CHAPTER III
RESEARCH METHODS

3.1 Research Location and Time

Making ethanol extract of green tea was done in the Pharmacy Laboratory, Department of Pharmacy, Faculty of Health Science, University of Darussalam Gontor. While the treatment of ethanol extract of green tea against *Staphylococcus epidermidis* was carried out at the Microbiology Laboratory, Faculty of Medicine, Surakarta State University (UNS). The study was conducted for 2 months, starting from December 2019 until January 2020.

3.2 Material and Instruments

The instruments used in this research were blender, mesh No. 14, beaker glass, porcelain cup, erlenmeyer, measuring cup, analytical balance, stirring rod, funnel, spatula, whatman paper No. 1, rotary evaporator, waterbath, large glass jar, small glass jars, ose needles, sterile disc paper, chloramphenicol disk paper, sterile stick cotton, blue tip, yellow tip, vial bottle, micropipette, callipers, CO_2 incubator, bunsen, matches, petri dishes, vortex, test tubes, tweezers drops, autoclaves, aluminium foil, permanent markers and labels.

The material needed for this research was green tea (*Camellia sinensis*). The green tea used was obtained from Jamus Tea Plantation, Sine, Ngawi, East Java. Other ingredients were ethanol 96%, sterile aquadest, aquadest, *Staphylococcus epidermidis* ATCC 12228 bacteria obtained from the Microbiology Laboratory of Surakarta State University, 0.5 Mc. Farland concentration of 10^5-10^8 CFU/mL, NaCl 0.9% solution, MHA (Muller Hinton Agar) media, MCA (Mac Conkey Agar) media, blood agar media, and NA (Nutrient Agar) media.

3.3 Research Design

The method used in this study was an experiment using 6 treatments :

K -: Negative control (sterile Aquadest)

K +: Positive control (chloramphenicol 30 µg)

P-1: Ethanol extract 60% green tea

P-2: Ethanol extract 70% green tea

P-3: Ethanol extract 80% green tea

P-4: Ethanol extract 90% green tea

Repetition was done in this study 4 times through the following formula :

$$(t-1).(r-1) \geq 15$$

Information :

t = number of treatment groups

r = number of repetitions of each treatment group

In this study, t = 6, thus obtained:

$(6-1).(r-1) \geq 15$

$5.(r-1) \geq 15$

$5r-5 \geq 15$

$5r \geq 15+5$

$5r \geq 20$

$r \geq 20/5$

$r \geq 4$

This study used a Completely Randomized Design or CRD on the placement of petri dishes in an incubator. The placement of petri dishes in the incubator is as follows:

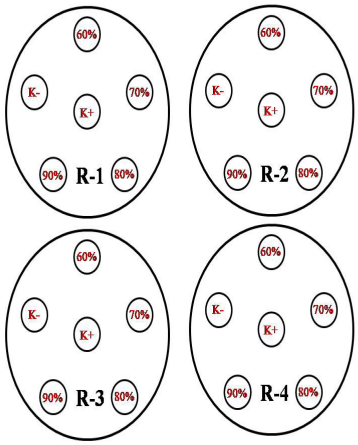

Figure 4. Placement of Petri Dishes in Incubators Using CRD

Information:

R= Replication or repetition

K= Control

The independent variable in this research was the ethanol extract of green tea. The ethanol extract of green tea used was in the form of green tea simplicia macerated with various concentrations of ethanol solvents, namely concentrations of 60%, 70%, 80% and 90%. The dependent variable in this study was the inhibitory zone value. In this research, the observed variables were the diameter of the bacterial inhibitory zone produced from various treatments to determine the concentration of the solvent in green tea extract which had the most optimal antibacterial activity.

3.4 Research Procedures

3.4.1 Sample Preparations

Green tea was obtained from Jamus Tea Plantation, Sine, Ngawi, East Java. Then the determination was conducted at UPT Materia Medika Batu, Malang. Determination aims to ensure the correctness of the plants used. Green tea is the top 3 leaves in tea plants which

in the post-harvest process do not undergo fermentation or enzymatic oxidation. Sample preparation was done by destroying green tea simplicia using a blender until it became powder. All powders were sieved with a mesh No. 14 and weighed up to 800 grams.

