dose 750 mg/kg/day

Toxicity test in animals

Acute oral toxicity

Acute oral toxicity was studied with oral administration of curcuminoids, using the OECD 2006 guidelines. As per the limit test, male Sprague Dawley rats were fasted overnight and given 5000 mg/kg of curcuminoid*s* orally. The next day, animals were observed for 48 hours for any signs of toxicity or mortality. In induced toxicity test, the alcohol-induced toxicity rats were induced by daily gastric intubations for 60 days and following given 5000 mg/kg of curcuminoids extract orally. The next day, animals were observed for 48 hours for any signs of toxicity or mortality.

Blood collection and serum preparation

Blood was collected from tail vein of rats by collection needle No. 25 and collected in centrifuge tube without an anticoagulant. Serum was separated by centrifugation at 3,000 rpm for 10 minutes, stored at 4°C and used within 3 days.

Biochemical analysis

During experiment, rat serums were measured for liver function enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) by enzymatic assay kit (Human™). Briefly, the sample were filled into a 96 well plate then were added working reagents. The kinetic method was used for the determination of AST, ALT, ALP and LDH activities by spectrophotometer. The AST, ALT, ALP and LDH activities were calculated using the formulations below to calculate enzyme activities.

1. Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) catalyzes the oxidation of lactate to pyruvate with stimultaneous reduction of NAD to NADH. The rate of NAD reduction can be measured as an increase in absorbance at 340 nm. This rate is directly proportion to LDH activity in serum. The equation of LDH activity was derived from:

$$\text{Pyruvate} + \text{NADH} + \text{H}^{+} \xrightleftharpoons{\text{LDH}} \text{Lactate} + \text{NAD}$$

$$\text{LDH activity (U/l)} = \text{Slop X } 8095^{a} \text{ X } 1.9^{b}$$

2. Aspartate aminotrasferase activity

Aspartate aminotransferase (AST) formerly called serum glutamate oxaloacetate transminase (SGOT) catalyses the reversible transfer of an amino group from aspartate to α-ketoglutarate forming glutamate, and oxaloacetate. The oxaloacetate is reduced to malate by malate dehydrogenase (MDH) and NADH. The rate of NADH decrease in concentration, represent AST present in the serum.

$$\alpha\text{-Ketoglutalate} + \text{L-aspartate} \xrightleftharpoons{\text{AST}} \text{L-glutamate} + \text{oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^{+} \xrightleftharpoons{\text{MDH}} \text{L-malate} + \text{NAD}$$

$$\text{AST activity (U/l)} = \text{Slop X } 952^{a} \text{ X } 3.8^{b}$$

3. Alanine aminotransferase activity

Alanine aminotransferase (ALT) formerly also called serum glutamate pyruvate transminase (SGPT). The amino group is enzymatically transferred by ALT present in the sample from alanine to the carbon atom of 2-oxoglutarate yielding pyruvate and L-glutamate. Pyruvate is reduced to lactate by LDH present in the reagent with the simultaneous oxidation of NADH to NAD. The reaction is monitored by measuring the rate of NADH decrease in absorbance at 340nm due to the oxidation of NADH. Notably endogenous sample pyruvate is rapidly and completely reduced by LDH during the initial incubation period so that it does not interfere with the assay.

$$\text{2-Oxoglutarate + L-alanine} \xrightarrow[\text{ALT}]{} \text{L-glutamate + pyruvate}$$

$$\text{Pyruvate + NADH + H}^{+} \xrightarrow[\text{LDH}]{} \text{L-lactate + NAD}^{+}$$

$$\text{ALT activity (U/l)} = \text{Slop X } 952^{a} \text{ X } 3.8^{b}$$

4. Alkaline phosphatase activity

Alkaline phosphatase (ALP) in serum is measured by alkaline phosphatase assay kit. The method utilizes ρ-nitrophenyl phosphate that is hydrolyzed by ALP into a yellow colored product absorbed at 405 nm. The rate of reaction is directly proportional to ALP enzyme activity.

$$\text{p-Nitrophenyl + AMP} \xrightarrow[\text{ALP}]{} \text{AMP-PO}_4 \text{ + p-nitrophenol}$$

$$\text{ALP activity (U/l)} = \text{Slop X } 2757^{a} \text{ X } 2.5^{b}$$

a: constant number from the company data sheet

b: the measured value differences constant number between spectrometer and microplate reader.

