CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and instruments are shown in Appendix A and B

2.2 Preparation of *Cleistocalyx nervosum*

The fruit of *C. nervosum*, as present in Figure 1-10, was collected from Tambol Choeng Doi, Amphur Doi Saket, Chiang Mai, Thailand, in July-August, 2008. This plant was identified and confirmed by comparing with voucher specimens of known identities (QBG 7290, QBG 17340, QBG 25139 and Panell and Chantaranothai, 2002) in the Queen Sirikit Botanic Garden. The voucher herbarium specimen (Punvittayagul and Taya 1) was deposited at the Queen Sirikit Botanic Garden, Chiang Mai, Thailand. The pulp was manually separated from seed, weighed and stored in a freezer at -20 °C until analyzed.

2.3 Extraction of *Cleistocalyx nervosum*

One hundred gram of *C. nervosum* pulp was ground using a blender and followed by extraction with 50 ml of distilled water. The extract was centrifuged at 3,000 rpm for 15 minutes, filtered through a Whatman no. 1 filter paper and dried by lyophilizer. The final yield of aqueous extract was 8.48 g per 100 g fresh weight. Finally, the aqueous extract was kept in -20 °C freezer under light protection.

2.4 Determination of some chemical constituents in aqueous extract of *C. nervosum*

2.4.1 Determination of total phenolic compounds

The amount of total phenolic compounds was measured by the method described by Emmons *et al.* (1999) with some modifications. Briefly, the aqueous extract or standard solution of gallic acid was added to reaction mixture composed of 7% Na_2CO_3 and Folin-Ciocalteu reagent and then incubated at 45 °C for 15 minutes. After incubation, the reaction mixture was left at room temperature for 5 minutes and determined the absorbance at 750 nm. Total phenolic content of aqueous extract was relatively expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight.

2.4.2 Determination of total flavonoids

Determination of the flavonoid content was achieved using the method described by Maksimovic *et al.* (2005). After incubating 5% $NaNO_2$ with the extract or standard catechin solution for 10 minutes, 10% of $AlCl_3$ was added. Ten minutes later, 1 M NaOH was sequentially added to the reaction solution. After the final reaction mixture was left at room temperature for 5 minutes, the optical density at 532 nm was determined. Total flavonoid of the aqueous extract was relatively expressed as mg of catechin equivalents (CE) per 100 g of fresh weight.

2.4.3 Determination of condensed tannins

Condensed tannin content was evaluated using modified Vanillin-HCl assay, described by Sun *et al.*, (1998). The aqueous extract or standard catechin solution was added to mixture solution containing methanol and vanillin solution. The reaction mixture was incubated for 30 minutes at 30 °C and the absorbance at 500 nm was measured. Condensed tannin content of aqueous extract was relatively expressed as mg of catechin equivalents (CE) per 100 g of fresh weight.

2.4.4 Determination of anthocyanins

Anthocyanins content was determined by Assoc. Prof. Dr. Sugunya Wongpornchai. The detail is presented in Appendix D.

2.5 Determination of *in vitro* antioxidant activity

2.5.1 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity

The free radical-scavenging activity of the aqueous extract was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH (Braca *et al.*, 2001). Aqueous extract (1 mg/ml) or standard solution of trolox (0.1 IU) was incubated with 0.3 mg/ml DPPH solution for 30 minutes. Then, the reaction mixture was determined an absorbance at 517 nm. The decreasing of absorbance indicated an increase of the DPPH radical-scavenging activity, which could be calculated as follows:

$$\text{DPPH radical-scavenging activity (\%)} = \frac{A_{control} - A_{sample} \times 100}{A_{control}}$$

Where $A_{control}$ was an absorbance without extract and A_{sample} was an absorbance with extract. Trolox was used as a positive control.

2.5.2 Non-site-specific and site-specific hydroxyl radical-scavenging activity by deoxyribose assay

The colorimetric deoxyribose method can determine both non-site-specific and site-specific hydroxyl radical-scavenging activities of plant extracts (Soobrattee *et al.*, 2005). 2-Deoxyribose was oxidized when exposed to hydroxyl radicals generated by Fenton reagent (Cheng *et al.*, 2003 and Sakanaka and Ishihara, 2008). The oxidation degradation can be detected by heating the products with thiobarbituric acid (TBA) under acid conditions to develop a pink chromogen of thiobarbituric acid reactive substance (TBARs) (Cheng and Chang, 2003). The 650 μl of reaction mixture

composed of 120 mM KH_2PO_4-KOH, 33.6 mM deoxyribose, 12 mM $FeCl_3$: 2.4 mM EDTA, 1.2 mM ascorbic acid, 2.8 mM H_2O_2 and 50 mg/ml aqueous extract. Reaction mixture was incubated at 37 °C for 45 minutes. Then, 0.67 % (w/v) TBA was added to each mixture followed by the addition of 2.8% (w/v) trichloroacetic acid. The solutions were heated on a water bath at 100 °C for 15 minutes to develop the pink color of malondialdehyde-thiobarbituric acid (MDA–2TBA) adduct, and the absorbance was measured at 532 nm. Uric acid (1 mg/ml) was used as positive control for scavenging hydroxyl radicals and singlet-oxygen (Shukla *et al.*, 1996).

