CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and instruments are shown in APPENDICES A and B

2.2 Preparation of glutinous purple rice extracts

Glutinous purple (*Oryza sativa* L. var *indica*) rice, known as Kum Doisaket was cultivated in Maehia Agriculture Research Demonstrative and Training Center of Chiang Mai University, Aumphur Muang, Chiang Mai, Thailand, during August-December, 2010. The rice hull was separated from its grain by a rice-hulling machine. Glutinous purple rice hulls were soaked in dichloromethane or 0.1% HCl in absolute methanol while rice seed was immersed in absolute methanol for 48 hours at room temperature. The extracts were filtered through Whatman No. 1 filter paper and then evaporated to dryness under reduced pressure on a rotary evaporator at 40 °C. The dried extracts were stored at -20 ºC until use.

2.3 Determination of the contents of chemical constituents in glutinous purple rice extracts

The total phenolic content was determined using the Folin-Ciocalteu method (Ma and Cheung, 2007). The extract was oxidized with Folin-Ciocalteu reagent and neutralized by 7% sodium carbonate. Then the solution mixture was incubated for 15 min at 45 ^{o}C. The absorbance of the resulting blue color was measured at 760 nm. The phenolic contents were determined using a standard curve obtained from various concentrations of gallic acid. The total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram extract.

The total flavonoid content was determined using the aluminum chloride colorimetric method (Maksimovic *et al.,* 2005) with several modifications. After the extract was incubated with 5% sodium nitrite for 10 min, 10% trichloroaluminium hexahydrate was added and the mixture was incubated at room temperature. Then 1 M sodium hydroxide was added and the absorbance of the reaction mixture was measured at 532 nm. The flavonoid contents were determined using a standard curve obtained from various concentrations of catechin. Total flavonoid content was expressed as milligrams of catechin equivalents (CE) per gram extract.

The total anthocyanins content was evaluated by the pH differential method (Lee *et al.,* 2005). The extracts were dissolved in 0.025 M potassium chloride and sodium acetate as pH 1.0 and pH 4.5 buffer, respectively. The absorbances of the extracts were determined at 520 and 700 nm. The amounts of anthocyanins were express as cyanidin-3-glucoside equivalents per gram extract using extinction coefficient 26,900 $M^{-1}cm^{-1}$.

2.4 Animals

Male Wistar rats weighing 80-100 g, were used for clastogenic and anticlastogenic studies and female Wistar rats weighing 190-200 g were used for acute toxicity test. All rats were purchased from the National Laboratory Animal Center, Nakhon Pathom. They were housed under standard environmental conditions of temperature at 25±1°C under a 12 h dark-light cycle and allowed free access to drinking water and standard pellet diet. All experimental protocols were approved by the Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University.

2.5 Acute oral toxicity test

The acute toxicity for glutinous purple rice extracts was followed OECD guideline TG 425 (OECD, 2008). Female Wistar rats were divided to 5 groups. Group 1 was orally fed with 5 ml/kg bw of distilled water as a vehicle control. Groups 2 and 3 were received a single dose of 2000 mg/kg bw of acidified methanol extract of glutinous purple rice hull and methanol extract of glutinous purple rice seed, respectively, which were dissolved in distilled water. Group 4 was orally fed with 8 ml/kg bw of 5% Tween-80 as a vehicle control and group 5 was received a single dose of 2000 mg/kg bw of dichloromethane extract of glutinous purple rice hull which was dissolved in 5% Tween-80 (Figure 2-1). Body weight, signs of toxicity, behaviors and mortality were observed for the initial 24 hours after administration and once daily for 14 days. At the end of the experimental period, all rats were sacrificed by intraperitoneal injection of sodium pentobarbital. The internal organs were excised and weighed. Gross pathological observation of tissues was evaluated.

