CHAPTER 2

Materials and methods

2.1 Chemicals and instruments are shown in Appendices A-B

2.2 Animals

Three-week-old male Wistar rats weighed approximately 60-70 gram were acquired from National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand and were acclimated for one week prior to start the experiment in the Animal House, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. Each three rats per cage were allowed free access to pellet diet and drinking water in a controlled environment at temperature 22-25 ^{o}C with 12-hr light/dark cycle till the termination.

2.3 Extraction and isolation of purple rice bran

Purple rice (*Oryza sativa*), cv. Kum Phayao, was planted in Chiang Mai University farm, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand during the growing season in June-August, 2013. The rice bran was obtained by milling process of husk. The procedure of purple rice bran extraction was performed using two sequential solvents. One kilogram of purple rice bran was soaked in 6 L of dichloromethane for 72 hr in dark at room temperature. The filtrate was separated by vacuum filtration technique through Whatman No. 1 sheet. The residues were then subsequently soaked in methanol with a similar procedure. Each filtrate was concentrated by a rotary evaporator at 42-45^{o}C and dehydrated by a lyophilizer. The dried dichloromethane and methanol extacts were kept at -20^{o}C until used and analyzed (Figure 15).

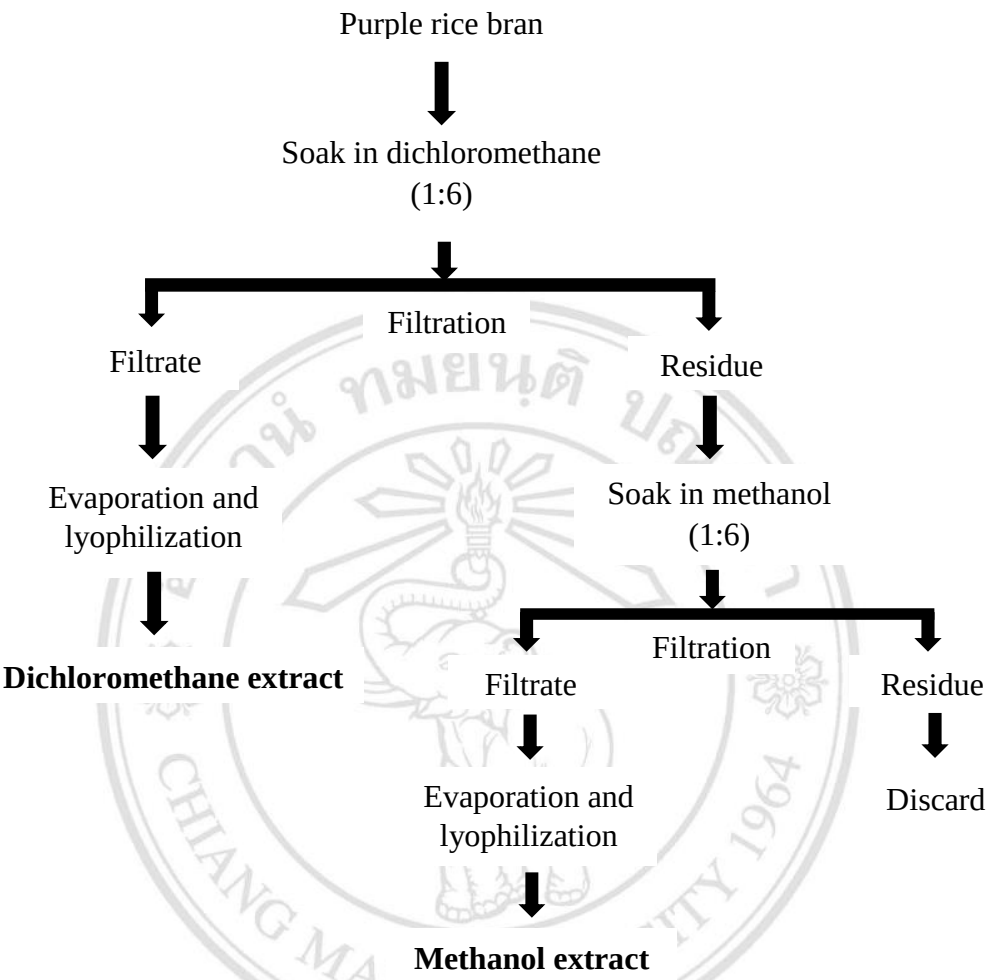

Figure 15. The extraction of purple rice bran

2.4 Determination of major constituents of purple rice bran extracts

To measure the amount of total phenolic compounds in purple rice bran, the dichloromethane and methanol extracts were diluted with dimethyl sulfoxide (DMSO) to 1, 2.5 and 5 mg/ml. The standard gallic acid and samples were incubated with mixture of distilled water and Folin reagent at room temperature for 10 min. The 7% sodium carbonate was added and continuously incubated at 45^{o}C for 15 min. Total phenolic compounds were measured at wavelength 765 nm (Inboot, 2012). The values are expressed in mg/g extract.

To quantify total flavonoids contents, standard of catechin or diluted extracts at 1, 2.5 and 5 mg/ml were mixed with 5% sodium nitrite. After 10 min incubation at room temperature, 1 M sodium hydroxide was added. Total flavonoids contents were evaluated at wavelength 532 nm (Inboot, 2012). The values are expressed in mg/g extract.