Site-specific hydroxyl radical-scavenging activity assay was employed to give supporting proofs of the iron-chelating properties for aqueous extract. The procedure was similar to an above assay without EDTA.

2.6 The evaluation the oral acute and subacute toxicities of *Cleistocalyx nervosum* in Wistar rats

2.6.1 Experimental animals

Male and female Wistar rats, weight range 150-180 g, were obtained from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. They were housed under standard environmental conditions of temperature at 24 °C under 12 hr dark-light cycle, and allowed free excess to drinking water and pelleted diet. An experimental protocol was approved by The Animal Ethics Committee of Faculty of Medicine, Chiang Mai University.

2.6.2 Acute toxicity test

The acute toxicity of the aqueous extract of *C. nervosum* was evaluated in rats using the fixed dose procedure (OECD, 2001). Ten rats were randomly divided into two groups of five animals per each gender. The aqueous extract at a single dose of 5000 mg/kg body weight was given orally to treated group, the control group received vehicle. Body weight, signs of toxicity and mortality were observed after the administration at the first, second, fourth and sixth hour and once daily for 14 days. On the day 14, all rats were fasted for 16–18 h, and then anesthetized with diethyl

ether. The internal organs were excised and weighed. The gross pathological observations of the tissues were performed.

2.6.3 Subacute toxicity test

The subacute toxicity of the aqueous extract of *C. nervosum* was evaluated in rats using repeated dose 28-day oral toxicity study in rodents (OECD, 2009). Thirty rats were randomly divided into three groups of five animals per each gender. Treatments were administered orally by gavage 5 times a week for 4 weeks. The first group, as a control, was received 4 ml/kg bw of water. The second and third groups were received the aqueous extract of *C. nervosum* at doses of 100 and 500 mg/kg bw, respectively. All rats were weighed and observed daily for physiological and behavioral changes.

Rats were anesthetized with diethyl ether on day 28. The heparinized blood samples were taken for determining white blood cell, hemoglobin, hematocrit, platelets, neutrophil, lymphocyte, monocyte, eosinophil and basophil. The serum from non-heparinized blood was carefully collected for blood chemistry and enzyme analysis. All rats were sacrificed after the blood collection. The internal organs were weighed to determine relative organ weights and observed for gross lesions.

2.7 The effect of *Cleistocalyx nervosum* extract on oxidative status in Wistar rats

Liver samples, obtained from male rats in subacute toxicity test, were determined for the effect of *C. nervosum* extract on oxidative status. Lipid peroxidation was a marker of oxidative stress while glutathione, glutathione peroxidase, glutathione reductase, catalase and heme oxygenase were antioxidant markers. The procedures for measurement these markers are presented in page 44 to 50.

2.8 The preliminary study for protocol of carcinogens induced oxidative stress in early stages of rat hepatocarcinogenesis

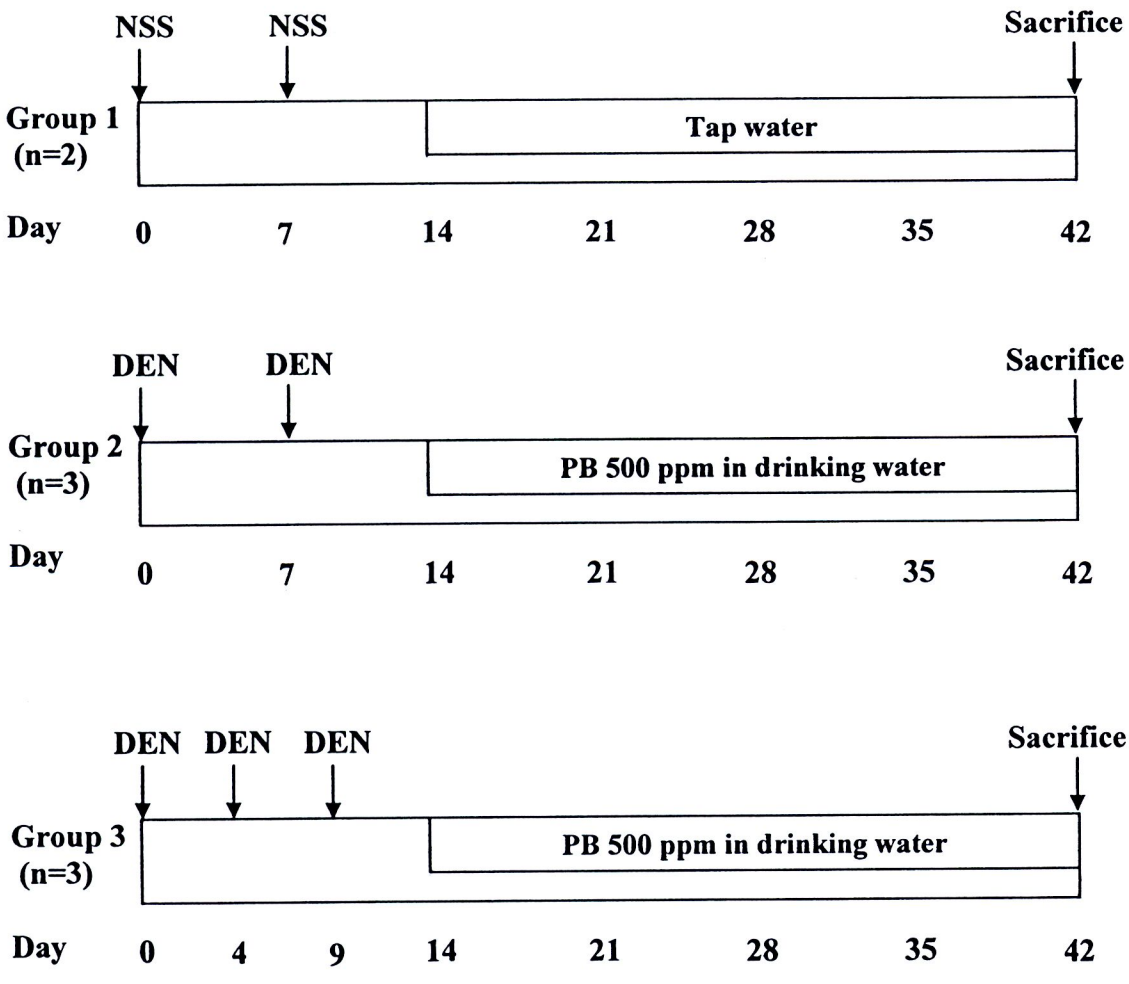
Dithylnitrosamine (DEN), a genotoxic carcinogen and Phenobarbital (PB), a nongenotoxic carcinogen were used in this study. The aim of this study was determined concentration of DEN to induce maximum oxidative stress in male rats

