Potential Antimicrobial Test Using Extract From Rosella (*Hibiscus sabdariffa L.*) Petals Against *Salmonella Typhi* Conducted In Vitro.

FINAL PROJECT

To fulfill the requirements for the degree in *Bachelor* of Medicine

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ELIJAH JAYARAJ PETER

Abstrak

Hibiscus sabdariffa L. atau umumnya dikenal sebagai rosela adalah tanaman berbunga yang berasal dari famili *Malvaceae*. Dalam sejarah pengobatan tradisional dan pengobatan alternatif rosela telah digunakan untuk menurunkan tekanan darah tinggi, menurunkan kolesterol, mencegah masalah jantung dan sebagai antioksidant. Rosela juga merupakan agen antibakteri. Penelitian ini dilakukan untuk membuktikan bahwa rosela memiliki efek antibakteri terhadap *Salmonella Typhi*, penyebab penyakit demam tifoid. Penelitian ini dilaksanakan di Laboratorium Mikrobiologi Fakultas Kedokteran, Universitas Brawijaya. Metode yang digunakan dalam penelitian ini adalah difusi sumuran dengan konsentrasi ekstrak 0%, 3,125%, 6,25%, 12,5%, 25%, 50% dan 100%. Data yang diperoleh dari Kruskal Wallis menginformasikan bahwa probabilitasnya <0,05, oleh karena itu dapat dinyatakan bahwa ada perbedaan yang signifikan dalam efek ekstrak rosela terhadap penghambatan pertumbuhan *Salmonella Typhi*. Uji korelasi Pearson menunjukkan koefisien sebesar 0,962, menunjukkan ada hubungan yang kuat antara rosela dengan *Salmonella Typhi*. Semakin tinggi konsentrasi ekstrak *Hibiscus sabdariffa L.*, semakin tinggi tingkat penghambatan pertumbuhan *Salmonella Typhi* yang diamati. Penelitian ini membuktikan bahwa ekstrak rosela (*Hibiscus sabdariffa L.*) memiliki efek antibakteri terhadap pertumbuhan *Salmonella Typhi* secara in vitro.

Kata Kunci : *Hibiscus sabdariffa L.*, rosela, *Salmonella Typhi*, difusi sumuran.

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Abstract

Hibiscus Sabdariffa L. or generally known as rosella is a flowering plant that derive from the family *Malvaceae* family. In the history of traditional medicine and alternative treatment rosella had been used for lowering high blood pressure, lower cholesterol, prevent heart problems, as well as an antioxidant. It is also an antibacterial agent. This research is performed to prove rosella has an antibacterial effect towards *Salmonella Typhi*, which is often the cause of typhoid fever. This experiment was conducted at Laboratorium of microbiology, Faculty of Medicine, Brawijaya University. Method used in this research was Well's diffusion with the different extract concentration of 0%, 3.125%, 6.25% 12.5%, 25%, 50% and 100%. The data obtained from Kruskal Wallis informs that the probability is < 0.05, therefore it can be stated that there is a significant different in the effect of rosella extract on the growth inhibition of *Salmonella Typhi.* The Pearson correlation test shows a coefficient of 0.962, indicating there is a strong relationship between rosella and *Salmonella Typhi*. The higher the concentration of *Hibiscus sabdariffa L*. extract, the higher the degree of growth inhibition of *Salmonella Typhi* observed. This research proves that rosella (*Hibiscus sabdariffa L.*) extract has an antibacterial effect towards the growth of *Salmonella Typhi* in vitro.

Keywords : *Hibiscus sabdariffa L.*, rosella, *Salmonella Typhi*, Well's diffusion method.

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CHAPTER 1

INTRODUCTION

1.1 Background

Salmonella Typhi is a gram negative rod bacteria that can infect humans. This bacteria is able to invade tissues outside the intestine, causing enteric fever, and the more severe being typhoid fever (Dzen *et al*., 2010). Salmonella is a facultative anaerobic by nature, and the Vi antigen found on the surface of the bacteria is restricted to the *S.Typhi* and *S.Paratyphi C* strain of the bacteria (Harrison's, 2015).

The disease resulting from *Salmonella Typhi* in an endemic infectious disease, affecting many people and is still a health problem in the tropics, especially in developing countries, including Indonesia (Musnelina *et al*., 2004). This disease causes problems because it is rarely able to bring the impact of increase morbidity and mortality. The disease is estimated to strike 22 million people per year with a mortality rate of up to 200,000 people per year. According to WHO, in 2003 there were about 900,000 cases in Indonesia, and about 20,000 from that total died (Rahmawati, 2010). Since 1948, chloramphenicol has been the drug of choice for typhoid fever. The clinics in some countries observed their child typhoid fever cases that were severe and even fatal, which were caused by *Salmonella Typhi* strains resistant to chloramphenicol.

Chloramphenicol is a broad spectrum antibiotic, which has bacteriostatic properties against most bacterial species. Chloramphenicol inhibits bacterial protein synthesis by penetrating into the bacterial cells and binds reversibly to the 50s ribosomal subunits. However, there has been an increasing prevalence of multiple drug-resistance strains of *Salmonella Typhi* which endanger those infected. Chloramphenicol also can cause an adverse effect on the bone marrow, causing hematological toxicity. Dose-related toxicity could cause effects such as anemia, leukopenia, thrombocytopenia and pancytopenia (Goodman & Gilman's, 2011).