To determine the amount of total anthocyanins, various concentrations of the extracts, 1.25, 2.5, 5 and 10 mg/ml, were diluted with potassium chloride and sodium acetate buffers of pH 1.0 and pH 4.5, respectively. Sample solutions were estimated at wavelength 520 and 700 nm (Lee, 2005). The contents of total anthocyanins are calculated by;

$$\text{cyanidin-3-glucoside (mg/100 g extract)} = \frac{[(OD_{520-700})_{pH1.0} - (OD_{520-700})_{pH4.5}] \times MW \times \text{dilution factor} \times 1000}{26900 \times 1}$$

2.5 Identification of some phenolic compounds in purple rice bran extracts

2.5.1 Phenolic acids, flavonoids and anthocyanins

These contents were identified by HPLC technique using C18 column (4.6 x 250 mm, 5 µm) at flow rate 1 ml/min. To determine phenolic acids and flavonoids, the standards consisted of gallic acid, protocatechuic acid, catechin, vanillic acid, epicatechin, *p*-coumaric acid, ferulic acid, rutin and quercetin are used. The mobile phase contained methanol and 3% acetic acid. The phenolic acids and flavonoids were detected at wavelength 260, 280, 320 and 360 nm (Punvittatyagul, 2014). To analyze anthocyanin, the standards comprise of cyanidin-3-O-glucoside (C3G), cyanidin-3-rutinoside (C3R), peonidin-3-O-glucoside (P3G), malvidin-3-glucoside (M3G). The mobile phase included 0.1% trifluoroacetic acid (TFA)-H_2O and 0.1% TFA-methanol. They were evaluated at wavelength 520 nm (Ryu, 1998). The values were expressed in mg/g extract.

2.5.2 Gamma-oryzanol

After dissolving the extracts by DMSO, they were injected through C18 column (Rainin Instrument Company, Woburn, MA). The mobile phase composed of methanol, acetonitrile, dichloromethane and acetic acid (50:44:3:3) at flow rate 2 ml/min. Gamma-oryzanol content was quantified at wavelength 330 nm (Sankam, 2013). The values were expressed in mg/g extract.

2.5.3 Tocopherols and tocotrienols

The extracts were applied to Develosil C30 UG column with mobile phase including methanol and deionized water (90:10) at flow rate 1 ml/min. The Shimadzu fluorescence detector was used at wavelength of excitation 280 nm and emission 325 nm (Punvittatyagul, 2014). The values were expressed in mg/g extract.

2.6 Clastogenicity of purple rice bran extract

To investigate an *in vivo* genotoxic effect of extracts, three weeks old male Wistar rats (six per group) were tested. A vehicle group, Group 1, received regular diet. Groups 2-3 orally received dichloromethane extract while Groups 4-5 were fed with methanol extract. Each extract was given at the concentration of 100 and 500 mg/kg bw, for 28 days. On day 29, rats were anesthetized before partial hepatectomy to induce hepatic cell growth and the liver section was performed for enzyme analysis. Four days after surgery, rats were injected with 70 mg/kg bw of thiopental and isolated hepatocytes using liver perfusion technique to observe liver micronucleus formation (Figure 16).

