Chapter 2
Materials and Methods

2.1 Chemicals Show in appendix 1.

2.2 Extraction of *Murdannia loriformis*

Murdannia loriformis was obtained from the *Murdannia loriformis* field at Mae Rim District, Chiang Mai, Thailand. The whole fresh plant was washed with tap water, cut in small pieces, freeze-dried and ground to fine powder. 100g dried powder was extracted with 1 liter of 80% ethanol for 24 hr. by stirring at room temperature. After filtrating by suction, the residue was re-extracted again with 80% ethanol. The combined filtrate was evaporated to dryness by vacuum rotatory evaporator at 50°C. The stickily residue was weighed, dissolved in known amount of 25% dimethylsulfoxide (DMSO) and kept at 4°C until used. The scheme of extraction method is shown in Figure 4.

2.3 Animal

Four-week-old male F344 rats (weight 80-100g) were purchased from SLC Japan (Hamamatsu, Japan). The rats were housed in plastic cages with wire tops and sawdust bedding, under a controlled temperature (23 ± 2°C), humidity (55 ± 10%) and a 12 hr light 12 hr dark photoperiod at the Institute of Animal Experimentation, School of Medicine, The University of Tokushima, Japan. The animals were acclimated to the standard laboratory chew diet for 1 week before beginning the experiment.

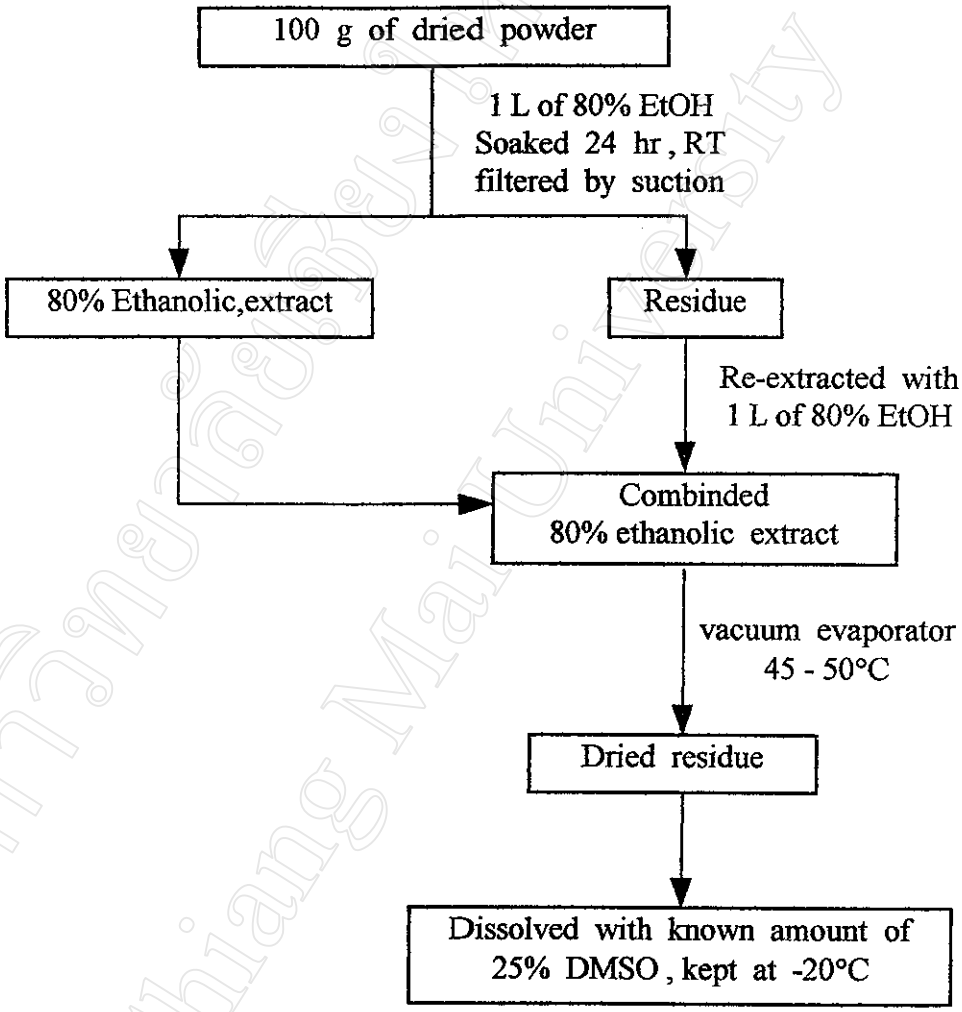

Figure 4. Protocol for extraction of *Murdannia loriformis*

2.4 Effect of *M. loriformis* Extract on Aberrant crypts foci (ACF) formation

The protocol of the study effect of *M. loriformis* on AOM-induced ACF formation at initiation stage is shown in Figure 5. The rats were devided into 7 groups with 6-8 rats per group (group 1 and 5 received 25% DMSO and group 2, 3, 4, 6 and 7 received *M. loriformis* extract) The rats were fed by P.O. intubation in the morning, then were provided diet and water *ad libitum*. After 1 week of the treatment, the rats in groups 1, 2, 3 and 4 received azoxymethane (AOM) at 15 mg/kg bw. by subcutaneous injection once per week for 2 weeks. Other groups were administered an equal volume of saline as a vehicle control. Three weeks after the second AOM injection they were sacrified by cervical dislocation or anesthesized by dimethyl ether.

The protocol for studying the effect of *M. loriformis* at promotion stage is shown in Figure 6. Two weeks after having been injected with the second dose of AOM the rats were then given *M. loriformis* extract at 0.1 or 1.0 g/kg bw. by P.O. intubation in the morning for the next 12 weeks. The rats were weighed every week. The experiments were terminated 16 weeks after initial dose of AOM.