when co-treated with PB. The rats were randomly selected for 3 groups, comprising of 2-3 animals in each group. Group 1, as a negative control, administered a twice intraperitoneal injection of 0.9% normal saline solution and received tap water instead of PB administration. Group 2 was intraperitoneally injected by 100 mg/kg bw of DEN for 2 times on day 0 and 7 of an experiment. Group 3 was intraperitoneally injected by 100 mg/kg bw of DEN for 3 times on day 0, 4 and 9 of an experiment. On day 14 of an experiment, PB was incorporated in the drinking water of animals in these groups at 500 ppm for 4 consecutive weeks. At the end of experiment, rats were anesthetized under diethyl ether. Treatment protocol was shown in Figure 2-1. The TBARs formation in liver and serum as parameters of oxidative stress and Glutathione-*S*-transferase placental form (GST-P) positive foci, a preneoplastic lesion of rat hepatocellular carcinoma, in liver were determined.

2.9 Effect of aqueous extract of *C. nervosum* on oxidative stress induced early stage of hepatocarcinogenesis

2.9.1 Protocol I

Male wistar rats were divided into 5 groups. Group 1, a negative control, was injected with 0.9% normal saline solution for 3 times on day 0, 4 and 9 of an experiment. Rat received tap water on day 14 of an experiment. Group 2 to 5 was intraperitoneally injected by 100 mg/kg bw of DEN for 3 times on day 0, 4 and 9 of an experiment. On day 14 of an experiment, rats were received 500 ppm of PB in drinking water for 4 weeks. Group 2, a positive control, was orally fed 4 ml/kg bw of distilled water before the first injection for 2 weeks until the end of an experiment. Group 3 was fed with the aqueous extract before the first injection for 2 weeks until the end of experiment, while Group 4 and 5 were fed with 500 mg/kg bw of aqueous extract and 100 mg/kg bw of silymarin in the same time of PB administration for 4 weeks. Silymarin is used as a standard antioxidant agent for comparison in the evaluation of hepatoprotective effects of plant extract (Dhiman and Chawla, 2005). Treatment protocol is shown in Figure 2-2. All rats were weighed once a week. The amount of diet and water consumption was measured twice a week. At the end of the



NSS: 0.9% Normal saline solution 4 ml/kg bw, ip

DEN: Diethylnitrosamine 100 mg/kg bw, ip

PB: Phenobarbital 500 ppm in drinking water

Figure 2-1 The protocol for the preliminary study of effect of diethylnitrosamine concentration on oxidative stress induced early stage hepatocarcinogenesis.