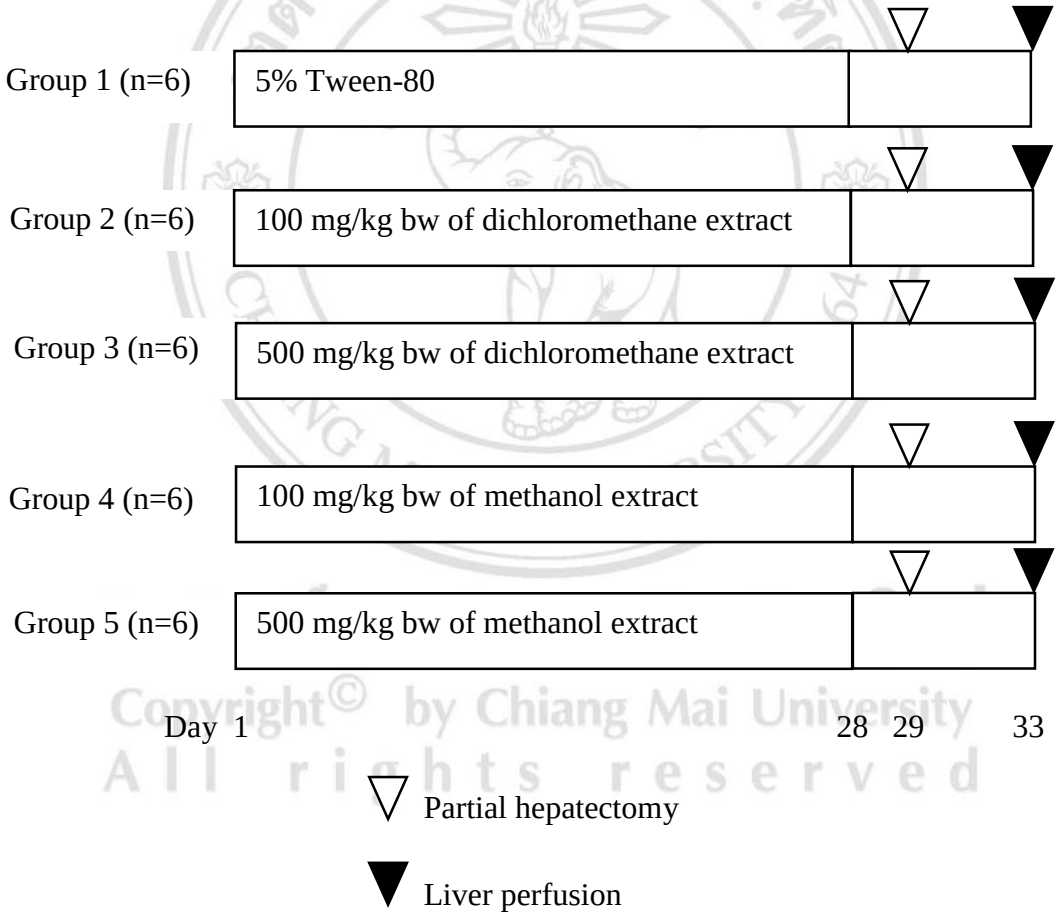

Figure 16. Clastogenicity of purple rice bran extracts in liver of rats

2.7 Partial hepatectomy

This operation was aimed to enhance hepatocytes undergoing mitosis during cell injury. Rats were exposed to the anesthetic diethyl ether. The abdominal area was shaved and sterilized with 70% ethanol. Then the outer skin layer was excised about 1-2 cm long to the xiphoid cartilage and shorter excision of the inner skin layer. Left lateral and median lobes, two-third of liver (Figure 17), were moved out with mild forced of thumb and forefinger and tied with thread before excision. The removal liver was rinsed with normal saline solution and was cryo-preserved in liquid nitrogen and stored at -80ºC until analysis. After that, the surgical wound was closed by suturing technique and symptom observation was done subsequently (Higgins and Anderson, 1931) (Figure 18).

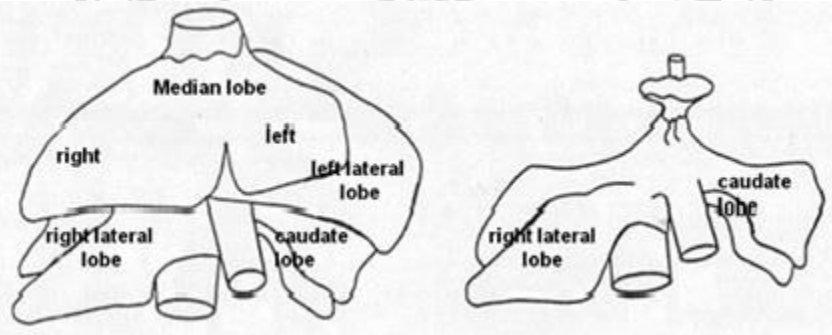

Figure 17. Anatomy of rat liver (Teixeira, 2010)

2.8 Liver perfusion and isolation of hepatocytes

The hepatocytes were separated from blood circulation using portal vein catheterization with preperfusion buffer. The hepatocytes were isolated in the process of directly 0.05% collagenase type IV digestion. Cell suspension was centrifuged at 400 g for 5 min at 4ºC and adjusted volume to 12 ml with PBS. This step was done for 3 times to get the last volume of cell pellet at 3 ml. Cells were treated with 10% formalin and cold-centrifuged at 400 g for 5 min. Isolated hepatocytes were fixed with 10% formalin and stored at 4ºC until measurement (Charoensin, 2010) (Figure 19).