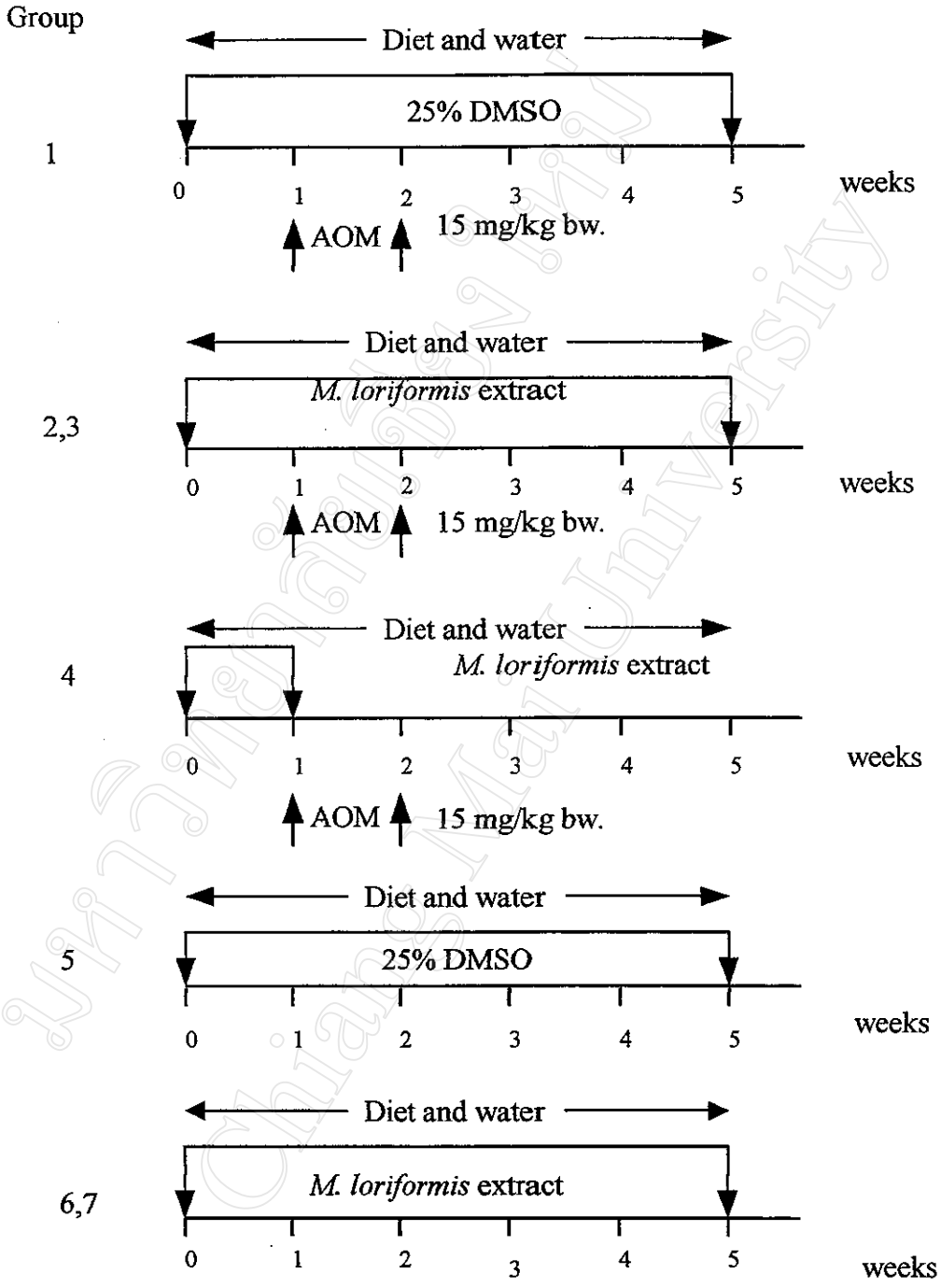

Figure 5. Protocol for studying the effect of *M. loriformis* extract on AOM-induced ACF formation at initiation stage

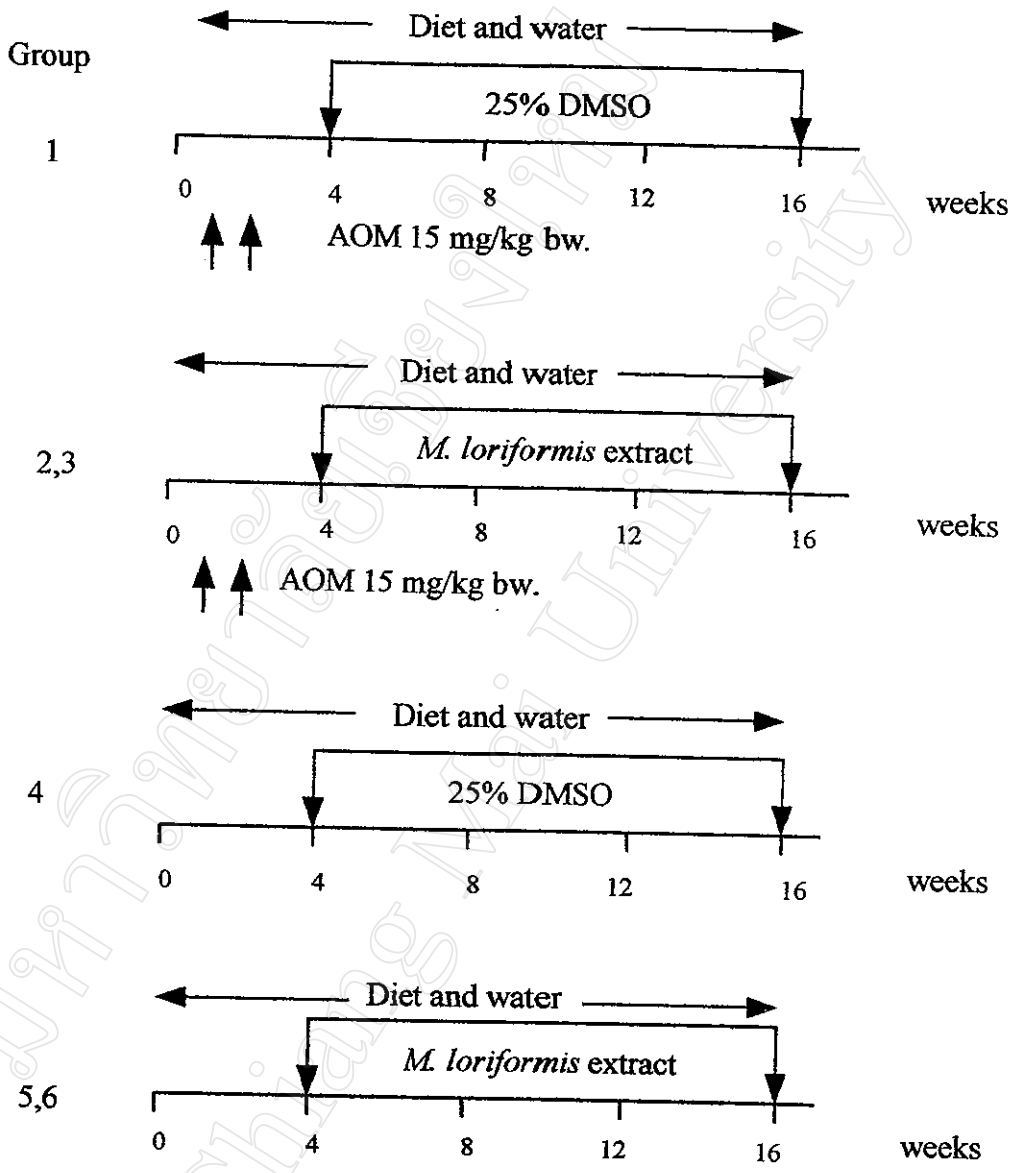

Figure 6. Protocol for studying the effect of *M. loriformis* extract on AOM-induced ACF formation at the promotion stage

2.4.1 Aberrant crypt foci (ACF) evaluation

The colons were evaluated for aberrant crypts foci formation as described by Bird (Bird, 1987). The colons were removed after 10% formalin-phosphate buffer saline (10% formalin-PBS) pH 7.4 was injected into them so as to be fixed in an expanded state. They were then split open along the longitudinal axis and cut separately into three parts, rectum (2 cm. from annus) and remaining colon into proximal and distal colon segments. The pieces of colon were fixed flat between filter papers and kept in the same 10% formalin-PBS pH 7.4 mentioned above. The ACF was obtained after staining with 0.2% methylene blue under a light microscope at a magnification of 10x.

