CHAPTER III

MATERIALS AND METHODS

3.1 Animal

Male Wistar rats weighing 180-200 g, 6-8 weeks old were obtained from The National Laboratory Animal Center, Mahidol University, Salaya, Nakorn pathom. Rats were housed in animal care facility at the Faculty of Pharmaceutical Sciences, Chulalongkorn University under room temperature 25±1°C with 12-hour light/dark cycle. They were received free standard pellet diet (Jareorn Pokphand Co., Ltd., Bangkok) and tap water, and were acclimatized for 7 days before the experiment.

3.2 The extract

The aerial part of *Phyllanthus amarus* Schum. et. Thonn. (PA) were collected from the Faculty of Pharmaceutical Sciences, Chulalongkorn University during May to June, 2005. The plant was identified by Assoc. Prof. Dr. Chaiyo Chaichantipyuth, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Preparation of the extract (Figure 7)

1.) Fresh aerial part was dried thoroughly under shade and cut into small pieces

2.) The small pieces were added with deionized water about 2 cm over, boiled for 15 minutes and filtered through 3 layers of fine cleaned gauze.

3.) Residues was boiled again in deionized water for 15 minutes and then filtered.

4.) The filtrate of both extractions were combined and centrifuged at 2,000 rpm. for 10 minutes.

5.) The supernatant was filtered using the filter paper No.1.

6.) The clear filtrate was lyophilized by a Freeze dryer.

The yield of the aqueous extract of PA was 17.42 % (w/w) from the starting dried aerial part. The extract was kept in the protected light bottle at 4 °C. Afresh preparation was prepared by dissolving in distilled water before each administration Silymarin (SL) was kindly provided by The British Dispensary (L.P.) Co.Ltd.

3.3 Equipments and Instruments

Surgical equipments, Fine cleaned gauze, Whatman Filter paper No.1, Test tube, Beaker, Eppendrof tube, Syringes and needle No. 21, Aluminum foil, Timer, Aspirator, Automatic pipette (Gilson, U54756, France), refrigerator (Thermo electron corporation, 995, U.S.A.), Digital weighting machine (Mettler Toledo, AB204-S, Switzerland), pH meter (Beckman, 12 pH/ISE, U.S.A.),Vortex mixer (Vortex genie, 2-G-5OE, USA.), Laboratory mixer (Ikamag, NR 245060, Germany), Water bath (Heto, 21AT, Denmark), Freeze dryer (FTS system, FD-6-85DMPO, U.S.A.), Spectrophotometer (LKB Biochrom, Ultraspec II, England), Cuvettes (Starna, IG6 3UT, U.S.A.), Automatic high speed refrigerated centrifuge (Hitachi, Himac SCR 20B/18B, Germany), Homogenizer with pestle and glass (Heidolph, RK 3,Germany), Sonicator (Elma, D7834, Germany), Cryocut (Leica, Cm 1800, Germany), Light microscope (OLYMPUS, U-ND25, Japan), Microplate reader (Anthos, htll, U.S.A.).

3.4 Chemicals

Diethyl ether (Merck, Germany), Ethanol (Merck, Germany), Sodium hydroxide (Merck, Germany), Sodium phosphate monobasic anhydrous (Sigma, Japan), Sodium phosphate dibasic anhydrous (Sigma, U.S.A.), 5'5 Dithiobis (2-nitrobenzoic acid) (Fluka chemie, Switzerland), Sulfosalicylic acid (Sigma, U.S.A.), Isopropanol (Merck, Germany), Potassium hydroxide (Sigma, Sweden), Glacial acetic acid (Merck, Germany), Heptane (Merck, Germany), Hydrochloric acid (Merck, Germany), Sulfuric acid (Merck, Germany), Trichloroacetic acid (Fisher Scientific,U.K.), Triobarbituric acid (Sigma, U.S.A.), Potassium dihydrogen phosphate (Riedel-de Haen, Germany), Potassium Chloride (Sigma, U.S.A.), Sodium Chloride (Merck, Germany), Acetyl acetone (Analar, England), Formaldehyde (Merck, Germany).

All other chemicals used throughout this study were analytical grade.