Microsomal preparation

Rats were injected with 50 mg/kg pentobarbital and sacrificed by heart puncture, after that a rat liver was collected for microsomal preparation. One gram of liver was cut into pieces and homogenized with 3 ml of phosphate buffer, pH 7.4. The liver homogenates were centrifuged at 10,000 g for 30 minutes at 4 °C. Then, the supernatants were transferred into ultracentrifuge tubes and centrifuged at 100,000 g for 60 minutes at 4 °C. Finally, the pellet microsomal subfraction was resuspended with phosphate buffer, pH 7.4, containing 20% v/v glycerol. The microsomal suspension was aliquoted, kept in microcentrifuge tube and stored at -80 °C.

Protein assay

The bicinchoninic acid (BCA) protein assay kit was performed to determine the protein content of each microsome preparation sample. The microsome extractions were diluted 100 folds by using 0.1 M phosphate buffer saline, pH 7.4. The microsome samples or standard proteins were pipetted into a 96 well plate and then added working reagent. The 96 wells plate was incubated at 60 °C for 30 minutes. The absorbance was measured on microplate reader at 595 nm and the protein concentration of each microsome sample was determined by using an albumin standard curve and expressed as μg/ml.

Lipid peroxidation measurement

Lipid peroxidation was estimated by the thiobarbituric acid reactive substances (TBARs) assay. The TBARs assay quantifies oxidative stress by measuring the peroxidative lipid damage that occurs with free radical generation. Free radical lipid damage results in the production of malondialdehyde (MDA), which reacts with TBA under conditions of high temperature and acidity, generating a chromogen that can be measured either spectrophotometrically or spectrofluorometrically.

In microsome measurement, the reaction mixture contains 500 μl of 2.5 mg/ml of rat microsome, 30% TBA, 10% TCA and 8% HCl as the ratio 1:2:1. The mixture was incubated at 90 °C for 1 hour and then it was cooled at room temperature. It was centrifuged at 3,000 g for 10 minutes and the fluorescences were read at excitation 485 nm, emission 535 nm. MDA levels were calculated by using standard curve and express as micromolar (μmol) of MDA.

Cell culture, cells plated into 24-well plates. The TBARs reagent was added to each wells and incubated at 90 °C for 1 hour. Fluorescences were read at excitation 485 nm, emission 535 nm. MDA levels were calculated as above mentioned.

Superoxide dismutase measurement

Superoxide dismutase (SOD) is one of the most important antioxidant enzymes which catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen. The principle base on using a competitive-inhibition assay that will be used xanthine/xanthine oxidase-generated superoxide radicals. The water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl) - 3-(4-nitrophenyl)-5-(2, 4-disulfophenyl) - 2H-tetrazolium, monosodium salt), produces formazan dye upon reduction with a superoxide anion.

Rat liver microsomes were prepared and assayed for SOD activity by using a commercially SOD determination kit. Briefly, 1 mg of microsomes were added to a 96 well plate and then added WST working solution to each well and mixed. Afterwards, an enzyme working solution was also added to each sample and then mixed thoroughly. After incubation for 20 minutes at 37 °C, absorbance was read at 450 nm by using microplate reader. The SOD activity (% inhibition rate) was calculated using the following equation.

$$\text{SOD activity (\% inhibition rate)} = \frac{[(A_{blank1} - A_{blank3}) - A_{sample} - A_{blank2})]}{(A_{blank1} - A_{blank3})} \times 100$$

Absorbance blank 1 is a reaction mixture that without a sample. Blank 2 is a reaction mixture that contains sample without enzyme working solution. Blank 3 is a reaction mixture without sample and enzyme working solution.

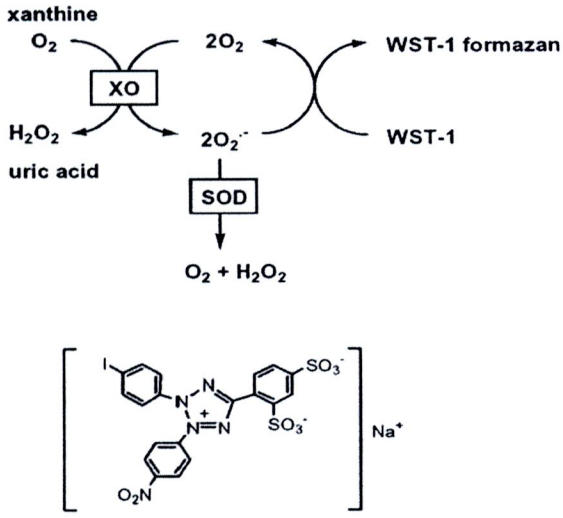

Figure 11 SOD assay reaction utilizing a water-soluble tetrazolium salt, WST, a highly water-soluble formazan dye upon reduction with a superoxide anion.