2.5 Effect of *M. loriformis* Extract on DNA adduct formation

2.5.1 Induction of DNA adduct by AOM

Groups 4 and 6 rats were fed with laboratory chew diet and water *ad libitum*. Groups 1 and 5 were fed with 25% DMSO as control. Groups 2, 6 and 3, 7 were fed by P.O. intubation in the morning with *M. loriformis* extract at 1.0 and 0.1 g/kg bw., respectively, while group 4 was given 1.0 g/kg bw. of the extract during 1 week only. After receiving the extract for 1 week, groups 1, 2, 3 and 4 were injected with AOM as in experiment 2.4. All rats were sacrified 12 hr after the second AOM injection. The protocol of the experimental design is shown in Figure 7. The perfused liver and colon were immediately removed. The colon was cut open longitudinally and washed with saline to remove the colonic contents. The livers and colons were kept at -80°C until analysis for DNA adducts was performed.

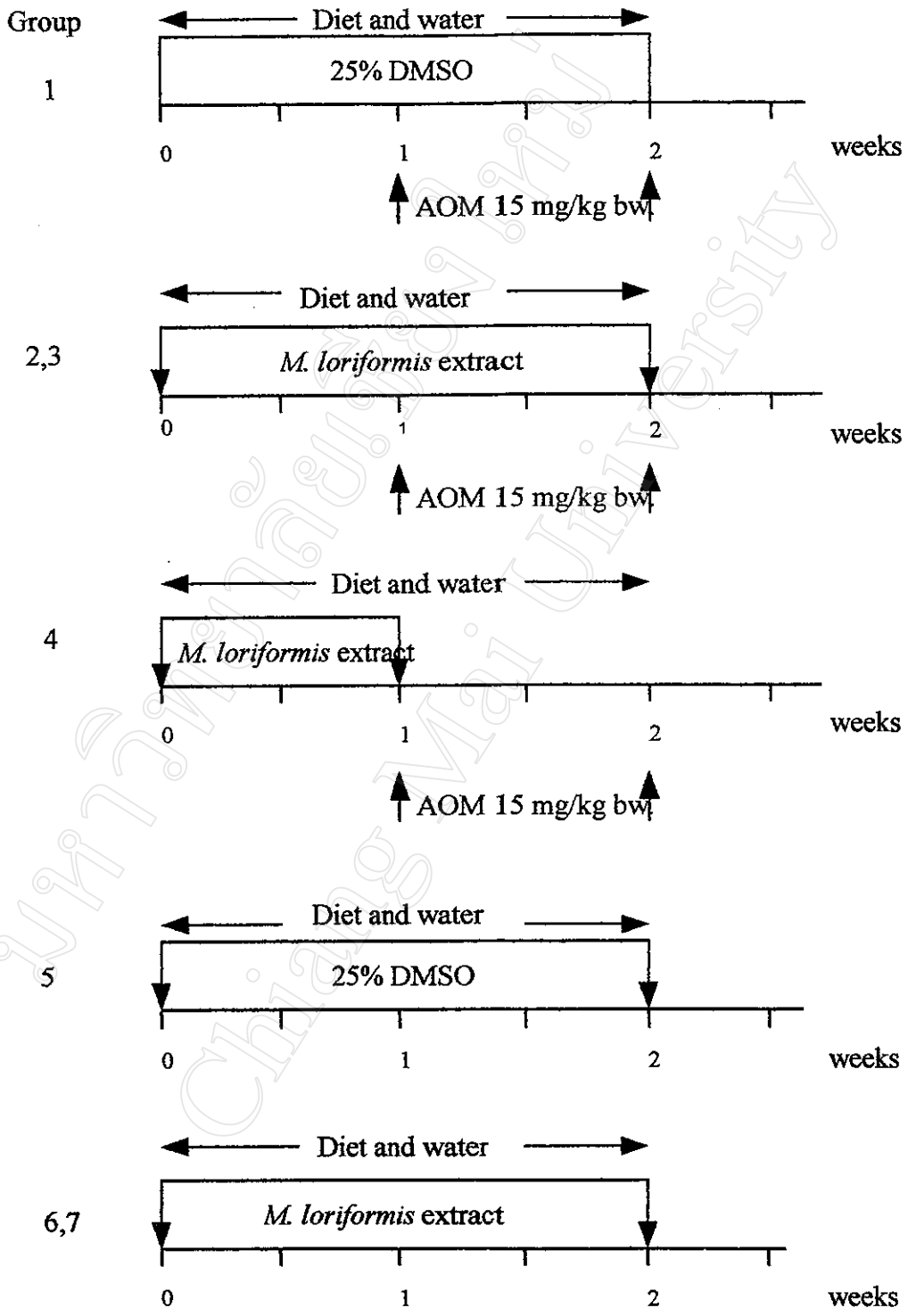

Figure 7. Protocol for studying the effect of *M. loriformis* extract on AOM-induced DNA adduct formation

2.5.2 Determination of DNA adducts formation

2.5.2.1 Isolation of DNA

The colon was laid flat on a glass plate and the mucosa was scraped off with a glass slide. Since the transportation of AOM-metabolites to colon occurred either via bile or the blood stream, DNA adducts in both colonic mucosa and colonic muscle were analyzed. Tissue DNA from the liver and colon were isolated with a phenol extraction method, as follows:

1. The frozen tissue (0.5 g) was thawed in 3 ml of 1.0% sodium dodecyl sulfate (SDS), 10 mM EDTA and 20 mM Tris-HCl (pH 7.4), and then homogenized with polytron homogenizer for 30 seconds.

2. The homogenate was incubated at 30°C for 30 min with RNase A and RNaseT1 at a mixture concentration of 200 µl/ml and 33.4 U/ml, respectively.

3. Proteinase K (500 µg/ml) was added and subsequently incubated at 37°C for 30 min.

4. The mixture was extracted successively with 1 vol (3 ml) of phenol saturated with 0.1 M Tris-HCl (pH 8.0), and a 1:1 mixture of phenol/sevag (chloroform/isoamyl alcohol, 24:1), and sevag. The extraction step was done in 15 ml polypropylene tubes.