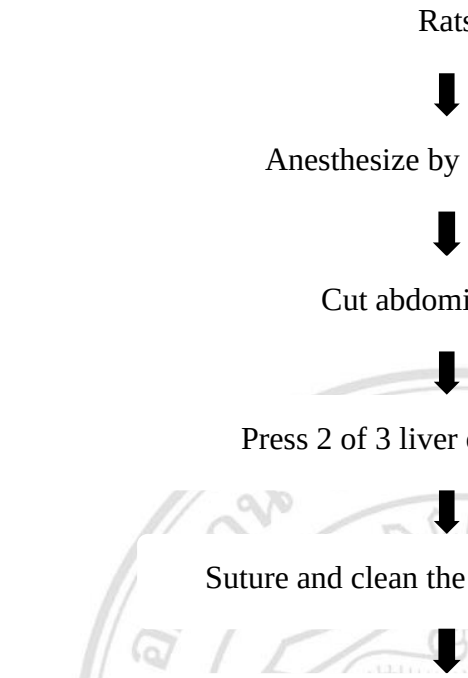

Figure 18. The procedure of partial hepatectomy

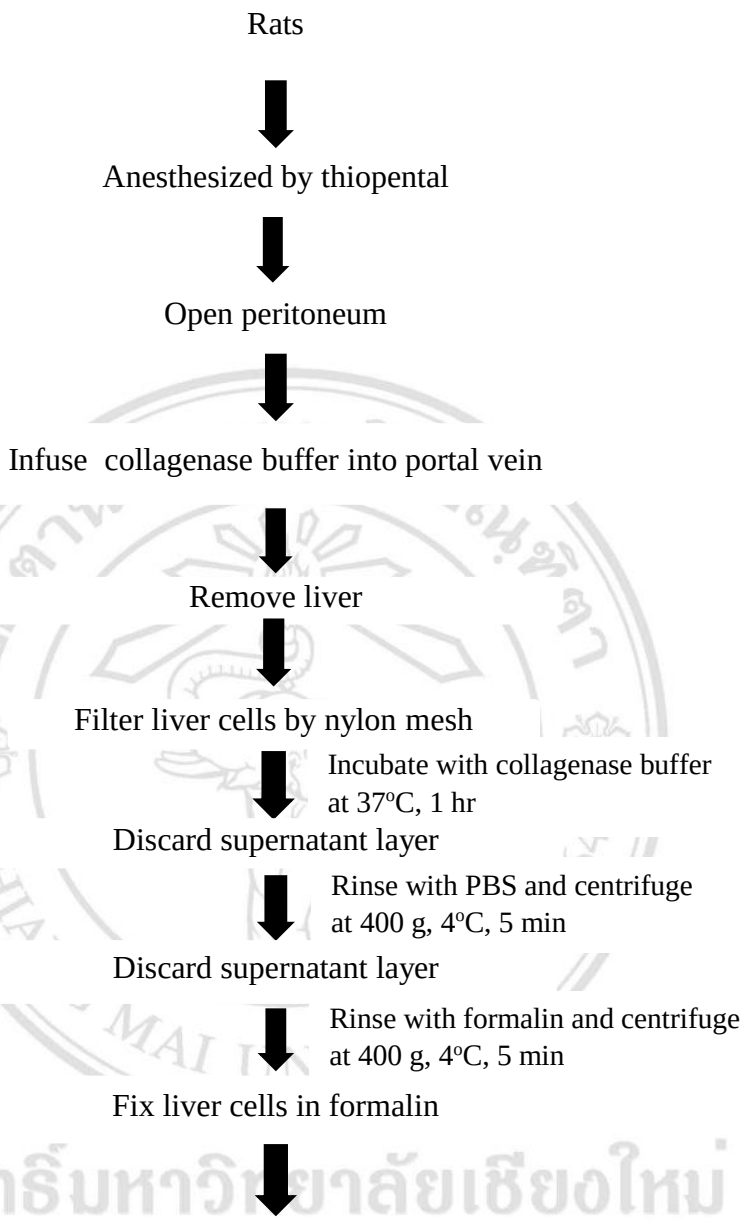

Figure 19. The procedure of liver perfusion and isolation of hepatocytes

2.9 Determination of micronucleus

The mutation in hepatocytes was proved using liver micronucleus assay. One hundred µl of cell suspension were pipetted and stained with 50 µl of 20 µg/ml 4',6-diamidino-2-phenylindole (DAPI). The solution was taken on to a glass slide and covered before observed with 400X fluorescence microscopy. The 2,000 isolated hepatocytes were counted including mono- and bi-nucleated cells with or without micronuclei. The micronucleated hepatocytes (MNHEPs) were identified as cell containing the round one-third smaller than and similar staining as the parent nucleus. Mitotic index (MI) was determined as an indirect assess of cell proliferation during cell division stage, from prophase to telophase, which will be a common indicator of cancer cell growth (Charoensin, 2010) (Figure 20).

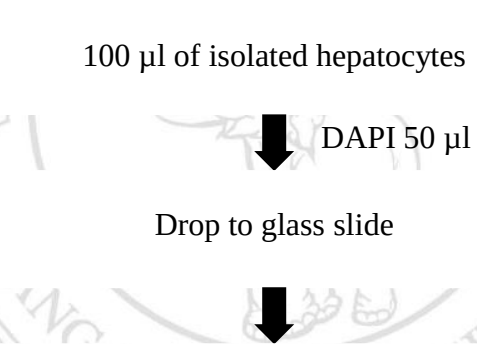

Figure 20. The procedure of microscopic observation and micronucleus determination

2.10 Optimization of genotoxic concentration of AFB_1 induced liver micronucleus formation in rats

The previous data showed the single injection of aflatoxin B_1 ($AFB_{1)}$ at the concentrations between 125 to 250 μg/kg bw before partial hepatectomy for 3 days could induce micronucleus in the liver of male F344 rats (Puatanachokchai, 1996). To induce the maximum frequency of liver micronuclei without toxicity, male Wistar rats were used. Group 1, a negative control, was intraperitoneally injected with 4 ml/kg bw of 5% Tween-80. Groups 2 and 4 were single injected with 100 and 200 μg/kg bw, respectively at day 4. Groups 3 and 5 were double injected with 100 and 200 μg/kg bw, respectively at day 4 and 8. Four days after last injection, a portion of liver was excluded using partial hepatectomy method. Day 12, all rat liver cells were isolated by perfusion process for measurement of micronucleated hepatocytes. The livers of each group were also investigated for some xenobiotic metabolizing enzymes involved in AFB_1 metabolism including cytochrome P450 1A2 and glutathione *S*-transferase (Figure 21).

