CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and instruments are shown in Appendix A and B

2.2 Animals

Male Wistar rats were purchased from National Laboratory Animal Center, Mahidol University, Salaya, Nakorn-Prathom, Thailand and were kept in the Animal House, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Rats were given an acclimatization period of week before each experiment. They were housed at a maximum of three per cage with a light–dark cycle 12–12 hours, at temperatures of 21–25°C and relative humidity 50–60% throughout the study. Each animal had free access to diet and tap water.

2.3 Extraction and isolation of pinocembrin from *Boesenbergia pandurata* (Roxb.) Schltr. rhizome.

Pinocembrin from ethyl acetate extract isolated form fingerroot (voucher specimen: BKF 68909) and according to the method of Tuchinda *et al.* (2002) and Jaipetch *et al.* (1983) was obtained from Assist. Prof. Wilart Pompimon, Faculty of Science, Lampang Rajabhat University, Thailand. The method and data of the isolation are presented in Appendix D. Pinocembrin was dissolved in 5% Tween-80 for further studies.

2.4 Mutagenicity study of pinocembrin in rat liver

According to treatment protocol (Figure 2-1), 24 male wistar rats were divided into 4 groups, 6 animals per group. Group 1 was a vehicle control group, while groups 2 to 4 were intragastrically fed with pinocembrin concentration at 1, 10 and 100 mg/kg bw, respectively for 7 days. All rats were partially hepatectomized at day 8 to induce mitotic stimulation. The incidence of micronucleated hepatocytes was determined 4 days after partial hepatectomy.

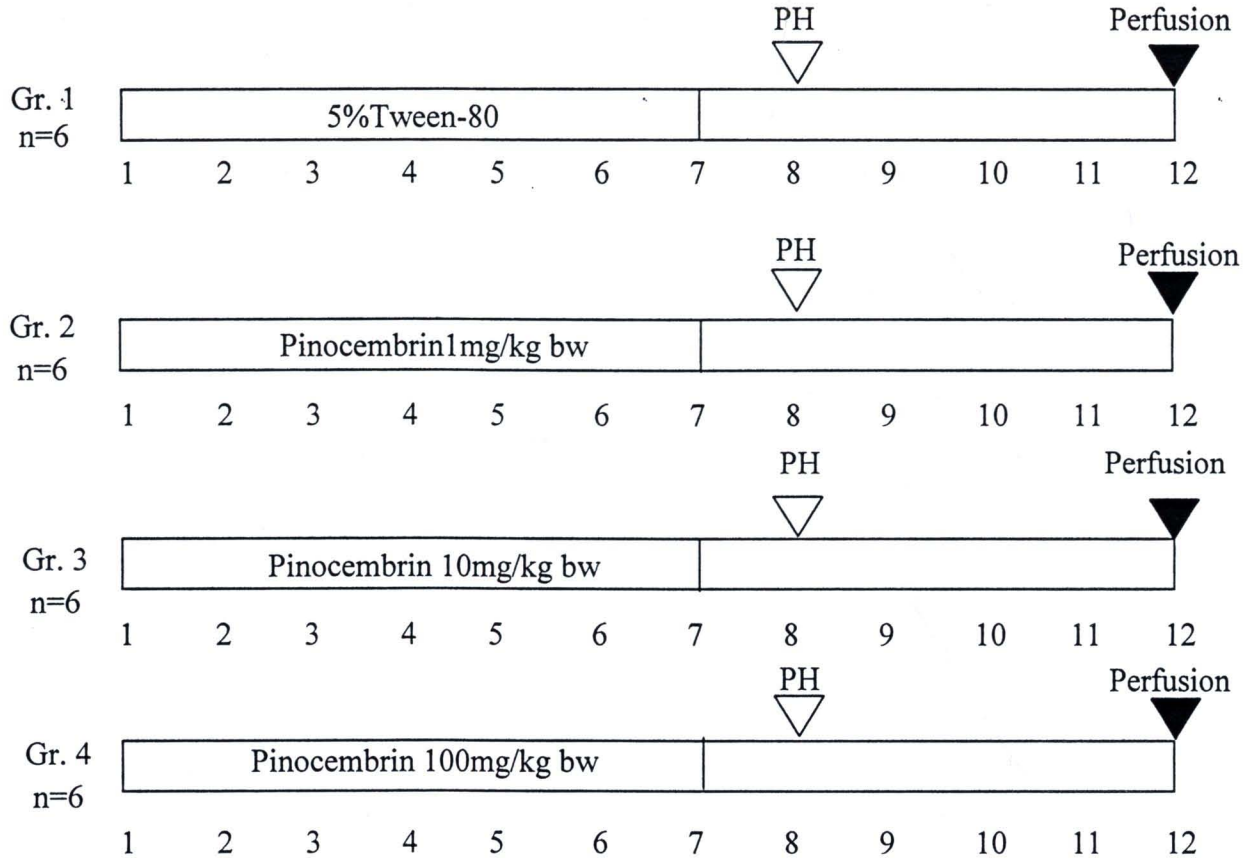


Figure 2-1 The protocol for mutagenicity study of pinocembrin in male wistar rat

2.5 Partial hepatectomy

In order to stimulate the hepatocytes into mitosis, rats were anesthetized light with diethyl ether, and two-thirds of the liver of each animal, comprising the left lateral and median lobes, was excised using the technique described by Higgins Anderson (1931). The abdominal region was shaved and disinfected with 70% ethanol. Incisions of the midline ventral abdominal skin and muscles, less than 2 cm long, were made from just above the xiphoid cartilage. Sterile gauze was placed along the edge of the skin incision to prevent blood from dripping into the abdominal cavity, and the median and left lateral lobes of the liver were then squeezed out of the abdominal cavity through the incision by pushing the lobes gently with the 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 gauze was folded over the lobes, which were then raised, placed under slight tension and severed with blunt-ended curved scissors. The incisions were 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 clipped by the operation.

