

CHAPTER IV

RESEARCH METHOD

IV.1. Scope of study, place and time of the research

Scope of the study was included Pathobiology, histopathology.

IV.2. Place and time of the study

The study was conducted in Animal Laboratory at Gadjah Mada University and the liver tissues examination were done at the Pathology Anatomy Department of Diponegoro University Semarang between September–November 2011.

IV.3. Research design

The study design sign was experimental study, namely post test only control group design. The samples were randomly divided into 3 intervention groups and 1 group for control .

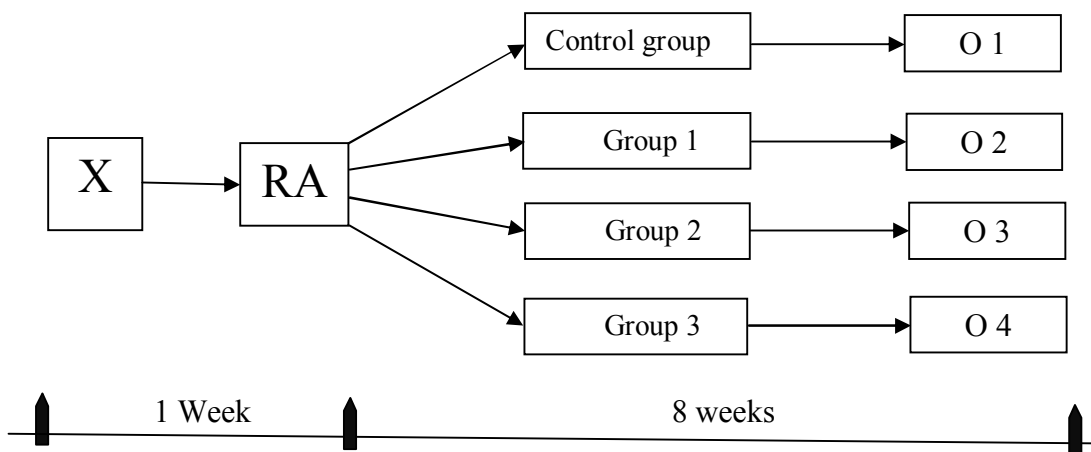


Figure 6 . The scheme of the study

Notes :

X = Adaptation time.

RA = Random Allocation into 4 treatment groups.

Control Group = Each rat were received ethanol (12 g/kg of b.w per day) by using the intragastric feeding model for 8 weeks

Group 1 (G1) = Each rat were received extract of *Nigella sativa* seeds in a dose (0.5g/kg of b.w per day) along with ethanol (12 g/kg of b.w per day) by using the intragastric feeding model for 8 weeks.

Group 2 (G2) = Each rat were received extract of *Nigella sativa* seeds in a dose (1g/kg of b.w per day) along with ethanol (12 g/kg of b.w per day) by using the intragastric feeding model for 8 weeks.

Group 3 (G3) = Each rat were received extract of *Nigella sativa* seeds in a dose (1.5 g/kg of b.w per day) along with ethanol (12 g/kg of b.w per day) by using the intragastric feeding model for 8 weeks.

Every group gets (1) additional rat for replacing rat which die during the study period.

O1 = Output from the control group.

O2 = Output from the group 1.

O3 = Output from the group 2.

O4 = Output from the group 3.

The doses of *Nigella sativa* seeds extract were devided according of experimental groups to three doses in two times per day by using the intragastric feeding model (sonde tube) for 8 weeks. Through this period the Wistar rats were

treated with *Nigella sativa* seeds extracts as follows : - Group 1, Rats were received 1ml of *Nigella sativa* extract given in two doses daily (0.5ml in the morning and 0.5ml in the afternoon), equivalent to 0.5 g/kg b.w. Group 2, Rats were received 1ml of *Nigella sativa* extract given in two doses daily (0.5ml in the morning and 0.5ml in the afternoon), equivalent to 1 g/kg b.w. Group 3, Rats were received 1ml of *Nigella sativa* extract given in two doses daily (0.5ml in the morning and 0.5ml in the afternoon), equivalent to 1.5 g/kg b.w. This research used doses gradual of *Nigella sativa* extract (0.5, 1, 1.5 g/kg b.w) according of previous studies were used doses gradual.¹⁶