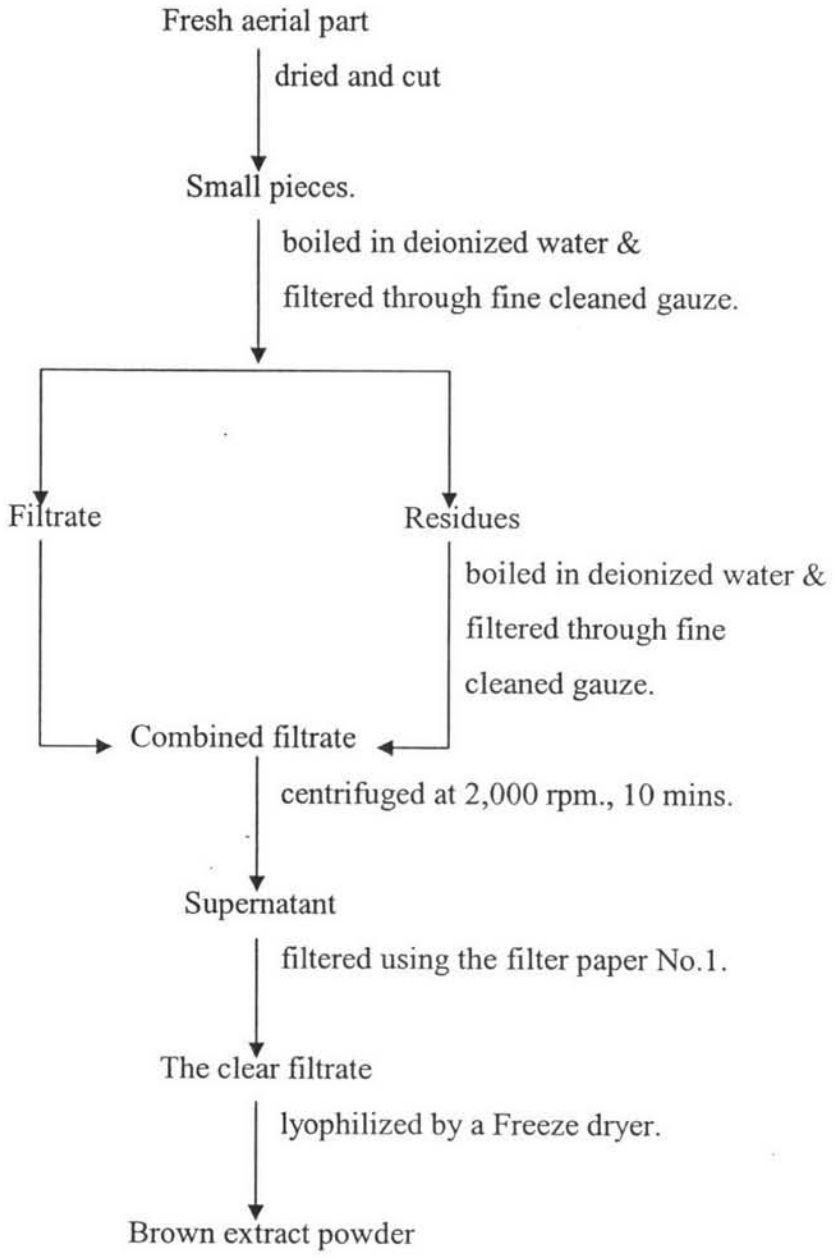


Figure 7. Diagram illustrated the preparation of aqueous extract of *Phyllanthus amarus.*

3.5 Experimental design

Experiment I: Effect of PA extract given 24 hours before single oral dose of ethanol (5 g/kg) (acute toxicity study).

Rats were divided randomly into six groups of six rats in each group.

Group 1 received distilled water (5 ml) orally (Control rats).

Group 2 received single dose of ethanol (5 g/kg) orally.

Group 3-5 received single oral dose of PA (25, 50 and 75 mg/kg, repectively) 24 hours before single oral dose of ethanol (5 g/kg).

Group 6 received single oral dose of SL (5 mg/kg) 24 hours before single oral dose of ethanol (5 g/kg).

Two hours after ethanol, rats were sacrificed under diethyl ether anesthesia. Blood and liver sample were collected for serum and liver homogenate.

Experiment II: Effect of PA extract given 7 days after administration of ethanol (4 g/kg/day) for 21 days (sub-acute toxicity study).

Rats were divided randomly into six groups of eight rats in each group.

Group 1 received distilled water (5 ml) orally for 21 days (Control rats).

Group 2 received ethanol (4 g/kg/day) orally for 21 days.