2.6 Isolation of hepatocytes

Hepatocytes were isolated from anesthetized rats by the collagenase perfusion method according to Puatanachokchai *et al.* (1996). The regenerated liver was removed from the abdominal cavity. The canula was inserted into portal vein. To rinse 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 resuspended in PBS and rinsed for 3 times in the same way. The final pellet was rinsed with 10% neutral buffered formalin for 2 times and centrifuged at 1000 rpm for 5 min. Hepatocytes were suspened with 10% neutral buffered formalin and stored in a refrigerator until analysis

2.7 Microscopic observation and micronucleus determination

Evaluation of the micronucleated hepatocytes (MNHEPs) was recorded based on analysis of 2000 hepatocytes from each animal. Briefly, 50 μl of hepatocyte suspensions were mixed with 50 μg/ml of 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) stain solution. Approximately, 50μl of stained hepatocytes suspension was dropped onto a glass slide and covered with a coverslide. Hepatocytes were analyzed under fluorescent microscope (x400 or higher) equipped with a UV excitation system. For the identification of micronuclei, the following criteria were used: 1. a diameter smaller than one-quarter of that of the nucleus; 2. a clearly marked perimeter; and 3. the same color or luster as the nucleus. The number of mitotic cells was also counted in 2000 hepatocytes in each animal to determine the mitotic index (MI) for administration route or treatment time differences. Mitotic cells were defined as cells at any stage from prophase to telophase.

2.8 Inhibitory effect of pinocembrin on diethylnitrosamine-induced micronucleated hepatocyte formation in rat

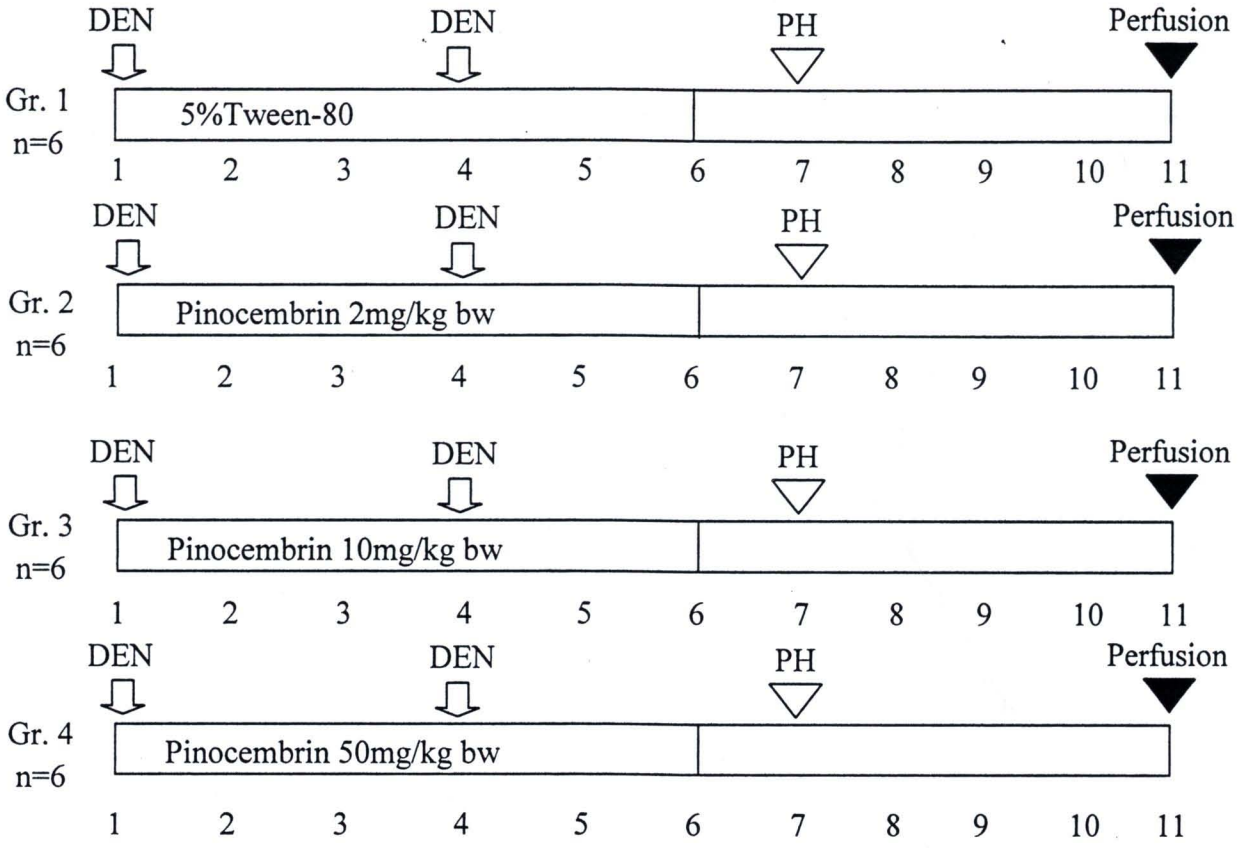
According to treatment protocol (Figure 2-2), male wistar rats were divided into 4 groups, 6 animals per group. All rats were given a double intraperitoneally injected with 30 mg/kg bw of diethylnitrosamine (DEN) at day 1 and day 4. Group 1 was a positive control group receiving 4 ml/kg bw of 5% Tween-80. Various concentrations of pinocembrin, 2, 10 and 50 mg/kg bw, were administered to group 2, 3 and 4, respectively, started from the first day of an experiment and continued for 6 days. All rats were partially hepatectomized at day 7 of experiment and hepatocytes were isolated after the operation for 4 days.

2.9 Preventive effect of pinocembrin on 30 mg/kg bw of diethylnitrosamine-induced micronucleated hepatocyte formation in rat

The experimental design is shown in Figure 2-3, male wistar rats were classified into 4 groups, 6 animals per group. Prior to the first DEN injection for 6 days, group 1 were orally fed with 5% Tween-80 and considered as positive control group. While group 2 to 4 were intragastrically fed with pinocembrin concentration at 10, 25, 50 mg/kg bw, respectively, until days 12 of an experiment. All rats were intraperitoneally administered with 30 mg/kg bw of DEN for 2 times at day 7 and 10 of an experiment. The incidence of micronucleated hepatocytes was determined 4 days after partial hepatectomy.