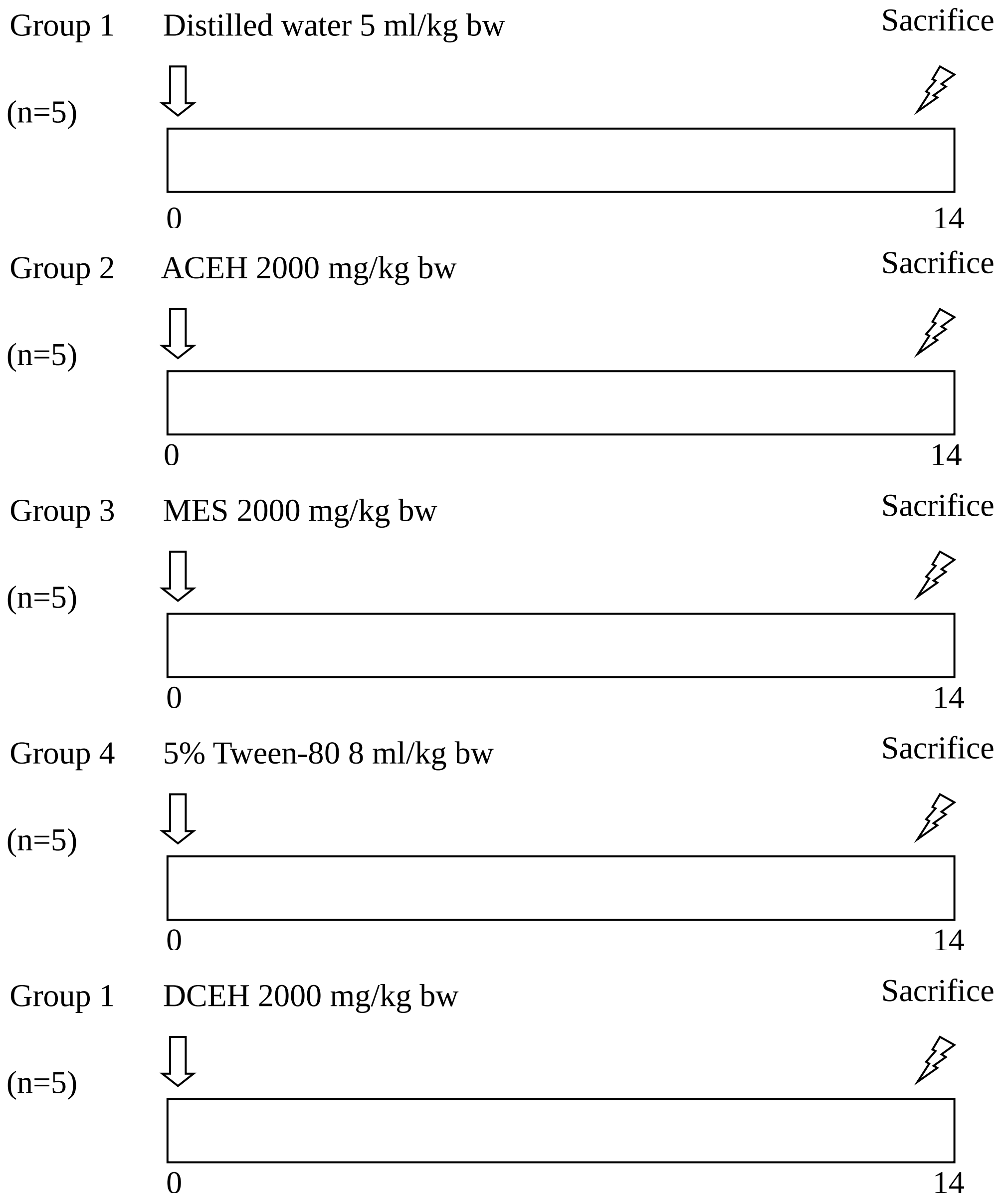

Figure 2-1 The protocol of acute oral toxicity study of glutinous purple rice extracts

ACEH: acidified methanol extract of glutinous purple rice hull

MES: methanol extract of glutinous purple rice seed

DCEH: dichloromethane extract of glutinous purple rice hull

2.6 Clastogenicity and anticlastogenicity studies of glutinous purple rice extracts using rat liver micronucleus test

2.6.1 Clastogenicity study of glutinous purple rice extracts

According to our previous study, acidified methanol extract of glutinous purple rice hull presented mutagenicity in Salmonella mutation assay, the concentrations of 100-1000 mg/kg bw of the extract were validated. Dichloromethane extract of glutinous purple rice hull and methanol extract of glutinous purple rice seed had no mutagenicity. Thus, the higest concentration of these extracts was tested in animal model. As shown in Figure 2-2, male Wistar rats were divided into 7 groups and orally fed for 28 days following experiment. Group 1 orally fed with distilled water as a vehicle control. Groups 2-4 were intragastrically fed with 100, 300 and 1000 mg/kg bw of acidified methanol glutinous purple rice hull, respectively. Group 5 was intragastrically fed with 1000 mg/kg bw of methanol extract of glutinous purple rice seed. Group 6 was received 5% Tween-80 as vehicle control of dichloromethane extract of glutinous purple rice hull. Group 7 was intragastrically fed with 500 mg/kg bw of dichloromethane extract of glutinous purple rice hull.