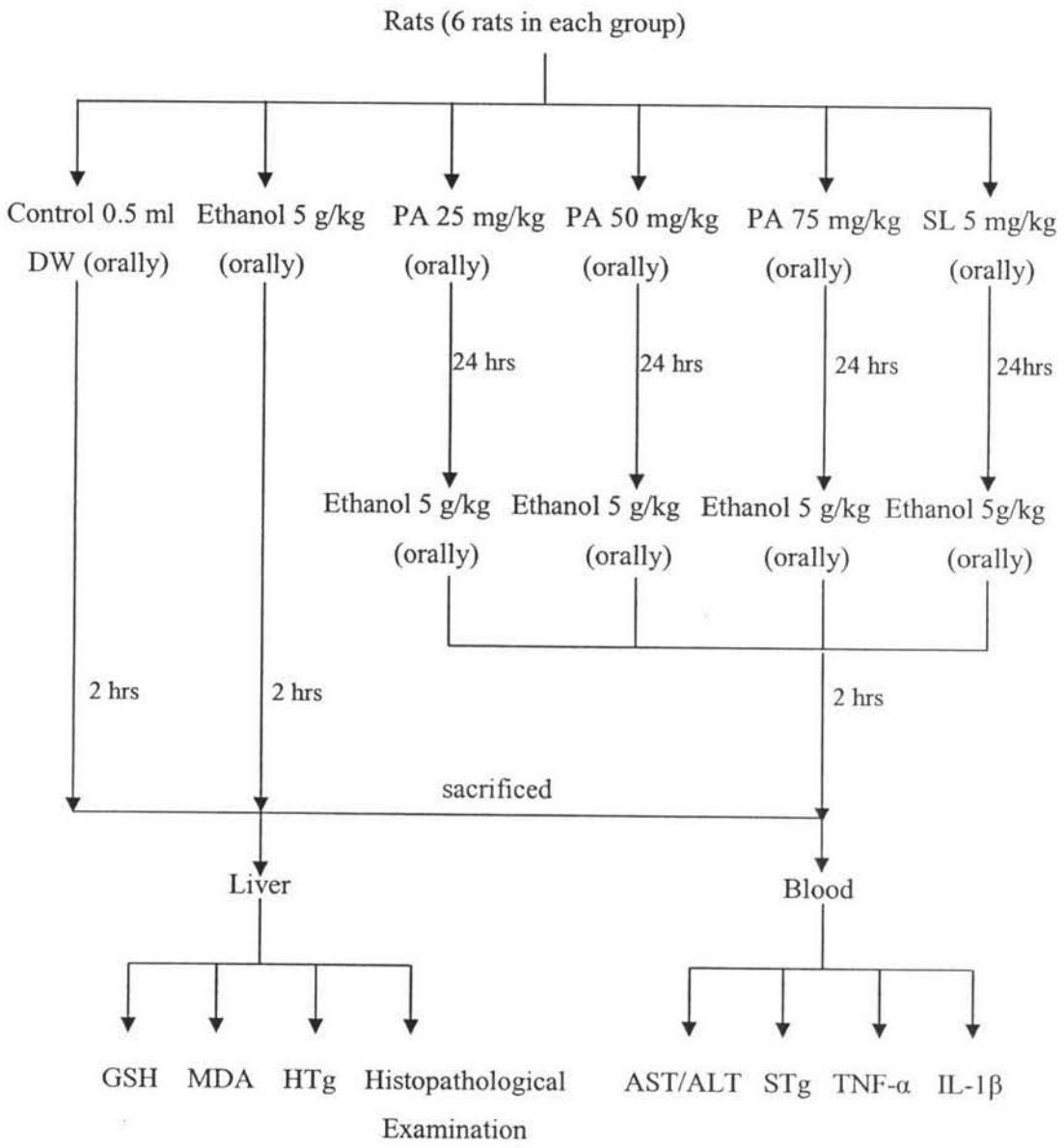
Group 3 received ethanol (4 g/kg/day) orally for 21 days, self recovery for 7 days.

Group 4 received ethanol (4 g/kg/day) orally for 21 days and then treated with PA at the most effective dose from acute toxicity study (75 mg/kg/day) orally for 7 days.

Group 5 received distilled water (5 ml) orally for 21 days and then received PA (75 mg/kg/day) orally for 7 days.