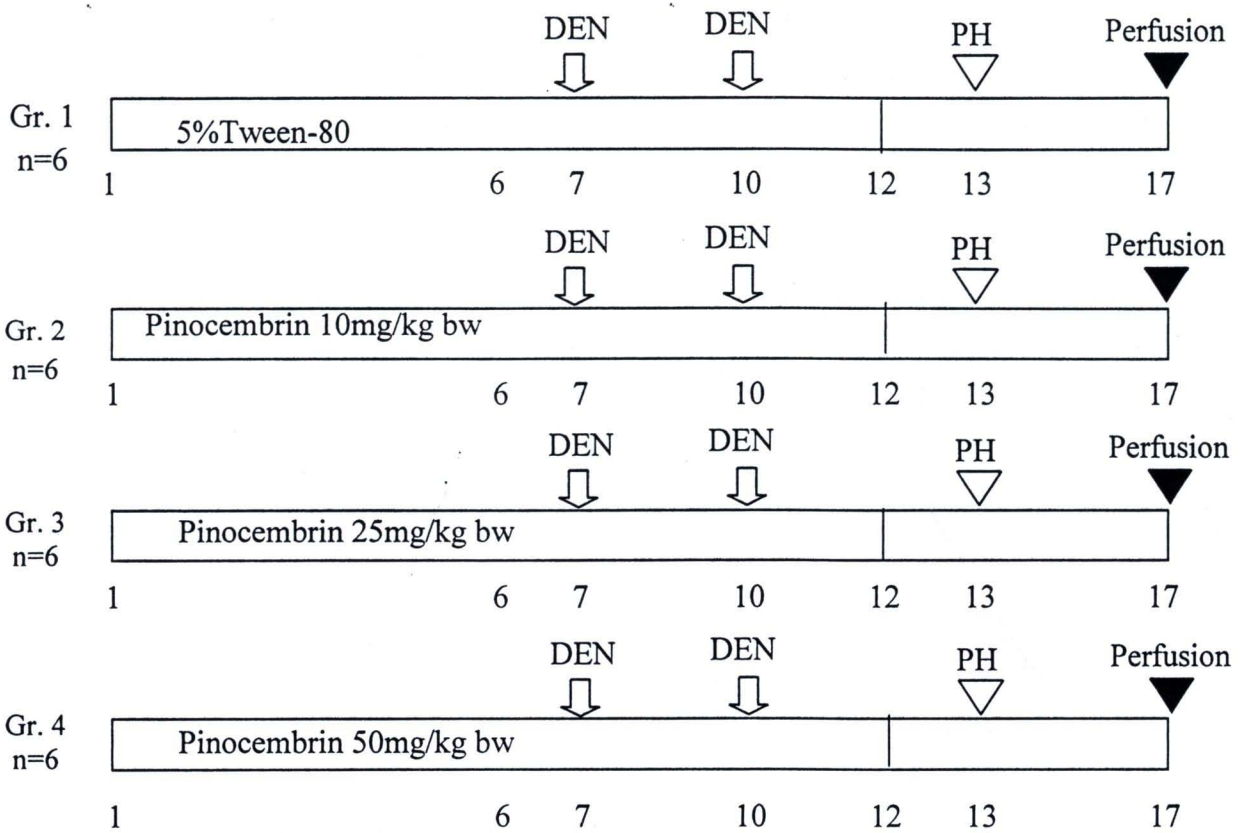
2.10 Protective effect of pinocembrin on 20 mg/kg bw of diethylnitrosamine-induced micronucleated hepatocyte formation in rat

As shown in Figure 2-4, male wistar rats were classified into 4 groups. Group 1 and 3 were intragastrically fed with 5% Tween-80 as vehicle control, while group 2 and 4 were orally fed with 10 mg/kg bw of pinocembrin for 14 days before DEN injection until days 21 of an experiment. Group 1 and 2 were intraperitoneally injected with 0.9% normal saline solution as a negative control groups, while group 3 and 4 were injected with 20 mg/kg bw of DEN at day 15 and day 18. All rats were partially hepatectomized 1 day after the last treatment and hepatocytes were isolated after the operation for 4 days.



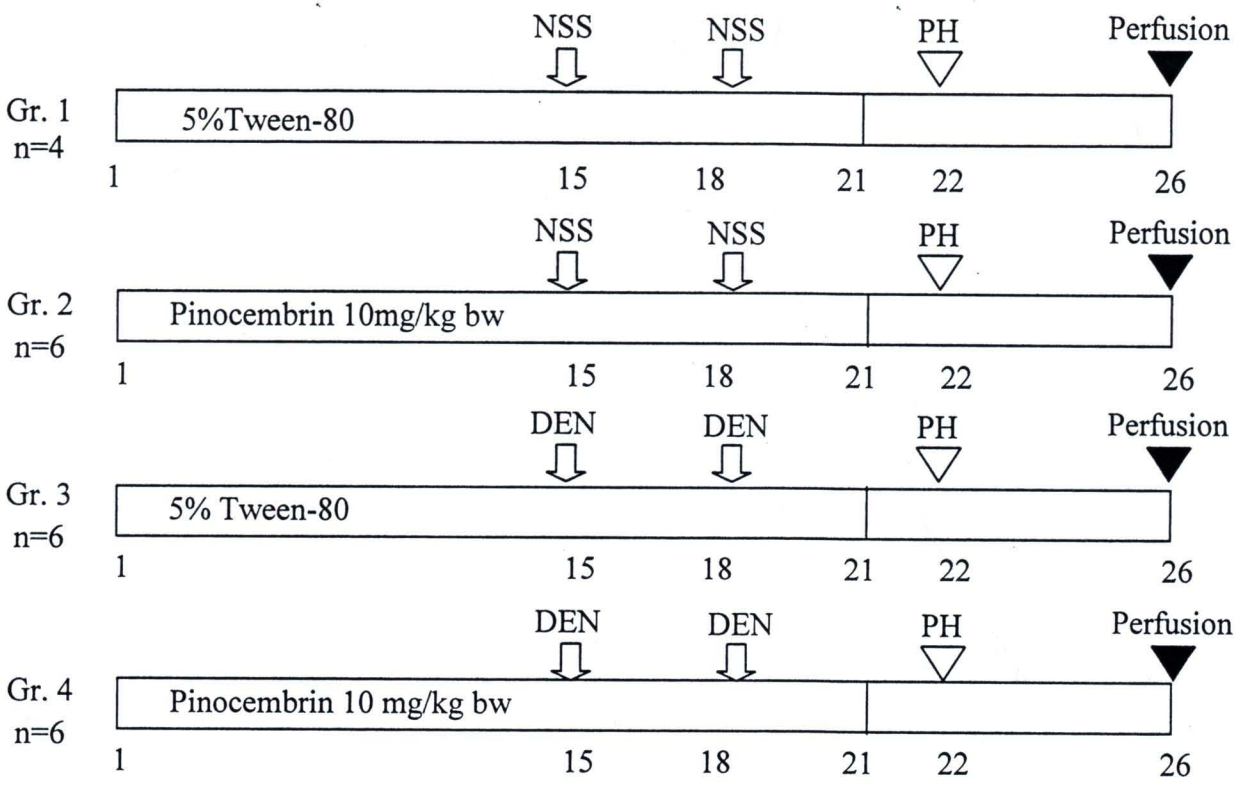
DEN: 30 mg/kg bw; i.p.