All rats were intraperitoneal injected by normal saline at days 22 and 25. All rats were subjected to partial hepatectomy to amplify initiated cells on day 28. Isolation of hepatocytes was performed by the 2-step collagenase perfusion method (Charoensin *et al.*, 2010) after complete compensatory hepatocyte proliferation.

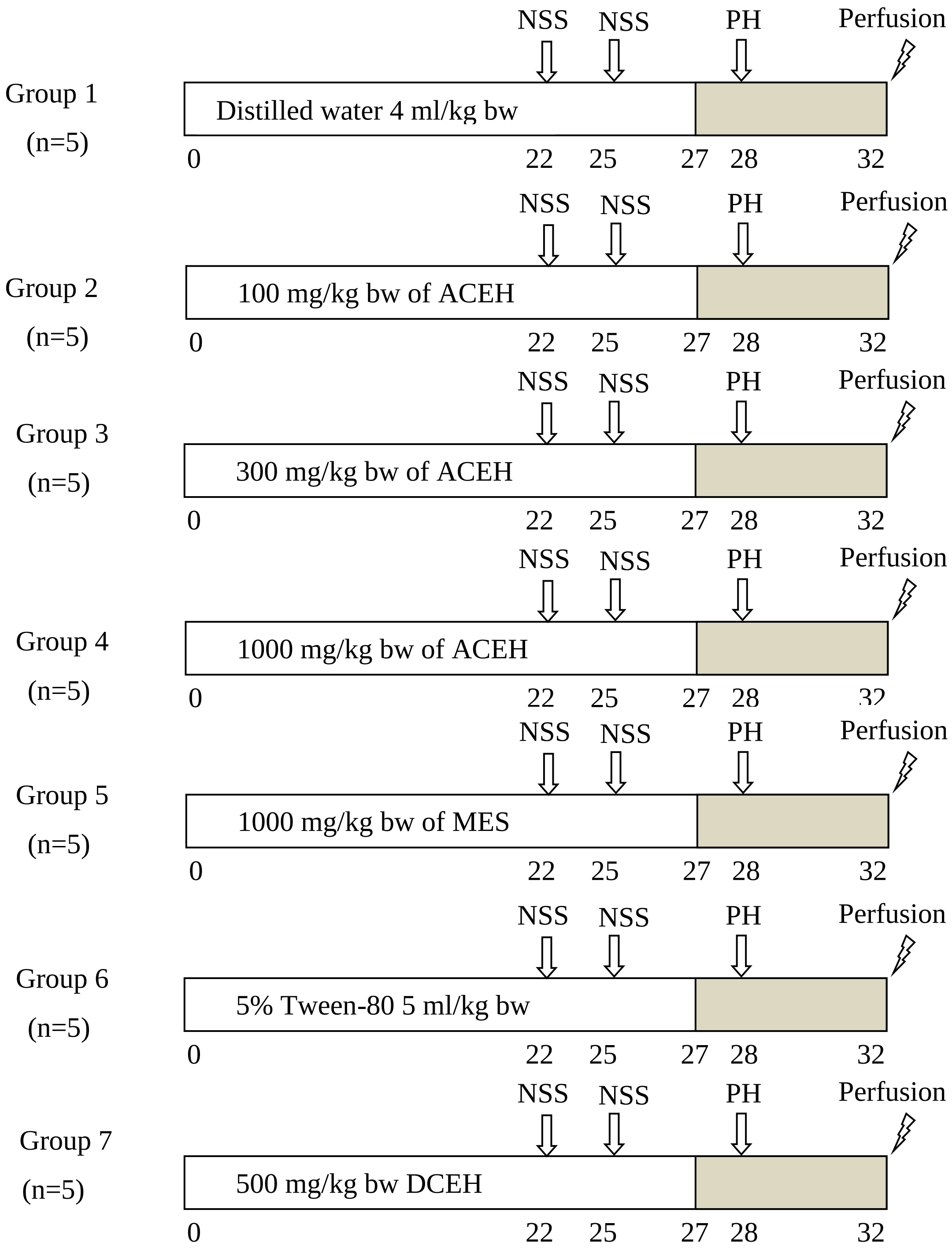

Figure 2-2 Treatment protocol for clastogenicity study of glutinous purple rice extracts