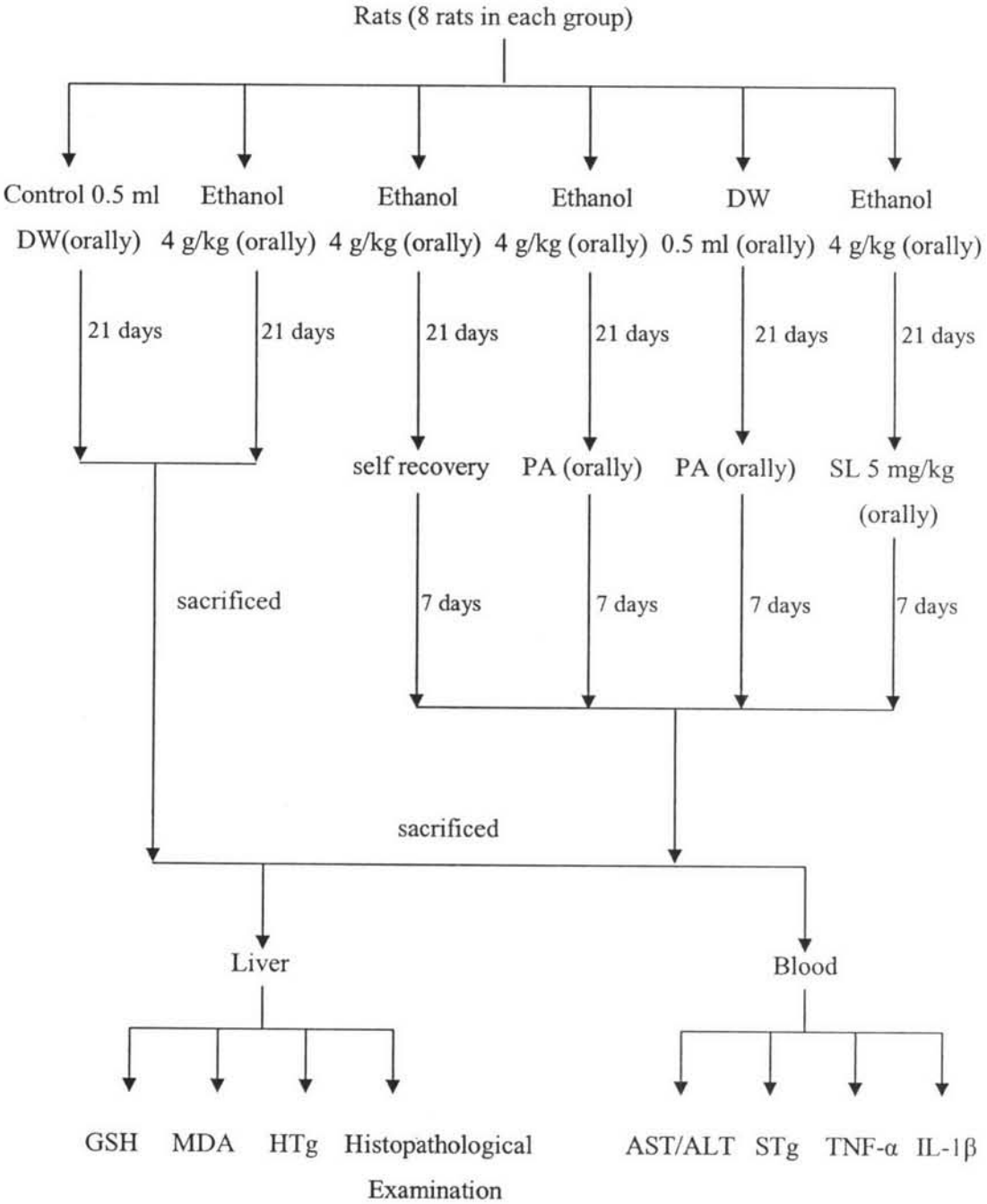
Group 6 received ethanol (4 g/kg) orally for 21 days and then treated with SL (5 mg/kg/day) orally for 7 days.

On the 21 st day, rats in group 1 and 2 were sacrificed and rats in group 3-6 were sacrificed on day 29. Blood and liver sample were collected for serum and liver homogenate.



* DW: Distilled water, SL: Silymarin, GSH: Glutathione, MDA: Malondialdehyde, HTg: Hepatic triglyceride, STg: Serum triglyceride, AST: Aspartate aminotransaminase, ALT: Alanine aminotransaminase, TNF-α: Tumor necrosis factor alpha, IL-1β: Interleukin 1 beta.

Figure 8. Diagram illustrated administration of PA extract 24 hours before single oral dose of ethanol (5 g/kg) in acute toxicity study.