5. The aqueous phase was then separated by centrifugation at 3000 rpm. at 4°C for 30 min.

6. After addition of 0.1 vol of 5 M NaCl, DNA was precipitated by the gradual addition of 1 vol of cold absolute ethanol (pre-cooled at -20°C).

7. The resulting emulsion was inverted several times, and then kept at -20°C, 4-5 hours, for complete DNA precipitation.

8. The DNA was washed by centrifuge at 3000 rpm., at 4°C for 10 min with 70% ethanol, 2 times.

9. DNA pellet was dissolved in 1 ml of 1/10 NaCl/Citrate - 0.1 mM EDTA and re-digested. Extraction steps 2 through 9 were then repeated again.

10. The DNA content was determined spectrophotometrically at 260 nm using an extinction coefficient at 50 μg/ml. The solution was stored at -80°C until hydrolysis.

The protocol for DNA extraction is shown in Figure 8.

2.5.2.2 DNA hydrolysis

The purified DNA was subjected to neutral hydrolysis by adding 10 mM sodium cacodylate (pH 7.0, 5 mg DNA/ml buffer) and boiled at 100°C for 30 min to release N^7-methylguanine (Beaker *et al.*, 1981). The partially apurinic DNA was precipitated by addition of 0.1 vol cold 1.0 M HCl, and then centrifuged at 0°C, 3000 rpm for 20 min. The pellet was suspended in 50 mM Bis-Tris 1 mM $MgCl_2$ (pH 6.5) and further hydrolyzed by acid hydrolysis using 0.1 vol of 1.0 N HCl. It was then boiled at 70°C for 30 min to yield pyrimidine nucleotide and free purine bases. Neutral thermal and acid hydrolysate were analyzed for specific adducts by High Performance Liquid Chromatography (HPLC). The scheme for this method is shown in Figure 9.

2.5.2.3 HPLC analysis of DNA-adduct

50 μl of the DNA hydrolysate was analyzed with a Shimadzu LC-5A HPLC, 7 SCX cation exchange chromatography column, with 4 mM ammonium formate (pH 3.0) as the mobile phase, at flow rate 1.0 ml/min. Elution of fluorescing base was monitored with a Shimadzu fluorescence HPLC detector using a 280 nm excitation wavelength and a 365 nm emission wavelength.