Today, alternative medicine is often a choice in the community. There are various types of alternative medicine, one of which is an alternative treatment that is based on herbs. This type of treatment uses leaves, or other parts of a plant. Rosella (*Hibiscus sabdariffa L.*) petals are one of the herbal remedies that can be used. Besides being very easy to obtain, rosella petals are also very useful as anti-inflammatory antiseptic, antibacterial, astringent, analgesic and anti-cancer, a high anti-oxidant, lowering both cholesterol and uric acid (Maryani, 2005). Rosella contains flavonoid, which inhibits the DNA synthesizing enzyme. Inhibition of this enzyme will result in the failure of DNA replication (Rizki & Muhammad, 2017). This process could greatly benefit the treatment of multiple drug-resistance strains of *Salmonella Typhi.*

Therefore the aim of the research is to study the effect of rosella with its antimicrobial effect towards *Salmonella Typhi,* as an alternative treatment for diseases caused by *Salmonella Typhi.*

1.2 Problem Formulation

Based on the background of study, problem formulations are generated as follows:

- Does *Hibiscus sabdariffa L.* extract have an antibacterial effect towards *Salmonella Typhi* in vitro?

1.3 Objective of the Research

The objectives are divided into general objective and specific objectives.

1.3.1 General Objective

To determine the antibacterial effect of *Hibiscus sabdariffa L.* extract on the growth of *Salmonella Typhi* in vitro.

1.3.2 Specific Objectives

1. To evaluate the difference in the effect of different concentrations *of Hibiscus sabdariffa L.* extract on the growth of *Salmonella Typhi*.

2. To determine the value of the Minimum Inhibitory Concentration (MIC) of *Hibiscus sabdariffa L.* extract on *Salmonella Typhi*.
3. To evaluate the relationship between the concentration of *Hibiscus sabdariffa L.* extract and the growth of *Salmonella Typhi.*

1.4 Significance of the Research

The significances are divided into academic significance and practical significance.

1.4.1 Academic Significance

1. To gain more knowledge about alternative medicine on curing typhoid fever using natural agents that have an antibacterial effect.

1.4.2 Practical Significance

1. To contribute to the medical world about the antibacterial effect of *Hibiscus sabdariffa L.* extract towards *Salmonella Typhi*.
2. To increase the use of alternative treatment to replace antibiotic to overcome antibiotic side effects.

CHAPTER 2
REVIEW OF RELATED LITERATURE

2.1 *Salmonella Typhi*

2.1.1 Classification of *Salmonella Typhi*

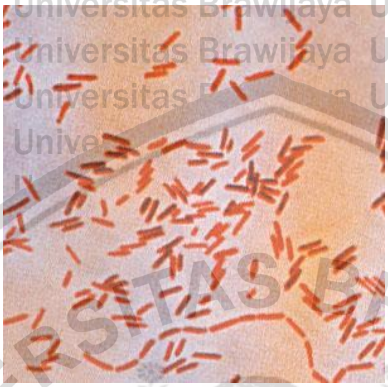


Figure 2.1 *Salmonella Typhi* microscopic image (Todar, 2008)

Scientific classification:

Kingdom	:	Bacteria
Phylum	:	Proteobacteria
Class	:	Gamma proteobacteria
Order	:	Enterobacteriales
Family	:	Enterobacteriaceae
Genus	:	*Salmonella*
Species	:	***Salmonella Typhi***

2.1.2 Morphology and Physiology

Salmonella is a straight rod-shaped bacteria with a size of 0.7-1.5 x 2-5 micrometers that does not form spores, is Gram negative, moves with a peritrichous flagella, and is a facultative anaerobic (Bergey *et al*., 1994). In gram-negative bacteria, the cell wall consists of a peptidoglycan layer and an outer membrane consisting of lipoproteins and lipopolysaccharides (Brooks *et al*., 2008). In Bismuth Sulfite Agar, bacterial colonies are metallic black and shinny, due to H_2S formation (Rasmilah, 2001).

2.1.3 Antigen Structure

The *salmonella* genus has 3 major antigens, comprising of somatic antigen (O), surface antigen and flagella antigen (H). Somatic antigen (O) is an antigen present in the cell wall and is able to withstand heat and alcohol temperatures. The surface antigen is an antigen that can be found in bacterial capsules. One specific surface antigen is the Vi antigen found in *Salmonella Typhi*. Flagella antigen is an antigen found in bacterial flagella and is a non-heat-resistant protein (Todar, 2008).

2.1.4 Clinical Manifestation

Systemic infection caused by *Salmonella enterica* bacteria, especially serotype *Salmonella Typhi* is typhoid fever (Rahmawati, 2010). The first week of infection, the symptoms are lethargy, fever, malaise and body aches. Constipation is more common than diarrhea. During this time, bacteria can penetrate into the intestinal walls, and infects the lymphatic system. Others will enter the bloodstream and infect the reticuloendothelial system. In both places, the bacteria will be eaten by monocyte cells but not killed, but multiply in the monocyte cells (Dzen *et al*., 2003).