* DW: Distilled water, SL: Silymarin, GSH: Glutathione, MDA: Malondialdehyde, HTg: Hepatic triglyceride, STg: Serum triglyceride, AST: Aspartate aminotransaminase, ALT: Alanine aminotransaminase, TNF-α: Tumor necrosis factor alpha, IL-1β: Interleukin 1 beta.

Figure 9. Diagram illustrated administration of PA extract for 7 days after ethanol (4 g/kg/day) for 21 days in sub-acute toxicity study.

3.6 Collection of sample and preparation of serum and liver homogenate

After the last oral dose of each experimental, rats were anesthesized with diethyl ether. The abdomen was opened and blood was collected (2.5 ml) via inferior vena cava and kept in eppendrof tube. Serum was separated from blood by centrifugation at 3000 rpm, 4 °C for 10 min which was used for the determination of alanine transaminase (ALT), aspartate transaminase (AST) and serum triglyceride (STg), tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β). After blood collection, the liver was perfused with 0.9% cold saline to completely remove all the red blood cells. Then it was removed, rinsed in 0.9% cold saline and weighed. The liver was divided into 2 parts. First, the largest lobe was divided into 2 parts for histopathological examination by fixing in 10% neutral buffer formalin and wrapping with aluminum foil and keeping in -80 °C. Second, the residual liver was homogenized with 15 ml iced-cold 0.1M phosphate buffer (pH 7.4) using homogenizer. The homogenate was used for determination of reduced glutathione (GSH), malondialdehyde (MDA) and hepatic triglyceride (HTg).

3.7 Determination of clinical chemistry parameters

3.7.1 Determination of serum ALT and AST

ALT and AST levels were determined by using the reagent kits (Human Gesellschaft fur Biochemica und Diagnostica mbH, Germany). Method based on kinetic reaction of the International Federation of Clinical Chemistry (IFCC) (Bergmeyer *et al.*, 1986).

Procedures

1.) The reagent for ALT or AST 500 µl in each tube was incubated in water bath at 37 °C.

2.) Serum 50 µl was pipetted to reagent tube.

3.) After mixing, the absorbance after 1, 2, 3 and 4 minute was measured by spectrophotometer at wavelength 340 nm.

Calculations

The ALT and AST levels were given by:

$$\text{ALT or AST} = \frac{(A_4-A_1) \times 1745}{(T_4-T_1)}$$

A = absorbance

T = time

The unit expressed as unit per liter (U/L)

3.7.2 Determination of serum triglyceride (STg)

STg was determined by using the triglyceride reagent kits (Human Gesellschaft fur Biochemica und Diagnostica mbH, Germany).

Procedures

1.) The reagent was warmed to room temperature and dispensed into each tube 1 ml.

2.) Standard 10 µl or Serum 10 µl or distilled water (blank) 10 µl was added to the reagent tubes, then mixed and incubated in water bath at 37^0C for 5 minutes.

3.) The absorbance was measured by Spectrophotometer at wavelength 500 nm.

Calculations

The STg levels were given by:

$$\text{STg} = \frac{A_{\text{sample}} - A_{\text{blank}} \times 200}{A_{\text{standard}} - A_{\text{blank}}}$$

The unit expressed as milligram per deciliter (mg/dl).

3.7.3 Determination of hepatic triglyceride (HTg)

HTg was determined by the method of Mendez *et al.* (1975).

Procedures

1.) Liver homogenate 0.5 ml and distilled water (blank) 0.5 ml were added with Heptane 2 ml, Isopropanol 3.5 ml and 0.004 M Sulfuric acid 1 ml respectively, then mixed thoroughly for 30 seconds and allowed to stand at room temperature for 15 minutes.

2.) The upper layer 0.2 ml and standard (10, 20, 30 and 40 µl) were reacted with isopropanol 2 ml and saponification reagent 0.6 ml respectively, then mixed and allowed to stand at room temperature about 5 minutes.

3.) Sodium metaperiodate 1.5 ml was added to each tube and mixed thoroughly, then added with acetyl acetone reagent 1.5 ml and mixed thoroughly.

4.) All tubes were incubated in water bath at 65 ^{0}C for 15 minutes.

5.) The absorbance was measured by Spectrophotometer at wavelength 415 nm within 45 minutes.

Calculations

HTg level were obtained from the standard curve plotted between the absorbance of standards against the amount of standard. The unit expressed as milligram per gram liver (mg/g liver).

3.7.4 Determination of hepatic reduced glutathione (GSH)

GSH level was determined by the method of Ellman (1959) and Jollow *et al.* (1977).