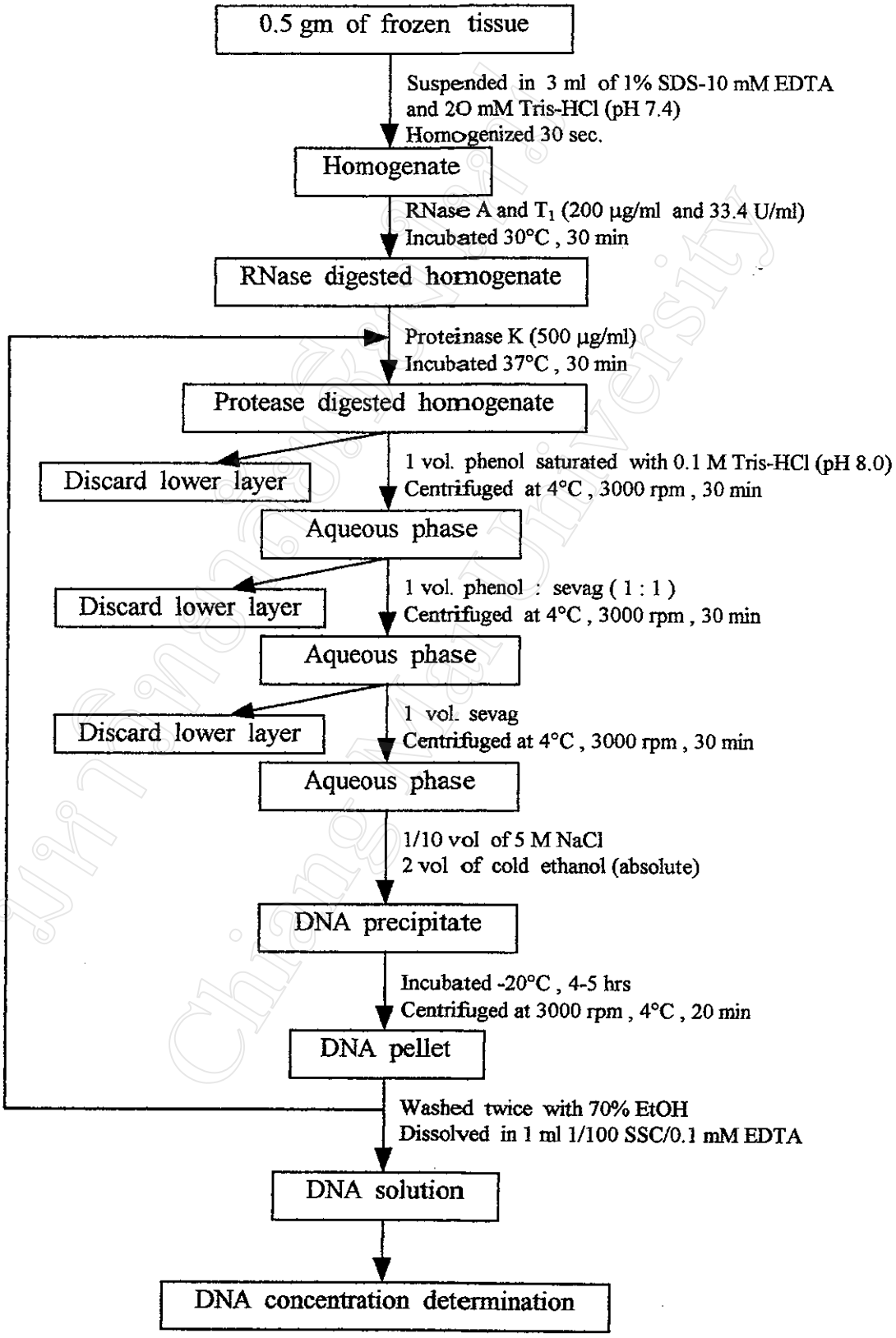

Figure 8. Protocol for DNA extraction

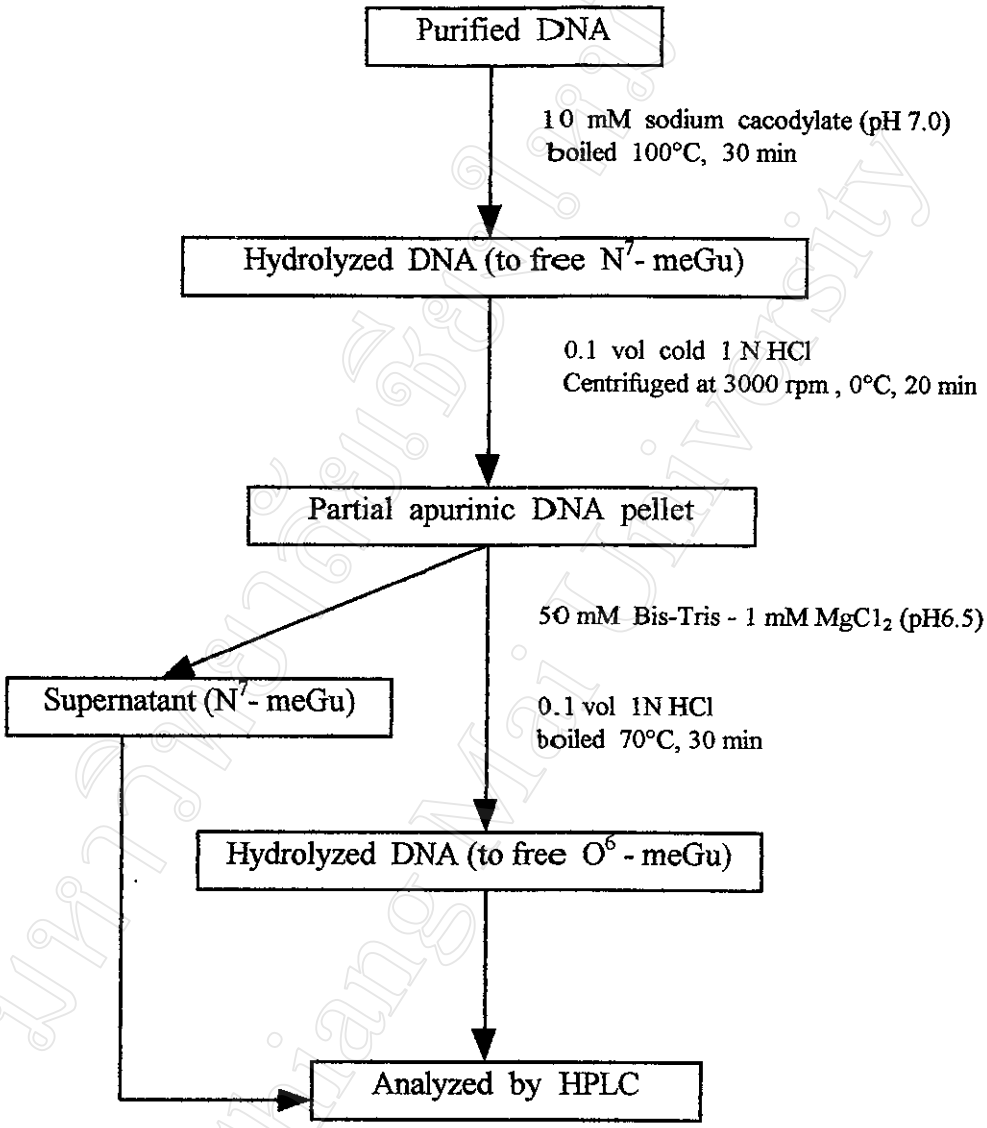

Figure 9. Protocol for preparing DNA solutions for HPLC analysis

2.6 Assay for antioxidant activity of *M. loriformis* extract

Rabbit erythrocyte membrane ghost was prepared by the method of Osawa (Osawa *et al.*, 1987). The erythrocyte membrane ghost suspension in PBS (pH 7.4) was incubated with 20 mM *tert*-buthylhydroperoxide (*t*-BHP) at 37°C for 30 min in the presence and absence of *M. loriformis* extract. Malondialdehyde (MDA) formation in the reaction mixture was determined by thiobarbituric acid (TBA) assay (Draper *et al.*, 1990). The reaction mixture was added with 0.35 ml of 20% trichloroacetic acid (TCA) and 0.35 ml of 0.8% TBA, then mixed well, and boiled at 100°C for 15 min. After cooling under tap water, malondialdehyde formation was calculated from the absorbance at 532 nm by using an extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$ (Aust and Buege, 1978). The scheme for the experiment is shown in Figure 10.

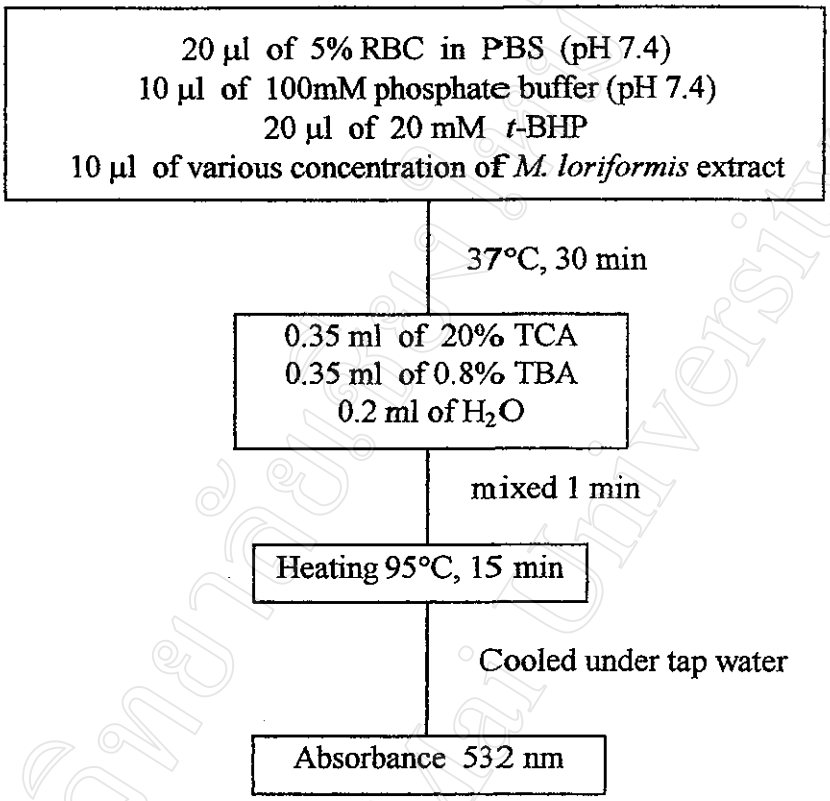

Figure 10. Diagramatic represent a protocol for antioxidant assay

2.7 Effect of *M. loriformis* extract on some drug metabolizing enzyme

Male Wistar rats, weighing 70-80 g, from National Laboratory Animal Centre, Mahidol University, Thailand were used in this study. The protocol for this experiment is shown in Figure 11. All rats were divided into 4 groups, with 20 rats in each group. The rats were fed with laboratory chew diet (Charuenphokaphun Comp., Bangkok, Thailand) and water *ad libitum*. Group 1 was fed with 25% DMSO as control. Group 2 and 3 were fed with *M. loriformis* extract at 1.0 and 0.1 g/kg bw. respectively, while group 4 was given 1.0 g/kg bw. of the extract for 7 days only. After receiving the extract for 10 days, ten rats from each group were sacrified. The remaining rats were sacrified after the received the extract for 30 days.