During the second week of illness, the bacteria reentered the bloodstream, causing a second bacteremia. Infections of the bile ducts and others occur at this time. Patients appear to be severely ill with fever up to 40^{o}C. Diarrhea can occur in the second or third week of the illness. After the third week the patient appears tired and still hot but shows improvement if no complications are experienced. Complications that can occur include bowel perforation, severe bleeding, pneumonia, and abscess formation. The mortality rate ranges from 2% - 10%. Approximately 20% of patients will experience a relapse (Dzen *et al*., 2003).

2.1.5 Treatment

Since 1948 chloramphenicol is the drug of choice for typhoid fever. The dose of chloramphenicol in adults is 4 times 500mg daily orally or intravenously for 4-5 days free of fever with the duration of treatment ranging from 17-23 days. Clinically in some countries

observing cases of typhoid fever in their children is very severe and even fatal, which is caused by strains of *Salmonella Typhi* that are resistant to chloramphenicol. Indian researchers reported cases of typhoid fever that were resistant to chloramphenicol in 1070, while in Mexico for the first time reported in 1972 on further development of *Salmonella Typhi* resistance, some countries reported their *Salmonella Typhi* multi-drug resistance strain against two or more antibiotics commonly used are ampicillin, chloramphenicol and cotrimoxazole. Thailand (1984) was the first country to report their child's MDR in typhoid fever, followed by other countries such as China (1987), Pakistan (1988) and Egypt (1993) (Musnelina *et al*., 2004).

Until now, chloramphenicol is still the treatment of choice for typhoid fever because of its effectiveness against *Salmonella Typhi* in addition to its relatively inexpensive medicinal price (Musnelina *et al*., 2004). However, there has been an increasing prevalence of multiple drug-resistance strains of *Salmonella Typhi* which endanger those infected. Resistance to chloramphenicol is usually caused by plasmid-encoded acetyltransferase found in the resistant strains which inactivates the drug. Acetylated derivatives of chloramphenicol fail to bind to bacterial ribosomes and therefore, failing to inhibit protein synthesis. Chloramphenicol also can cause an adverse effect on the bone marrow, causing hematological toxicity. Dose-related toxicity could cause effects such as anemia, leukopenia, thrombocytopenia and pancytopenia (Goodman & Gilman's, 2011).

2.2 *Hibiscus sabdariffa L.*

2.2.1 Taxonomy

Figure 2.2 *Hibiscus sabdariffa L.*

Scientific Classification based on ITIS :

Kingdom	:	Plantae
Subkingdom	:	Viridiplantae
Infrakingdom	:	Streptophyta
Superdivision	:	Embryophyta
Division	:	Tracheophyta
Subdivision	:	Spermatophytina
Class	:	Magnoliopsida
Superorder	:	Rosanae
Order	:	Malvales
Family	:	Malvaceae
Genus	:	Hibiscus L.
Species	:	*Hibiscus sabdariffa L.*

2.2.2 Origin of Rosella

Roselle (Hibiscus Sabdariffa L.) belongs to the family Malvaceae (Mahadevan *et al*., 2009; Anjah *et al*., 2012). It is believed to have originated from Asia (India to Malaysia) or tropical Africa. The plant grows well especially in the tropics such as the Caribbean, India, Africa, Hawaii and Philippines, as a home garden crop (Mahadevan *et al*., 2009).

2.2.3 Morphology

Rosella is a plant with a taproot, growing straight and branched. This plant is less than a year old, and has a height ranging between 3–4m. Dark green colour to red stems. The leaves finger (3-7 fingers) with a serrated periphery and petiole length. Capsule-shaped flowers with a 5cm long and 5.3cm width (Suharmiati dan Hanyadani, 2005; Rukmana, 2001). Rosella has only one flower bud on each flower stalk. This flower has 8-11 hairy petal

strands with a length of 1cm, each base attaches and is red in colour. Rosella petals are red to yellow with a darker colour in the centre. The stalks are sari-sized, short and thick. The pistil is tubular shaped and is yellow or red in colour. Rosella is hermaphrodite so that it can perform its own reproduction (Maryani & Kristiana, 2005). Flowers of the rosella are solitary and axillary, nearly sessile. The bracteoles 8-12 colored red, fleshy, lanceolate-shaped, 5-10mm long, 2-3mm wide, covered with sparsely hirsute, with thorn-like appendages near the top, base and calyx connate, yellow flower (Achlana, 2013).

2.2.4 Geographical Distribution and Habitat

Rosella grows well in the tropical and subtropical (25-35˚C) and a humidity of 70% and at an altitude of 0–500m above sea level (Maryani & Kristiana, 2005). Although these plants need abundant rainfall during the vegetative period for maximum yields, Rosella also grows in areas with low rainfall. Rosella can grow in different types of soil as long as it has a nice texture and drainage (Mahadevan & Shivali, 2009). This plant has a natural habitat in an area stretching from India to Malaysia. However, now the plant is already widespread in tropical and subtropical regions around the world. It is no wonder that this plant has a common name which varies (Maryani & Kristiana, 2005).

