CHAPTER 4

METHODOLOGY

4.1 Research Design

The study design used is the in vitro experimental laboratory study using the tube dilution test. The purpose was to observe the antifungal effect of *Annona muricata* extract on the growth of *Candida albicans*. The tube dilution test on Sabouraud's dextrose broth medium is used to determine the minimum inhibitory concentration (MIC) and the streaking stage on a Sabouraud's dextrose agar plate to determine the minimum fungicidal concentration (MFC).

4.2 Research Sample

The sample used is the *Candida albicans* culture readily available at the Microbiology Laboratory of Brawijaya University, Malang, from vaginal secretions of a patient with candidiasis.

4.3 Repetition Estimation

The total number of repetition used in this study is calculated using the formula (Loekito, 1988):

$$p (n – 1) \geq 15$$

$$7 (n – 1) \geq 15$$

$$7 n \geq 22$$

$$n \geq 3.15$$

Explanation: p = number of treatment

n = number of repetition needed

Therefore, in this study the number of repetition needed is 4.

4.4 Location and Time of Study

The study was conducted in the Microbiology Laboratory of the Medical Faculty of Brawijaya University, Malang in the months of May and June 2013.

4.5 Variable Identification

4.5.1 Independent Variable

The independent variables in this study were the *Annona muricata* leaf extract with concentrations of 45%, 42.5%, 40%, 37.5%, 35% and 32.5%. These concentrations were obtained through random exploration.

4.5.2 Dependent Variable

The dependent variables in this study were the turbidity of the test tubes and the number of colonies of *Candida albicans* yeast on the solid media.

4.5.3 Confounding Variable

The confounding variables in the study were the working process, such as the sterilization technique between repetitions and the time interval between the production of the extract and the treatment with the extract.

4.6 Operational Definition

- The soursop leaves used were from the soursop plant obtained from Kediri.
- The soursop leaf ethanol extract was the concentrate of the soursop leaves which have undergone the extraction process using 96% ethanol.
- The fungal isolate of *Candida albicans* was obtained from readily available cultures in the Microbiology Laboratory of the Medical Faculty of Brawijaya University, Malang from vaginal secretions of a patient with candidiasis.
- The *Candida albicans* inoculum was the inoculum with a concentration of 1-5 x 1,000,000 CFU/ml.
- Minimum Inhibitory Concentration is the concentration at which the soursop leaf extract solution is able to inhibit the growth of the fungal sample (*Candida albicans*), marked with the absence of turbidity in the soursop leaf extract solution which has been inoculated.
- Minimum Fungicidal Concentration is the concentration at which the soursop leaf extract is able to kill the fungal sample (*Candida albicans*), marked by the number of colonies on the solid agar medium which has undergone streaking with one inoculating loop of the soursop leaf extract solution.

4.7 Instruments and Materials

4.7.1 Instruments

- Blade
- Bunsen burner
- Inoculating loop
- Analytic weighing apparatus
- Reaction tube
- Filter paper
- Beaker
- Incubation equipment
- Evaporator set
- Colony counter
- Microscope
- Object glass
- Immersion oil
- Cotton bud
- Vortex
- Calibrated pipette
- Matches
- Forceps
- Tray with rack

4.7.2 Materials

- Soursop leaves
- 96% ethanol

- Pure *Candida albicans* culture
- Sabouraud Dextrose Broth liquid media
- Sabouraud Dextrose Agar solid media
- 0.85% sterile NaCl solution
- Gram stain dyes: crystal violet, Lugol's iodine, 96% alcohol, safranin
- Distilled water

4.8 Study Procedures

4.8.1 Soursop Leaf Extract

A. Extraction Process

The soursop leaves were sun-dried then finely ground using the blender. Once fine, the ground soursop leaves were wrapped with filter paper and soaked in 96% ethanol overnight (± 12 hours). The 96% ethanol used for soaking was replaced several times until the extract solution is clear. The extracted product was then ready for evaporation.

B. Vaporization Process

The evaporator set was fixed onto a permanent pillar for it to hang at a 30-40° slant towards the table arranged with the water heater at the base, followed by the collecting flask, the rotary evaporator and finally the cooling tower at the top. The cooling tower was then connected to the cold water circulation pump linked to the water basin through a plastic pipe. The cooling tower was also joined with the vacuum pump and the vapor collector.

The extraction product was then collected in the collecting flask once the rotary evaporator, cold water circulation pump, and the vacuum pump has started.

The distilled water heater was kept running until the extract in the vapor collecting tube boiled at a temperature of 80°C (according to ethanol's boiling point) and the ethanol started to evaporate.

The ethanol vapor was then condensed towards the ethanol collecting flask so that it did not mix with the other vapors sucked in by the vacuum pump.

The evaporation process was conducted until the extract volume decreased and thickened. Once viscous, the evaporation was stopped and the product was collected. The vapor was placed into a vapor cup and then heated in the oven for 2 hours at 80°C to vaporize the remnants of the solution until the extract obtained is 100%. The wet mass of the leaves used in the experiment was around 1 kg and the amount of extract obtained in this process was about 100 ml.

