CHAPTER III

MATERIALS AND METHODS

Apparatuses and Equipments

All apparatuses and equipments are listed in Appendix A.

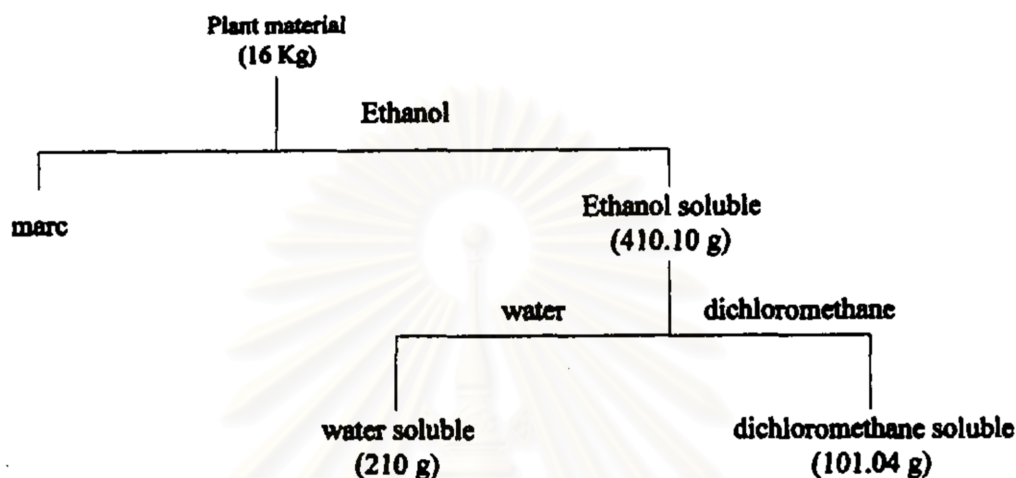
Chemical Agents

All chemical agents are listed in Appendix A.

Extraction Procedure of *Derris trifoliata* leaves

The ground sun-dried leaves (16 kg) were extracted with ethanol by soxhlet extraction apparatus. After 12 hours extraction, the solvent became dark green. The ethanol solution were concentrated and removed under reduce pressure by rotary evaporator gave an ethanol crude extract. The same procedure of soxhlet extraction was repeated four times and removed out the solvent gave the ethanol crude extract of 410.10 g (2.56 % wt. by wt.) as dark green gum. The ethanol crude extract was further separated by partition between dichloromethane : water in the liquid-liquid extractor for 48 hours. Most of the polar compounds are dissolved in water. The water soluble part was dried by the freeze drier gave a water soluble crude extract of 210 g (1.3125 % wt. by wt.) as a pale brown crystal. The non-polar compounds are dissolved in dichloromethane. The dichloromethane solution was evaporated the solvent under reduce pressure gave a dichloromethane crude extract of 101.04 g (0.6315 % wt. by wt.) as a dark green gum. The result of extraction of *D. trifoliata* leaves are shown in Appendix B. The scheme of extraction procedure is shown in Figure 3.1.

Figure 3.1 Extraction procedure of the leaves of *Derris trifolia* .



Experimental Animal

Nile tilapia *Oreochromis niloticus*, were kindly supplied by Bangkant Inland Fisheries Center, Patumtanee. Two days old Nile tilapia were cultured in hatching tank at the department of biology, Chulalongkorn University. The fish were fed by boiled yolk three times a day for 3 days. After that, they were fed ad libitum by the commercial feed ration (38% protein) until they were 30 days old. Abnormal, injured, and dead individuals were discarded.

The appropriate numbers of equal-sized (fingering size) fish were transferred to each test chambers, 4 days prior to the experiment for acclimation period. The fish were acclimated in fresh and clean dilution water.

Dilution Water

Dechlorinated tap water was used as dilution water. The tap water was passed through carbon filter to remove toxic metals and organic matters, and to control hardness and alkalinity. The dilution water was kept in 200 L tank and aerated for maintain the dissolved oxygen concentration during the holding time.

Preparation of Test Solution

There are two parts of *Derris trifoliata* crude extract in this experiment; the water soluble part and the water insoluble part. The water soluble part is easily dissolved in dilution water. On the other hand, the water insoluble part (dichloromethane soluble) can not dissolve in dilution water thus the solvent (95% ethanol) must be added to make it suspend in the dilution water.