2.2.5 Chemical Substances of *Hibiscus sabdariffa L.*

Rosella contains anthocyanin pigments that form the flavonoids that act as antioxidants. Flavonoid in Rosella consists of the pigment anthocyanins and flavonols. Anthocyanins in rosella flowers are in the form of glucosides consisting of cyanidin-3-sambubioside, delphinidin-3-glucose and delphinidin-3-sambubioside. Meanwhile flavonols contain gossypetin, hibiscetin and quercetiaii (Mardiah *et al*., 2009). Rosella also contains alkaloids, L-ascorbic acid, anisaldehyde, beta carotene, protocathecuic acid, cyaniding-3-rutinoside, mucopolysaccharides, beta cytosterol, nitric acid, galactose, polyphenols, pectin, polysaccharides, sterat acids and waxes (Hirunpanich *et al*., 2005). The antibacterial property of rosella is possible due to it containing alkaloids, allicin, saponin, flavonoids and

tannin, the dominant one being flavonoid. Flavonoid accounts for about 60% of the antimicrobial property of rosella (Rizki & Muhammad, 2017). The vitamin content in the rosella flower is complete, namely vitamin C, A, D, B1, B2 and amino acids. Rosella contains 18 amino acids that the body needs. Among them are arginine and lignin that play a role in the process of rejuvenation of body cells. In addition Rosella also contains protein and calcium (Harmanto 2007). Riboflavin also known as Vitamin B2 has proved to exhibit antimicrobial properties against gram positive and negative bacteria (Ahgilan & Sabaratnam, 2016). Vitamin D activates CAMP and DEFB4 genes, which are biologically important for innate immune response towards wounds and infections (Gombart AF, 2009). Vitamin C enhances the inhibitory effect of quercetin, which is a natural flavonoid (Johanna, 2012).

2.3 Mechanism of antimicrobial

Antimicrobial drugs act in one of serval ways, an ideal antimicrobial agent should be selectively toxicity. The mechanisms of action can be divided into: inhibition of cell wall synthesis, inhibition of cell membrane function, inhibition of the protein synthesis and inhibition of nucleic acid formation.

2.3.1 Inhibition of cell wall synthesis

Bacteria consists of a rigid outer layer knowns as the cell wall, the cell wall plays a role in maintaining the shape and size of the bacteria. Inhibition of its formation or injuring to the cell wall may cause the cell lysis (Brooke *et al.*, 2007).

2.3.2 Inhibition of the cell membrane function.

The cytoplasmic membrane serves as a selectively permeability barrier. It carriers out active transport function and regulate the internal composition of the cell. If the function of the cytoplasmic membrane is interrupted, macromolecules and ions escape from the cell, cell might be damage or even resulting cell death (Brooke *et al.*, 2007).

2.3.3 Inhibition of protein synthesis.

The bacteria carries a ribosome which consists of genetic information of the cell. The interruption of the protein synthesis causes the genetic code to be interrupted and causes cell death (Brooke *et al.*, 2007).

2.3.4 Inhibition of Nucleic Acid Formation

For many microorganisms, p- aminobenzoic acid (PABA) is an essential metabolite. The specific mode of action of PABA involves an adenosine triphosphate (ATP). PABA is involved in the synthesis of folic acid. In the mechanisms of antibacterial the structural analogs PABA and inhibit dihydropteroate synthetase resulting failure of synthesis of folic acid and the cell not able to form ribosome and genetic information code interrupted. (Brooke *et al.*, 2007)

2.4 Measurement of Antimicrobial Activity In Vitro

There are two principle methods when it comes to determine the susceptibility of a pathogen to antimicrobial substance; dilution or diffusion.

2.4.1 Dilution Method

The media are subsequently inoculated with the test bacteria and incubated. The amount of antimicrobial substance required to inhibit the growth of bacteria are noted as the end point (Brooke *et al*., 2007).

2.4.2 Diffusion Method

A filter paper disk containing a measured quantity of a drug is placed on the surface of the solid medium that has been inoculated with the test bacteria. After incubation, the diameters of the clear zones are measured for the inhibitory power of the substance against the specific organisms (Brooke *et al*., 2007).

