CHAPTER 4

METHODOLOGY

4.1 Research Design

The study design used is the in vitro experimental study using the broth macrodilution test. The purpose was to observe the antifungal effect of *Daucus carota* extract on the growth of *Candida albicans*. The macrodilution test comprises of two stages which included the testing stage on a broth medium to determine the minimum inhibitory concentration and the streaking stage on a nutrient agar plate to determine the minimum fungicidal concentration.

4.2 Research Sample

The sample used was the *Candida albicans* isolates from vaginal secretion obtained from the culture readily available at the Microbiology Laboratory of Brawijaya University, Malang.

4.3 Repetition Estimation

The total number of repetition used in this study was calculated using the following formula:

$$p\,(n-1) \geq 15 \qquad \text{(Loekita, 1988)}$$

$$7\,(n-1) \geq 15$$

$$7n \geq 22$$

$$n \geq 3.15$$

Where: p = number of treatment

n = number of repetition needed

Therefore, in this study the number of repetition needed was 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 March – June 2013.

4.5 Variable Identification

4.5.1 Independent Variable

The independent variables in this study were the *Daucus carota* extract with concentrations of 100%, 90%, 80%, 70%, 60%, and 50%. 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 factors occurring in 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 carrots used were obtained from the *Pasar Besar* (Central Market) of Malang town.
- The carrot extract was the concentrate of the carrot which have undergone the extraction process using 96% ethanol.
- The fungal isolates of *Candida albicans* were isolates from vaginal secretion obtained from the readily available cultures in the Microbiology Laboratory of the Faculty of Medicine Brawijaya University, Malang.
- The *Candida albicans* inoculum was the inoculum with a concentration of 1-5 x 10^3 CFU/ml.

- The extraction degree was a certain degree or concentration of carrot substance which is used in this experiment.
- Minimum Inhibitory Concentration is the smallest concentration at which the carrot extract solution was able to inhibit the growth of the fungal sample (*Candida albicans*), marked with the absence of turbidity in the carrot extract solution which has been inoculated.
- 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, then a score of 0 was given; if the marking appeared blurry, then a score of 1 was given; if the marking appeared very blur or difficult to see, then a score of 2 was given; if the marking could not be seen at all, then a score of 3 was given. The lowest concentration showing no turbidity (0 score) was the minimum inhibitory concentration.
- Minimum Fungicidal Concentration is the value at which the carrot 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 carrot extract solution after being inoculated.

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

- Carrots
- 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 Carrot Extract

A. Extraction Process

The carrots were sun-dried then finely ground using the blender. Once fine, the ground carrots 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 inthe oven for 2 hours at 80°C to vaporize the remnants of the solution until the extract obtained was 100%.

The weight of dried and ground carrot was 148 gram. Later after the extraction and vaporization the carrot becomes a 10 milliliter of extract. It has thick consistency with dark brown color.

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 get rid of 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 then rinsed off with tap water.
- The preparation was flooded with Lugol's iodine for 1 minute then rinsed off with tap water.
- The preparation was flooded with 96% alcohol for 5-10 seconds or until the stain faded then rinsed off with tap water.
- The preparation was flooded with safranin for 30 seconds, then 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* mold 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**.**
- 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. This procedure determined the optical density of the *Candida albicans*.
- With the optical density determined, the formula $N^1V^1 = N^2V^2$ was then used to determine the volume of *Candida albicans* to be mixed with sterile NaCl solution to produce a 10ml suspension of 1 x 10^6 CFU/ml.
- A sterile NaCl solution was prepared in 2 test tubes, each of which 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 1 x 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* became 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* became 1-5 x10^3 CFU/ml and ready to be used.

4.8.3 Antifungal Testing of Carrot Extract

- 8 sterile test tubes were prepared, labeled 100% (1), 90% (2), 80% (3), 70% (4), 60% (5), 50% (6), CF (control fungus), and CS (control substance). The control substance was the carrot extract and the control fungus was the *Candida albicans* culture of concentration 0.5-2.5 x 10^3 CFU/ml.
- 1ml of carrot extract was filled into tube labeled 100%, extract (tube 1).
- 0.10ml of distilled water was filled into tube labeled 90% then 0.90ml of carrot extract was added (tube 2).
- 0.20ml of distilled water was filled into tube labeled 80% then 0.80ml of carrot extract was added (tube 3).

- 0.30ml of distilled water was filled into tube labeled 70% then 0.70ml of carrot extract was added (tube 4).
- 0.40ml of distilled water was filled into tube labeled 60% then 0.60ml of carrot extract was added (tube 5).
- 0.50ml of distilled water was filled into tube labeled 50% then 0.50ml of carrot extract was added (tube 6).
- 1ml of distilled water was filled into tube labeled CF.
- 1ml of liquid *Candida albicans* culture was added into each test tube except for the tube labeled CS.
- 2ml of carrot extract was filled into tube labeled CS.
- After the addition of the *Candida albicans* culture, the extract concentration for each test tube was halved to give final concentrations of 50% (1), 45% (2), 40% (3), 35% (4), 30% (5), 25% (6), and CF (control fungus), and CS (control substance).
- All test tubes were incubated at temperature 37-37.5°C for 18-24 hours.
- To determine the minimum fungicidal concentration, one ose (0.005ml) from each of the test tubes was 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.

4.10 Data Analysis

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

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