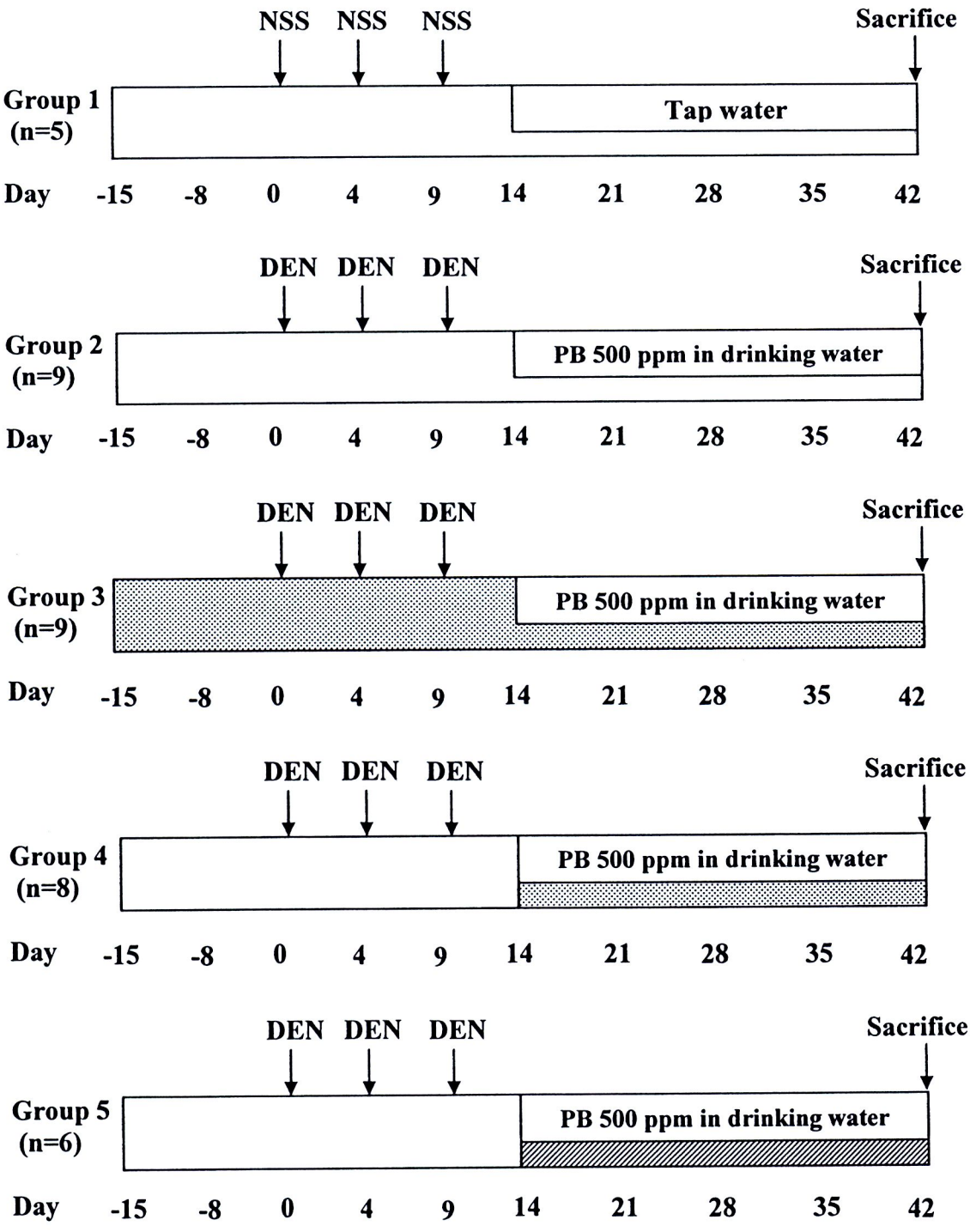


Figure 2-2 Treatment protocol I for the effect of aqueous extract of *C. nervosum* on oxidative stress induced early stage of hepatocarcinogenesis

study, the rats were anesthetized under diethyl ether. The whole blood was taken from abdominal vein. The serum was separated from whole blood by centrifugation at 3,000 rpm for 15 min at 4 °C and collected into microcentrifuge tube for biochemical determination. The liver, spleen and kidney tissues were removed and weighted in gram unit express in relative organ weight. Three lobes of the liver were fixed in 10% formalin solution for histological evaluation and the remaining portion was frozen and stored at -80 °C until analysis.

2.9.2 Protocol II

Male wistar rats were divided into 4 groups. Group 1, a negative control, was injected with 0.9% normal saline solution twice a day on day 0 and 7 of the experiment. Rat received tap water on day 14 of experimental days and throughout the experiment. Groups 2 to 4 was intraperitoneally injected with 100 mg/kg bw of DEN twice a day on day 0 and 7 of experimental days. On day 14 of an experiment, rats received 500 ppm of PB in drinking water for 4 weeks. Group 2, a positive control, was orally fed with 4 ml/kg bw of distilled water for 2 weeks before the first injection of DEN and throughout the experiment. Groups 3 and 4 were fed with 500 and 1000 mg/kg bw of aqueous extract, respectively for 2 weeks before the first injection of DEN and throughout the experiment. Treatment protocol was shown in Figure 2-3. All the rats were weighed once a week. The amount of diet and water consumption was measured twice a week. At the end of the study, the rats were anesthetized under diethyl ether. The whole blood was taken from the abdominal vein. The serum was separated from the whole blood by centrifugation at 3,000 rpm for 15 min at 4 °C and collected into microcentrifuge tube for biochemical determination. The liver, spleen and kidney tissues were removed and washed thoroughly with saline and weighed in gram unit express in relative organ weight. Three lobes of the liver were fixed in 10% formalin solution for histological evaluation and the remaining portion was frozen and stored at -80 °C until analysis.