2.5 Minimum Inhibitory Concentration (MIC)

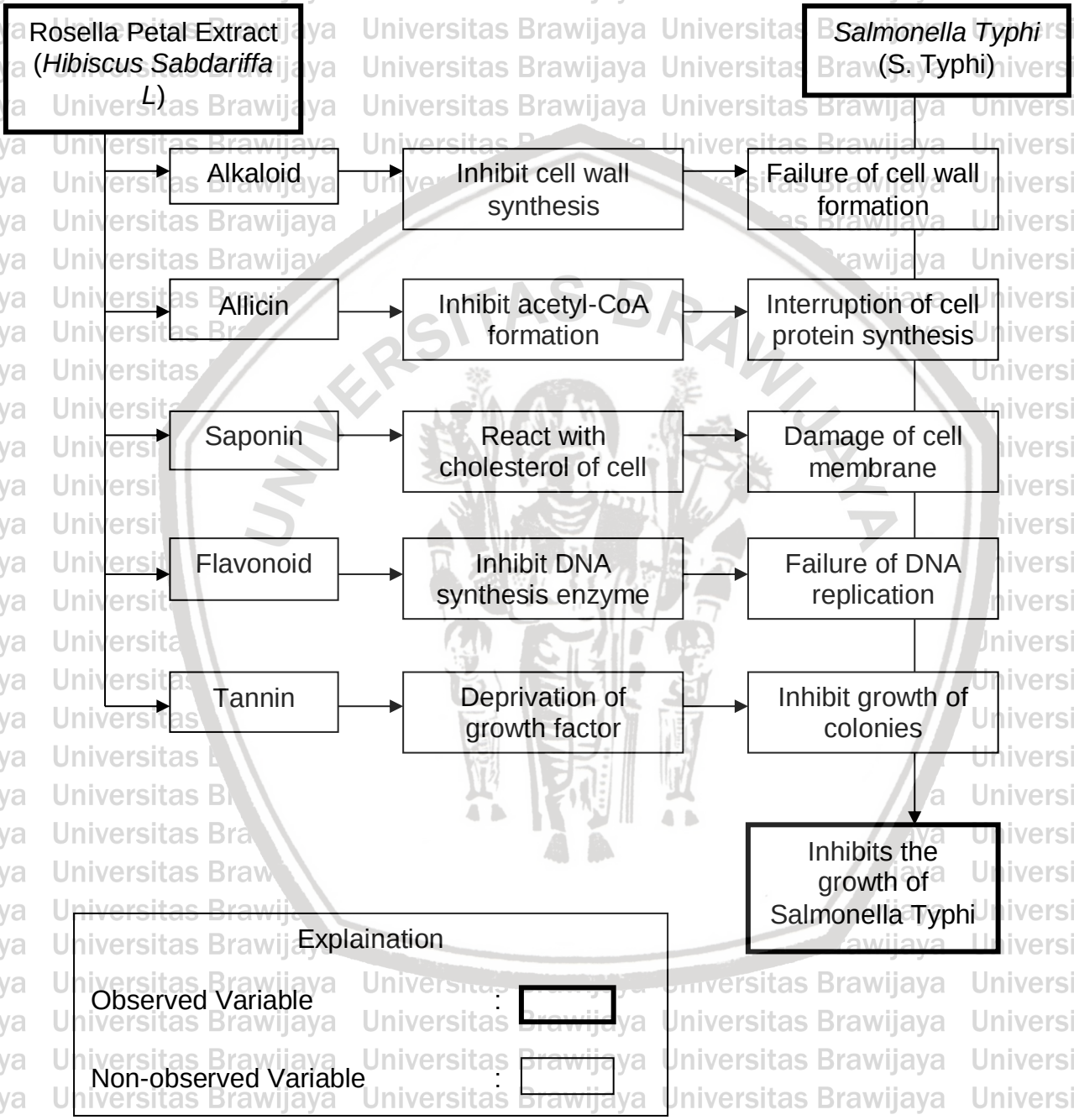
The Minimum Inhibitory Concentration is defined as the lowest concentration of an antimicrobial agent that is bacteriostatic. MIC evaluates the antimicrobial efficacy of a compound by measuring the effect of decreasing concentrations of antibiotics over a defined

period in regards to inhibition of microbial population growth. These evaluations are useful in R&D phase of a product to determine the appropriate concentrations of the drug, required to produce an effect (Goins, 2017).

CHAPTER 3

CONCEPTUAL FRAMEWORK AND HYPOTHESIS

3.1 Conceptual Framework



Figur 3.1 Conceptual framework

Hibiscus sabdariffa L. contains tannin which works as an antibacterial. It's mechanism of action is to inhibit the enzyme reverse transcriptase of DNA topoisomerase, so that

bacterial cells cannot be formed (Robinson, 1995). Tannins can also wrinkle cell wall or cell membrane so that it can interfere with the permeability of the bacterial cell wall. As a result of the disruption, the cell cannot perform life activities that its growth becomes stunted and eventually dies (Ajizah, 2004). As for alkaloids as an antibacterial, the mechanism is through inhibition of cell wall synthesis that would lead to lysis of the cells so that the cells of the bacteria will die (Lamothe, 2009). Meanwhile, saponin is able to bind to lipopolysaccharide bacterial cell wall, causing increased permeability of the bacterial cell wall (Arabski *et al*., 2009). Allicin has the ability to inhibit acetyl-CoA from forming. This is response interrupts the synthesis of bacterial cell protein. Flavonoid inhibits DNA enzyme synthesis which causes the failure of bacterial DNA to replicate.

The inhibition of *Salmonella Typhi* growth is made possible due to five factors which are the failure of the cell wall to form, interruption of the cell protein synthesis, damage of the cell membrane, failure of DNA replication and the inhibition of cell growth.

3.2 Hypotheses

- *Hibiscus sabdariffa L.* possesses and demonstrates antibacterial activities towards *Salmonella Typhi growth* in vitro.

CHAPTER 4

METHODOLOGY

4.1 Research Design

The study design used is the in vitro experimental study using the Well's Diffusion test. The purpose was to observe the antimicrobials effect of *Hibiscus sabdariffa L.* extract on the growth of *Salmonella typhi*.

4.2 Research Sample

The sample used was the isolated *Salmonella Typhi* culture available at the Microbiology Laboratory of dr Saiful Anwar General Hospital, Malang.

4.3 Repetition Estimation

The total number of repetition used in this study is calculated using the formula: (Notobroto,2005)

$$p (n – 1) \geq 15$$

$$7 (n – 1) \geq 15$$

$$7 n \geq 22$$

$$n \geq 3.15$$

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 month of November 2017.

4.5 Variable Identification

4.5.1 Independent Variable

The independent variables in this study were the *Hibiscus sabdariffa L.* extract with concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100%. The 100% concentration is the positive control, and 0 % is the negative control.