4.8.2 Candida albicans Preparation

4.8.2.1 Identification with Gram Stain

- The object glass was cleaned with a piece of sterile cotton then passed briefly over the flame to remove the fat and allowed to cool.
- One drop of distilled water or saline solution was dropped on the object glass.
- With a sterile inoculating loop, a small amount of *Candida albicans* colony growing on a solid media was taken and suspended into the drop of distilled water or saline solution on the object glass. The smear was done thinly.

- The smear was allowed to air-dry. Once dried, the smear was fixed by passing it briefly over the flame 3 times. The preparation was ready for staining.
- The preparation was flooded with crystal violet for 1 minute before being rinsed off with tap water.
- The preparation was flooded with Lugol's iodine for 1 minute before being rinsed off with tap water.
- The preparation was flooded with 96% alcohol for 5-10 seconds or until the stain faded before being rinsed off with tap water.
- The preparation was flooded with safranin for 30 seconds, before being rinsed off with tap water.
- The preparation was dried with a blotting paper.
- The preparation was observed under the microscope using 100x objective lens magnification.
- Positive result: oval-shaped *Candida albicans* yeast stained purple (Gram positive).

4.8.2.2 Identification with Germinating Tube Test

- 10 colonies of *Candida albicans* were inserted into 1ml of mammalian serum**.**
- The culture was incubated at temperature 37°C for 2-4 hours.
- The culture was collected with an inoculating loop, placed on an object glass, and covered with a cover slip.
- The preparation was observed under the microscope.
- Positive result: detection of *Candida albicans* pseudohyphae.

4.8.2.3 Preparation of *Candida albicans* Testing Suspension

- 10 colonies of *Candida albicans* measuring 1mm in diameter were removed from the solid media.
- The colonies were inserted into 9ml 0.85% sterile NaCl and then vortexed.
- Using spectrophotometry at 530nm wavelength, the colonies were measured to determine the optical density of the *Candida albicans*. 0.1 spectrophotometry units is equal to 0.5 McFarland constant which is equivalent to 10^6 CFU/ml. of *Candida albicans.*
- Sterile NaCl solution was prepared in 2 test tubes, each contained 9ml and labeled tube A and B respectively, and 1 test tube contained 9ml of SDB liquid media.
- The volume of *Candida albicans* determined was mixed with sterile NaCl solution to produce 10ml suspension of 10^5 CFU/ml (tube A).
- 1ml of the *Candida albicans* suspension from tube A was transferred into tube B until the concentration of *Candida albicans* becomes 1-5 x 10^4 CFU/ml.
- 1ml of suspension from tube B was then transferred into the SDB liquid media until the concentration of *Candida albicans* becomes 1-5 x 1,000 CFU/ml and ready to be used.

4.8.3 Antifungal Testing of Soursop Leaf Extract

- 7 sterile test tubes were prepared, using extract concentrations of 90% (1), 85% (2), 80% (3), 75% (4), 70% (5), 65% (6), and 0% (7) (control fungus).