The dose of ethanol was 4 ml of 40% ethanol given as two doses daily (2ml in the morning and 2 ml in the afternoon), equivalent to 12 g/kg b.w as an aqueous solution, using the intragastric feeding model (sonde tube) for 8 weeks. This research used a fixed dose (12 g/kg b.w) that is the optimal dose of ethanol on rats. It is based on preliminary experiment that shows this dose did not kill the rats according of previous studies were used doses gradually, which was considered an optimal dose. All rats both of Control group, group 1, group 2 and group 3 were received 4 ml of 40% ethanol given in two doses daily (2 ml in the morning and 2 ml in the afternoon) by using the intragastric feeding model (sonde tube) for 8 weeks.^{11,12}

IV.4. Population of The study

The population of the study was healthy male Wistar rats inbred strain of Animal Laboratory at Gajah Mada University. Samples were taken from such rats which fulfill to the following criteria :

IV.4.1. inclusion criteria

Wistar rat (male), in healthy condition (move actively), measuring the temperature of the rats and 12-16 weeks old and weights is between (250-300 g).

IV.4.2 exclusion criteria

The Wistar rats were showed behavioral changes (e.g., Low activity or lazy)

IV.5. Sample size

The sample size of the study was determined according to the WHO requirement for traditional medical study. According to WHO guideline minimal 5 rats are necessary in each group. In this study, according to Federer's formula, the sample size in each group were 6 of Wistar rats. The total animals were 24 of Wistar rats.

Federer's formula = (T-1) (N-1) >15

Abbreviation: **T** = Number of the group

N = Number of the rats

$$= (3N) - (3) >15 \quad = (3N) < 15+3 \quad = (N) < 6$$

By using sampling techniques (simple random sampling), samples as many as 6 tails. Determination of the treatment of the sample was randomly by using a random table.

IV.6. Research variable

IV.6.1. Independent variable

The independent variable was doses of *Nigella sativa* seeds extract.

IV.6.2. Dependent variable

The dependent variable was hepatic tissue changes (hepatic steatosis state, hepatic inflammation state, Mallory body state).

IV.7. Operational definition

IV.7.1. Hepatic steatosis state

The hepatic steatosis state is accumulation of either small or large fat droplets in hepatocytes. It was diagnosed by evaluating the range of hepatic steatosis in hepatocytes stained with HE staining with light microscope by using NASH activity scoring system. (The NASH activity scoring system was attached at appendix 2).

The scale is ordinal.

IV.7.2. Hepatic inflammation state

The hepatic inflammation state is infiltration of inflammatory cells that consists of white blood cells. It was diagnosed by counting the number of white blood cells of hepatic tissue stained with HE staining under light microscope by using the Knodell scoring system. (The Knodell scoring system was attached at appendix 2).

The scale is ordinal.

IV.7.3. Mallory bodies

Mallory bodies are large irregular masses which occur in the cytoplasm of damaged liver cells. It was diagnosed by using Mallory bodies present or none present in hepatocytes stained with HE staining under light microscope.

The scale is nominal.

IV.7.4. *Nigella sativa* seeds

Three doses of *Nigella sativa* seeds extract were (0.5 g/kg of b.w, 1 g /kg of b.w, 1.5 g /kg of b.w daily) given daily by intragastrically for 8 weeks.¹⁶

IV.8. Research tools and material

IV.8.1. Research material

- A. Ethanol.
- 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.

IV.8.2. Research tools

- A. Glass slides and cover slides.
- B. Light Microscope for examination of tissues.
- C. Hot plate or Water bath.
- D. Wash bottle.
- E. Racks for slides.
- F. Marker for marking of slides.

- G. Microtome.
- H. Hot plate or water bath.
- I. Syringe.
- J. Sonde tube.

IV.9. Research procedure

IV.9.1. Preparation of extract of the thymoquinone-rich fraction (TQRF) of *Nigella sativa* seeds :-

Nigella sativa seeds were cleaned and dried in an oven at 40°C until a constant weight was attained. Identification of *Nigella sativa* seeds were conducted and the extraction have done by using ethyl acetate by maceration procedure. *Nigella sativa* extracts at three doses, 0.5, 1 and 1.5 g/kg of b.w. All doses were given to rats by intragastrically for 8 weeks.¹⁶ *Nigella sativa* seeds extract were prepared in the Pharmacology Department of Gadjah Mada University.

IV.9.2. Preparation of ethanol.