2.11 Anticlastogenicity of purple rice bran extract

In anticlastogenic study, protective effect of extracts was examined when treated with AFB_1. Male Wistar rats were divided into six groups. The negative and positive groups, Groups 1 and 2 respectively, were orally fed with 5% Tween-80. Groups 3 and 4 were administrated with the dichloromethane extract at 100 and 500 mg/kg bw. Groups 5 and 6 were obtained the 100 and 500 mg/kg bw of methanol extract via gavage feeding for 28 days. Groups 2-6 were injected with 200 μg/kg bw AFB_1 for two times at day 21 and 25 to initiate liver micronuclei. Group 1 was replaced by 5% Tween-80 injection (Figure 22).

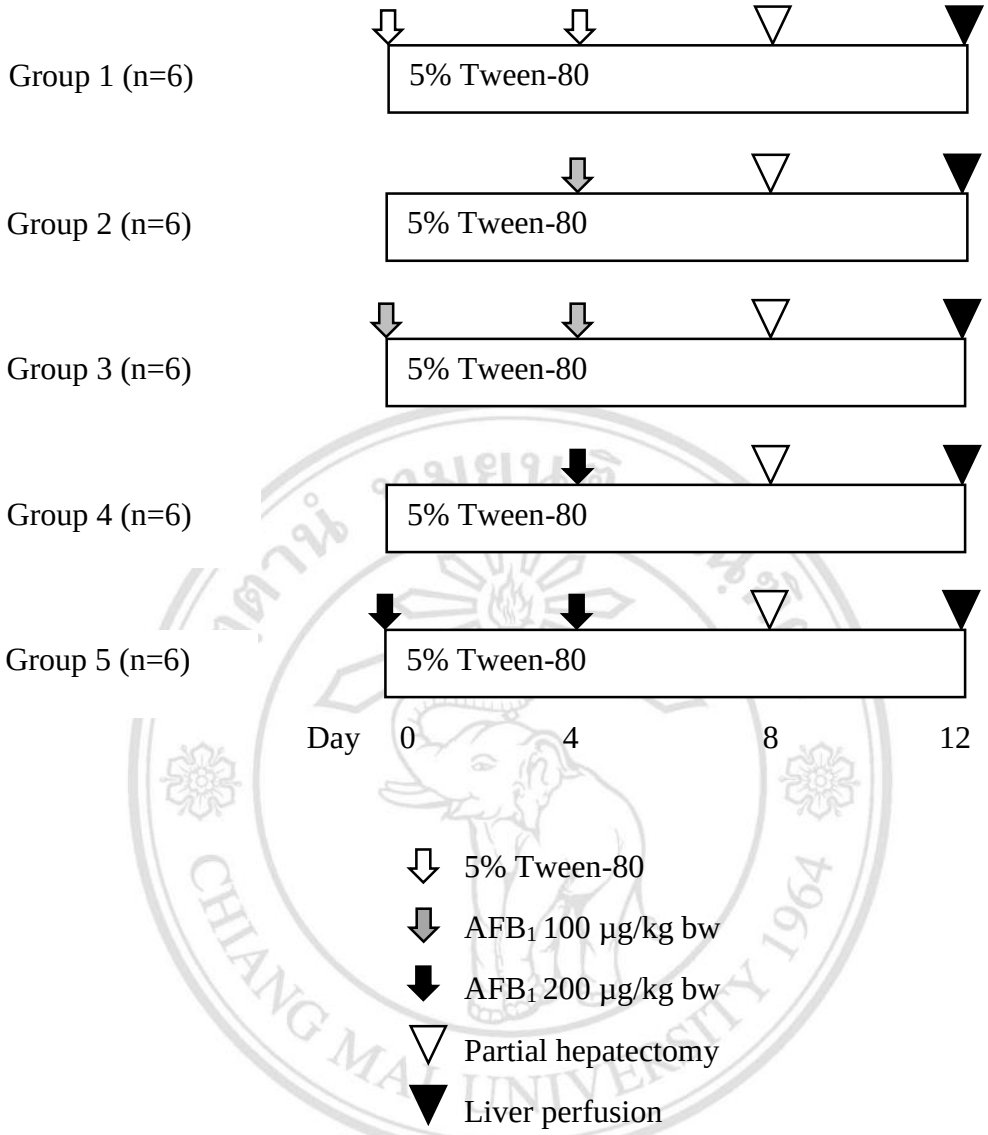

Figure 21. Effect of AFB_1 treatment on liver micronucleus formation in rat

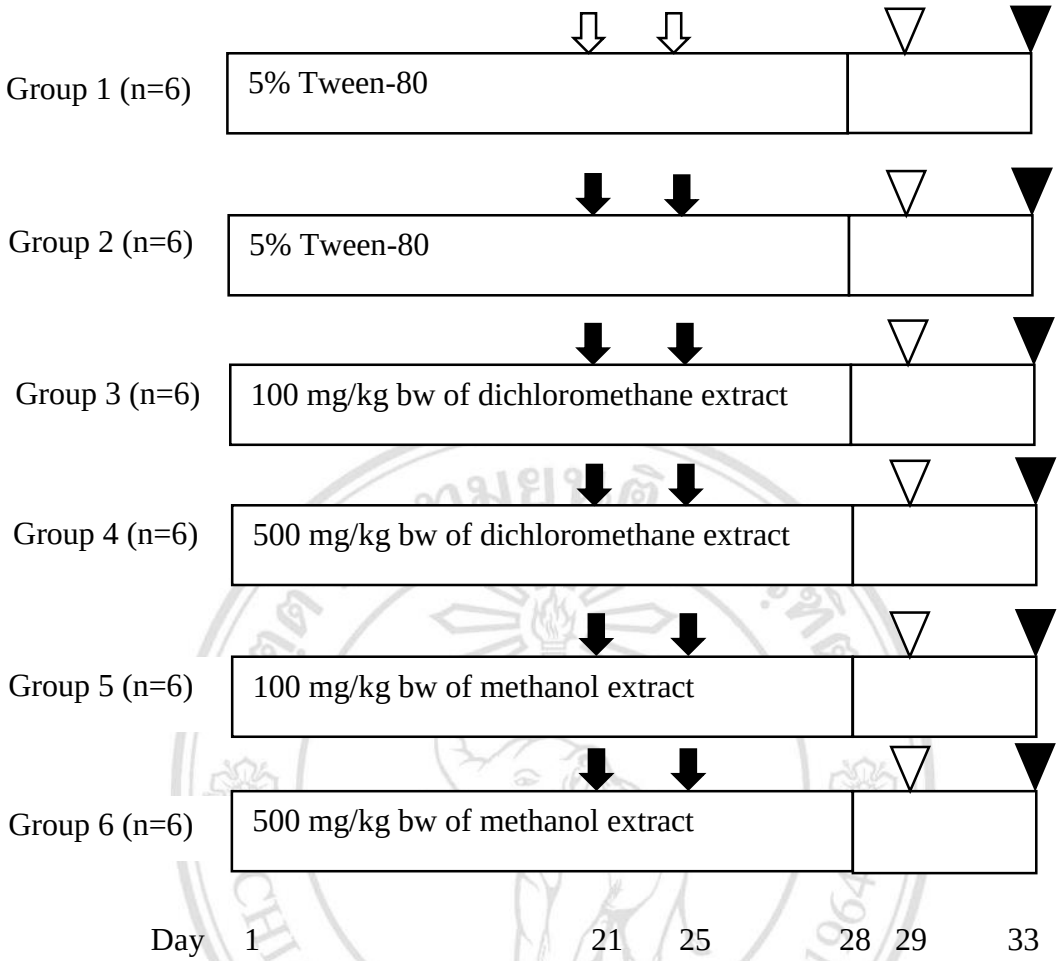

Figure 22. Anticlastogenicity of purple rice bran extracts

2.12 Preparation of cytosolic and microsomal fractions