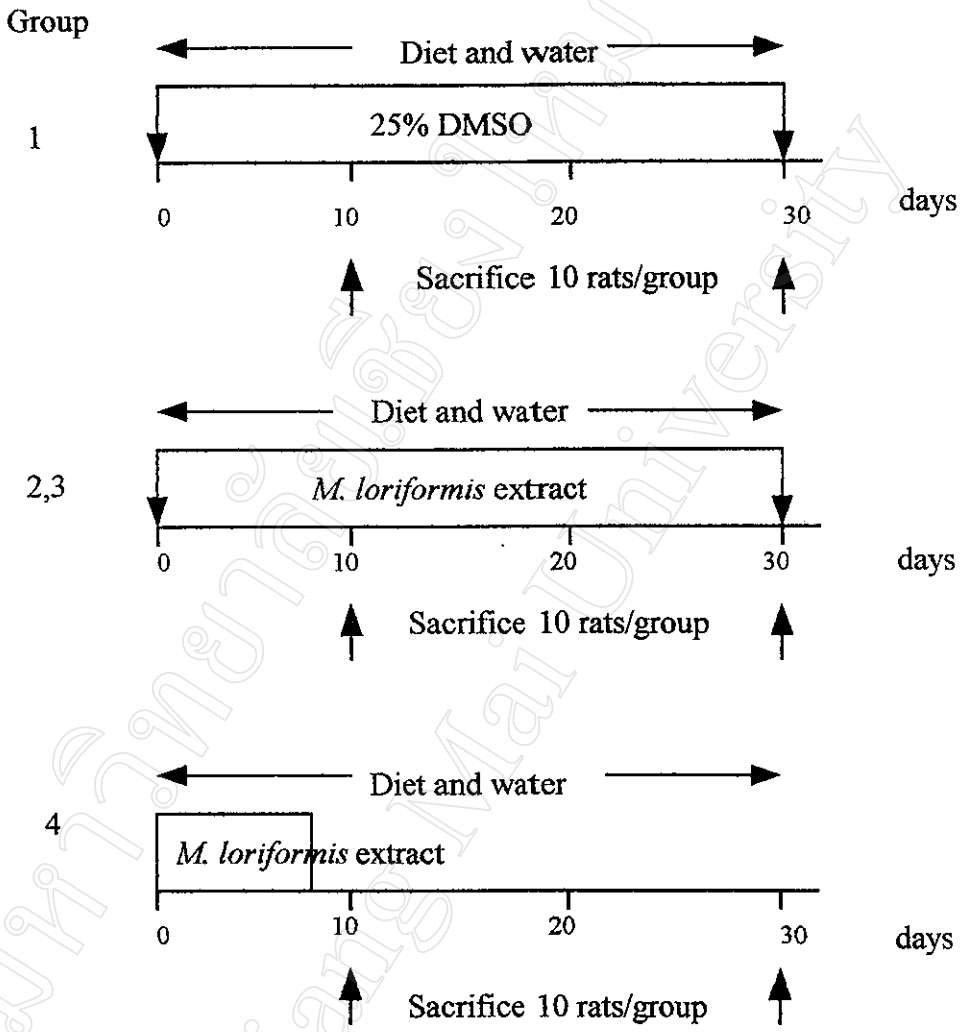

Figure 11. Protocol for studying the effect of *M. loriformis* extract on drug metabolizing enzyme

2.7.1 Preparation of hepatic microsomal and cytosolic fractions

Preparations were done according to the method of Ruangyttikarn (Ruangyuttikarn *et al.*, 1991). All steps of the procedure were carried out at 4°C. The perfused livers was quickly removed, weighed, minced and homogenized with a Potter homogenizer fitted with a Teflon pestle in 3 vol ice-cold 0.05M Tris buffer (pH 7.4) containing KCl at 1.15% (w/v) for isotonicity. The homogenate was centrifuged at 9,000g for 30 min. The supernatant was subsequently centrifuged at 105,000g for 60 min by ultracentrifuge. Supernatant fractions were added to liver cytosol and was used to determine glutathione-*S*-transferase and DT-diaphorase activities. The microsomal pellet was resuspended in the same buffer by gently using a teflon pestle homogenizing. The concentration of protein content in microsome fraction was determined and the microsomal samples were diluted to 1 mg protein/ml. The aliquoted microsomal fractions were kept frozen at -80°C and were used to measure the content of cytochrome P450, aminopyrine demethylase and UDP-glucuronyl transferase activities. The scheme of the preparation is shown in Figure 12.

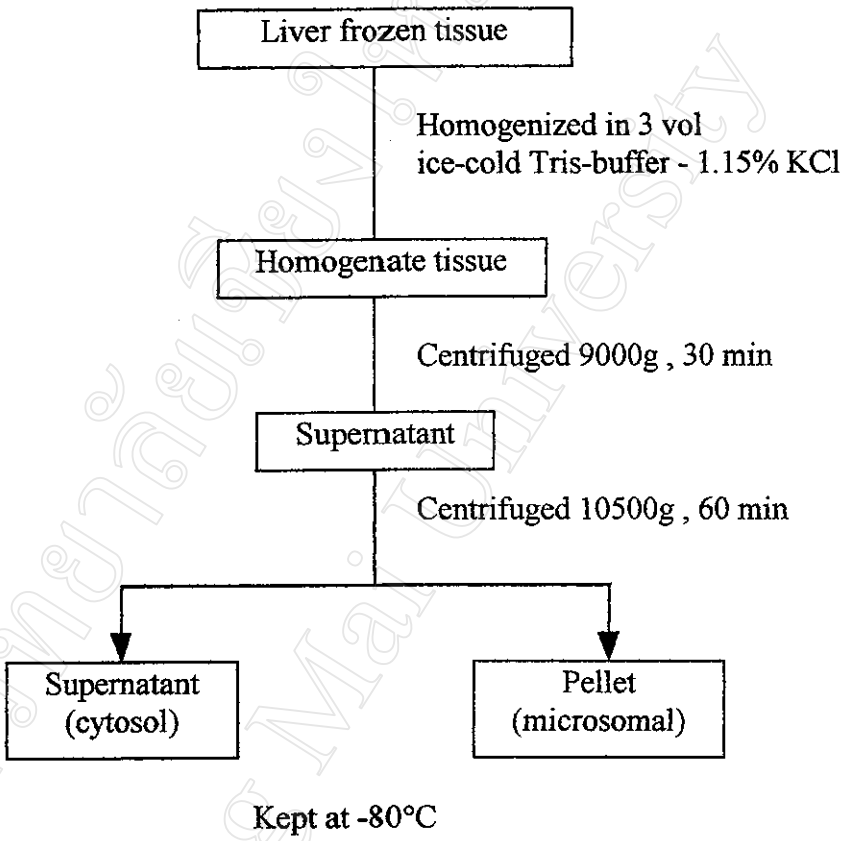

Figure 12. Diagramatic represent a protocol for microsomal and cytosol preparation

