CHAPTER IV

METHODS

4.1. Research fields

The fields of this research were Herbal Medicine and Immunology.

4.2. Research locations and periods

The periods of research were March-April 2013. The research was conducted at the Parasitology Laboratory Faculty of Medicine Diponegoro University for the BALB/c mice handling, adaptation and termination.

Processes of peritoneal macrophages isolation, macrophages culture and Phagocytic Index measurements were done at the Center for Biomedical Research (CEBIOR) Faculty of Medicine Diponegoro University Semarang Indonesia.

4.3. Research design

This research was experimental analytic with the Randomized Post-test Only Control Group Design.

4.4. Population and Samples

4.4.1. Targeted Population

Targeted Population of this research was the population of BALB/c mice (*Rattus rattus* strain BALB/c*)*.

4.4.2. Accessible Population

Reached Population of this research was the population of BALB/c mice (*Rattus rattus* strain BALB/c*)* in Biology Laboratory, Faculty of Mathematics and Natural Sciences, Semarang State University.

4.4.3. Sample

Samples of this research were BALB/c mice (*Rattus rattus* strain BALB/c*)* from the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Semarang State University which met the research criteria.

4.4.3.1. Inclusion Criteria

Samples were taken with inclusion criteria:

- Adult male BALB/c mice (*Rattus rattus* strain BALB/c*)*
- Age between 4-6 weeks
- Body weight 20-40 gram.
- Appeared healthy and active
- Physically normal

4.4.3.2. Drop Out Criteria

Samples were dropped out with criterion such as BALB/c mice which were sick during research process.

4.4.4. Sampling Method

Samples were obtained with the simple randomized sampling method.

4.4.5. Sample Magnitude

Sample-taking was based on World Health Organization Guidance (WHO Guidance) for the herbal medicine research. it required 5 mice with 1 substitute for each group.[30] There were 24 BALB/c mice in all four different groups.

4.5. Research Variables

4.5.1. Dependent Variable

Peritoneal Macorphages Phagocytic Index.

4.5.2. Independent Variable

Combination Extract of *Phaleria* and *Phyllanthus*.

4.5.3. Confounding Variable

- Bacterial infection.
- Stressors for the BALB/c mice.

4.6. Operational Definitions

Table 2. Operational Definitions

No	Variable	Unit	Scale
1	*Phaleria* Extract *Phaleria macrocarpa* (Scheff) Boerl extract which was provided by Sidomuncul Inc. Dossage 0.14 mg/0.5mL/day	mg	Nominal
2	*Phyllanthus* Extract *Phyllanthus Niruri* Linnaeus extract which was provided by Sidomuncul Inc. Dossage 0.4 mg/0.5mL/day	mg	Nominal
3.	Combination Extract of *Phaleria macrocarpa* and *Phyllanthus niruri* The combination of both herbs extracts. Dossage in 0.14 mg/0.25mL/day & 0.4 mg/0.025 mL/day	mg	Nominal
4.	Peritoneal Macrophages Phagocytic Index Actively Latex-Beads phagocytizing macrophages counted per 200 macrophages. [31]	/200 cells	Ratio

4.7. Data Collection

4.7.1. Lab Work Materials

1. Animal handling materials: animal feeding, distilled water
2. Animal treatment materials: extract of *Phaleria macrocarpa* (0.14 mg/0.5 mL/day), extract of *Phyllanthus niruri* (0.4 mg/0.5 mL/day), and combination extracts of *Phaleria macrocarpa* and *Phyllanthus niruri* (0.14 mg/0.25mL/day and 0.4 mg/0.25 mL/day).
3. Macrophage isolation and culture materials: ethanol 70%, Rosewell Park Memorial Institute (RPMI-1640) solution, Phosphate-buffered Saline (PBS) solution, Fetal Bovine Serum (FBS) 10%, Penicillin-Streptomycin 50ug/mL, acetic acid 3%, and RPMI Complete Medium (RPMI, FBS, Penicillin).
4. Phagocytic Index measurement materials: Latex beads, PBS, absolute methanol, Giemsa 20%, and distilled water.

4.7.2. Lab Work Equipment

- Animal handling and treatment: scale, experimental animal cages, gastric tube, disposable syringe, and Erlenmeyer glass.
- Peritoneal macrophages isolation and culture: Falcon tubes 15 cc, centrifuge, hemocytometer, microplate, coverslip, incubator, and wells.
- Peritoneal macrophages phagocytosis examination: microscope slide, coverslip, object glass, and microscopes.