In order to prepare cytosolic and microsomal fractions of rat liver, two grams of crushed liver were homogenized in 6 ml of homogenizing buffer and were centrifuged at 10,000 g for 30 min at 4^{o}C. The supernatant was cold-centrifuged at 100,000 g for 1 hr. The supernatant was collected as a cytosolic fraction. The pellet was rinsed with homogenizing buffer and was ground in microsomal buffer solution before stored at -80 ^{o}C. Ten µl of each fractions were taken into 90 µl of deionized water for protein measurement (Figure 23).

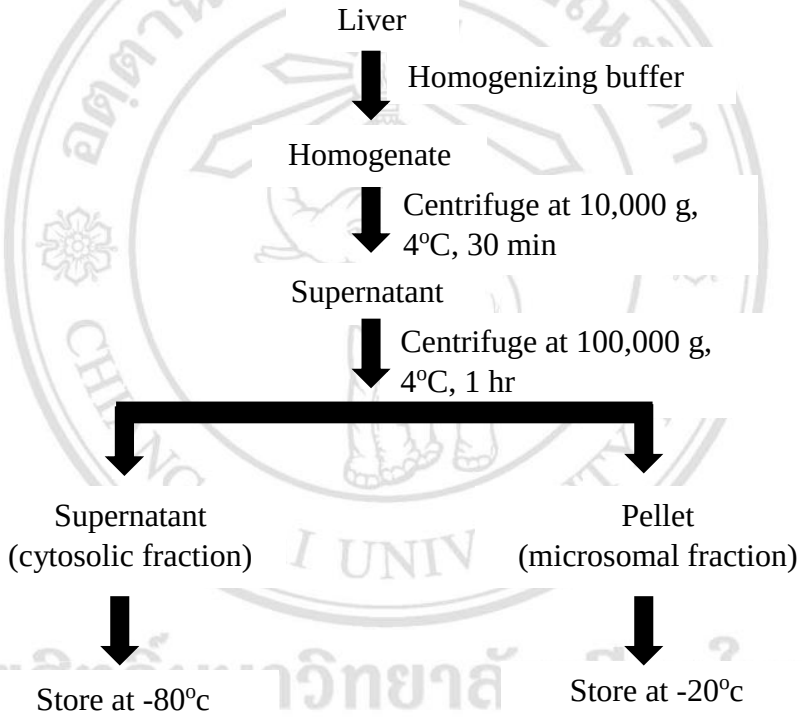

Figure 23. Preparation of cytosolic and microsomal fractions

2.13 Determination of protein concentration

Both cytosolic and microsomal fractions of hepatocytes were diluted with distilled water and incubated with the mixture of sodium carbonate, copper sulfate and potassium sodium tartrate at room temperature for 10 min. To stop reaction, Folin-ciocalteus was added and incubated for 30 min. The resulting mixture was measured at wavelength 750 nm by spectrophotometer with standardized bovine serum albumin.