ACEH: acidified methanol extract of glutinous purple rice hull

DCEH: dichloromethane extract of glutinous purple rice hull

MES: methanol extract of glutinous purple rice seed

NSS: normal saline PH: partial hepatectomy

2.6.2 Anticlastogenicity studies of glutinous purple rice extracts

The study of anticlastogenicity of acidified methanol extract of glutinous purple rice hull and methanol extract of glutinous purple rice seed was depicted in Figure 2-3. Male Wistar rats were divided into 6 groups and orally fed for 28 days following experiment. Groups 1 and 2 were orally fed with distilled water as a vehicle control. Group 3 and 4 were intragastrically fed with 300 and 1000 mg/kg bw of acidified methanol extract of glutinous purple rice hull. Groups 5 and 6 were received with 300 and 1000 mg/kg bw of methanol extract of glutinous purple rice seed. Groups 1 were intraperitoneal injected with normal saline while groups 2-6 were intraperitoneal injected by 30 mg/kg bw diethylnitrosamine at days 22 and 25.

Anticlastogenicity of dichlorometane extract of glutinous purple rice hull was evaluated as shown in Figure 2-4. Male Wistar rats were divided into 3 groups and orally fed for 28 days following experiment. Groups 1 and 2 were orally fed with 5% Tween-80 as a vehicle control. Group 3 was intragastrically fed with 500 mg/kg bw of dichloromethane extract of glutinous purple rice hull. Groups 1 were intraperitoneal injected with normal saline while groups 2-3 were intraperitoneally injected by 30 mg/kg bw diethylnitrosamine at days 22 and 25.

All rats were subjected to partial hepatectomy to amplify initiated cells on day 29. Isolation of hepatocytes was performed by the 2-step collagenase perfusion method (Charoensin *et al.*, 2010) after complete compensatory hepatocyte proliferation.