The stock solution were prepared by ;

(1) The water soluble part, dissolved 1 g of the water soluble extract direct into 10 L dilution water to a final concentration of 100 mg/L (100 ppm).

(2) The dichloromethane part, dissolved 1 g of the water insoluble extract into 5 ml of 95% ethanol and then diluted to 10 L with dilution water to a final concentration of 100 mg/L (100 ppm).

Both of the stock solutions were diluted by dilution water to prepare the appropriate concentration of testing solution.

Acute Toxicity Testing

Preliminary Toxicity Range-Finding Tests : Consists of control, solvent-control and a down-scaled of widely-spaced sample dilution five concentration levels based on a logarithmic ratio, three replicates a group, abbreviated static acute test in which groups of five Nile tilapia in each replicate. The test concentrations were 0.01, 0.1, 1, 10, 100 ppm., selected the part in which more effective to the fish for continue testing in definitive tests. The amount of mortal fish of range-finding tests were recorded.

Multi-Concentration (Definitive) Toxicity Tests : Use in determining (1) a point estimate of toxicity in terms of LC_{50}, (2) a no-observed-adverse-effect concentration (NOAEC) defined in terms of mortality, and obtain by hypothesis testing. The crude from water soluble part is not effect to juvenile Nile tilapia at any concentration, so the definitive test performed only the crude from water insoluble part. The tests consist of three group (1) control, (2) solvent-control and (3) treatment group are at least five concentrations commonly selected a geometric series. There

were three replicates per group, in which groups of ten fish in a replicate. The first test concentrations were 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 ppm., the second test concentrations were 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5 ppm. The amount mortal fish of definitive tests were record.

Test Procedure : The acute toxicity tests were static non-renewal technique. The experiment chambers were filled with 10 L dilution water and or testing solutions. Only dilution water filled the chambers as the control group. The dilution of 95% ethanol (3 ml of 95% ethanol dilute to 10 L with dilution water, 0.03%) was solvent control group. And the appropriate concentrations of testing solutions as the treatment group. Nile tialapia aged 30 days with an average of 3.187 (± 0.282) cm in length, and an average of 0.446 (± 0.155) g in weight were randomly put into each chamber. Loading of test organisms were approximate 0.446 g/L. The fish were not fed 24 hours before starting and 96 hours during the experiment. The symptoms in which showed and the number of dead fish were recorded every 24 hours from beginning throughout the end of the experiment (96 hours). Dissolved oxygen, pH, temperature and hardness were measured every 24 hours.

Data Analysis : The end point of acute toxicity testing is (1) an estimate of the concentration which is lethal to 50% of the test organisms in the time period prescribed by the test, expressed as the LC_{50}, or (2) the highest concentration at which survival is not significantly different from the control (No-Observed-Effect Concentration, NOEC). The LC_{50} determined by probit analysis method (Finney, 1971), the NOEC determined by hypothesis testing.

Sub-acute Toxicity Testing

Test Concentration : The threshold concentration that produces statistically significant deleterious effects is expressed as the maximum acceptable toxicant concentration (MATC). The MATC is hypothetical concentration and is in a range of no-observed-effect concentration (NOEC) and the lowest observed-effect concentration (LOEC).

Therefore, the MATC can be represented as NOEC < MATC < LOEC (Mount and Stephan, 1967). The test concentration was estimated by use of the application factor (AF) concept. The AF is a unitless chemical-specific measure calculated as the threshold chronically toxic concentration of a chemical divided by its acutely toxic concentration. In practice, AF is calculated by dividing the limits (NOEC and LOEC) of the MATC by the LC_{50} 96-h.

$$AF = MATC / LC_{50} = (NOEC \sim LOEC) / LC_{50}$$

The range of NOEC~LOEC determined by hypothesis testing of mortality on the acute toxicity testing. The median lethality concentration (LC_{50}) at 24-h, 48-h, 72-h, 96-h were calculated by probit method (Finney, 1971). The average of upper and lower AF was used to estimate the MATC by this equation; $MATC = AF \times LC_{50}$. The test concentration determined by using of 0.5 dilution series from MATC. Because limitation of the budget, the 12.5% of MATC approximately 0.2 mg/L was selected to the test concentration.