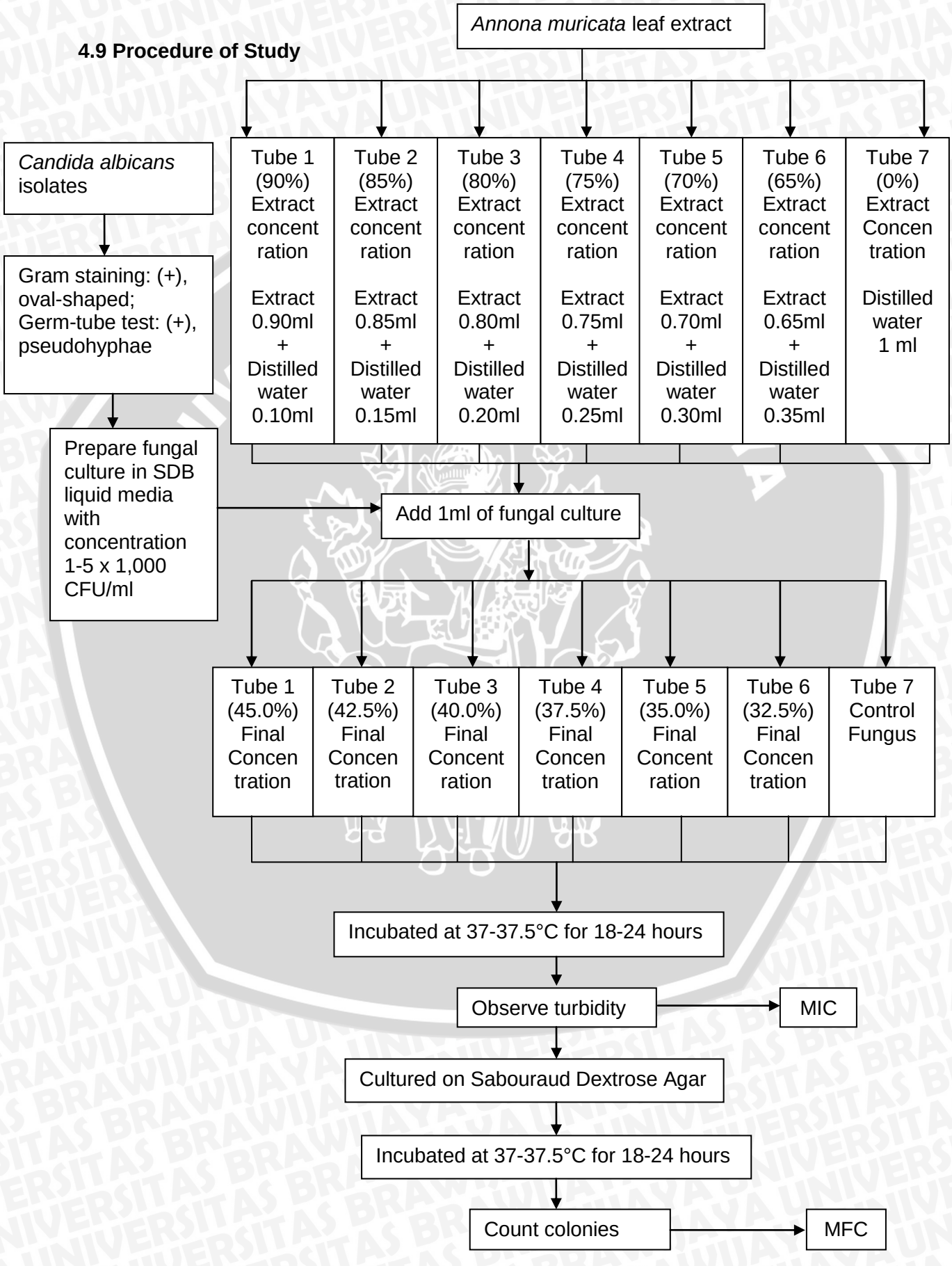
The control fungus was the *Candida albicans* culture of concentration 0.5-2.5 x 1,000 CFU/ml.

- 0.10ml of distilled water was filled into a tube labeled 90% then 0.90ml of soursop leaf extract was added (tube 1).
- 0.15ml of distilled water was filled into a tube labeled 85% then 0.85ml of soursop leaf extract was added (tube 2).
- 0.20ml of distilled water was filled into a tube labeled 80% then 0.80ml of soursop leaf extract was added (tube 3).
- 0.25ml of distilled water was filled into a tube labeled 75% then 0.75ml of soursop leaf extract was added (tube 4).
- 0.30ml of distilled water was filled into a tube labeled 70% then 0.70ml of soursop leaf extract was added (tube 5).
- 0.35ml of distilled water was filled into a tube labeled 65% then 0.65ml of soursop leaf extract was added (tube 6).
- 1ml of distilled water was filled into a tube labeled 0% (tube 7).
- 1ml of 1-5 x 1,000 CFU/ml liquid *Candida albicans* culture was added into each test tube.
- After the addition of the *Candida albicans* culture, the extract concentration for each test tube was halved to give final concentrations of 45% (1), 42.5% (2), 40% (3), 37.5% (4), 35% (5), 32.5% (6), and 0% (7).
- All test tubes were incubated at temperature 37-37.5°C for 18-24 hours.
- After 18-24 hours, the degree of turbidity for all the tubes was observed and recorded. The method of determining the degree of turbidity was by placing a white paper with black marking behind a tube and then observing the black marking from the front of the tube. If the marking

could be clearly seen therefore a score of 0 was given; if the marking appeared blurry, a score of 1 was given; if the marking appeared very blur or difficult to see, a score of 2 was given; if the marking could not be seen at all, a score of 3 was given. The lowest concentration showing no turbidity (0 score) was the minimum inhibitory concentration.

- To determine the minimum fungicidal concentration, one ose (0.005ml) from each of the test tubes was streaked and incubated on the Sabouraud Dextrose Agar at the temperature of 37-37.5°C for 18-24 hours.
- After 18-24 hours, the number of growing *Candida albicans* colony was counted with the colony counter. The lowest concentration showing no *Candida albicans* colonial growth was the minimum fungicidal concentration.

4.9 Procedure of Study



4.10 Data Analysis

The study data is the number of *Candida albicans* colony and the type of data analysis used is the one way ANOVA. With the one-way ANOVA, the effect of different concentrations of soursop leaf extract on Candida albicans can be determined and concluded whether or not this extract has any significance towards the growth of *Candida albicans* in vitro.

The correlation statistical analysis is also conducted to determine the size of the effect and the correlation between the soursop leaf extract concentrations and the growth of *Candida albicans* in vitro. In this study, the internal credibility used is 95% for a significant level of (α) = 0.05.