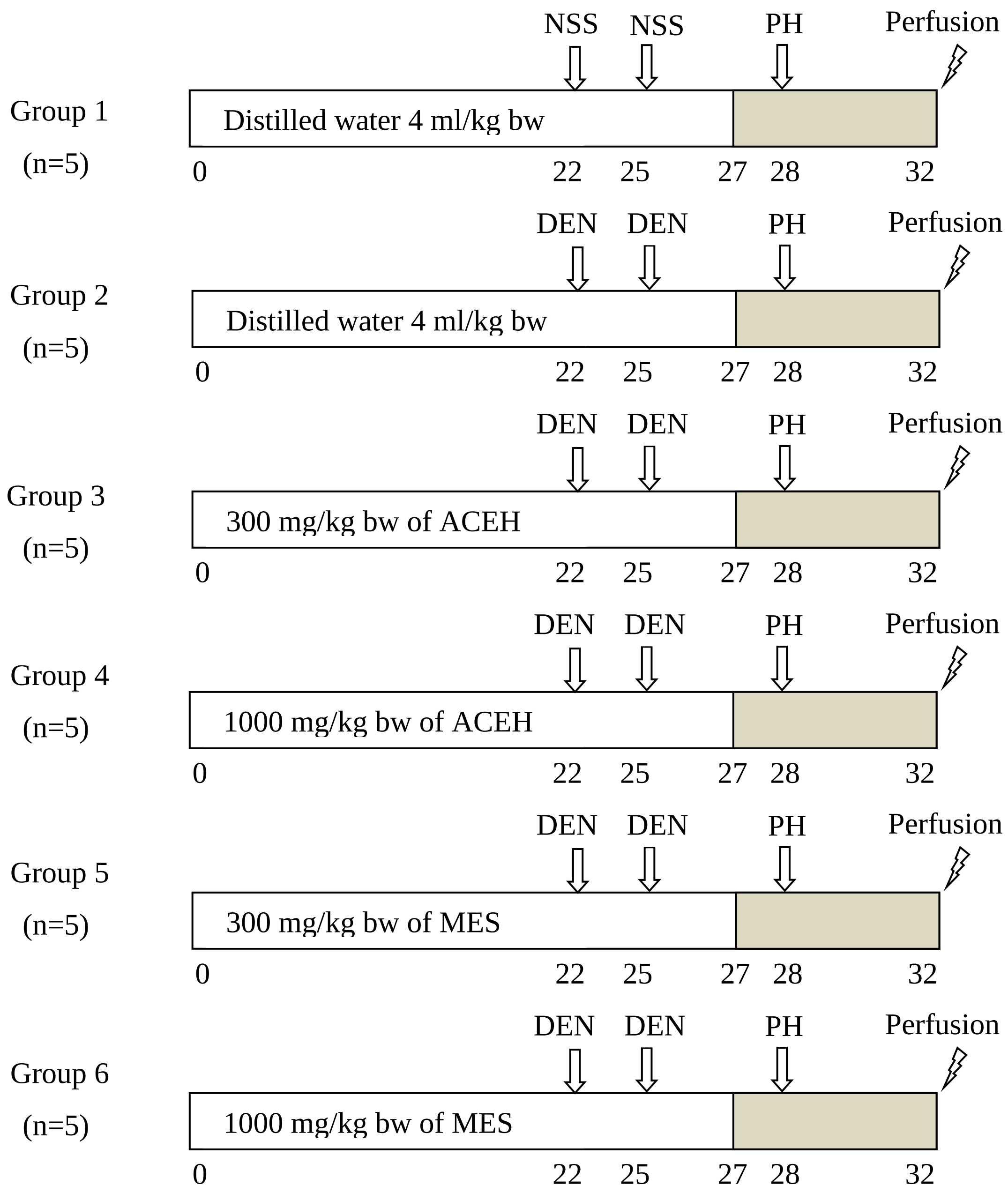

Figure 2-3 Treatment protocol for anticlastogenic study of acidified methanol extract of glutinous purple rice hull and methanol extract of glutinous purple rice seed

ACEH: acidified methanol extract of glutinous purple rice hull

MES: methanol extract of glutinous purple rice seed

NSS: normal saline

DEN: 30 mg/kg bw diethylnitrosamine

PH: partial hepatectomy

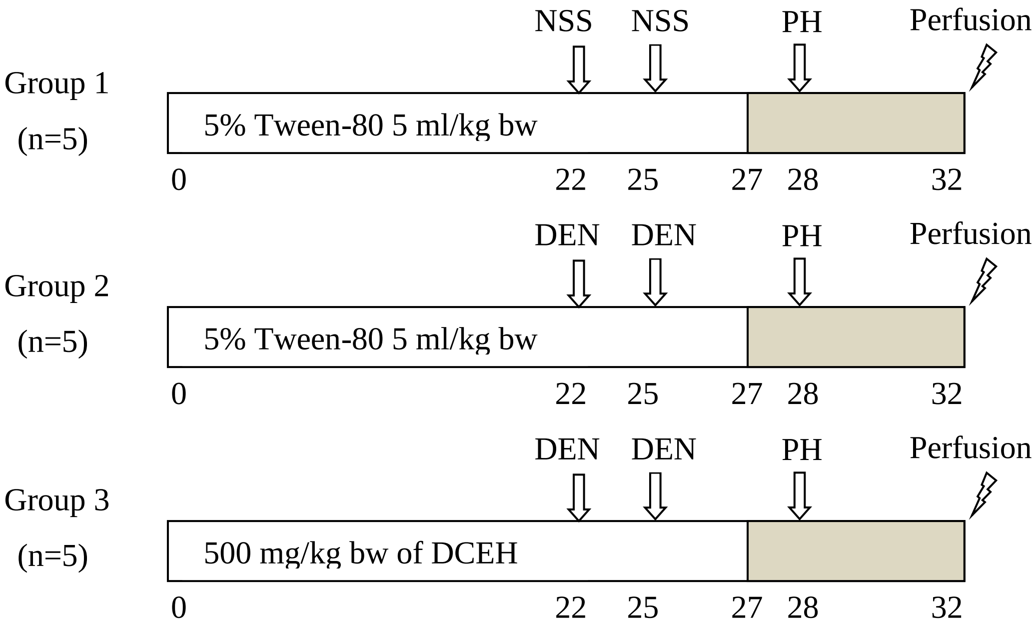

Figure 2-4 Treatment protocol for anticlastogenic study of dichloromethane extract of glutinous purple rice hull

DCEH: dichloromethane extract of glutinous purple rice hull

NSS: normal saline

DEN: 30 mg/kg bw diethylnitrosamine

PH: partial hepatectomy

2.6.3 Partial hepatectomy

The rat liver is divided into four main lobes (Figure 2-5) caudate lobe (8 %), right liver lobe (22 %), median lobe (38 %) and the left lateral lobe (30 %) (Maertins *et al*., 2008).