2.7.2 Determination of Phase I enzymatic activity

2.7.2.1 Determination of total cytochrome P450 centent

The total cytochrome P450 concentration was estimated by CO-difference spectrophotometry of the dithionite-reduced samples. Concentrated formic acid and sulfuric acid for setting the CO generating system was used CO gas bubbled through the reaction mixture for about 3 min, and a baseline was recorded. After that a small amount of sodium dithionite was added to the contents in a cuvette and the spectrum was immediately ran between 400-500 nm. The concentration of cytochrome P450 was determined from the absorbance difference between 450 nm (peak) and 490 nm (isobestic point), based on an extinction coefficient of 91 $mM^{-1}cm^{-1}$ (Omura and Sato, 1964).

2.7.2.2 Determination of Aminopyrine demethylase

The activity of enzyme was determined by quantitation of formaldehyde released by Nash reagent (Nash, 1953). The mixture was composed of 0.5 ml of a solution in water of 50 mM aminopyrine and 25 mM $MgCl_2$ (final concentration 8.33 and 4.17 mM respectively). 1.34 ml of 0.60 mM $NADP^+$ and 3.33 mM G6P in 50 mM Tris-HCl (pH 7.4) and 20 µl of G6P-DH (final concentration 0.93 U/ml), were incubated at 37°C for 5 min. The reaction was started by adding 150 µl of microsome samples, then incubated again at 37°C for 5 min. After the reaction was stopped by adding 1 ml of 25% $ZnSO_4$ and saturated by $Ba(OH)_2$, the reaction mixture was centrifuged at 3,000 rpm for 10 min. 1 ml of Nash's reagent was added to the supernatant fraction, mixed well and incubated at 37°C for 40 min. The absorbance was measured at 412 nm and molar absorbtivity of 8,000 was used for the calculation. The protocol for this assay is shown in Figure 13.

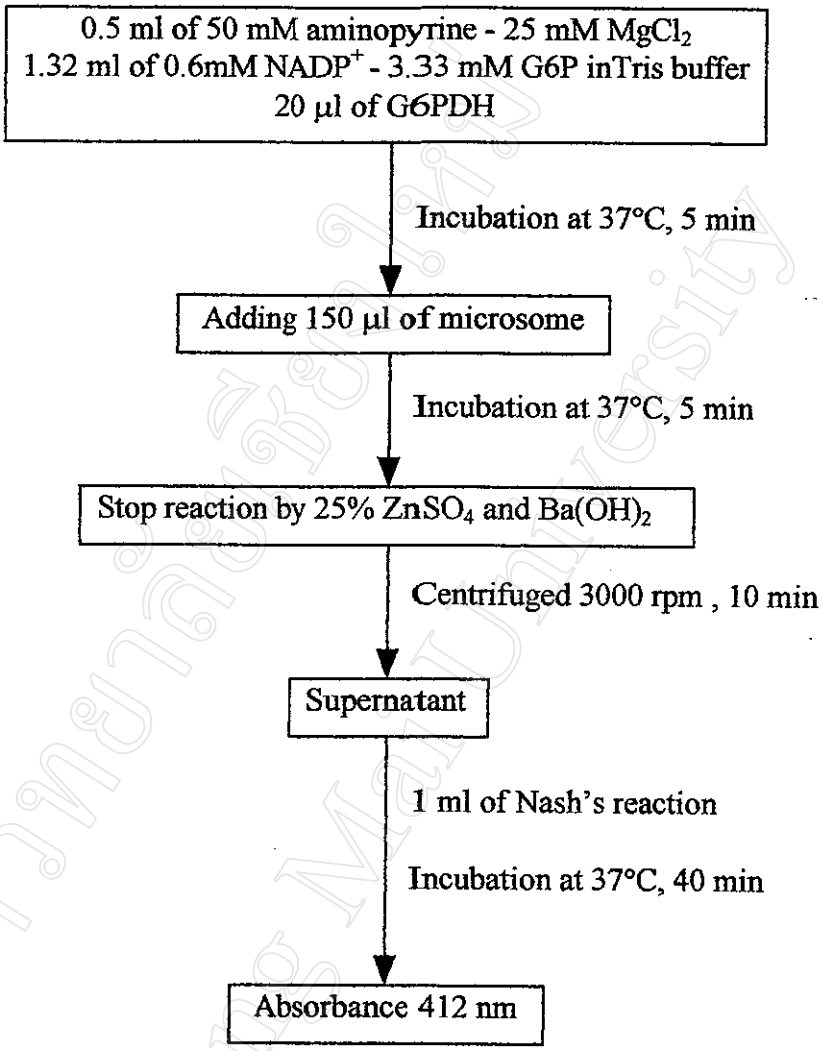

Figure 13. Protocol for the determination of aminopyrine N-demethylase activity

2.7.3 Determination of Phase II enzymatic activity

2.7.3.1 Determination of glutathione *S*-transferase (GST) activity

GST was measured kinetically at 25°C, (pH 6.5) in the presence of 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (Habig *et al.*, 1974). A completed assay mixture without the enzyme was used as a control. The total incubation volume was 3 ml which was composed of 0.1 M phosphate buffer (pH 6.5), 25 µl of 20 mM reduced glutathione (GSH), 25 µl of 20 mM 1-chloro-2,4-dinitrobenzene in 95% ethanol, and 5 µl of cytosolic sample. The absorbance was measured at 340 nm after incubation at 30°C for 10 min The concentration of GST was determined from an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$. The scheme is shown in Figure 14.

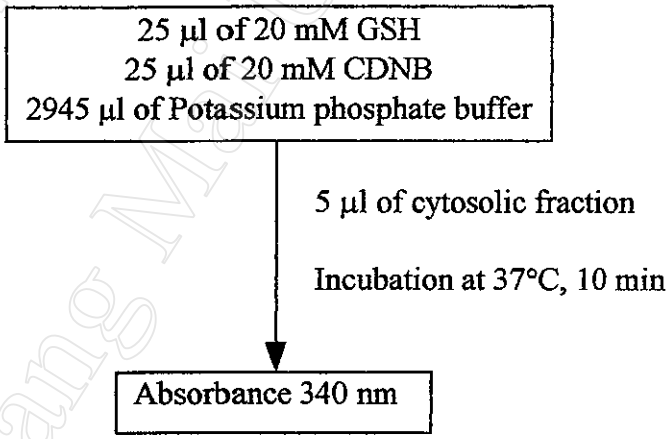

Figure 14. Assay for Glutathione *S*-transferase activity

2.7.3.2 Determination of DT-diaphorase activity

The concentration of DT-diaphorase was determined by the method of Ernster (Ernster, 1967). The mixture containing 5 µl of cytosolic sample, 200 µl of 1% BSA and 30 µl of 1% tween 20. After incubation at 25°C for 8 min, 25 mM Tris buffer (pH 7.5) and 200 µl FAD-and NADH-solutions were added. Then, 100 µl of 25% DMSO was added to the first tube, and to a second tube 100 µl of 0.3 mM dicumarol was added. After incubation at 25°C for 5 min, the reaction was started by adding 100 µl of 2,6-dichlorophenolindophenol (DCPIP). The reaction was incubated at 25°C for 3 min exactly and the absorbance was measured at 600 nm. An extinction coefficient 2.1×10^4 was used in the calculation. The scheme is shown in Figure 15.