4.5.2 Dependent Variable

The dependent variables in this study were the diameter of the clear zone 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 Definition of Key Terms

- The rosella extract is the concentrate of rosella obtained from Materia Medica, Batu, which had undergone the extraction process using 96% ethanol.
- The isolates of *Salmonella typhi* were isolates obtained from readily available cultures in the Microbiology Laboratory of dr Saiful Anwar General Hospital, Malang
- The *Salmonella typhi* inoculum was the inoculum with a concentration of 1 x10 ^{8} CFU/ml.

4.7 Instruments and Materials

4.7.1 Instruments

- Blade
- Bunsen burner
- Inoculating loop
- Incubation equipment
- Rotatory evaporator
- Cork borer (4-mm diameter)
- Microscope
- Object glass
- Immersion oil
- Mortar and pestle
- Vortex
- Spectrometer
- Calibrated pipette
- Matches
- Forceps
- Ruler
- Petri dish
- Glass jar
- Test tube

4.7.2 Materials

- Rosella
- 96% ethanol
- Isolated *Salmonella Typhi* culture
- Bismuth sulfite agar
- Mueller Hinton agar (with 5% Sheep blood)

- 0.9% sterile NaCl solution
- Gram stain dyes: crystal violet, Lugol's iodine, 96% alcohol, safranin
- Distilled water

4.8 Study Procedures

4.8.1 Rosella Extract

The rosella are finely crushed using the motar and pestar blender. Once fine, the crushed rosella were wrapped with filter paper and soaked in 96% ethanol overnight. The 96% ethanol used for soaking was replaced several times until the extract solution is clear. The extracted product was then ready for evaporation. For organic solvents, the extract is concentrated by evaporation under reduced pressure (using a rotary evaporator) at a low temperature to minimize the degradation of thermolabile compounds (Seidel, 2012).

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%.(Sarker *et al*., 2006)

4.8.1.1 Dilution of Rosella Extract

- This process used crude rosella extract and distilled water
- Solution was made in several concentrations ranging from 3.125%, 6.25%, 12.5%, 25% and 50 % with distilled water

- 387.5 µL of distilled water were pipetted into a tube, add 12.5 µL of 100% rosella extract in Whisk to blend the mixture above. The tube now contained the solution of rosella extract 3.125%
- 375 µL of distilled water were pipetted into a tube, add 25 µL of 100% rosella extract in Whisk to blend the mixture above. The tube now contained the solution of rosella extract 6.25%
- 350 µL of distilled water were pipetted into a tube, add 50 µL of 100% rosella extract in Whisk to blend the mixture above. The tube now contained the solution of rosella extract 12.5%
- 300 µL of distilled water were pipetted into a tube, add 100 µL of 100% rosella extract in Whisk to blend the mixture above. The tube now contained the solution of rosella extract 25%
- 200 µL of distilled water were pipetted into a tube, add 200 µL of 100% rosella extract in Whisk to blend the mixture above. The tube now contained the solution of rosella extract 50%

4.8.2 Salmonella Typhi 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 *Salmonella Typhi* 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, and 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: Rod shape bacilli stained red (Gram negative).

4.8.2.2 Identification with TSI testing

- The inoculating needle was sterilized in the blue flame from a bunsen burner till red hot, and was allowed to cool.
- The Trypticase soy broth tube containing the 24-48 hour culture was taken from the rack, removing the cap and flaming the neck of the tube.
- Using the aseptic technique, the culture of the organism from the TSB (tryptic soy broth) was taken using a needle.
- The neck of the tube was flamed again.
- A sterile TSI slant tube is removed from the rack, its capped was removed and the neck of the tube was flamed.
- The needle containing the pure culture was stabbed into the medium, up to the butt of the TSI tube, and the needle was streaked back and forth along the surface of the slant.
- The neck of the TSI tube was flamed, capped and placed back into the rack. It was incubated at 37^{o}C for 18-24 hours.
- Positive result: Red alkaline slant, yellow butt (*Salmonella Typhi*).

4.8.2.3 Preparation of *Salmonella typhi* Testing Suspension

- 5 colonies of *Salmonella Typhi* measuring 1mm in diameter were removed from the BHIA media.
- The colonies were inserted into 9ml 0.9% sterile NaCl and then vortexed.
- Using spectrophotometry at 625nm wavelength, the colonies were measured. This procedure determined the optical density of the *Salmonella Typhi*.
- With the optical density determined, the formula $N^1V^1 = N^2V^2$ was then used to determine the volume of *Salmonella Typhi* to be mixed with sterile NaCl solution to produce a 10ml suspension of 1 x 10^8 CFU/ml. (Balouiri *et al*., 2015)

4.8.3 Antimicrobial Testing of Rosella Extract

4 sterile petri dishes were divided in to 7 zones using marker pen to mark the backs of the petri dish. The petri dish were labeled 100%(1), 50%(2), 25%(3), 12.5%(4), 6.25%(5), and 3.125%(6) .

