CHAPTER III

RESEARCH METHOD

III.1. Scope of Study,Place and Time of The Research

Scope of study include Pathobiology, biochemistry, histopathology and The study conducted in Animal Laboratory at Gajah Mada University between October -December 2011.

III.2.Research Design

Study design is experimental study, namely post test only control group design. The samples randomly divided into 3 intervention group and 1 group for control.

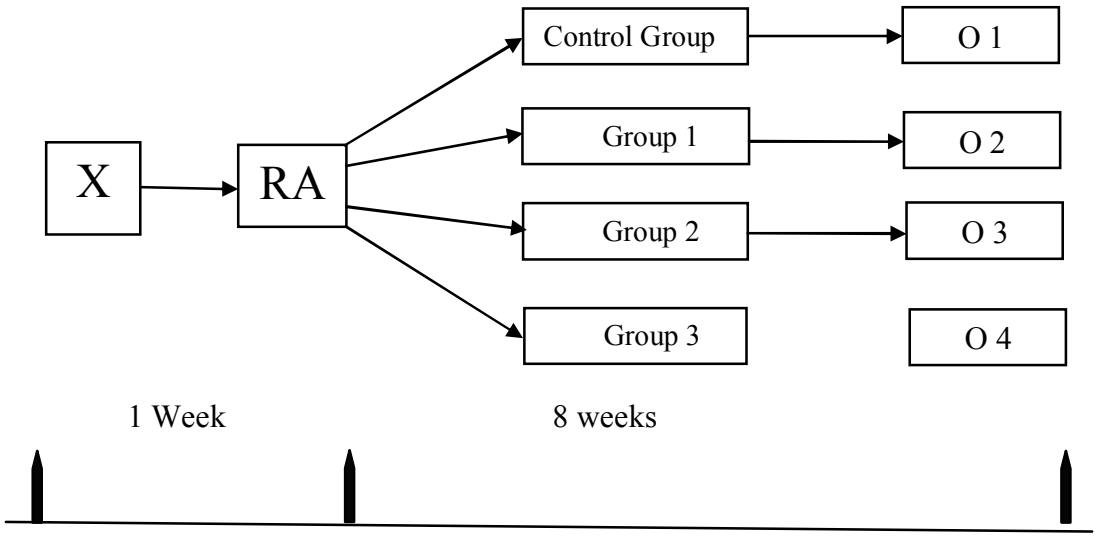

Figure 7 : The scheme of the study

Notes :

X = Adaptation time.

RA = Random Allocation into 4 groups.

Control Group =each rat received ethanol (8g/kg of b.w per day) by orally.

Group 1 (G1) = each rat received extract of *Nigella sativa* seeds in a dose (0.5 g/kg of b.w per day) by orally then ethanol (8g/kg of b.w per day) by orally.

Group 2 (G2) = each rat received extract of *Nigella sativa* seeds in a dose (**1** g/kg of b.w per day) by orally then ethanol (8g/kg of b.w per day) by orally.

Group 3 (G3) = each rat received extract of *Nigella sativa* seeds in a dose (1.5 g/kg of b.w per day) by orally then ethanol (8g/kg of b.w per day) by orally.

Every group get (1) additional rat for replacing rat which dies during the study period, all the groups receive extract of *Nigella sativa* seeds and ethanol for 8 weeks.

O1 = Output from the control group.

O2 = Output from the group 1.

O3 = Output from the group 2.

O4 = Output from the group 3.

III.3. Population of The study

Population of the study are healthy male Wistar rats inbred strain of Animal Laboratory at Gajah Mada University. Samples were taken from such rats which fullfill to the following criteria :

III.3.1 Inclusion Criteria

Wistar rat (male), in active condition and 12-16 weeks old and weight is about (250-300 g).

III.3.2 Exclusion Criteria

Wistar rat shows different activities and died before experiment ends.

III.4. Sample Size

Determine the number of samples based on WHO requriement with a sample of at least 5 . Sample in this research is Wistar rats (Male) .The number of the rats is 24 rats , divided into four groups, each of group consist of 6 rats .

III.5.Research Variable

III.5.1. Independent variable

The independent variable is various doses of *Nigella sativa* seeds extract .