Procedures

1.) Liver homogenate 0.5 ml and distilled water (blank) 0.5 ml were added with 4% sulfosalicylic acid 0.5 ml, then mixed thoroughly.

2.) Centrifuged at 3,000 rpm. for 10 minutes.

3.) Supernatant 0.5 ml was reacted with 0.1 mM 5'5 Dithiobis (2-nitro benzoic acid) in 0.1 M phosphate buffer solution 4.5 ml, then mixed thoroughly.

4.) The absorbance was measured by Spectrophotometer at wavelength 412 nm.

Calculations

GSH level were calculated by using the extinction coefficient ($E = 1.36x10^{4}\ M^{-1}cm^{-1}$) and expressed as micromole per gram liver (µmol/g liver).

3.7.5 Determination of hepatic malondialdehyde (MDA)

MDA, as an index for lipid peroxidation, was determined by the thiobarbituric acid reactive substances (TBARs) method (Budge and Aust, 1978).

Procedures

1.) Liver homogenate 1 ml and distilled water (blank) 1 ml were added with TCA-TBA solution 3 ml, then mixed thoroughly and heated in boiling water(100°C) for 15 minutes.

2.) Centrifuged at 3,000 rpm for 10 minutes.

3.) The absorbance of the supernatant was measured by Spectrophotometer at wavelength 535 nm.

Calculations

MDA level were calculated by using the extinction coefficient ($E = 1.56 \times 10^5 M^{-1}cm^{-1}$) and expressed as nanomole per gram liver (nmol/g liver).

3.7.6 Determination of serum TNF-α

TNF-α levels were determined by rat TNF-α kit (Biosource International, Inc., U.S.A.). The method based on Enzyme Linked-Immuno-Assay (ELISA). The antibody specific for TNF-α has been coated onto the wells of the plate. Standards and samples were pipetted into these wells, followed by addition of a biotinylated second antibody. After removal of excess second antibody, streptavidin-peroxidase was added to wells for binding to the biotinylated antibody. After washing to remove all the unbound enzyme, a substrate solution was added, which was acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the level of TNF-α in the standards and samples.

Procedures

1.) All wells were added with incubation buffer 50 µl.

2.) Five wells of standard were added with 100 µl of standard 15.6, 31.2, 62.5, 125, 250 pg/ml, respectively, but well of standard 0 pg/ml was added with standard diluent buffer 100 µl.

3.) Wells of samples were added with standard diluent buffer 50 µl to each well followed by serum 50 µl. Tap gently on the side of the plate to mix.

4.) All wells were added with biotinylated anti-TNF-α (biotin conjugate) solution 50 µl. Tap gently on the side of the plate to mixed, covered plate with plate cover and incubated for 90 minutes at room temperature.

5.) Solution were decanted from wells and washed 4 times with wash buffer.

6.) Streptavidin-HRP working solution 100 µl was added into each well, then covered plate with plate cover and incubated for 45 minutes at room temperature.

7.) Solution were decanted from wells and washed 4 times with wash buffer.

8.) Stabilized chromogen 100 µl was added into each well. The liquid in the wells begin to turn blue. Plate was covered with plate cover and incubated in the dark for 30 minutes at room temperature.

9.) Stop solution 100 µl was added into each well. Tap gently on the side of the plate to mix. The solution in the wells were changed from blue to yellow.

10.) The absorbance was measured by Microtiter plate reader at wavelength 450 nm within 2 hours after adding the stop solution.

Calculations

TNF-α levels were obtained from the standard curve plotted between the absorbance of standards against the amount of standard. The unit expressed as picogram per milliliter (pg/ml).

3.7.7 Determination of serum IL-1β

IL-1β levels were determined by rat IL-1β kit (Biosource International, Inc., U.S.A.). The method based on Enzyme Linked-Immuno-Assay (ELISA). The antibody specific for IL-1β has been coated onto the wells of the plate. Standards and samples were pipetted into these wells. After washing, a biotinylated second antibody was added and washed excess second antibody. Streptavidin-peroxidase is added to wells for binding to the biotinylated antibody. After washing to remove all the unbound enzyme, a substrate solution was added, which was acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the level of IL-1β in the standards and samples.

Procedures

1.) Five wells of standard were added with 100 µl of standard 31.2, 62.5, 125, 250 and 500 pg/ml respectively, but well of standard 0 pg/ml was added with standard diluent buffer 100 µl.