- 1ml of bacterial suspension were dropped into each petri dish and followed by 20ml of MHA medium with 5% of sheep blood, waited for it to solidify.
- A hole were punched at each zone of the petri dish using a cork bores (4 mm diameter).
- The calibrated pipetted 1 ml of 100% extract was dropped into the holes of petri zone 1.
- The calibrated pipetted 1 ml of 50% extract was dropped into the holes of petri zone 2.
- The calibrated pipetted 1 ml of 25% extract was dropped into the holes of petri zone 3.
- The calibrated pipetted 1 ml of 12.5% extract was dropped into the holes of petri zone 4.
- The calibrated pipetted 1 ml of 6.5% extract was dropped into the holes of petri zone 5.
- The calibrated pipetted 1 ml of 3.125% extract was dropped into the holes of petri zone 6.
- The calibrated pipetted 1 ml of distilled water was dropped into the petri zone 7.
- The petri dish was incubated at 37°C for 24 hour.
- After 24 hour, the diameter of clear zone was observed and measured. The method of determining the diameter of clear zone was by placing a white paper at the front of the petri dish and measuring the diameter of clear zone from the holes with a ruler from the bade.

4.9 Procedure of Study

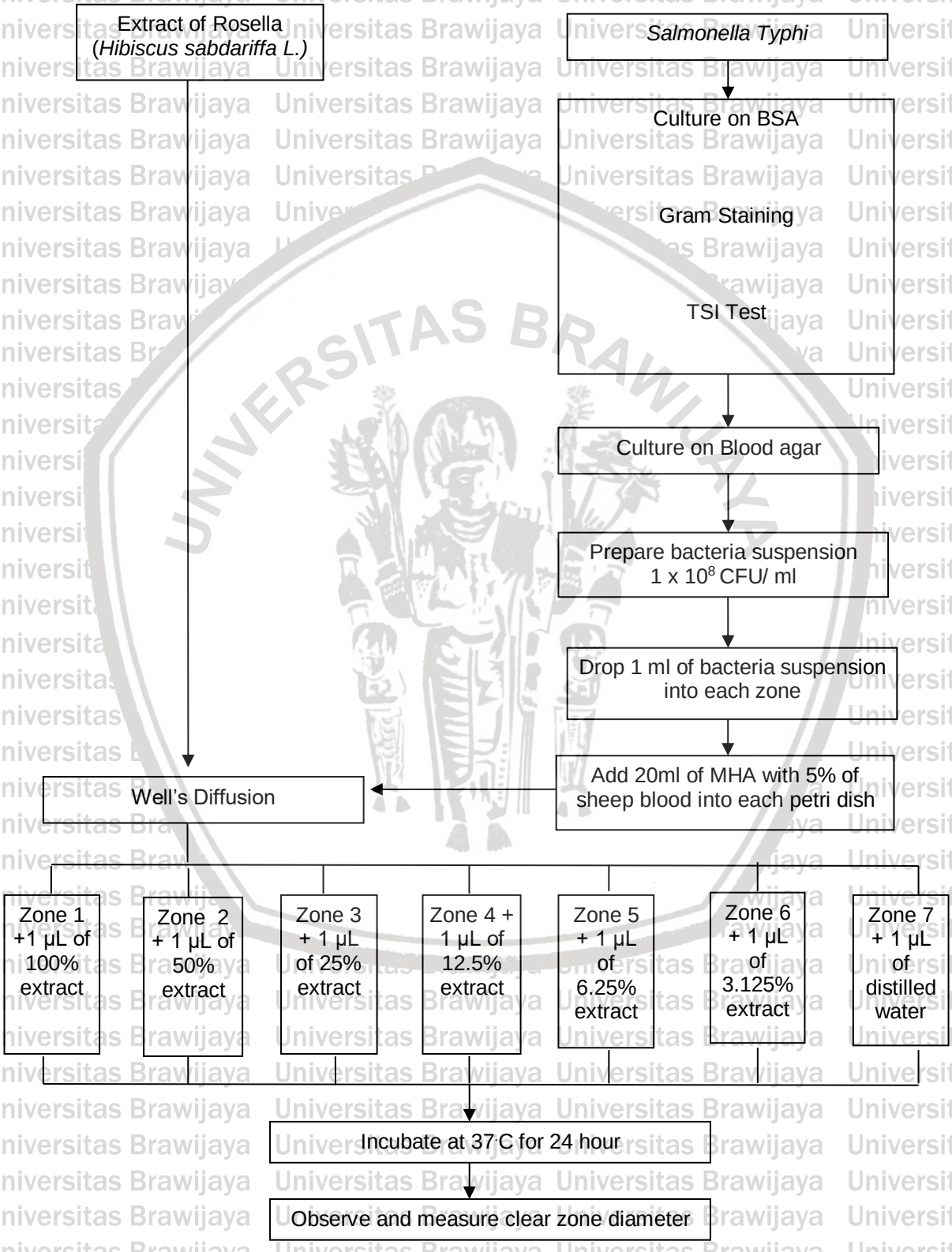


Figure 4.1 Procedure of Study

4.10 Data Analysis

The study data were the number of *Salmonella Typhi* colony and the type of data analysis used was the Kruskal Wallis method. This method was used because the residual normality test was fulfilled, however the residual homogeneity test was not fulfilled. With the Kruskal Wallis method, the effect of different concentrations of rosella extract on *S.Typhi* could be determined and concluded whether or not this extract had any significance towards the growth of *S.Typhi* in vitro. The linear regression correlation statistical analysis was also conducted to determine the size of the effect and the correlation between the rosella extract concentrations and the growth of *S.Typhi* in vitro. The Pearson correlation method was used to determine the strength and direction between both variables.