III.5.2. Dependent variable

Dependent variable are hepatic tissue damage and expression of TNFα

III.6. OPERATIONAL DEFINITION

III.6.1. Expression of TNFα

TNFα immunohistochemical expression quantified in accordance to Allred score [50] by two independent pathologists and compared across histological categories using Kappa test. Allred score was established using a 0–8 scale based upon the sum of a proportion score (percent of stained cells) and intensity score (weak, intermediate, and strong) (Table 3). The possible values of Allred score are: 0 – Allred 0*; 1 – Allred 2, 3, 4; 2 – Allred 5, 6; 3 – Allred 7, 8 (*Allred score 1 is not possible). each slide rated 5 field of view with magnification 400x, TNFα (52B83) is a mouse monoclonal antibody raised against purified full length native TNFα of human origin[53]. The scale of this variable is ordinal (full score attach in appendix 1).

II.6.2. Liver Tissue Damage.

Histopathological examination carried out according to the standard methods (Humason, 1979). The pathological changes of fatty liver and degeneration of hepatocytes, graded as follows:

• Level 0 (normal): normal liver morphology, hepatocytes had the round nucleus centrally and homogenous cytoplasm, the flat endothelial cells around central vein and sinusoid.

• Level +1 (mild degree): some of 1–2 hepatocyte rows around central vein demonstrated hepatic cell degeneration, necrosis (loss of nucleus), less injury of endothelial cells around central vein and less fat vacuoles in hepatocytes.

• Level +2 (moderate degree): hepatocyte rows around central vein had swelling, intracytoplasmic vacuolar degeneration in centrilobular, midzonal and periportal areas, endothelial cells around central vein more injury than level +1 and increasing of fat vacuoles in hepatocytes as compared with level +1.

• Level +3 (severe degree): three to four hepatocyte rows around central vein showed hepatocytic degeneration and necrosis (loss of nucleus), degeneration cells including centrilobular, midzonal and periportal areas (diffuse intracytoplasmic vacuolar degeneration), endothelial lining of central vein exhibited more cell injury, as well as increasing of fat vacuoles in hepatocytes as compared with level +2. Besides, focal necrosis and bile duct proliferation were marked.[51]

III.6.2 .*Nigella sativa* seeds

Herb of the Mediterranean region originally from Syria having pungent seeds .Three doses of *Nigella sativa* seeds extract are (0.5 g/kg of b.w , 1 g /kg of b.w , 1.5 g /kg of b.w daily) by orally using sonde tube for 8 weeks.

III.7. RESEARCH TOOLS AND MATERIAL

III.7.1 RESEARCH MATERIAL

A. Eethanol .

B. *Nigella sativa* seeds extract .

C. Wistar rats.

D. Food and drink for Wistar rats.

E. Hematoxylin Eosin staining.

F. Paraffin wax .

G. Formalin % .

H. Xyline.

I. Albumin.

III.7.2 RESEARCH TOOLS

A. Glass slides.

B. Light Microscope for examination of tissues.

C. Hot plate or Water bath.

D. Wash bottle.

E. Racks for slides.

F. Marker of marking of slides.

G. Cedar oil.

H. Microtome.

I. Hot plate or water bath.

III.8. RESEARCH PROCEDURE

III.8.1.Preparation of *Nigella sativa* seeds extract.

Nigella sativa seeds cleaned and dried in an oven at 40°C until a constant weight was attained. Identification of the seeds conducted and the extraction done using Ethel acetate by Maceration process (steady-state extraction), (full method attach in appendix 1).

III.8.2. Preparation Of Ethanol.

Rats received 40% ethanol . Liver cell damage induced in rats by administering 2 mL of 40% ethanol two time daily , equivalent to 8 g/kg bodyweight as an aqueous solution, using sonde tube for 8 weeks[49].

III.8.3. Histology Specimen Preparation and staining.

After the total duration of the experiment 8 weeks, at the end of it, the animals subjected to whole-body perfusion using normal saline under light ether anaesthesia. way to termination pay attention to the principles stated in Helsinki Declaration of 1972 and the and National Guidelines for Health Research Ethics- Indonesia (PNEPK),The liver removed and stored immediately in buffered formalin 10 % for histopathological examination. The tissue fixed for at least 48 hours in buffered formalin 10%.(Full procedure attach in appendix 1). [11]

III.9. DATA COLLECTION AND ANALYSIS

III.9.1 Data analysis

Data collected from observation of hepatic tissue changes among all group descriptively analysed by counting the standard deviation and median Number of liver hepatocyte changes of each group showed in box-plot graph. For ordinal variable the results presented in the form of a cross table and analyzed, Hepatic tissue damage and TNFα expression data analyzed using Kruskal-Wallis test followed by Mann Whitney U test. Assisted statistical analysis with SPSS 17.0 , The significant level used is $p < 0.05$.