Experimental Procedure : Sub-acute toxicity test was static renewal technique. All aquarias were renew every 72-hours. The experiment were carried out in 7 glass aquarium tanks, 50x120x50 cm., containing 150 L of dilution water and test solution, with continuous aeration. The control group and solvent control group (95% ethanol 3 ml in 150 L dilution water, 0.002 %) were two replicates, the treatment group (0.2 ppm) was three replicates. Nile tialapia aged 30 days were randomly allocated into aquarium tanks, each aquaria contained 100 fish. Loading of test organisms were approximate 0.297 g/L. The fish were fed twice a day with 38 % protein commercial feed ration except on the sampling day.

Physical and Chemical Properties of dilution water : For maintenance the physical and chemical properties of dilution water and test solution, all aquarias were renew every 72 hr. The following parameters were measured in all aquarias.

- Temperature (°C) was recorded every day.
- DO, pH, and hardness were determined every 3 days.

Data Sampling : Every month, randomly collected 8 fish in each experimental unit measuring the following parameters, that are described below.

- Growth test : The fish in each group were individually measured the standard body length and body weight (wet weight).

- Liver collecting : The part of fish liver were fixed in 10% buffer formalin for paraffin technique and fixed in 4% glutaraldehyde in Millonig's phosphate buffer and 1% osmium tetroxide for electron microscope study.

- Relative weight index : Measured the liver weight. The relative weight index were calculated by dividing the fish liver weight by its body weight.

Data Analysis : (1) The tissue studies were examined the histopathological alterations and ultrastructural change of the fish livers between and among the experiment groups, and described the histopathological alterations which were found in each month throughout five months of experiment.

(2) Determine sub-acute toxicity by one-way analysis of variance method for comparison of the body length and body weight (growth rate) and relative liver weight index between and among group.

Histopathological Study

Light Microscopic Study : There are two categories of light microscope study technique; the standard paraffin technique and the frozen technique.

(1). The preparation of fish liver for standard paraffin technique, the liver was cut into small pieces about 0.5-1 cm^3 , and fixed in 10% formalin in phosphate buffer pH 7.2 for 24 hours at room temperature. After that the tissues were dehydrated in the series of ethanol and embedded in paraplast. The paraffin blocks were cut at 5 µm thickness by the rotary microtome, the sections were attached with the slides and dry at 40 °C on warm plate. These section were deparaffined with xylene and hydrated in the series of ethanol before staining by heamatoxylin and eosin.(Gurr, 1963), and then examined under light microscope. The tissue embedding procedure is shown in Figure 3.2 and heamatoxylin/eosin staining procedure is shown in Figure 3.3.

(2). The frozen technique are used for study on the histochemistry. The liver was cut into a small pieces approximate size at 3 mm^3, and then rapidly picked up the tissue pieces in foil molds and filled it with the frozen medium (tissuetek). The molds

were immediately cooled down at -20 °C. The frozen tissues were cut at 5 μm thickness by the cryostat microtome, the section were attached with the slides and air dry before staining. There were used of two staining techniques in this study for histochemical investigation.

- The Oil red O staining technique were used for study on the lipid composition of the fish liver (Culling, 1963). The staining procedure is shown in Figure 3.4.

- The Periodic acid / Schiff (PAS) technique were used for study on the value of glycogen composition (Gurr, 1963). The staining procedure is shown in Figure 3.5.

Ultrastructural Study : The fish liver were minced and fixed in 4% glutaraldehyde in Millonig's phosphate buffer pH 7.2 at 4°C for 30 minutes. Following 3 minutes x 3 times washed with Millonig's phosphate buffer, the tissues postfixed in 1 % osmium tetraoxide in Millonig's phosphate buffer, at 4°C for 45 minutes. After rinsing with distilled water 3 minutes x 2 times, the tissues were exposed to 2% aqueous uranyl acetate for 20 minutes and dehydrated in the series of ethanol, and cleared in propylene oxide. The dehydrated tissues were infiltrated with propylene oxide : Epon 812 resin (50:50) at 37°C for 30 minutes and transferred into Epon 812 only for 2 hours and finally embedded in Epon 812 resin with polyethylene beam capsule block, at 70°C overnight. The capsules were squeezed to release the plastic blocks and then continued hardened in hot air oven at 100°C for 1 hour before sectioning. Ultrathin sections were cut by glass knives, collected on copper grids and stained with uranyl acetate and lead citrate. The tissue processing procedure of ultrastructure investigation is shown in Figure 3.6. The ultrastructural of histopathological alteration of the fish livers were examined under the transmission electronmicroscope (JEOL model JEM-2004CX) and described the pathological effect in which found in treatment group.