2.) Wells of samples were added with standard diluent buffer 50 µl to each well followed by serum 50 µl. Tap gently on the side of the plate to mix. Plate was covered with plate cover and incubated for 3 hours at room temperature.

3.) Solution were decanted from wells and washed 4 times with wash buffer.

4.) All wells were added with biotinylated anti-IL-1β (biotin conjugate) solution 100 µl. Tap gently on the side of the plate to mixed, covered plate with plate cover and incubated for 1 hour at room temperature.

5.) Solution were decanted from wells and washed 4 times with wash buffer.

6.) Streptavidin-HRP working solution 100 µl was added into each well, then covered plate with plate cover and incubated for 30 minutes at room temperature.

7.) Solution were decanted from wells and washed 4 times with wash buffer.

8.) Stabilized chromogen 100 µl was added into each well. The liquid in the wells begin to turn blue. Plate was covered with plate cover and incubated n the dark for 30 minutes at room temperature.

9.) Stop solution 100 µl was added into each well. Tap gently on the side of the plate to mix. The solution in the wells were changed from blue to yellow.

10.) The absorbance was measured by Microtiter plate reader at wavelength 450 nm within 2 hours after adding the stop solution.

Calculations

IL-1β levels were obtained from the standard curve plotted between the absorbance of standards against the amount of standard. The unit expressed as picogram per milliliter (pg/ml).

3.8 Histopathological examination

The histological study was supervised and analyzed by Assistant Professor Somlak Poungshompoo, a pathologist at Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University. The standard procedures for

histopathological examination (ศุภลักษณ์ โรมรัตนพันธ์, 2539; Humason, 1979) follow a logical and specific sequence as follows:

3.8.1 Fixation: Liver sample was fixed in 10% neutral buffer formalin for prevention of cell destruction by bacteria and the process of autolysis (self-digestion), protection of cells from shrinkage and distortion during dehydration, embedding and sectioning, and improvement of staining potential of cell parts including alteration refractive indices of cell for better visibility.

3.8.2 Washing: After fixation, the excess of 10% neutral buffer formalin were washed out from tissue to prevent interference with subsequent process using running water.

3.8.3 Dehydration: During fixing and washing, tissues have a high water content which can obstruct later processing. Therefore, it is necessary to remove water from tissues by immersing in gradually increasing percentages of ethyl alcohol for reduction of the shrinkage in the tissues.

3.8.4 Clearing: Alcohol used for dehydration did not dissolve or mix with melted paraffin, so the tissues were immersed in clearing agent such as xylene which dissolved in both alcohol and paraffin before infiltrating with paraffin.

3.8.5 Infiltration: After clearing, tissues were replaced the fluid by melted paraffin which harden to a firm and easily sectioned material.

3.8.6 Embedding: The tissue is thoroughly infiltrated with paraffin, placed in embedding mold, filled with melted paraffin and the whole was cooled rapidly for paraffin to solidity around and within the tissues.

3.8.7 Sectioning: The tissue blocks were sectioned thickness 6 μ by Microtome, then mounted on glass slides which prepared for staining.

For the examination of tissue fat, tissue blocks were sectioned (6 μ) as frozen sections by Cryostat to prevent the solvents used in conventional histological preparations dissolved the fat.

3.8.8 Staining

1.) Hematoxylin and Eosin (H&E) staining.

Hematoxylin is the basic dye which stains the acid components in cells such as nucleus and nucleolus, resulting in deep blue appearance. On the other hand, eosin is the acid dye which stains the basic components present in cytoplasm,

resulting in pink appearance. This staining make various tissue components conspicuous and permit distinctions to be made between them.

Procedures

1.) Immerse in xylene I and II for 10 minutes in each step.

2.) Immerse in 100% alcohol I and II for 2 minutes in each step.

3.) Immerse in 95% alcohol for 2 minutes.

4.) Immerse in 70% alcohol for 2 minutes.

5.) Wash in running water for 5 minutes.

6.) Stain in hematoxylin for 6 minutes.

7.) Wash in running water for 5 minutes.

8.) Dip 1 time in 1% acid alcohol.

9.) Wash in running water for 5 minutes.

10.) Dip 4 times in litium carbonate ($LiCO_3$).

11.) Wash in running water for 5 minutes.

12.) Stain in eosin for 5 minutes.

13.) Dip 5 times in 95% alcohol.

14.) Immerse in 100% alcohol I and II for 2 minutes in each step.