4.7.3. Type of data

The type of data was primary data obtained from the result of experiment.

4.7.4. Laboratory Work

4.7.4.1. BALB/c Mice Handling and Termination

1. 24 BALB/c mice with age around 4-6 weeks were acclimatized at the Department Parasitology Faculty of Medicine Diponegoro University and kept in the cages with enough lighting, animal feed and hygiene.
2. Post acclimatization phase, the mice were divided into 4 groups, with simple randomized allocation and treatment as:
    a. Control Group (C): consisted of 6 mice treated with freshwater as placebo.
    b. Treatment I Group (T1): consisted of 6 mice received *Phaleria macrocarpa* extract only with the dose 0,14 mg/0.5mL/day.
    c. Treatment II Group (T2): consisted of 6 mice received *Phyllanthus niruri* extract only with the dose 0.4 mg/0.5mL/day.
    d. Treatment III Group (T3): consisted of 6 mice received combination extract of *Phaleria macrocarpa* extract and *Phyllanthus niruri* with the dose 0.14 mg/0.25 mL/day and 0.4 mg/0.25 mL/day.

    This treatment was given in 7 days, based on the significance of previous researches results. At the day-8, the mice were terminated to isolate the peritoneal macrophages, which was being cultured and then counted for Macrophage Phagocytic Index.[31]

4.7.4.2. Peritoneal Macrophage Isolation and Culture[31]

1. Peritoneum was disinfected with alcohol 70%.
2. 10 cc cold RPMI solution (Rosewell Park Memorial Institute) was irrigated to the peritoneal cavity.
3. Peritoneum was massaged slowly to obtain more macrophages.
4. RPMI solution was aspirated with pipette, and then put into Falcon tube 15cc, centrifuged 1200rpm 4^oC (10 minutes).
5. Erythrocytes contamination was rinsed out with PBS (Phosphate-Buffered Saline).
6. Supernatant was removed, add 3 mL complete RPMI medium, consisted of RPMI 1640, FBS 10% (Fetal Bovine Serum) and penicillin to pallet.
7. Cells were counted with hemocytometer after diluted in 3% acetic acid (hemolysed), made suspension again with complete RPMI medium until obtained cell suspension of 2.5×10^6 cells/mL.
8. The cells were grown and cultured in complete medium in the micro-plate 24 wells (flat base) and put the coverslip at the base.
9. Each well was filled with 200 uL with density 5×10^5 cell/mL, incubated with CO_2, 37^oC, 30 minutes and then added 1 mL RPMI complete medium at each well, incubated in 2 hours.
10. Cells were washed with RPMI two times; added complete medium 1 mL each well and incubated in 24 hours.

4.7.4.3. Macrophage Phagocytic Index (Latex-Beads Method)[31]

1. Cultured macrophages were rinsed with RPMI two times.
2. Latex beads (Sigma-Aldrich Steinheim, Germany) were made into suspension (2.5 x 10^7/mL).
3. Each well was filled with 200 uL Latex/well, incubated 60 minutes, CO_2 5% 37^{o}C.
4. Incubated materials were made slide smear, rinsed with PBS (in purpose to remove the unphagocytized particles).
5. Dried slide smear was fixated with absolute methanol.
6. Slides were dyed in Giemsa 20% about 30 minutes.
7. Dyed smear slides were washed with distilled water and dried in room temperature.
8. Object glass was attached and under the microscope the phagocytosis activity was measured from the percentage of macrophages, which phagocytized the Latex beads, counted on 200 cells multiplied with average amount of particle at positive cells and stated as the Phagocytic Index.

4.8. Research Protocols

BALB/c mice were fostered and aclimatized to be adapted, and then randomizedly taken as samples. In this research the mice were divided into four groups. There were Group control(C), Treatment 1 (T1) *Phaleria* single extract, Treatment 2 (T2) *Phyllanthus* single extract treatment and Treatment 3 (T3) Combination extracts of *Phaleria* and *Phyllanthus* treatment. Dossage of *Phaleria*

extract given was 0,14 mg/0.5 mL/day, while for *Phyllanthus* extract 0.4 mg/0.5 mL/day. For the combination extracts each for 0.14 mg/0.25 mL/day and 0.4 mg/0.25 mL/day.