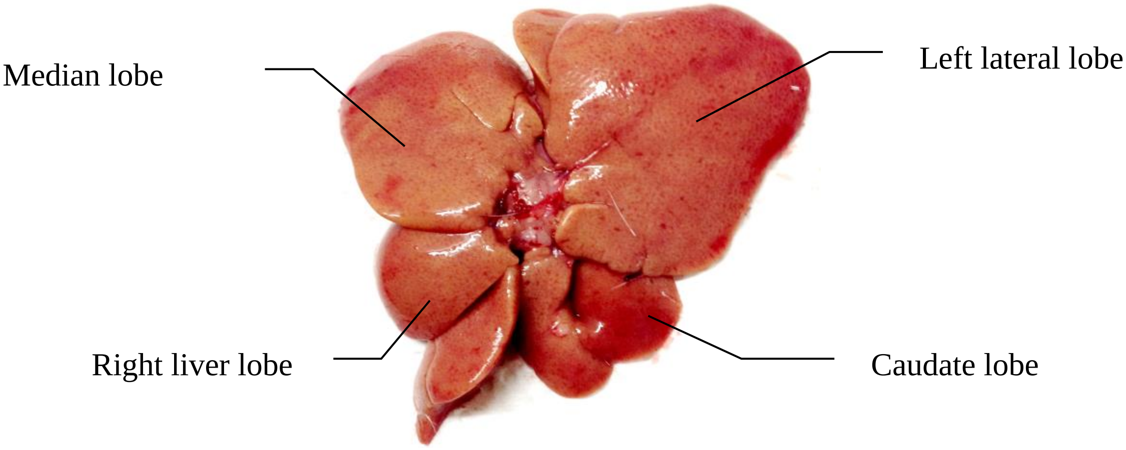

Figure 2-5 Anatomy of rat liver

In the order to stimulate the hepatocytes into mitosis, rats were anesthetized by diethylether, and two-third, comprising the left leteral and median lobes, of the liver of each animal, was excised using partial hepatectomy technique of Higgins and Anderson (Higgins and Anderson, 1931). All rats were anesthesia by diethylether. The abdominal region was shaved and disinfected with 70% ethanol. Incisions of midline ventral abdominal skin and muscles, less than 2 cm long, were made from just above the xipoid cartilage. Sterile guaze was placed along the edge of the skin incision to prevent blood from dripping into abdominal cavity, and the median and the left lateral lobes of liver were then squeezed out of the abdominal cavity through the incision by pushing the lobes gently with thumb and forefinger of each hand. A ligature with a double reef knot was placed tightly around the two lobes and their blood vessels at their base. The guaze was folded over the lobes, which were then raised, placed under slight tension and severed with blunt-ended curved scissors. The incision was closed with sutures for peritonium and staples for skin. The skin was painted by providine solution. The animals were usually conscious within 15 min after operation.

2.6.4 Isolation of hepatocytes

Hepatocytes were isolated from anesthetized rats by the collagenase perfusion (Charoensin *et al.*, 2010). The regenerated liver was removed from the abdominal cavity. The canula was inserted into a portal vein. To remove blood, 50 ml of preperfusion buffer were infused for 3 times. Then, to separate a single hepatocyte, medium containing 0.05 % collagenase type IV at 37 °C was continuously perfused until the surface of the liver appeared small cracks. The liver was cut into petridish and incubated with collagenase solution at 37 °C for 1 hour. The cell suspension was filtered through a 60-mm nylon mesh and centrifuged at 1000 rpm for 5 min. The pellet was resuspension in phosphate buffer saline and rinsed for 3 times in the same way. The final pellet was rinse with 10 % neutral buffered formalin for 2 times and centrifuged at 1000 rpm for 5 min. Hepatocytes were suspended with 10 % neutral buffered formalin and stored in refrigerator until analysis.

2.6.5 Determination of micronuclei

Hepatocyte suspension was mixed with 20 µg/ml 4′, 6-diamidino-2-phenylindole dihydrochloride stain solution for fluorescent microscopy. Stained hepatocyte suspension was dropped onto a glass slide and covered with a coverslip.

Hepatocytes were analyzed under a fluorescent microscope (x400 or higher) equipped with an ultraviolet excitation system. Round or distinct micronucleus stained with the same color as the nucleus, with diameters of ¼ or less than that of the main nucleus as shown in Appendix D. The number of micronucleated hepatocytes was counted and recorded based on analysis of 2,000 hepatocytes from each animal. The percent inhibition was calculated using the equation below:

$$\text{Percent inhibition} = \frac{(\text{MN1-MN2})}{\text{MN1}} \quad \text{X100}$$

MN1: number of micronuclei/1000 hepatocytes in DEN control group
MN2: number of micronuclei/1000 hepatocytes in treated group

The number of mitotic cell was also counted in 2,000 hepatocytes in each animal to determine the mitotic index (MI), indicator of mitotic activity. Mitotic cells were defined as cells at any stage from prophase to telophase (Cliet *et al.*,1989). Elevated frequencies of binucleated cells, a marker of genomic instability, were observed (Kennedy *et al.*, 1996).