III.10.FLOW CHART OF STUDY

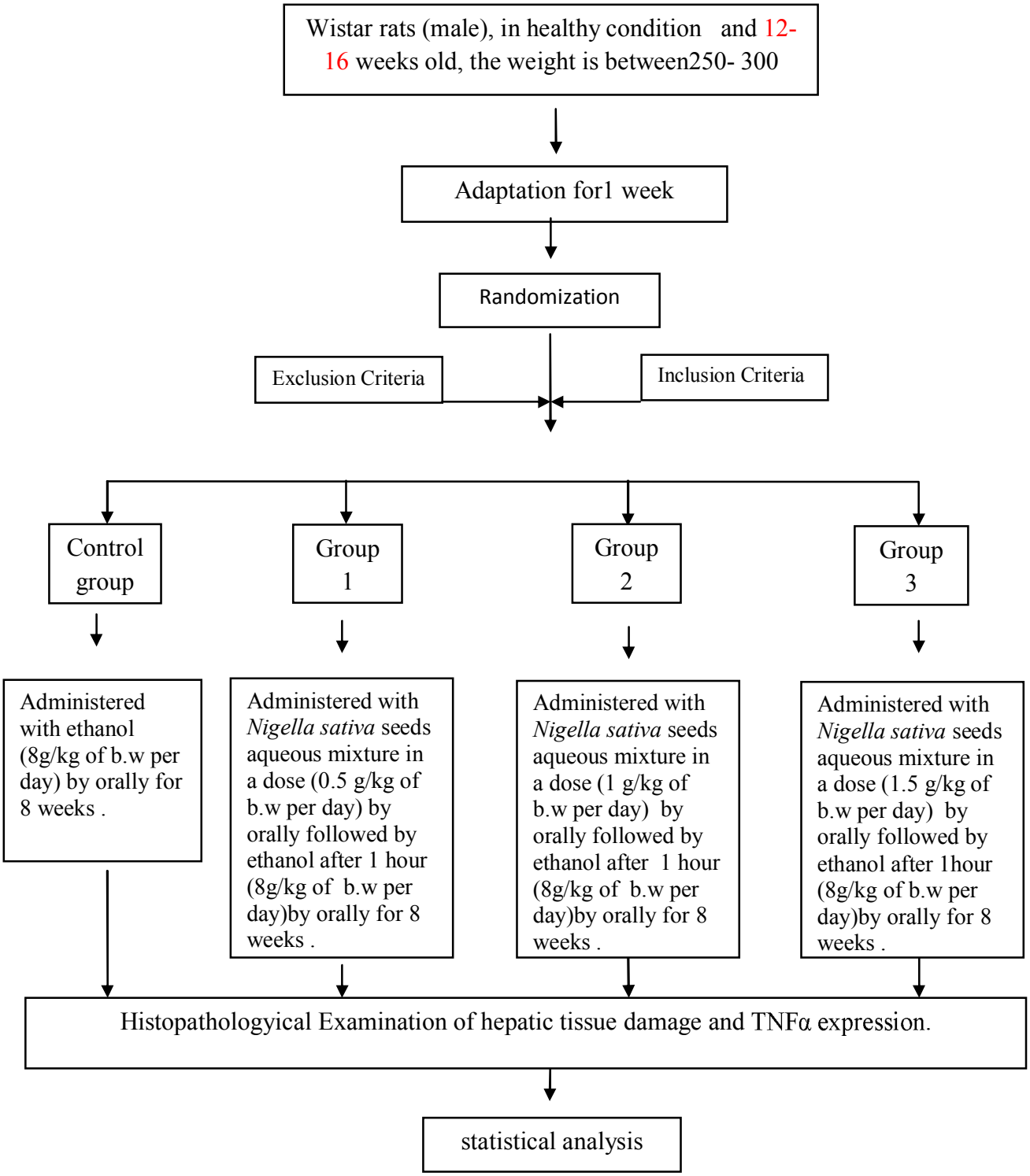

Fig 8: Flow Chart of Study