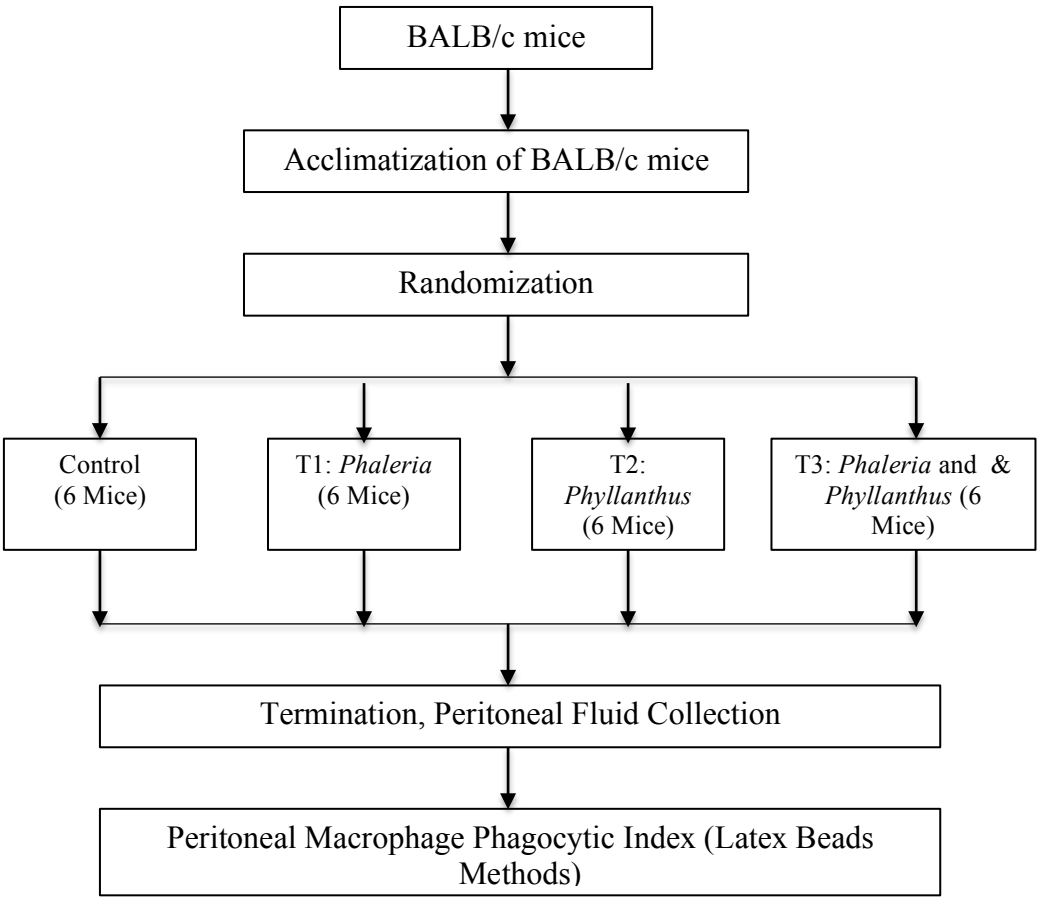

Figure 8. Research Protocol Framework

4.9 Data Analysis

Data was analyzed with the steps:

a. Editing the collected data

b. Data cleaning, to re-check the mistakes during data taking

c. Data tabulation, present the data in table

d. Data analysis

The data characteristics presented the values, mean, mode and deviation standart of the Peritoneal Macrophages Phagocytic Index. The data normality was analyzed with the Saphiro-Wilk test, since there were 20 samples.

The normally distributed data was analyzed with the Analysis of Variance (ANOVA) Test. The ANOVA test result were considered significant when the $p<0.05$. It was continued with *post-hoc* test to know the difference of each group.

4.10 Research Ethics

All of data collections and research was done under permission of Health Research Bioethics Commission Faculty of Medicine Diponegoro University/Dr. Kariadi General Hospital Semarang Indonesia.

4.11 Research Schedule

Table 3. Research Schedule

Activities	**Feb 2013**		**Mar 2013**				**April 2013**		**Jul 13**	**Aug 13**
Weeks	W3	W4	W1	W2	W3	W4	W1	W2	W3	W2
Research Proposal										
Research Preparation										
Research										
Data Analysis										
Research Report										
Report Presentation										