Figure 3.2 The tissue embedding procedure

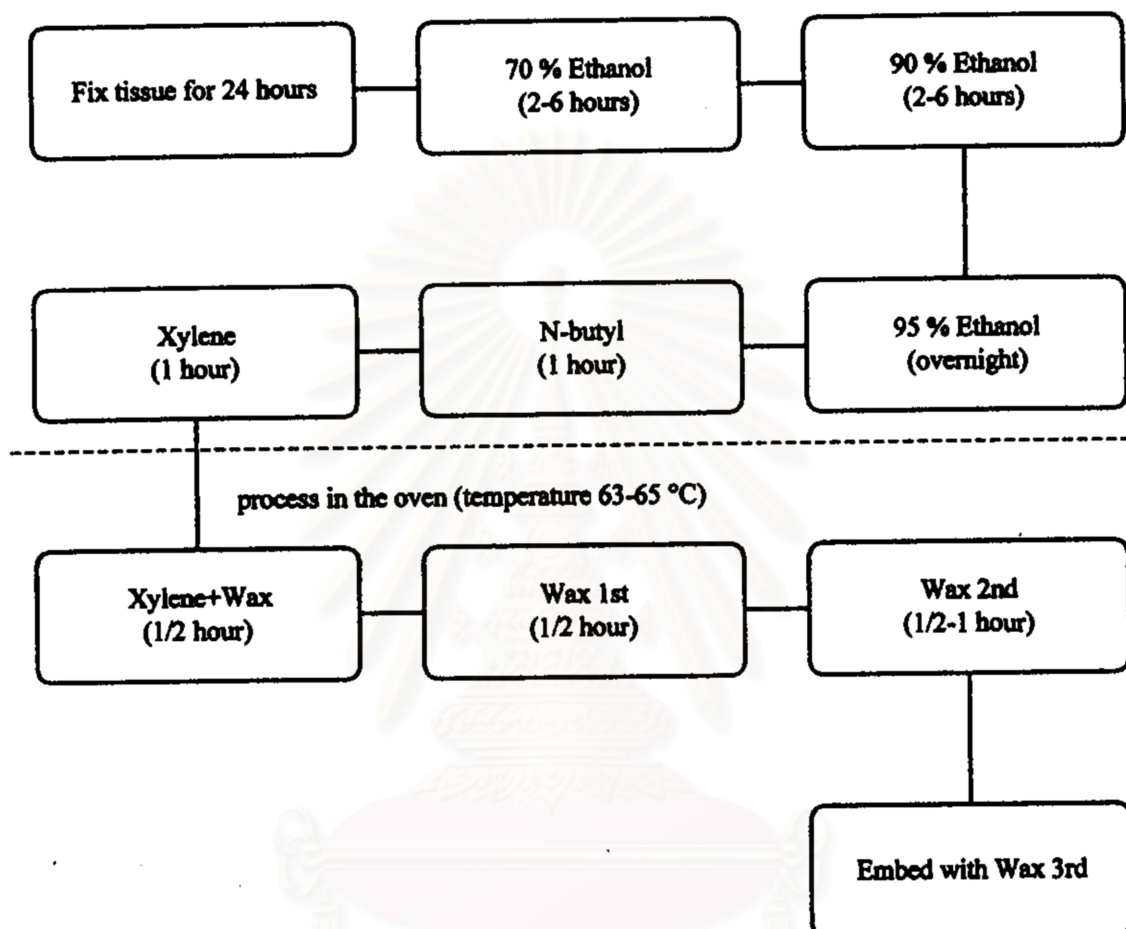


Figure 3.3 The heamatoxylin/eosin staining procedure

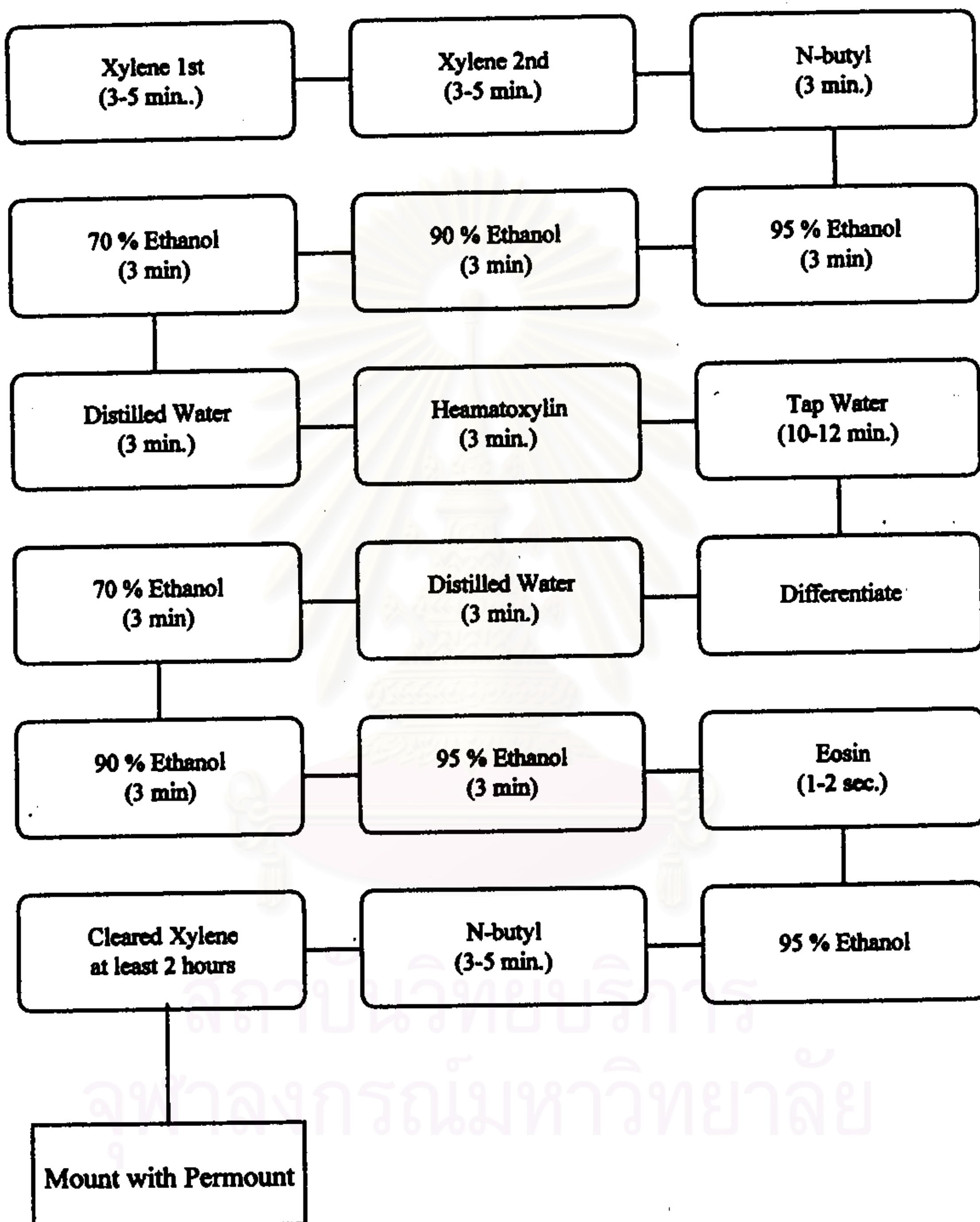