2.7 The study of activities and expressions of xenobiotic metabolizing enzymes

2.7.1 Preparation of hepatic microsome and cytosol

One gram of liver sample was homogenized in 3 ml of homogenizing buffer containing 1.15% w/v of potassium chloride and 0.25 mM of phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 10,000 *g* for 30 min at 4°C. The supernatant was further centrifuged at 100,000 *g* for 60 min at 4°C to obtain the clear cytosol fraction as a supernatant and microsomal fraction as a pellet. After the pellet was washed in homogenizing buffer, it was resuspended in 200 µl of microsome suspension buffer pH 7.4, containing 30 % v/v of glycerol and 1 mM dithiothreitol. The microsome and cytosol were stored at -80°C refrigerator prior to use. The protein concentration of each sample was determined by Lowry method (Lowry *et al.*, 1951).

2.7.2 Determination of cytochrome P450 reductase activity

NADPH-cytochrome P450 reductase (P450 reductase) is localized in the membrane of endoplasmic reticulum. It transfers electrons from NADPH to several oxygenases**,** the most important of which is the cytochrome P450 family of enzymes**,** responsible for xenobiotic metabolism.

$$\text{Cytochrome } c \text{ (oxidized)} + \beta\text{-NADPH} \longrightarrow \text{Cytochrome } c \text{ (reduced)} + \beta\text{-NADP}^+$$

P450 reductase activity was evaluated spectrophotometrically by measuring the rate of cytochrome *c* reduction at 550 nm (Vermilion *et al.*,1981). To analyze, the reaction mixture containing diluted microsome, 0.3 M potassium phosphate buffer pH

7.5, 1 mM cytochrome c, and 50 mM potassium cyanide was incubated for 2 min. After incubation, 5 mM β-NADPH was added as coenzyme for P450 reductase. P450 reductase activity was measured spectrophotometrically at 550 nm by calculation using a molar coefficient 21 $M^{-1}cm^{-1}$. The activity was expressed as U/mg microsome protein.

2.7.3 Determination of heme oxygenase activity

The heme oxygenase (HO) activity assay was determined with some modifications using hemin as a substrate (Tenhunen *et al.*, 1968). Bilirubin levels are then measured by a standard spectrophotometric method using the difference in absorption at 460 and 530 nm.

$$\text{Hemin} \xrightarrow[\text{NADPH}]{\text{HO-1}} \text{Biliverdin} \xrightarrow[\text{NADPH}]{\text{Billiverdin reductase}} \text{Billirubin}$$

The measurement of heme oxygenase activity assay was performed in a reaction containing 50 µM hemin, 0.8 mM NADPH, 2 mM glucose 6-phosphate, 0.2 U glucose 6-phosphate dehydrogenase and 10 mg of liver cytosol, a source of billiverdin reductase. The reaction was prepared in 0.1 M potassium phosphate buffer with 2 mM magnesium chloride, pH 7.4. After being incubated in dark at 37 ^{0}C for 1 hour, the reaction was stopped via the addition of chloroform and the generated bilirubin was measured in a spectrophotometer at 460 and 530 nm. Results were expressed as nmol bilirubin/mg protein/min ($\varepsilon = 40$ mM^{-1} cm^{-1}).

2.7.4 Determination of glutathione *S*-transferase activity

The glutathione *S*-transferase (GST) colorimetric activity assay is based upon the GST-catalyzed reaction between GSH and GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB). Under certain condition, GST catalyzes a formation of CDNB-GSH producing a dinitrophenyl thioether which can be detected by spectrophotometer at 340 nm. One unit of GST activity is defined as an amount of enzyme producing 1 mmol of CDNB-GSH conjugate/min under the condition of the assay.

$$\underset{\text{(reduced form)}}{\text{Glutathione}} + \text{CDNB} \xrightarrow{\text{GST}} \text{Glutathione conjugated CDNB}$$

GST activity was estimated by the method of Habig and Jakoby (Habig and Jakoby, 1981). The one milliliter of reaction mixture was consisted of 0.2 M potassium phosphate buffer (pH 6.5), deionized water, 10 mM GSH, diluted cytosol, and 10 mM CDNB. The changes in the absorbance were recorded at 340 nm and enzymes activity was calculated as mmol CDNB conjugate formed (U/mg protein) using a molar coefficient of 9.6 M^{-1} cm^{-1}.