2.14 Determination of Phase I metabolizing enzyme activities

2.14.1 Cytochrome P450 isozymes 1A1 and 1A2

Cytochrome P450 1A subfamily is involved in phase I xenobiotic metabolism of substances or toxicants. The major isozymes are found in mammals including CYP 1A1 and 1A2 which are inducible by polycyclic aromatic hydrocarbons and heterocyclic aromatic amines, respectively.

The activity of CYP 1A1 and 1A2 were analyzed using ethoxyresorufin-O-deethylation (EROD) and methoxyresorufin-O-demethylation (MROD) methods, respectively (Chatuphonprasert and Jarukanjorn, 2012). These methods are based on the measurement of fluorescence resorufin product of deethylation of 7-ethoxyresorufin or demethylation of 7-methoxyresorufin. One µM standard resorufin or 10 mg/ml of liver microsome was mixed with reactive solution including 0.01 M Tris and 0.05 mM of the substrate, ethoxyresorufin or methoxyresorufin, and then the reaction was stopped with 0.5 mM NADPH. The excitation wavelength at 520 nm and emission wavelength at 590 nm were evaluated using spectrofluorometer.

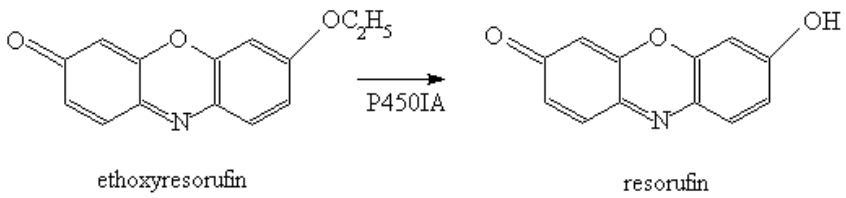

Figure 24. Principle of the measurement of CYP 1A activity

2.14.2 Cytochrome P450 isozyme 3A2

Cytochrome P450 3A enzymes are abundant and catalyze several drugs such as erythromycin, nifedipine, rifampicin and testosterone. A commonly isoform in human liver is CYP 3A4, while CYP 3A2 is found in rat liver.

The analysis of CYP 3A2 activity is erythromycin N-demethylation (ENDM) detected by colorimetry. The specific erythromycin substrate was demethylated by CYP 3A to produce formaldehyde. The reaction was started by incubating standard formaldehyde or 10 mg/ml of liver microsome with a reaction mixture containing phosphate-buffered saline, 10 mM erythromycin, 150 mM $MgCl_2$ and 5 mM NADPH at 37ºC in water bath shaker for 20 min. Then 12.5% trichloroacetic acid was added. After the mixture was centrifuged at 1,300 g for 15 min, the supernatant was mixed with Nash reagent containing ammonium acetate, acetic acid and acetylacetate, and incubated at 50ºC for 15 min in a shaker. The optical density at 405 nm was measured using spectrophotometer (Chatuphonprasert and Jarukanjorn, 2012).

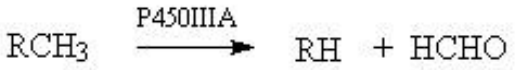

Figure 25. Principle of the measurement of CYP 3A activity

2.14.3 NADPH-cytochrome P450 reductase

NADPH-cytochrome P450 reductase (CPR) is a membrane-bound flavoprotein that is mostly found in endoplasmic reticulum of liver. It is capable of transfering electron from NADPH to CYP enzymes.

The measurement of CPR activity depends on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The liver microsome was preincubated with a reaction mixture comprised of 0.3 M potassium hydrogen phosphate buffer (pH 8.7), 1 mM cytochrome c Type VI and 50 mM potassium cyanide *at* 37^{o}C for 2 min. Ten mM NADPH were added to stop reaction. The activity was evaluated by spectrophotometer at wavelength 550 nm and calculated using a molar coefficient of 21 $mM^{-1}cm^{-1}$ (Yim SK, 2005).

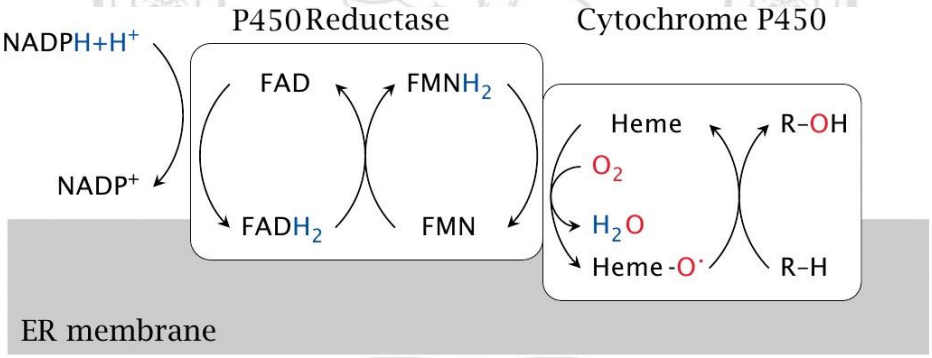

Figure 26. The function of NADPH-cytochrome P450 reductase

2.15 Determination of Phase II metabolizing enzyme activities

2.15.1 Glutathione-*S* transferase

Glutathione-*S* transferase (GST) is one of phase II detoxifying enzymes which is able to conjugate the substrates to the reduced glutathione (GSH) by its sulfhydryl group to be more water soluble substances.