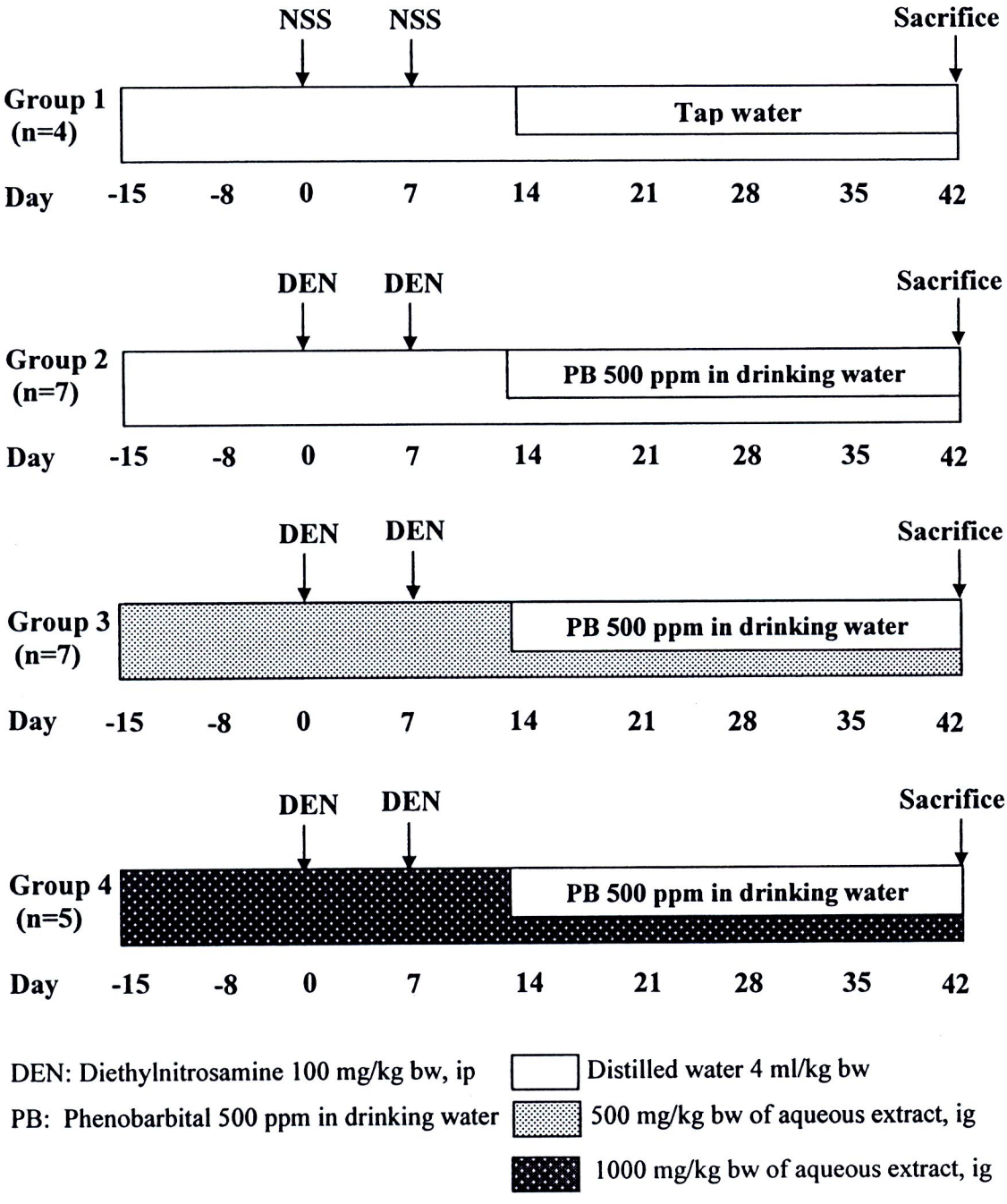


Figure 2-3 Treatment protocol II for the effect of aqueous extract of *C. nervosum* on oxidative stress induced early stage of hepatocarcinogenesis

2.10 Determination of oxidative status and antioxidant markers

2.10.1 Determination of lipid peroxidation

A thiobarbituric acid reactive substance (TBARs) is a well established assay for screening and monitoring lipid peroxidation. MDA forms a 1:2 adduct with thiobarbituric acid (Figure 2-4). The MDA-TBA adduct formed from the reaction of MDA in samples with TBA can be measured colorimetrically. TBARs levels are determined from a malondialdehyde equivalence standard (Esterbauer *et al.*, 1991).