2.7.5 Determination of UDP-glucuronyltransferase activity

The activity of UDP-glucuronyltransferase (UDP-GT) was determined using 4-nitrophenol as a substrate (Zhu *et al.*,1995). The reaction mixture consisting of 200 mM Tris/HCl, 40 mM magnesium chloride, 5 mM 4-nitrophenol (4-NP) and 2.5 mg protein/ml of cytosol was preincubated at 37 ^{0}C for 5 min. The reaction mixture and 20 mM UDP-glucuronic acid (UDP-GA) were incubated at 37 ^{0}C for 1 hour. The reaction was stopped by adding 10% TCA. The mixture was centrifuged at 10,000 rpm for 15 min. The supernatant was alkalinized with 0.5 M sodiumhydroxide. The absorbance of conjugated 4-NP was measured at 405 nm. The enzymes activity was expressed as µmol 4-NP conjugated formed/min/mg protein using a molar extinction coefficient 29 mM^{-1} cm^{-1}.

$$\text{UDP-GA + 4-NP} \xrightarrow{\text{UDP-GT}} \text{UDP-GA conjugated 4-NP}$$

2.7.6 Determination of NADPH-quinone oxidoreductase activity

NADPH-quinone oxidoreductase (NQO) was determined by the method of (Bensonr *et al.*, 1980) using 2,6-dichloroindophenol as an electron acceptor. The reaction mixture consisting of 37.5 mM Tris-HCl buffer, 30 mg/ml bovine serum albumin, 10% v/v Tween 20, 300 mM NADPH, 4.5 mM FAD, cytosolic protein and 18 mM 2,6-dichloroindophenol. The enzyme activity was quantified by measuring the disappearance of 2,6-dichloroindophenol at 600 nm. The activity was expressed as nmol of reduced 2,6-dichloroindophenol/min/mg protein using an molar extinction coefficient 2.1x10^{4} M^{-1} cm^{-1}.

$$\text{2,6- dichloroindophenol +NADPH} \xrightarrow{\text{NQO}} \text{reduced 2,6-dichloroindophenol + NADP}^{+}$$

2.7.7 Assay of protein expression of some xenobiotic metabolizing enzymes

The determination of GST-alpha expression was determined using cytosolic protein was used. Microsomal protein was used in the study of CYP2E1, NADPH P450 reductase and heme oxygenase-1 protein expression. The 50 mg/ml of cytosolic and 5 mg/ml of microsome proteins were seperated by 10%v/v sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrophoresed with 0.02 A/gel. Following SDS-PAGE, the gel was equilibrated in 20% v/v methanol blotting buffer for 30 min and proteins were transferred to nitrocellulose membrane (5.5 mA/cm^2) for 1 hour with semi-dry blotting system.

Prior incubation of primary antibody, the membrane-nonspecific binding sites were blocked by Tween 20-phosphate buffer saline pH 7.5 containing 5% w/v skim milk for 2 hours, followed by overnight incubation with the rabbit primary polyclonal antibody against rat-CYP2E1(1:5,000 dilution) or NADPH-P450 reductase (1:5,000 dilution) or glutathione *S*-transferase alpha (1:2,000 dilution), dissolved in 0.2% w/v skim milk-TPBS pH 7.5 at 4 °C. Then, the membrane was washed with washing solution for 3 times and incubated with the secondary antibody (horse raddish peroxidase-labeled goat anti-rabbit IgG) for 30 min. In detection, the antigen–antibody complexes were visualized by enhanced chemiluminescence (ECL) system (peroxide as substrate and luminol as enhancer) with short exposure of the membrane to X-ray film. The light emitted at the sites of substrate catalyzed can be captured on such film. Film was scaned and intensity of each band was evaluated by Image J program.

2.7.8 Determination of total protein

The principle of the Lowry method (Lowry *et al*., 1951) for determining protein concentrations lies in the reactivity of the peptide nitrogen with the copper (II) ion under alkaline condition. The subsequent reduction of Folin and Ciocalteu's phenol reagent yields blue solution by the copper-catalyzed oxidation of aromatic acids detected spectrophotometrically at 750 nm. The Lowry method is sensitive to low concentrations of protein.

According to this method, test samples were pretreated with copper (II) in an alkaline copper solution stabilized by sodium potassium tartrate for 10 min at room temperature. Folin and Ciocalteu’s phenol reagent was added to generate chromogen that give increasing absorbance at 750 nm. Total protein concentration in each sample was calculated using a bovine serum albumin calibration curve (0-100 µg/ml) and expressed as mg protein/ml.

2.8 Statistical analysis

Data are reported as means±S.D. of each variable for each group. The significance of difference between each group was analyzed using one way ANOVA and paired t-test with LSD significance level adjustment for post hoc tests. Values of $p < 0.05$ were considered to be significant.