The assessment of GST activity was performed following Punvittayagul, 2012. The reaction was started with incubation of liver cytosol and mixture of 0.2 M phosphate buffer, 10 mM GSH and 1- chloro-2,4-dinitrobenzene at 37^{o}C for 20 sec. The enzyme activity was measured at wavelength 340 nm using spectrophotometer and calculated by using a molar coefficient of 9.6 $M^{-1}cm^{-1}$.

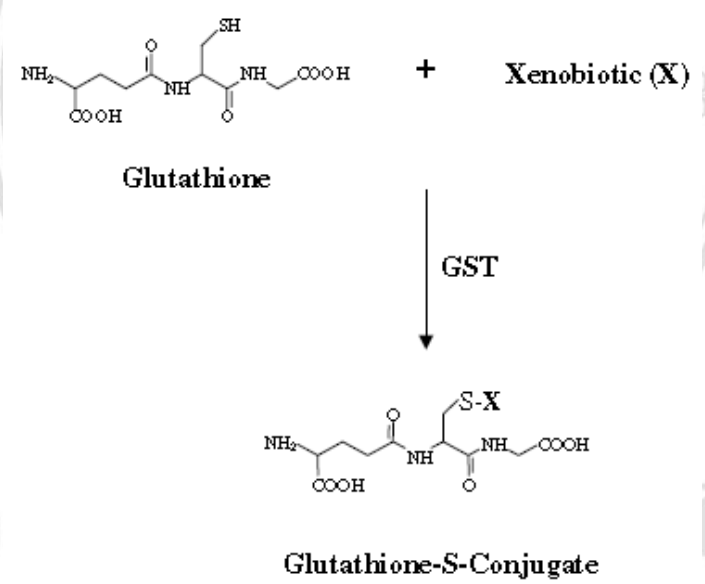

Figure 27. The reaction of glutathione-*S* transferase (Townsend, 2003)

2.15.2 UDP-glucuronyltransferase

UDP-glucuronyltransferase (UGT) is a tetrameric enzyme which is a major phase II xenobiotic metabolizing enzymes. The reaction is glucuronidation by transferring the glucuronosyl group from uridine 5'-diphospho-glucuronic acid (UDP-GA) to xenobiotic and producing the more polar substance, glucuronide.

According to Punvittayagul, 2012, the reaction of 5 mg/ml of liver microsome was started by adding the solution of 200 mM Tris, 40 mM magnesium chloride, 5 mM *p*-nitrophenol and 20 mM UDP-GA. After dark incubation at 37ºC for 20 min, the reaction was stopped by adding 10% TCA. The mixture was centrifuged at 10,000 g for 5 min. 1 M NaOH was mixed with the supernatant and the activity of UGT was evaluated at wavelength 405 nm.

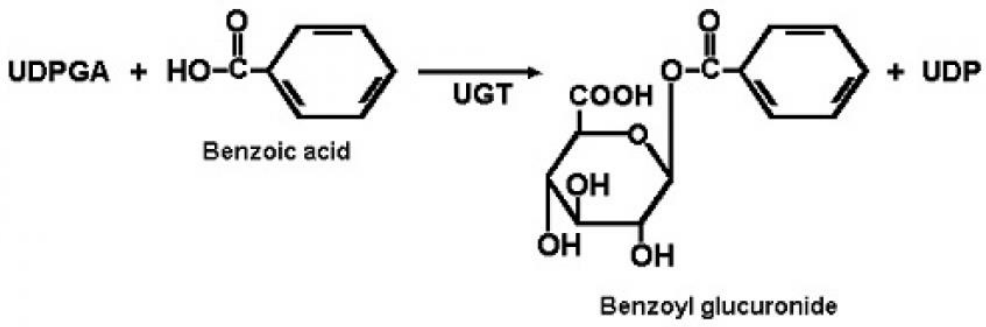

Figure 28. The reaction of UDP-glucuronyltransferase

2.16 Determination of protein expression of phase I and II metabolizing enzymes

This detection was aimed to consider the relation between the activity and expression of phase I and II metabolizing enzymes. The proteins were separated by their molecular weight using sodium dodecyl sulfate-polyacrylamide denaturing gel electrophoresis and transferred to nitrocellulose membrane for 1 hr and then washed with phosphate-buffered saline-0.05% Tween-20 (TPBS) for three times. In blocking step, membrane was incubated in non-fat dry milk with TPBS for 2 hr to avoid non-specific binding. After the membrane was incubated with primary specific antibody for 1 hr, it was subsequently incubated with secondary antibody consist of peroxidase-conjugated anti-rabbit IgG for 30 min and washed with phosphate-buffered saline. A chemiluminescent horseradish peroxidase substrate was used as a protein detector by 5 min incubation, then exposed to x-ray film. The intensity of protein bands were estimated by Image J program.

2.17 Statistical analysis

All data are presented as means±SD. The assessment of statistical significance was analyzed by One way analysis of variance (ANOVA). When significance was observed, Bonferroni's post hoc test was conducted. The level of significance was expressed as $p < 0.05$.