Figure 2-2 The protocol for inhibitory effect of pinocembrin on DEN-induced micronucleus formation in rat liver



DEN: 30 mg/kg bw; i.p.

Figure 2-3 The protocol for preventive effect of pinocembrin on 30 mg/kg bw of DEN-induced micronucleus formation in rat liver



DEN: 20 mg/kg bw; i.p.

Figure 2-4 The protocol for protective effect of pinocembrin on 20 mg/kg bw of DEN-induced micronucleus formation in rat liver

2.11 Effect of pinocembrin on promotion stage in diethylnitrosamine-induced rat hepatocarcinogenesis

The experimental design is shown in Figure 2-5, 63 male wistar rats, 4-week-old were divided into 7 experimental groups for the medium-term carcinogenicity test. At week 3 and 4 of an experiment, groups 1 to 5 were given double intraperitoneal injections of 100 mg/kg bw of DEN to initiate hepatocarcinogenesis, while group 6 and 7 were intraperitoneally administered a normal saline solution. Before 2 weeks of an injection, group 2 and 3 were orally received pinocembrin at 2 and 10 mg/kg bw, respectively. Group 4 and 5 were fed with pinocembrin at 2 and 10 mg/kg bw, respectively, after 1 week of DEN injection. Group 1 and 6 were treated with 5% Tween-80 as a vehicle control, while group 7 was fed with pinocembrin at 10 mg/kg bw. All animals were subjected to 2/3 partial hepatectomized at week 6. Surviving rats in each group were killed under mild anesthesia for examination at week 15. Blood samples were collected from the abdominal artery under mild anesthesia, serum alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase activities were analyzed. The liver samples, the remaining 3 lobes (right anterior lobe, right posterior lobe and caudate lobe) were fixed in 10% formalin and embedded in paraffin. They were used for immunohistochemical examination of glutathione-*s*-transferase placentral form (GST-P), a preneoplastic lesion of rat hepatocellular carcinoma, as the end point marker. Body weight, water and food intake were recorded weekly for all rats throughout the experimental period.

2.12 Immunohistochemistry for GST-P

Liver sections of 3 μm thickness were immunohistochemically assessment of GST-P positive foci with the avidin–biotin complex method according to Puatanachokchai *et al.* (2006) as shown in Figure 2-6. After deparaffinization, liver sections were treated sequentially with 3% H_2O_2, normal goat serum and then were incubated with rabbit polyclonal anti-rat GST-P antibody in a dilution 1: 2,000 at 37°C for 2 hours. Reactivity with the primary antibody was demonstrated with anti-rabbit IgG biotinylated antibody in a conjugated with the avidin-biotin-peroxidase complex. Finally, the sites of peroxidase binding were visualized with the substrate diaminobenzidine tetrahydrochloride. The tissue sections were lightly counterstained with hematoxylin to facilitate orientation under microscopic examination.

2.13 Quantitative assessment of GST-P positive foci

Quantitative analysis of GST-P positive foci was performed using 2-dimensional evaluation. The number of GST-P positive foci greater than 0.2 mm diameter was counted. The total area of the liver section was measured using indirect estimation by a 5 digit balance to give value per cm^2 of liver section.