Figure 3.4 The Oil red O staining procedure

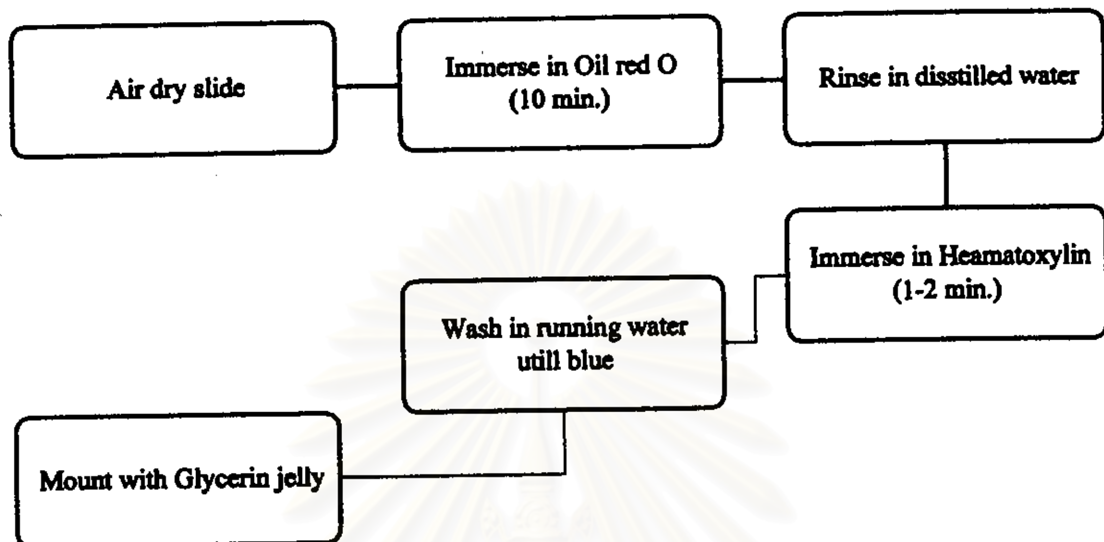


Figure 3.5 The Periodic acid / Schiff (PAS) procedure