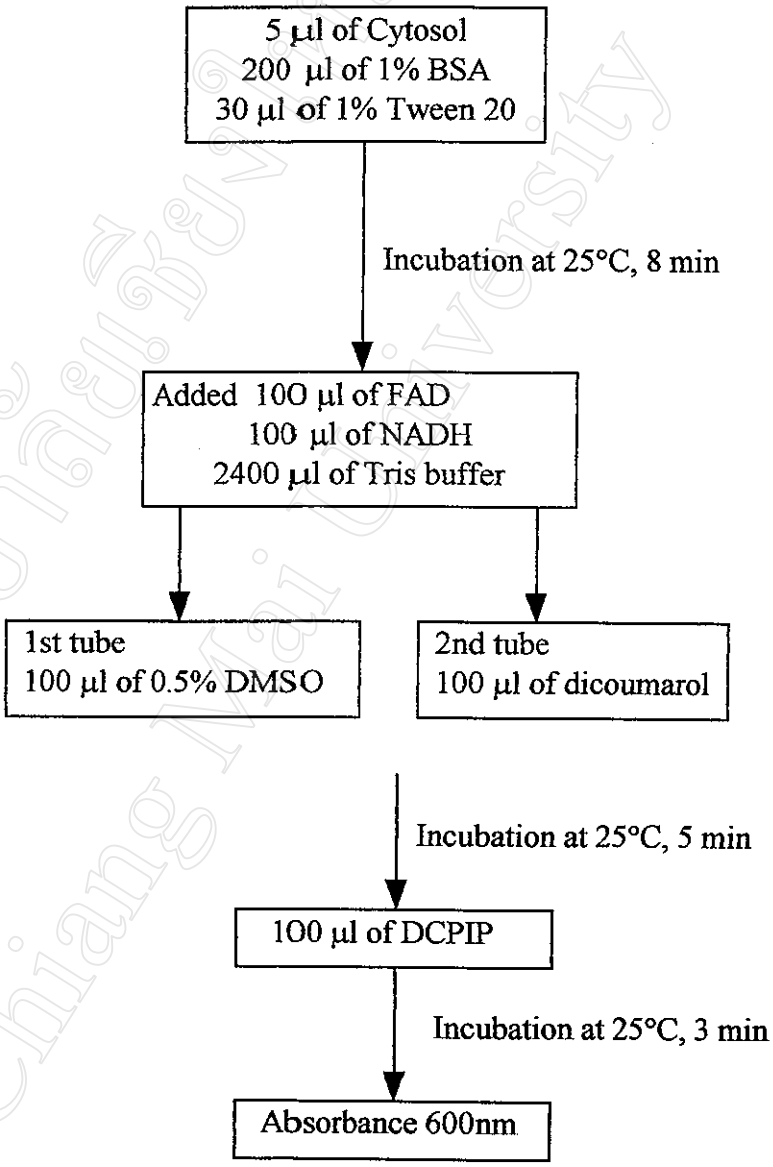

Figure 15. Assay for DT-diaphorase activity

2.7.3.3 Determination of UDP-glucuronyl transferase activity

The incubation mixture contained 2 mM *p*-nitrophenol, 50 mM UDP- glucuronic acid (UDPGA), 0.5 M Tris buffer (pH 7.4) and liver microsome (1 mg protein/ml). After 5 min preincubation at 37°C the reaction was started by the addition of microsome fraction. After exactly 30 min the protein in the incubation mixture was precipitated with 2 M TCA-NaH_2PO_4. Thereafter centrifugation at 3,000 rpm for 10 min, 1.25 ml diethyl ether was added and mixed well. Then the aqueous phase was removed and the absorbance was measured at 312 nm. The extinction coefficient for *p*-nitrophenyl-glucuronide formation is 8.5×10^3. The scheme is shown in Figure 16.

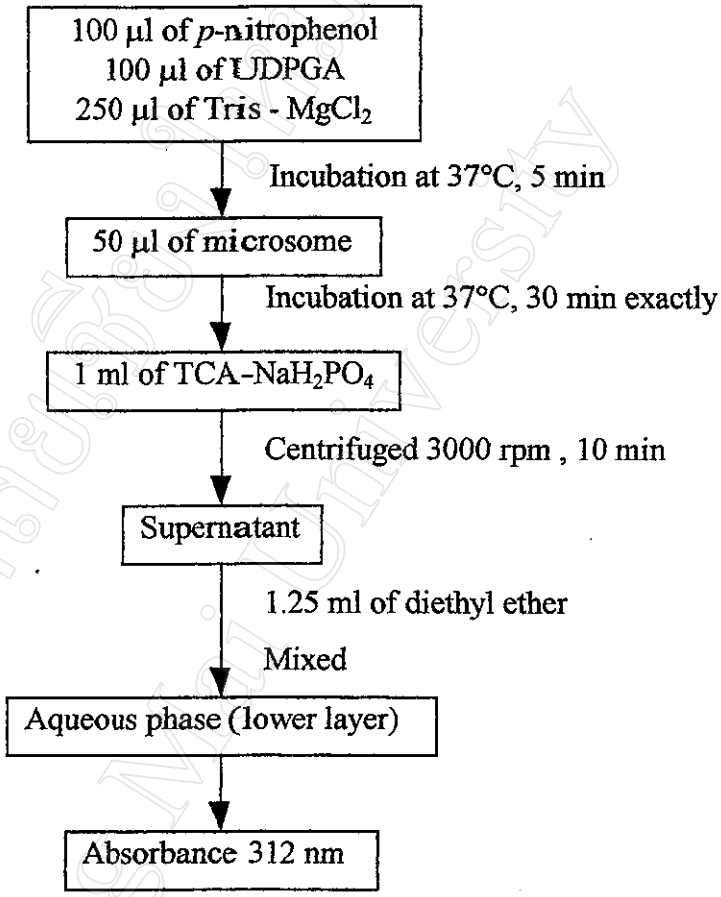

Figure 16. Assay for UDP-glucuronyl transferase activity