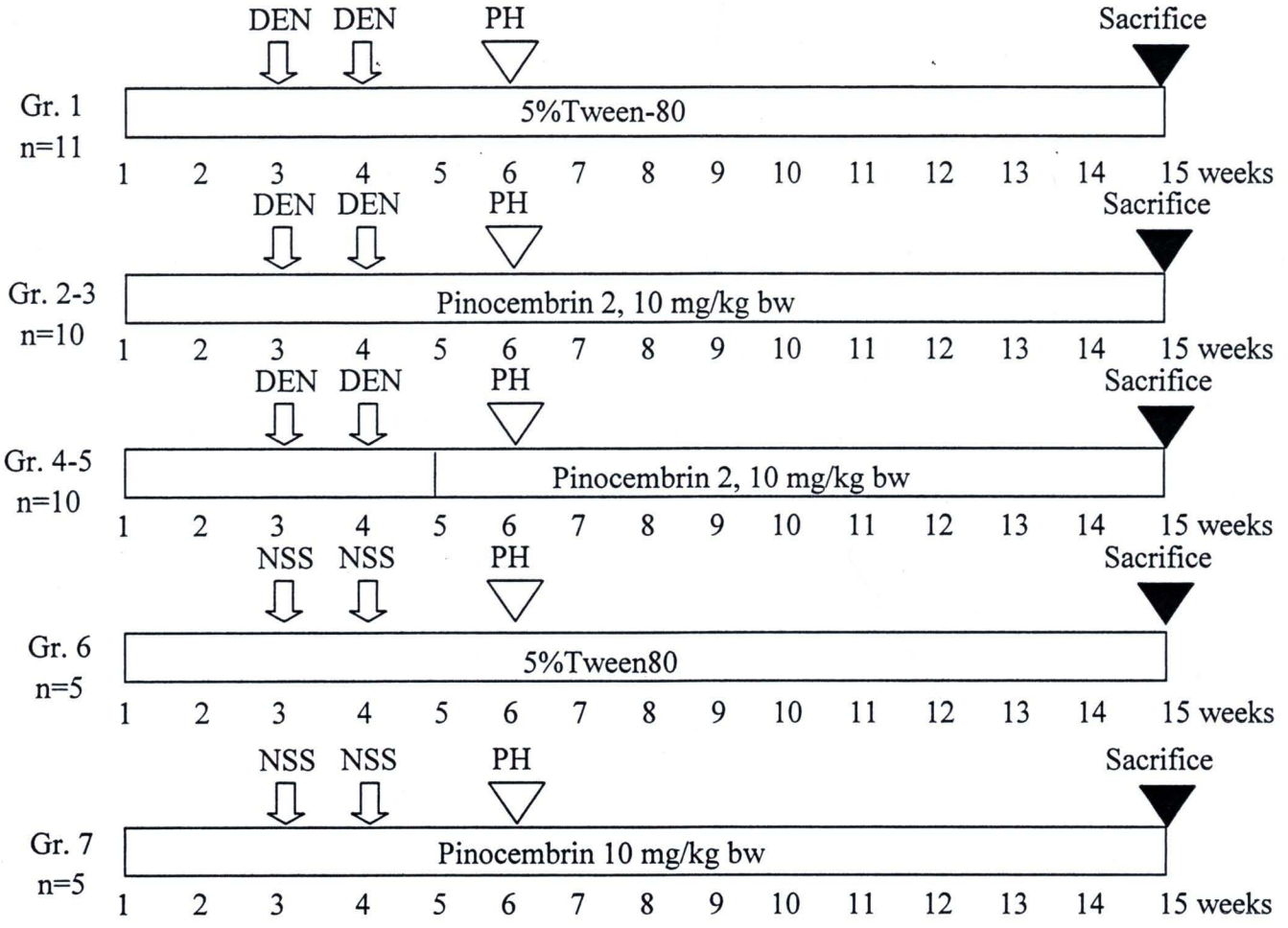


Figure 2-5 The protocol for the effect of pinocembrin on promotion stage in diethylnitrosamine-induced rat hepatocarcinogenesis