Rats received 12 g/kg b.w of 40 % ethanol. Liver cells damage were induced in rats by administering 4 ml of 40 % ethanol, equivalent to 12g/kg of body weight as an aqueous solution by using sonde tube for 8 weeks.¹²

IV.9.3. Histology specimen preparation

After the total duration of the experiment 8 weeks, at the end of it, the rats from all groups were terminated. Initially the rats were subjected to whole-body perfusion using normal saline and buffered 10%formalin under light ether

anesthesia. The procedure used for termination of rats pay attention to the principles stated in the Helsinki Declaration of 1975 and the National Guidelines for Health Research Ethics, and then were operated to take its liver tissue. The liver tissues were removed and stored immediately in 10% formalin to preparation of histology specimen for histopathological examination. The liver tissues were fixed for at least 48 hours in 10% formalin. The specimens were dehydrated in ascending grade solutions of alcohol and cleared in xylene and then impregnating and embedding in paraffin wax for 2 hours. Sections were cut at 5 μ m by using the rotary microtome and then stained with hematoxylin and eosin for examination using a light microscope.¹² (Procedure for preparing of liver histology slides was attached at appendix 1)

IV.10. Data collection and analysis

IV.10.1. Data collection

The study was conducted in Animal Laboratory at Gajah Mada University. After terminated the rats, the liver tissue of the rats were taken to do histopathology examinations. The slides for this examination were made in the Pathology Anatomy Laboratory of Kariadi hospital Semarang and the tissue examination at the Pathology Anatomy Department of Diponegoro University Semarang.

IV.10.2. Statistical analysis

At first, the data collected from examination of hepatic tissue changes in 5 fields for each slide were evaluated statistically by calculating median, then

Kappa Statistic (K) was used to assess inter- pathologists agreement for the reliability and validity to each diagnostic score. All of the grouped data from the examination of hepatic tissue changes among all groups were descriptively analyzed by calculating the standard deviation and median. The number of liver hepatocyte changes of each group showed in box-plot graph. For categorical variable (Mallory bodies) the results were presented in the form of a crosstabs and analyzed using the Kendall's tau-b test. Hepatic tissue changes data (inflammation and steatosis) were analyzed using nonparametric tests through Kruskal-Wallis test followed by Mann Whitney test. Assisted statistical analysis with SPSS. The level of statistical significance was set at $P < 0.05$.

IV.11. Ethics

Protocol of the study has been submitted to Ethical Committee of Medical and Health Research Medical Faculty Diponegoro University and then ethical clearance was obtained. All animals have been care according to Helsinki's Declaration, as well as, all invasive treatment have been conducted under anesthesia.

IV.12. Preparations followed for the study.

The study was conducted in Animal Laboratory at Gajah Mada University. Twenty four healthy, male, adult Wistar rats 12-16 weeks old and the weight was between 250 - 300 grams. They were procured from Animal Laboratory at Gajah Mada University.

All animals were cared on Animal Research Care Facility of Faculty Medicine Gajah Mada University. Adaptation time was done in one week to prepare the rats, that was weighing, examining the health status, and help the rats to adapt to new environments. They were housed in metal cages under controlled conditions, and raised by balancing feeding at room temperature and constant wetness degree according to the alteration of day and night. The rats were divided into four groups of six rats each. All groups of rats were received a normal diet of standard pellets. Liver cell damage was induced in rats of all Groups by administering 4 ml of 40% ethanol (2 ml in the morning and 2 ml in the afternoon) by intragastrically (sonde tube) for 8 weeks.^{11,12} Through of this period the Wistar rats were treated as follows for 8 weeks :-

The first group was an experimental control group of rats, which were received 4 ml of 40% ethanol given in two doses daily (2 ml in the morning and 2 ml in the afternoon), equivalent to 12 g/kg b.w as an aqueous solution, using the intragastric feeding model (sonde tube). This research used a fixed dose (12 g/kg b.w) that is the optimal dose of ethanol on rats.^{11,12} Group 1, Rats were received two doses of *Nigella sativa* seeds extract and ethanol at two times in the morning and in the afternoon. One dose was consisted of 0.5 ml of *Nigella sativa* seeds extract, along with 2 ml of 40% ethanol after two hours from receipt of *Nigella sativa* seeds extract by intragastrically.^{11,12,16} Group 2, Rats were received two doses of *Nigella sativa* seeds extract and ethanol at two times in the morning and in the afternoon. One dose was consisted of 0.5 ml of *Nigella sativa* seeds extract, along with 2 ml of 40% ethanol after two hours from receipt of *Nigella sativa*

seeds extract by an intragastrically.^{11,12,16} Group 3, Rats were received two doses of *Nigella sativa* seeds extract and ethanol at two times in the morning and in the afternoon. One dose was consisted of 0.5 ml of *Nigella sativa* seeds extract, along with 2 ml of 40% ethanol after two hours from receipt of *Nigella sativa* seeds extract by an intragastric tube .^{11, 12, 16}

The total of duration of the experiment was 8 weeks, at the end of it, the rats from all groups were terminated, which was according to the principles stated in the Helsinki Declaration of 1975 and the National Guidelines for Health Research Ethics. Initially the rats were subjected to whole-body perfusion using normal saline and 10% formalin under light ether anesthesia, and then were operated to take its liver tissue. The liver was removed and stored immediately in 10% formalin to preparation of histology specimen for histopathological examination using a light microscope by using magnification of 400x in 5 fields per slide on 2 times by two pathologists.