Histological examination

Pathological changes of ethanol induced toxicity rat liver tissues were evaluated by using Hematoxllin and Eoxin (H&E).

1. Fixation

The process of fixation after animal death is necessary, become of autolysis due to rupture of cells that lead to bacterial and fungal growth and finally result in complete destruction of tissue structures. Therefore, rapid and adequate liver fixation in 10% neutral buffered formalin before tissue processing is essential. Rat livers were cut into pieces. Samples were taken from all rats and fixed with 10% neutral buffered formalin.

2. Dehydration of tissue

Dehydration is important process. The tissue is in increased concentration of alcohol solution, then gradual replacement of alcohol by a paraffin solvent. This step is generally automated using tissue processors. Samples are transferred through automatic tissue processor that processes dehydration by computer program. We entered tissue samples in a solvent jar of equipment and set timing program for each

solvent jar. The tissue processor will be exchanged solvent jar automatically until complete dehydration process.

Table 5 The list of solutions and the duration used for dehydration by tissue processor.

Solvent	Time (minutes)
70% alcohol	30
80% alcohol	30
85% alcohol	30
90% alcohol	30
95% alcohol	30
95% alcohol	30
100% alcohol	60
100% alcohol	60
Xylene	120
Xylene	120
Paraplast	120
Paraplast	480

3. Embedding and section

The liver tissues were placed and then melted paraffin was poured into the mold. The base of original cassette was immediately on top of mold. The paraffin blocks were stored at 4 °C. The blocks were cut 3 μm-thicks by microtome and floated on a water bath maintained at 45 °C to streten the paraffin section. A microscope glass slide is placed under the sected tissue section and then dried at 70 °C for 30 minutes in oven.

4. Staining

Hematoxylin and Eosin (H&E) is the staining used to study histopathology changes in tissue and organs. Tissue sections were rehydrated to remove paraffin by using xylene, alcohol solution and water. The following protocol describes manual H&E staining: First of all, slides were deparaffinized in xylene, then, its were taken rehydrations in absolute ethanol and 95 % ethanol and then rinsed in running tap water. The slides were stained in Hematoxylin for 6 minutes and washed in running tap for 1 minute. The slides were immersed in lithium carbonate and rinsed in tap water. Next, it counterstained in Eosin and washed in running tap water. After eosin staining, slides were dehydrated with 95% ethanol and 100 % ethanol, respectively. Slides were cleared in xylene and cover glass was closed with slides. The H&E stained slides were further study under microscope for fatty liver and hepatitis markers.

Table 6 The following protocol describes detail manual H&E staining.

Solvent	Drips (times)
Xylene	20
Xylene	20
Absolute ethanol	20
Absolute ethanol	20
95% ethanol	20
95% ethanol	20
Water tap	1 (1 minute)
Haematoxylin	1 (6 minutes)
Water tap	1 (1 minute)
Lithium carbonates	20
Water tap	1 (1 minutes)
95% ethanol	20
Eosin	1 (20 seconds)
95% ethanol	20
95% ethanol	20
Absolute ethanol	20
Absolute ethanol	20
Absolute ethanol	20
Xylene	20
Xylene	20

Cell culture

Human hepatocellular carcinoma cell line, HepG2 cells were obtained from American Type Culture Collection (ATCC). HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and incubated at 37°C in humidified atmosphere with 5%

CO_2 until confluence. Cells were passaged, briefly they have been rinsed with 1xphosphat buffer saline (PBS) and added pre-warmed (37°C) 0.25% trypsin-EDTA solution for 3-5 minutes. Then, complete growth medium with 10% FBS, 1% penicillin-streptomycin were added into a flask and cells layer were dispersed 3-5 minutes. The split ratio is 1:4 every 3 days.