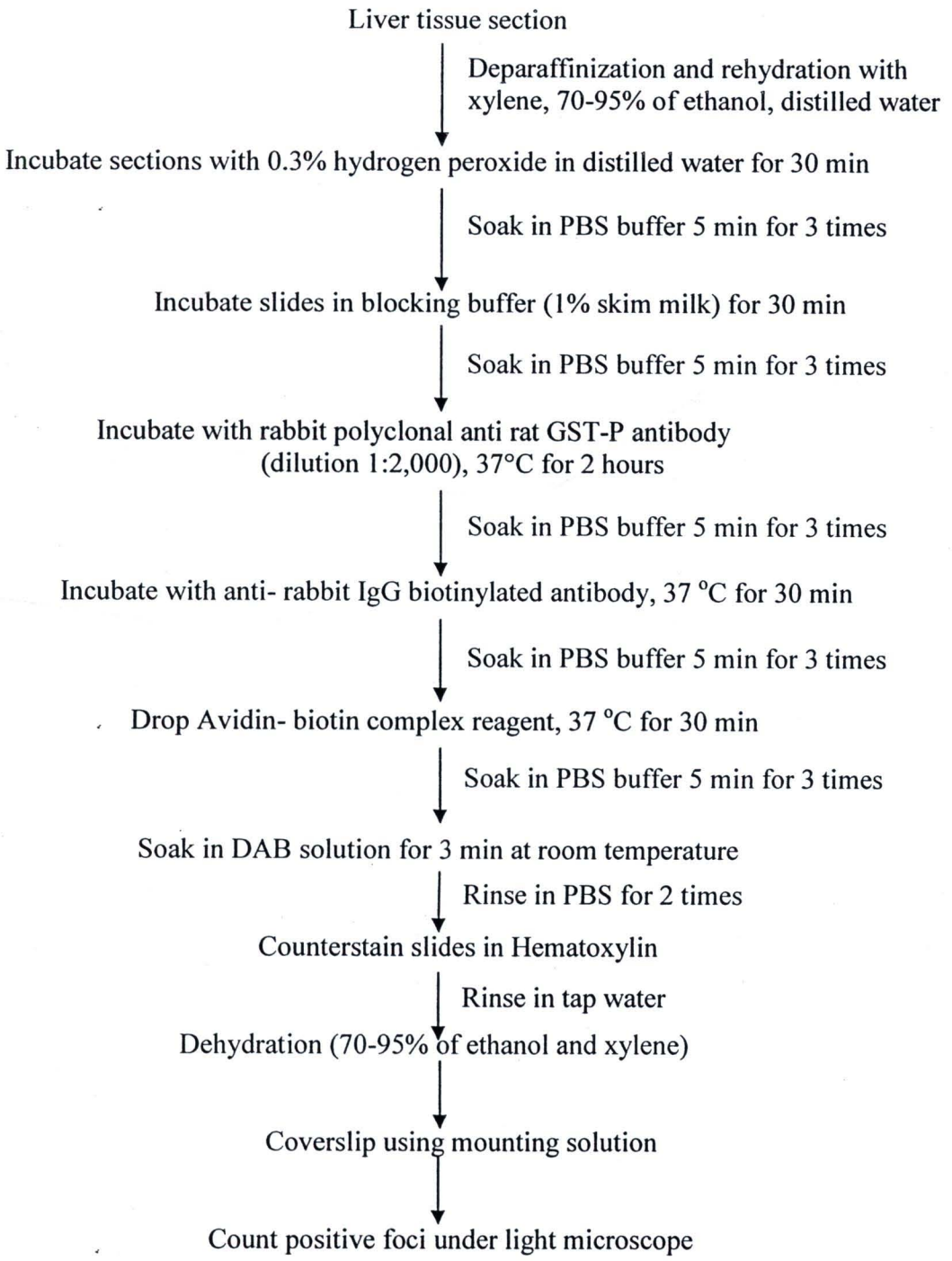


Figure 2-6 The procedure of GST-P immunohistochemistry in rat liver

2.14 Determination of lipid peroxidation by thiobarbituric acid reactive substances assay

The measurement of Thiobarbituric Acid Reactive Substances (TBARS) is a well-established assay for screening and monitoring lipid peroxidation. Malondialdehyde (MDA) forms a 1:2 adduct with thiobarbituric acid (TBA) as shown in Figure 2-7. The MDA-TBA adduct formed from the reaction of malondialdehyde in samples with thiobarbituric acid can be measured colorimetrically or fluorometrically. TBARS levels are determined from a malondialdehyde equivalence standard.

Lipid peroxidation was evaluated by measuring the TBARS according to the TBA test described by Ohkawa *et al.* (1979) with the following modifications. Liver tissue homogenates were prepared on ice and precipitated in 600 µl of 50% TCA and centrifuged at 6000 rpm and 4°C for 20 min. Two ml of 0.67% TBA was added to 2 ml of the resulting supernatant. This solution was afterwards heated at 100 °C for 10 min. After heating, the tubes were cooled on ice. The reaction mixture was centrifuged at 3000 rpm for 10 min after adding 2.6 ml of butanol. The resulting pink-stained MDA-TBA was determined in a spectrophotometer at 532 nm using a standard curve of MDA. The values are expressed in nmol MDA/mg protein.

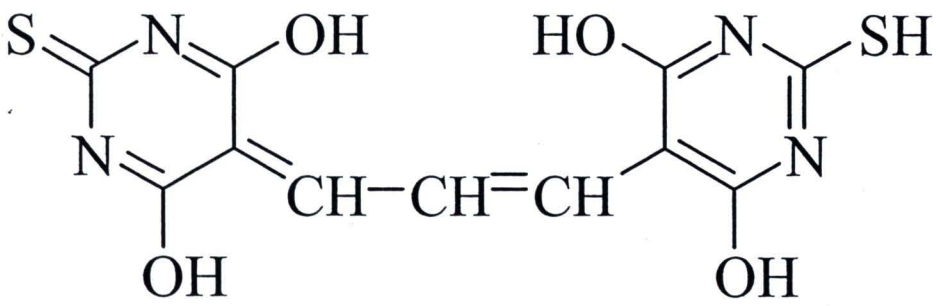


Figure 2-7 MDA-TBA adduct (Pink)

2.15 Determination of the expression and activities of phase I and phase II xenobiotic-metabolizing enzymes

2.15.1 Preparation of microsomes and cytosol

Cytosol and microsome of rat liver fractions were prepared at 0-4°C as shown in Figure 2-8. One gram of individual liver was homogenized in homogenizing buffer containing 1.15% w/v of KCl and 0.25 mM PMSF. Homogenates were centrifuged at 10,000 g for 30 min at 4°C. The resultant supernatants were then centrifuged at 100,000 g for 1 hour at 4°C yielded clear cytosol fractions as a supernatant and microsomal fraction as a pellet. A final pellet was washed in 3 ml of homogenizing buffer and resuspended in 200 µl of microsome suspension buffer pH 7.4, containing 30% v/v of glycerol and 1 mM of dithiothreitol. The resultant microsomes were stored at -80°C refringerator prior to use. Protein concentration for each sample was determined using the Lowry method.

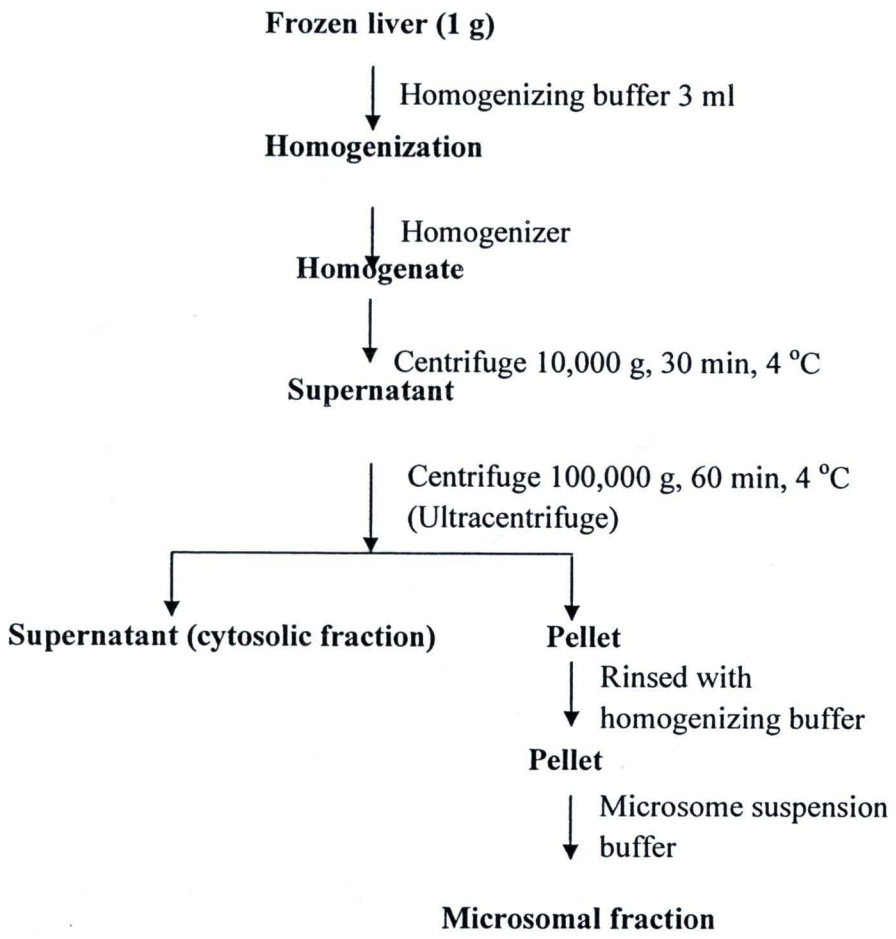


Figure 2-8 The preparation of microsomal and cytosolic fractions obtained from rat liver