The levels of hepatic MDA were determined using the method of Ohkawa *et al* (1979). Firstly, the homogenate was precipitated in 50% TCA. The supernatant was collected by using centrifugator at 6,000 rpm, 4 °C for 20 minutes. Two ml of 0.67% thiobarbituric acid was added into collected supernatant. The mixture was heated at 100 °C for 10 minutes. The reaction was stop by placing on ice and adding butanol. Finally, the absorbance of the resulting solution was determined spectrophotometrically at 532 nm. The amount of TBARs was quantified and used as an index of lipid peroxidation.

The levels of serum MDA were determined using the method of Fujiwara (2003). Briefly, serum was mixed with 3 N sulfuric acid and 10 % phosphotungstric acid. After standing at room temperature for 5 minutes the mixture was centrifuged at 3,000 rpm for 15 minutes. The supernatant was discarded and the sediment thoroughly mixed with 3 N sulfuric acid and 10 % phosphotungstric acid. Then the centrifugation was repeated. The sediment was then resuspended in fresh thiobarbituric acid reagent (0.33 % thiobarbituric acid in glacial acetic acid); mixed thoroughly, and heated at 100 °C for 1 hour in a water bath, cooled and extracted with 2-butanol. After centrifugation at 3,000 rpm for 15 minutes, supernatant was measured at 530 nm in a spectrophotometer. The amount of TBARs was quantified and used as an index of lipid peroxidation.

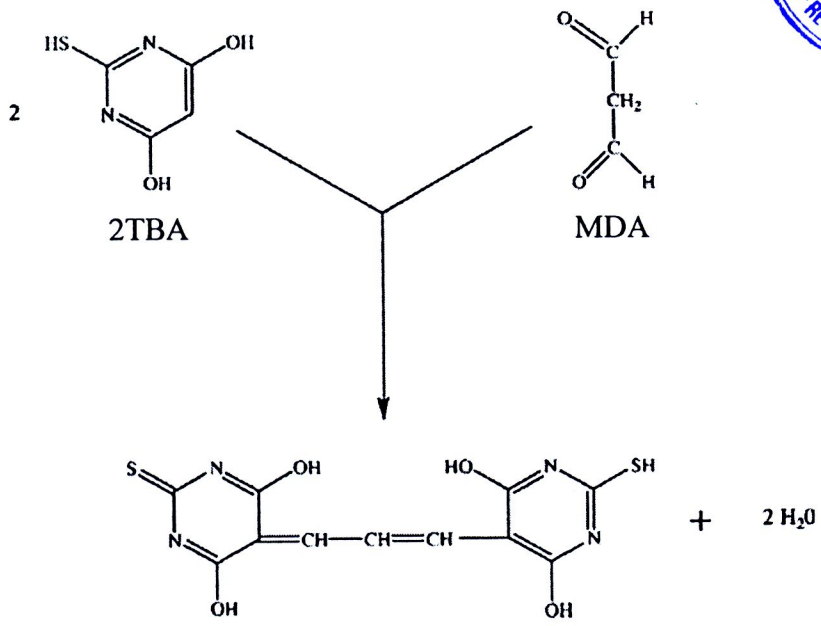


Figure 2-4 Principle of thiobarbituric acid reactive substances (TBARs) assay (http://books.google.co.th/books?id=6tdn_kdESXgC&printsec=frontcover&source=gbs_summary_s&cad=0#v=onepage&q&f=false)

2.10.2 Determination of total glutathione

Reduced glutathione (GSH) interacts with 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) to form the colored product 2-nitro-5-thiobenzoic acid and oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase to form GSH (Figure 2-5) (Shaik and Mehvar, 2006).

Glutathione was determined by the method of Akerboom and Sies (1981). Briefly, the liver homogenate was centrifuged at 14,000 rpm for 30 minutes at 4 °C and supernatant was collected. Two hundred µl of supernatant was deproteinized with 5% metaphosphoric acid (MPA), then the mixture was centrifuged at 14,000 rpm for 30 minutes at 4 °C. The reaction mixture was composed of deproteinized supernatant, 10 mM sodium phosphate buffer containing 5 mM EDTA (pH 7.5), 4 mM β-NADPH, 6 U glutathione reductase, and 10 mM DTNB in a total volume of 200 µl. The yellow color developed, was read immediately at 405 nm on a microplate

reader after incubation at 37 °C for 30 minutes. Glutathione concentration in samples was calculated using a calibration curve and expressed as nmol/mg protein.

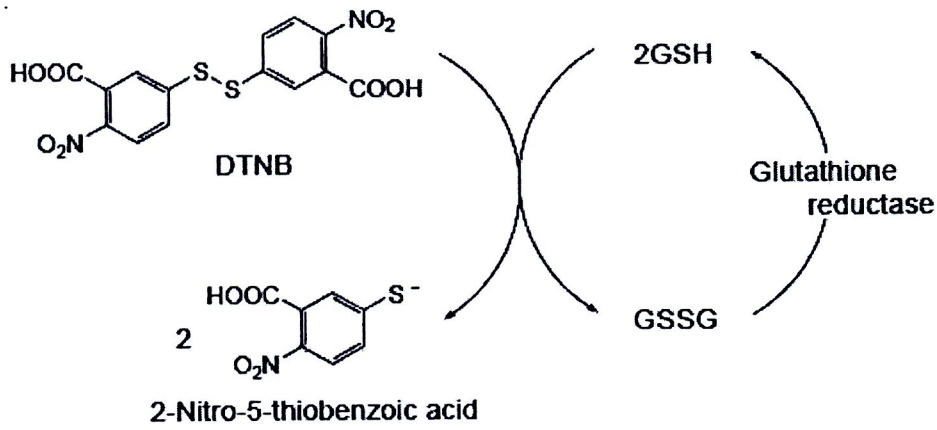


Figure 2-5 The principle of glutathione measurement (www.dojindo.com/newimages/TGQKTechnicalInformation.pdf)