15.) Immerse in xylene I, II and III for 5 minutes in each step.

16.) Mount with permount and put a cover glass onto the slide.

2.) Oil red O staining.

Oil red O is the oil-soluble dye which dissolves in a lipid contained within the tissues, but it must not be dissolved in water, the major constituent of cells. The result of staining, fat vacuole and nucleus appeared bright-red and blue, respectively.

Procedures

1.) Fix slide in 10% formalin for 10 minutes.

2.) Wash in running water for 5 minutes.

3.) Immerse in propylene glycol for 5 minutes.

4.) Stain in Oil red O for 10 minutes.

5.) Immerse in 85% propylene glycol for 3 minutes.

6.) Rinse in distilled water.

7.) Stain in hematoxylin for 1 minutes.

8.) Wash in running water for 5 minutes.

9.) Rinse in distilled water.

10.) Mount with aqueous mounting media, Glycerin Jelly and put a cover glass onto the slide.

3.) Periodic Acid Schiff (PAS) staining.

This staining is used to identify glycogen in tissues. The reaction of periodic acid selectively oxidizes the glucose residues, creates aldehydes that react with the Schiff reagent and creates a purple-red color. While nucleus appeared blue of hematoxylin.

Procedures

1.) Immerse in xylene I and II for 10 minutes in each step.

2.) Immerse in 100% alcohol I and II for 2 minutes in each step.

3.) Immerse in 95% alcohol for 2 minutes.

4.) Wash in running water for 5 minutes.

5.) Immerse in periodic acid for 5 minutes.

6.) Wash in running water for 5 minutes.

7.) Immerse in Schiff reagent for 30 minutes.

8.) Immerse in sodium bisulfite 3 times for 3 minutes in each step.

9.) Wash in running water for 10 minutes.

10.) Stain in hematoxylin for 1 minutes.

11.) Wash in running water for 5 minutes.

12.) Immerse in 95% alcohol for 5 minutes.

13.) Immerse in 100% alcohol for 5 minutes.

14.) Immerse in xylene for 15 minutes.

15.) Mount with permount and put a cover glass onto the slide.

4.) Masson's Trichrome staining.

The tissue is stained first with the acid dye, Biebrich scarlet acid fuchsin solution, which binds with the acidophilic tissue components. Then treated with the phosphor acids, the less permeable components retain the red, while the red is pulled out of the collagen. At the same time causing a link with the collagen to bind with the aniline blue. The results of this staining, the nucleus appeared black, cytoplasm and muscle appeared red and collagen appeared blue.

Procedures

1.) Immerse in xylene I and II for 10 minutes in each step.

2.) Immerse in 100% alcohol I and II for 2 minutes in each step.

3.) Immerse in 95% alcohol for 2 minutes.

4.) Wash in running water for 5 minutes.

5.) Modant in Bouin's solution for 1 hour at 56 ^{0}C.

6.) Wash in running water to remove the yellow color of Bouin's solution

7.) Rinse in distilled water.

8.) Stain in Weigert's iron hematoxylin solution for 10 minutes.

9.) Wash in running water for 10 minutes.

10.) Rinse in distilled water.

11.) Stain in Biebrich scarlet-acid fuchsin solution for 10 minutes.

12.) Rinse in distilled water.

13.) Immerse in Phosphomolybdic-phosphotungstic acid solution for 15 minutes.

14.) Stain in aniline blue for 10 minutes.

15.) Rinse in distilled water.

16.) Immerse in 1% acetic acid for 5 minutes.

17.) Immerse in 95% alcohol 2 times for 5 minutes in each step.

18.) Immerse in 100% alcohol 2 times for 5 minutes in each step.

19.) Immerse in xylene 2 times for 5 minutes in each step.

20.) Mount with permount and put a cover glass onto the slid

3.8.9 Microscopic finding and analysis.

After staining, the slides of tissues were analyzed and graded of the pathological changes under light microscope by a pathologist.

3.9 Data analysis

The data of clinical chemistry parameters were expressed as mean ± standard error of mean (SEM).

The data of histopathological examination were expressed as grading and light micrograph.

Results were analyzed statistically by one-way analysis of variance (ANOVA) followed by Tukey's test using the SPSS 11.5 for window software. P-value < 0.05 was regarded as statistically significant.

3.10 Ethical consideration

The protocols in this study were ethically approved by the Ethical Committee on Animal and Human Research Studies, Faculty of Pharmaceutical Sciences, Chulalongkorn University.