Cytoxicity test

Cytotoxicity was quantitated spectrophotometrically both by the metabolism of the (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide reduction (MTT) and by lactate dehydrogenase (LDH) released into the medium.

1. MTT assay

The direct effect of ethanol and curcuminoids were evaluated by conventional MTT reaction assay. Cell viability was determined by the reduction of MTT resulting from a mitochondrial succinate dehydrogenase. HepG2 cells were plated into a 96 well plate at density $3x10^4$ cells/well for 24 hours for complete adherence of the cells on a culture plate. The medium was removed and replaced medium without 10% FBS and penicillin/streptomycin. Next, cells were treated with various concentrations of ethanol 2.5, 5, 5.5, 6, 6.5, 7 and 7.5% ethanol (v/v) or curcuminoids 0.156, 0.313, 0.625, 1.25 and 5 µg/ml. After 4, 8, 24, 48, 72 hours incubation, the medium containing ethanol and curcuminoids was changed to 0.25 mg/ml MTT in PBS at final volume 100 µl/well. Cells were further incubated with MTT for 2 hours and then the MTT buffer was discarded. An extraction buffer (10% of 0.1 mol/L glycine in DMSO, pH 10) was added to each well and the plate was shaken for 20 minutes. Absorbance was read at 595 nm by a microplate reader.

In ethanol-induced toxicity and effect of curcuminoids concentration on % cell viability in HepG2 cells by MTT assay study, we used 5.5 % (v/v) ethanol to induce toxicity in HepG2 cells. Briefly, HepG2 cells were maintained as previous mention. The cells were pre-treated with various concentrations of curcuminoids (0.156, 0.313, 0.625, 1.25, 2.5, and 5 µg/ml) in serum free medium for 2 hours, after that cells were added 5.5% (v/v) ethanol final concentration for 22 hours. The HepG2 cells then were measured cell viability by MTT assay.

Calculations

$$\% \text{ Cell viability} = \frac{\text{sample absorbance} - \text{cell free sample blank}}{\text{Mean media control absorbance}} \times 100$$

2. Lactate dehydydrogenase assay

Lactate dehydrogenase (LDH) enzyme as a marker of remaining live cells, LDH assays can be performed by assessing LDH released into the medium as a marker of dead cells. The assay is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD. The formations of NADH from the above reaction result in change in absorbance 340 nm.

All cells were seeded into a 96 well plate at density $3x10^4$ cells/ well for 24 hours. The medium was remove and cells were treated with various concentration of ethanol or curcuminoids in non- phenol red serum free medium for 4, 8, 24, 48, 72 hour. After that, the medium containing ethanol and curcuminoids were removed from each well and transfer into another plate to measuring LDH enzymes. Briefly, the reagent preparations are mixed together with 0.3 mM and 3 mM pyruvate then, 50 µl medium samples were transferred to new plate and then 100 µl of LDH reagent were added to each well to start the reaction. The kinetic changes of the LDH enzyme absorbance were read at 340 nm every 10 minutes for 1 hour.

$$\text{LDH activity (ratio of product formation/minute)} = \frac{\text{Slope of sample}}{\text{Slope of control}}$$

Nitric oxide assay

Diaminofluorescein –2 diacetate (DAF-2DA) is used to measure real time production of NO. DAF-2DA is a non-fluorescent cell permeable in living cells under physiological conditions. Once inside the cell the diacetate groups on the DAF-2DA reagent are hydrolyzed by cytosolic esterases thus releasing DAF-2 and sequestering the reagent inside the cell. Production of nitric oxide converts the non-fluorescent dye, DAF-2, to its fluorescent triazole derivative, DAF-2T. This product can be observedby fluorescence microplate reader. Briefly, all cells were plated into a 96-black well plate at density $3x10^4$ cells/well for 24 hours. Then, the medium was removed and cells were pre-treated with various concentrations of curcuminoids in serum free medium for 2 hours. After that cells were added with 7.5% (v/v) of ethanol for 22 hours. The DAF-2 DA was added to the wells and incubated for 30 minutes in dark. Fluorescence was read at excitation 485 nm and emission 535 nm. The cells were lysed and measured protein

Data analysis

All data will be presented as means ± standard deviation (SD). The mean values of the various treatment groups were compared using one-way analysis of variance (one-way ANOVA) and student *t*-test will be employed. Differences will be considered statistically significant at $P<0.05$