2.10.3 Preparation of the cytosolic and microsomal fractions

One gram of liver sample was homogenized in 3 ml of homogenizing buffer containing 1.15% w/v KCl and 0.25 mM PMSF. The homogenate was centrifuged at 14,000 rpm for 30 minutes at 4 °C. The supernatant was further centrifuged at 30,000 rpm for 60 minutes at 4 °C to obtain the cytosolic and microsomal fractions. The cytosolic supernatant was aliquoted and frozen at -20 °C until use. The pellet was washed in homogenizing buffer. Finally, the pellet was resuspended in 1000 µl of microsome suspension buffer containing 30% v/v glycerol pH 7.4 and 1 mM dithiothreitol, and stored at -80 °C until analysis. The cytosolic and microsomal protein was determined by Lowry method.

2.10.4 Glutathione peroxidase activity assay

Glutathione peroxidase (GPx) plays important role in protecting of organisms from oxidative damage. It converts GSH to GSSG, to reduce lipid hydroperoxides to their corresponding alcohols, or reduce free hydrogen peroxide to water (Figure 2-6;

reaction 1). GPx activity was measured through a coupled reaction with glutathione reductase. GPx reduces *tert*-butylhydroperoxide (*t*-BHP), and oxidizes GSH to GSSG. The generated GSSG is reduced to GSH with consumption of β-NADPH by glutathione reductase. The decrease of β-NADPH is proportionally to GPx activity in the reactions. The decrease of β-NADPH can be estimated by absorbance at 340 nm.

GPx activity was determined according to method described by Nagalakshmi and Prasad (2001) with slight modifications. The reaction mixture was contained 0.1 M Tris-EDTA buffer (pH 8), 0.1 M GSH, 2 mM β-NADPH, 7 mM *t*-BHP, 10 U Glutathione reductase and 10 µl of sample. The oxidation of β-NADPH was followed by measuring the decrease in the absorbance at 340 nm.

2.10.5 Glutathione reductase activity assay

Glutathione reductase (GR) is a flavoprotein that is required for the conversion of GSSG to GSH which plays an important role in oxidoreduction processes and detoxification of biological substances which are produced during inflammatory processes in living cells. At the same time, it oxidizes β-NADPH (Figure 2-6; reaction 2). GR therefore plays a major role in glutathione peroxidase and glutathione-*S*-transferase reactions to control peroxides and free radicals. When level of catalase is decreased, the glutathione reductase becomes activated.

GR activity was determined according to Carlberg and Mannervik (1985). The reaction mixture contained with 100 mM Potassium phosphate buffer (pH 7.0), 1.2 mM GSSG, and 1.2 mM β-NADPH. Decrease in the absorbance of β-NADPH at 340 nm was monitored spectrophotometrically, at 37 °C. A unit of activity (U) was defined as the amount of enzyme that catalyzes the oxidation of 1 µmole of NADPH in 1 minute under these conditions. Specific activity is defined as units per mg of protein.

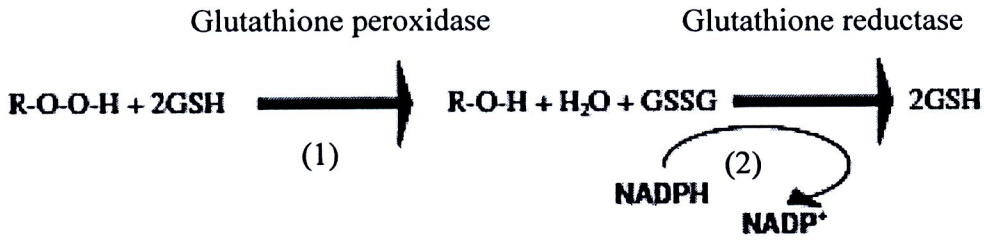


Figure 2-6 Principle of glutathione peroxidase (1) and glutathione reductase (2) activities assay (www.zeptometrix.com/0805002.pdf)

2.10.6 Catalase activity assay

Catalase (CAT) decomposes hydrogen peroxide into water and oxygen. CAT activity was measured according to Aebi, H (1984). The reaction mixture contained with 30 mM H_2O_2, 50 mM phosphate buffer, pH 7.0 and sample in a total volume of 750 µl. CAT activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm.