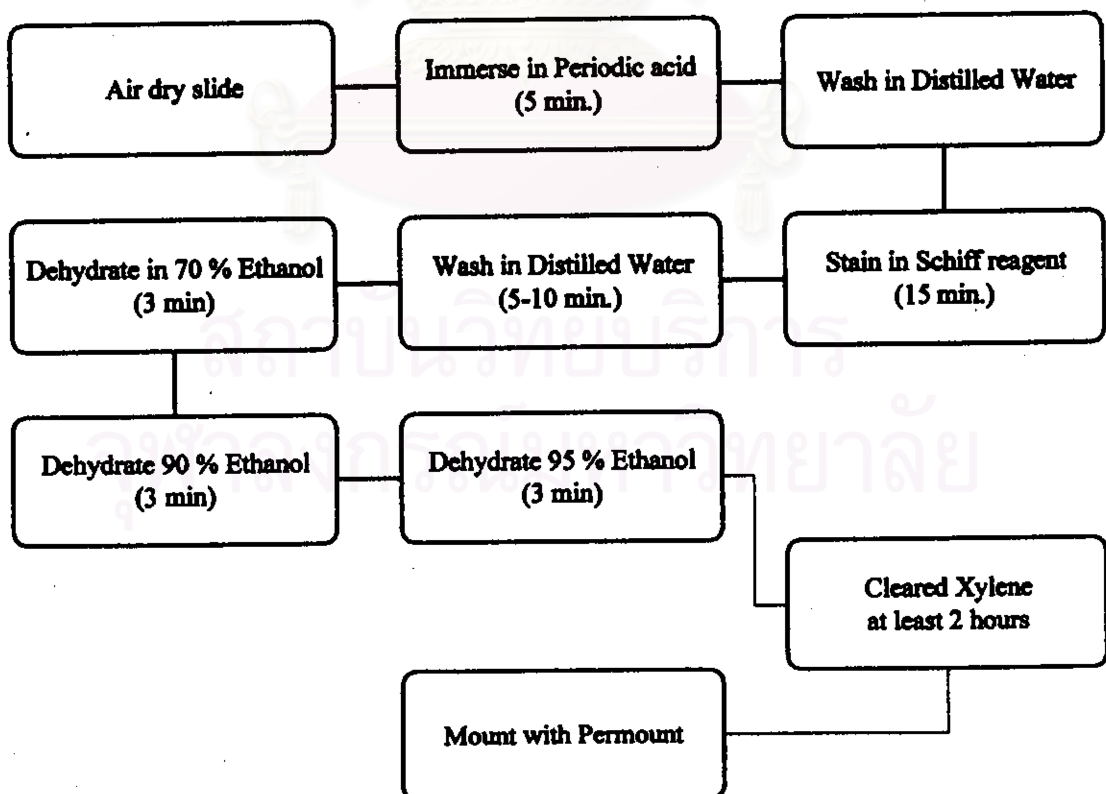


Figure 3.6 Diagram of Rapid Method for Electron Microscope Technique (Modified by Department of Pathology, Faculty of Medicine, Mahidol University)