3.4.2 Extraction

Simplicia powder of 800 grams was extracted by maceration method using 60%, 70%, 80% and 90% ethanol solvent respectively with several stirring. Maceration was chosen because the process is easy and requires simple tools. In this maceration, 200 grams of green tea powder simplicia was used for each solvent. The solvent used was 96% ethanol which was then diluted to ethanol 60%, 70%, 80% and 90%. The maceration process was carried out for 6 x 24 hours with stirring and replacement of solvents every 1 x 24 hours with a total of 2.4 L of solvent at each concentration of ethanol solvent. After the maceration process was completed, the maceration filtrate was filtered using whatman paper no.1 and funnel and collected in a large glass jar to then be concentrated with a rotary evaporator at a temperature of 78.4°C. The remaining extract filtrate was evaporated using a water bath at a temperature of 80°C until a fluid extract was obtained. The ethanol extract of green tea was stored in a small glass jar that had been sterilized.

3.4.3 Sterilization of Tools and Materials

Sterilization is to destroy microorganisms contained in tools and materials. Sterilization of the equipment was done by washing the tools that will be used with running water until clean and then dried. Tools that were clean and dry are then wrapped in paper and plastic. After that, all the wrapped devices were put into an autoclave for 20 minutes at 121°C.

3.4.4 Preparation of Agar Media

The media used in this study were blood agar media and MCA

(Mac Conkey Agar) media for extract sterility tests, Nutrient agar media for growth media and MHA (Mueller Hinton Agar) media for antibacterial testing.

The process of making media was shown below :

1. Blood Agar Media

   As much as 20 grams of media agar to be weighed and added to 500 mL aquadest then was heated and stirred until all the ingredients were homogeneous. Then to be sterilized by autoclaving at 121°C for 15 minutes. Media that has been sterilized were cooled to temperatures reaching 45°C-50°C and added as much as 5% sheep blood (25 mL) then stirred until homogeneous. After that, the agar media was poured into a sterile petri dish with a thickness of about 4 mm.

2. MCA Media (Mac Conkey Agar)

   A total of 53 grams of MCA media powder was dissolved with 1 L distilled water by heating so the powder was dissolved properly. Then, the media was sterilized by autoclaving at 121°C for 15 minutes and wait until the temperature reaches 45°C-50°C and then poured into a sterile petri dish with a gelatinous thickness of about 4 mm.

3. Tilting Agar (Nutrient Agar)

   Nutrient agar was weighed of 0.046 grams and added to 20 mL aquadest then heated while stirring until all the ingredients are homogeneous. Then as much as 5 ml of warm liquid Nutrient Agar (NA) media was put into clean test tubes, then the top of the test tube was closed or corked with a cotton swab and the final sterilized media uses an autoclave. After being sterile and removed from the autoclave, the tube containing NA was placed in a buffer at an angle of about 30° and the media was left to solidify.

4. MHA Media (Mueller Hinton Agar)

   A total of 19 grams of Mueller Hinton Agar (MHA) media was weighed and 500 mL aquadest added then heated while stirring until all the ingredients were dissolved properly and measured in pH. After that, the MHA media was sterilized by autoclaving at 121°C for 15 minutes and waited until the temperature reached 45°C-50°C then poured into a sterile petri dish with a gelatinous thickness of around 4 mm.

3.4.5 Extract Sterility Test

The concentrated green tea ethanol extract was tested for sterility to determine the possibility of contamination in the extract. The ethanol extract of green tea used was diluted with sterile aquadest at a concentration of 100% w/v. A total of 1 gram of green tea extract was inserted into a vial bottle that had been sterilized and given the label information, then added 1 mL of sterile aquadest using a micropipette and homogenized using a vortex. Homogeneous extracts were etched on MCA media and blood agar media by dividing MCA media and blood agar media into 4 parts and marked for each extract on the cup then the extract was applied to the media by the streak plate method using ose. This process was carried out in a sterile way by approaching a tool or material used on a burning bunsen. After each extract was etched on the media, the media were incubated in an incubator at 35°C for 1 x 24 hours. MCA media and blood agar media were chosen because they are universal growth media that could grow various kinds of bacteria.