2.10.7 Heme oxygenase activity assay

Heme oxygenase (HO) is a potent antioxidant enzyme and is important in the cellular response to oxidative injury. HO is the rate-limiting enzyme in the conversion of heme into biliverdin, releasing free iron and carbon monoxide. Biliverdin is rapidly metabolized to bilirubin, which is a potent antioxidant (Figure 16). It is likely that HO activity is a component of the cellular defense mechanism against oxidative stress (Chen *et al.*, 2005; Nuhn *et al.*, 2009). The end products of HO are carbon monoxide, biliverdin, and free iron. Biliverdin can be subsequently converted to bililubin by biliverdin reductase (Figure 2-7). Bilirubin levels are then measured by a spectrophotometric method using the difference in absorption at 460 and 530 nm (Farombi *et al.*, 2008).

The reaction mixture containing 50 µM of the substrate hemin, microsomal fraction and rat liver cytosol as a source of biliveridin reductase, 2 mM glucose-6-phosphate, 0.2 U/ml glucose-6-phosphate dehydrogenase, 0.8 mM NADPH and 100

mM potassium phosphate buffer containing $MgCl_2$, pH 7.4 was incubated at 37 ^{O}C for 1 hour. The reaction was stopped with chloroform and after extraction; the chloroform layer was measured spectrophotometrically. Bilirubin formation was calculated from the difference in absorption between 460 and 530 nm.

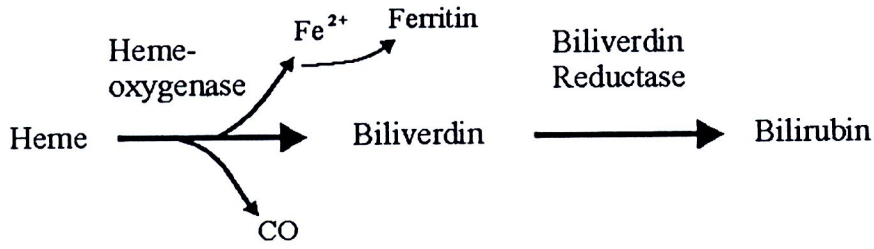


Figure 2-7 Enzymatic reaction of heme oxygenase (http://herkules.oulu.fi/isbn9514 266625/html/x494.html)

2.10.8 Total protein determination

Lowry method was the most widely used for protein quantitation. The procedure involves reaction of protein with cupric sulfate and tartrate in an alkaline solution, resulting in formation of tetradentate copper-protein complexes. The Folin-Ciocalteu reagent is added, it is effectively reduced in proportion to these chelated copper complexes, producing a water-soluble product whose blue color can be measured at 750 nm (Lowry *et al.*, 1951). The Lowry method is sensitive to low concentrations of protein between 1-100 μg/ml.

One ml of each standard solution and test solution were added to 1 ml of reagent composed of copper (II) in an alkaline copper solution stabilized with sodium potassium tartrate, mix and allowed standing at room temperature for 10 minutes. One hundred micro liters of the Diluted Folin-Ciocalteu's Phenol reagent was added, mixed and allowed standing at room temperature for 30 minutes. The absorbance of the solutions was determined at the wavelength of maximum absorbance at 750 nm using the solution from the blank to set the instrument to zero.

2.11 Glutathione-*S*-transferase P form positive foci

The rat glutathione-*S*-transferase placental form (GST-P) positive foci has been found to be dramatically up-regulated in its expression in preneoplastic and neoplastic cells and is widely used as a specific marker in the basic analysis of chemical carcinogenesis. However, the mechanism by which the expression of GST-P is induced in the rat liver during carcinogenic treatment has remained to be solved (Higashi *et al.*, 2004). GST-P in the rat is markedly induced in preneoplastic foci and nodules, and is detected in a single cell as early as 2-3 days after the administration of the chemical carcinogen. Although GST-P belongs to phase II detoxification enzymes and is a member of glutathione transferase isozymes, this enzyme is not induced by eletrophilic xenobiotics which induce the expression of other GST isozymes. These expression patterns indicate that the expression of GST-P is closely associated with the early stage of hepatocarcinogenesis (Sakai and Muramatsu, 2007).

Liver tissues were embedded in paraffin, cut at 3 μm thickness and stained for immunohistochemical process. Briefly, GST-P immunoreactivity detection was performed as follows: deparaffinized liver sections on poly-L-lysine-coated slides were successively treated with 3% H_2O_2 for 5 minutes, skim milk for 30 minutes, polyclonal anti-rabbit GST-P antibody (1:1000 dilution) at 4 °C overnight, biotinylated IgG anti-rabbit antibody for 30 minutes, and avidin-biotin-peroxidase solution for 30 minutes. Chromogen color development was accomplished with diaminobenzidine as the substrate to demonstrate the sites of peroxidase binding. The slides were counter stained with Harris's hematoxylin. The number of GST-P positive foci was counter under light microscope.

2.12 Statistical analysis

Data expressed as mean ± SD of each variable for each group were firstly tested for normality using Kruskal-Wallis test. The significance of differences between groups was analyzed using the analysis of variance (ANOVA) with Bonferroni for post hoc test. P value < 0.05 was regarded as significance.