2.15.2 Western blot analysis

Microsomal protein of each rat was separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The amount of microsomes loaded to the gel was varied in each experiment: 5 mg for NADPH: cytochrome P450 reductase, CYP2B1 and CYP2E1; 2 mg for CYP3A2; 3 mg for CYP2C11 and 24 mg for CYP1A1. One lane was usually reserved for a molecular weight marker. When voltage was applied along the gel (0.02 A/gel), proteins migrate into it at different speeds. These different rates of mobilities separate into bands within each lane. After electrophoresis, protein was transferred to nitrocellulose membrane (5.5 mA/cm^2) for 1 hour using semi-dry system. After blotting, membrane was incubated with non-fat dry milk for 2 hours to block the non-specific binding proteins and then incubated with the diluted primary specific antibody (1:12,000 dilution of anti-cytochrome P450 reductase and CYP3A2; 1:15,000 dilution of anti-CYP2E1; 1:10,000 dilution of anti-CYP2C11 and 2B1 and 1:8000 dilution of CYP1A1 in TPBS buffer containing 0.2% BSA) for 1 hour. Membranes were washed with washing solution and incubated with the secondary antibody solution consisting of peroxidase-conjugated anti-rabbit IgG for 30 min. Chemiluminescence detection was performed using horseradish peroxidase conjugated secondary antibodies and an Pierce enhanced chemiluminescent kit, as shown in Figure 2-9. Film was scanned and the intensity of each band was evaluated by Image J program.

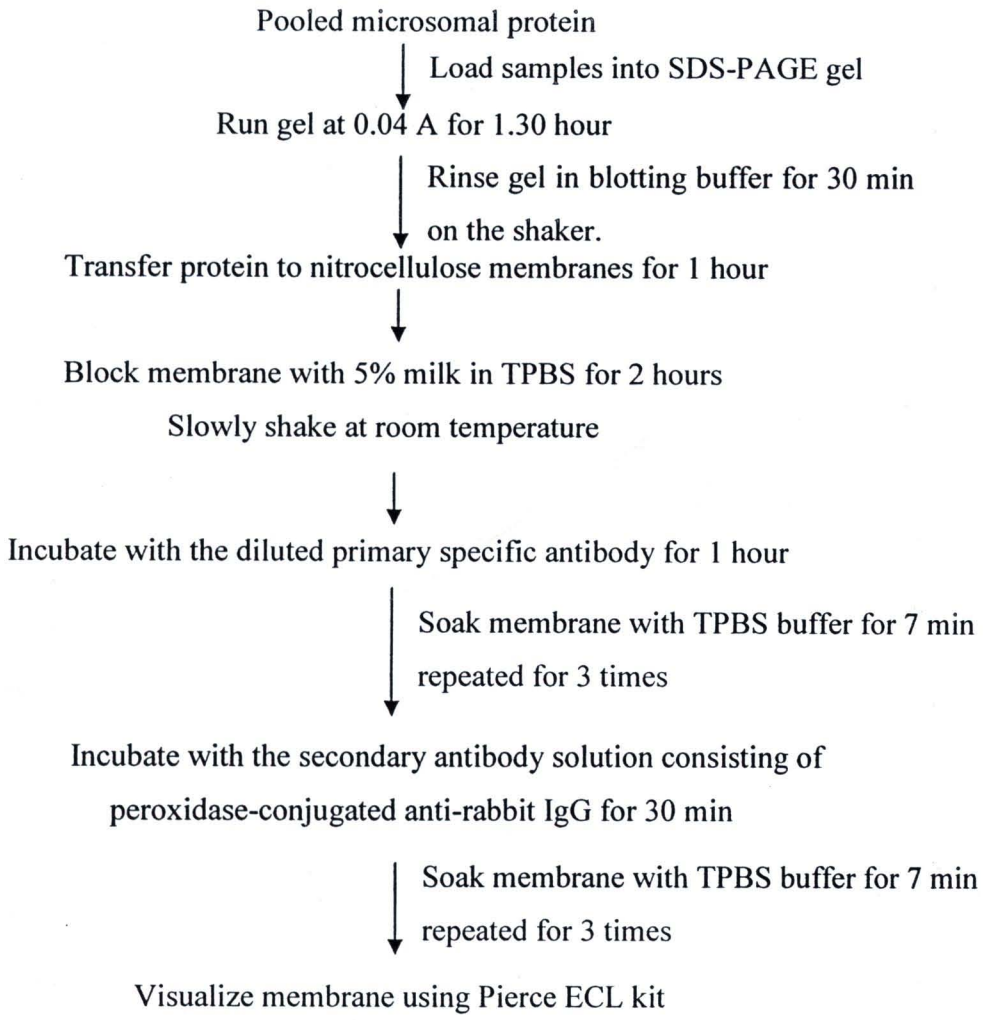


Figure 2-9 SDS-PAGE and Western blot procedures

2.15.3 The assay of NADPH- cytochrome P450 reductase activity

NADPH-cytochrome P450 reductase (CPR) transfers electrons from NADPH to cytochrome P450 and also catalyzes the one-electron reduction of many drugs and foreign compounds (Yim *et al.*, 2005). As the reduction of CPR is relatively difficult to measure directly, a simplified determination of enzyme activity is widely used utilizing exogenous oxidized form of cytochrome *c* as an artificial electron acceptor (Brankova *et al.*, 2007). It can be reduced to be a reduced form which has a maximal absorbtion at 550 nm. Therefore, the enzyme activity can be conveniently assayed by measuring the increase in absorbance at 550 nm.

NADPH: cytochrome P450 reductase activity was evaluated by using cytochrome *c* as an electron acceptor (Brankova *et al.*, 2007). The reaction was started by the addition of NADPH to the reaction mixture containing 0.3 M potassium phosphate buffer (pH 7.5), 1 mM cytochrome *c*, 50 mM KCN and microsomal protein. The rate of reduction was calculated by measuring the differential absorption coefficient of 21 $M^{-1} cm^{-1}$ at 550 nm. The enzyme activity was expressed as U/mg microsome protein.