3.4.6 Bacterial Regeneration

The pure culture of the *Staphylococcus epidermidis* bacterium was inoculated into a sloping medium, then incubated at 37°C for 24 hours in an incubator. 24 hours is the time of harvest, during which time the bacteria make a constant division and the number of cells increases. Regeneration of bacteria on the media to tilt function so that bacteria can last for a longer period compared to bacteria that grow in the blood agar.

3.4.7 Treatments

The treatment carried out in this antibacterial test is carried out in a sterile way by approaching a tool or material used on a burning bunsen. The ethanol extract of green tea that has been diluted with sterile aquadest, is dripped into an empty test disc that has been provided in a sterile petri dish. A total of 4 test discs dropped 20 µL of sterile aquades to be a negative control, and for treatment used 100% w/v of green tea ethanol extract from each solvent concentration was dropped into 4 test discs of 20 µL for each disc. Four discs were used for each treatment.

Bacteria were suspended by mixing 1 ose of the *Staphylococcus epidermidis* bacterial colony into a test tube containing 0.9% sterile NaCl. Then turbidity was observed and standarized with a concentration of 0.5 Mc Farland so that the number of bacteria met the requirements for a sensitivity test, namely: 10^5-10^8/mL. If the NaCl 0.9% solution is less turbid then some ose bacteria are added until the turbidity is the same as Mc Farland's standard.

MHA media used in the sensibility test were patterned so that there were distances between the discs to be tested. Suspension of bacteria that had been according to the standard, then planted in a cup so that the MHA with the streak plate method was by dipping a sterile cotton swab into the bacterial suspension, then the sterile cotton swab was removed and squeezed by stressing the inner tube wall while spinning and then sticking a sterile cotton swab on the surface the MHA cup until the entire surface was covered with even scratches.

Empty test discs that had been dripped with ethanol extract of green tea, were all placed on the surface of the MHA media that has been planted with bacteria according to the pattern found on the media. This step was carried out sterically by approaching a tool or material used on a burning bunsen. After that, the media was incubated into the incubator. Incubation was carried out at 35° C for 24 hours and

measured the diameter of the inhibition zone or the clear zone (clear zone) that was formed using a callipers.

3.4.8 Data Collection

Inhibited zones formed were observed around the test isolates. Inhibitory zone area was calculated by the formula :

$$\text{Average Lz} = \frac{(\text{Lav 1} - \text{Ld}) + (\text{Lav 2} - \text{Ld})}{2}$$

Information :

Lz : inhibition zone diameter (mm)
Lav : inhibition zone diameter with disc paper (mm)
Ld : paper disc diameter (6 mm)

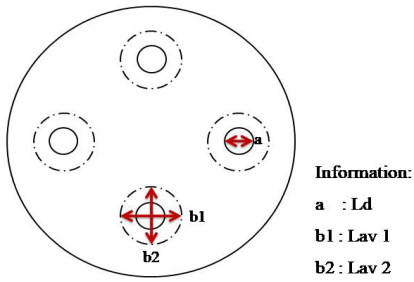

Figure 5. Method of Measurement of Inhibition Zone Diameter against Test Bacteria

3.5 Data Analysis

The results of the research were conducted a normality test and a homogeneity test. Normality test served to find out whether the data were normally distributed or not and homogeneity test functions to find out whether the data analyzed had homogeneous variants or not. Furthermore, the data that were declared normal and homogeneous were analyzed using parametric tests of more than two unpaired groups so that the statistical

test used was one way ANOVA. This parametric test was to determine the antibacterial activity of green tea ethanol extract at each concentration of the solvent. After the parametric test, the Post Hoc test was performed to determine any significant differences between the test groups. The test used for Post Hoc analysis in the one way ANOVA test was the Tukey test.