IV.13. FLOW CHART OF STUDY

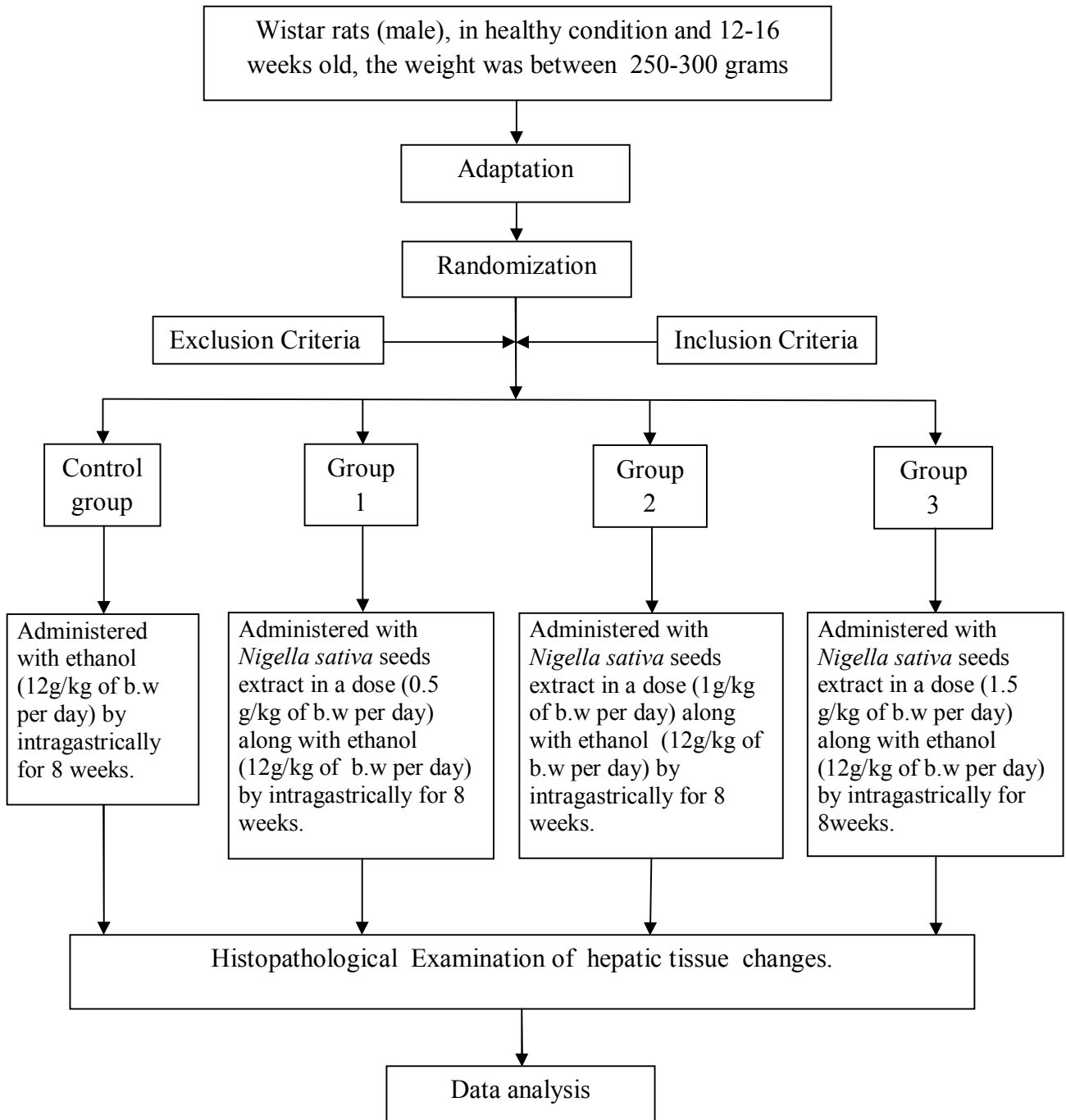


Figure 7. Flow Chart Of Study