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

2.15.4 The assay of Heme oxygenase activity assay

The heme oxygenase activity assay was determined as described by McNally *et al.* (2004) with modifications using hemin as a substrate. The reaction is performed at 37°C and pH 7.4. Bilirubin levels are then measured by a standard spectrophotometric method using the difference in absorption at 460 and 530 nm ($\varepsilon = 40\ mM^{-1}\ cm^{-1}$).

The heme oxygenase activity assay was determined by using hemin as a substrate. This assay was performed in a reaction mixture containing 50μM hemin, 0.8 mM NADPH, 2 mM G-6-P, 0.2U G-6-PD and 10 mg of liver cytosol, a source of biliverdin reductase. The reaction was prepared in 0.1 M potassium phosphate buffer with 2mM $MgCl_2$, pH 7.4. After being incubated in the dark at 37°C for 1 hour, the reaction was terminated 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.

2.15.5 The assay of UDP-Glucuronyltransferase assay

UDP-glucuronyltransferase activity assay was determined as described by Bratton and Marshall (1939) with modifications (Gordon and Skett, 1994). A useful compound to assess glucuronyl transferases activity is 2-aminophenol, because this phenol readily forms an *O*-linked glucuronide conjugate in the presence of microsomal fractions and UPD-glucuronic acid. The principle of the analytical method is based on the observation that when an aqueous solution of sodium nitrite is added to a cold, acidified solution of an aromatic amine, a dizonium salt is formed. Excess nitrite is removed by addition of ammonium sulfamate and the dizonium salt is finally reacted with *N*-naphthylethylene diamine, to produce a brightly colored azo compound that can analyzed spectrophotometrically at 540 nm.

The cofactor solution containing 0.1 M Tris buffer, 0.15 M $MgCl_2$, 1% Triton X-100, 0.02 M ascorbic acid and UDP-gucuronic acid was incubated with 1 mM 2-aminophenol and microsomal fraction at 37 °C for 30 min. The reaction was stopped with ice-cold 20% TCA. After adding 0.1% sodium nitrite, 0.5% ammonium sulfamate and 0.1% N-naphthylethylene diamine, the solution was mixed and kept at room temperature in the dark for 60 min. The reaction absorbance was measured in a spectrophotometer at 540 nm. The amount of product formed was calculated using a reference standard curve and the activity expressed as nmol product formed/min/mg protein.

$$\text{2-aminophenol + UDP-glucuronic acid} \xrightarrow{\text{UGT}} \text{2-aminophenyl glucuronide + UDP}$$

$$\text{2-aminophenyl glucuronide + sodium nitrite + ammonium sulfamate} \longrightarrow \text{dizonium salt}$$

$$\text{Dizonium salt + } \textit{N}\text{-naphthylethylene diamine} \longrightarrow \text{Azo compound (brightly color)}$$

2.15.6 The assay of NADPH quinone oxidoreductase assay

NQO activity was determined by the method of Benson *et al.* (1980), as described by Iqbal *et al.* (2003), using 2, 6-dichloroindophenol as an electron acceptor. The reaction mixture consisted of 0.025 M Tris-HCl buffer, 1.0 mg/ml BSA, 1 % Tween-20, 150 μM FAD, 30 mM β-NADPH, 24 mM 2, 6-dichloroindophenol, and cytosolic fraction for a total volume of 3.0 ml. 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 extinction coefficient of $2.1 \times 10^4\ M^{-1}cm^{-1}$. Protein concentration in all samples was determined by Lowry method.

$$\underset{\text{(Deep green)}}{\text{2, 6 dichloroindophenol+NADPH}} \xrightarrow{\text{NQO}} \underset{\text{(Colorless)}}{\text{reduced 2, 6 dichloroindophenol+NADP}^+}$$

2.15.7 The assay of Glutathione-*S*-transferase activity assay

The activity of cytosolic GST was determined by the method of Habig *et al.* (1974). GST catalyzes the reaction to conjugate glutathione to the CDNB substrate through the thiol group of the glutathione. The reaction product (GS-DNB Conjugate) absorbs at 340 nm.

The enzyme assay was carried out in a final volume of 1 ml containing 0.2 M phosphate buffer (pH 6.5), deionized water, 10mM GSH, diluted cytosolic protein and 10mM CDNB. The reaction started by the addition of the aromatic substrate. The change in absorbance was recorded at 340 nm at an interval of 30 sec for 5 min. The

enzyme activity was expressed as μmol CDNB-GSH conjugate formed/min/mg cytosolic protein using a molar coefficient of 9.6 $M^{-1}cm^{-1}$.

$$GSH + CDNB \xrightarrow{GST} GS\text{-}DNB\ Conjugate + HCl$$

2.15.8 Total protein determination

Protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The method combines the reactions of cupric ions with the peptide bonds under alkaline conditions with the oxidation of aromatic protein residues. This method is best used with protein concentrations of 0.01-1.0 mg/ml and based on the reaction of cuprous ion, produced by the oxidation of peptide bonds, with Folin's reagent in the Folin-Ciocalteu reaction. The concentration of the reduced Folin reagent is measured by absorbance at 750 nm.

According to this method, 200 μl of protein sample was pretreated with 1 ml of copper (II) in an alkaline copper solution stabilized with sodium potassium tartrate for 10 min at room temperature. After that 100 μl of diluted Folin Ciocalteu's phenol reagent was added to generate chromogens that give increasing absorbance at 750 nm. Total protein concentration in sample was calculated using a bovine serum albumin calibration curve (0-100 mg/ml) and express as mg protein/ml.

2.16 Statistical analysis

Data are reported as means ± SD of each variable for each group. Data were firstly tested for normality using a Kruskal-Wallis test. The significance of differences between groups was analyzed using one-way analysis of variance (ANOVA) with Bonferroni significance level adjustment for post hoc tests. Values of $p < 0.05$ were considered to be significant.