CHAPTER 5

STUDY RESULT

5.1 Study Result Data

5.1.1 Identification Results of *Salmonella Typhi*

The isolates were obtained from the Microbiology Laboratory of dr.Saiful Anwar General Hospital and subjected to Gram staining, TSI test, and BSA plant. The results of the mentioned procedures are shown in the following figures:

From Gram staining, the isolated bacterial were observed under a light microscope with the magnification of 1000X.The morphology shows that gram negative rod shaped bacillus. While TSI test shows a red alkaline slant, yellow butt with gas bubbles and blackening. The colonies formed on the Bismuth Sulfite Agar plate were noted to be round in shape and black in colour.

Figure 5.1 Rod-shaped, Gram-negative *Salmonella Typhi* observe by Gram staining

Figure 5.2 Red alkaline slant, yellow butt, *Salmonella Typhi* observe by TSI test

Figure 5.3 Black colonies on the Bismuth Sulfite Agar plate

5.1.2 Determination of the Growth Inhibition of *Salmonella Typhi*

From the inoculation of the *Salmonella Typhi* on Blood Agar medium, the diameter of clear zone of each concentration were measured by ruler and the result are tabulated as below.

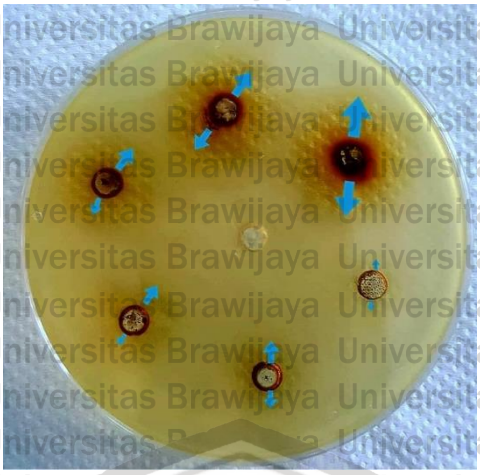


Figure 5.4 The clear zone of the *Salmonella typhi* in different extract concentrations

Table 5.1 The diameter of clear zone of Salmonella Typhi in different concentrations of extract (mm)

N	Concentration of *Salmonella Typhi* Extract						
	0%	3.125%	6.5%	12.5%	25%	50%	100%
1	0	10.0	12.0	13.0	16.0	20.0	21.0
2	0	9.0	11.0	13.0	15.0	20.0	22.0
3	0	8.0	12.0	14.0	15.0	19.0	21.0
4	0	8.0	11.0	13.0	16.0	18.0	22.0
Mean	0	8.8	11.5	13.3	15.6	19.3	21.5

Explanation: N = number of interventions

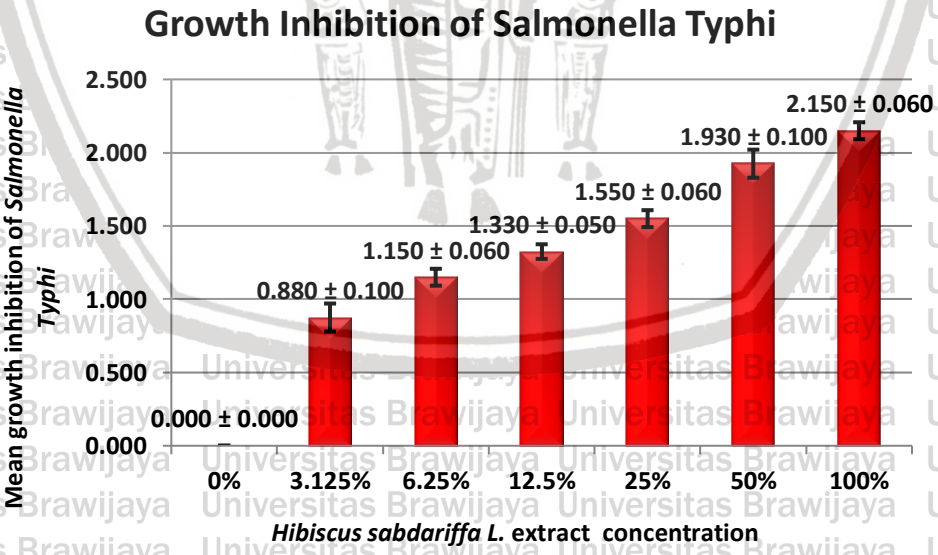


Figure 5.5 Mean Growth Inhibition Of *Salmonella Typhi* Based on different Extract Concentration

Based on the graphic above, it can be seen that isolated *Salmonella Typhi* culture which does not get Hibiscus sabdariffa L. extract (0%) has an average inhibition growth rate

of *Salmonella Typhi* at 0.000 ± 0.000. Then the isolated *Salmonella Typhi* cultures which obtained Hibiscus sabdariffa L. extract with concentration of 3.125% had an average inhibition growth rate of 0.880 ± 0.100, whereas isolated *Salmonella Typhi* culture which obtained Hibiscus sabdariffa L. extract of 6.5% concentration had an average inhibition growth *Salmonella Typhi* of 1.150 ± 0.060. While isolated *Salmonella Typhi* cultures which received Hibiscus sabdariffa L. extract of 12.5% concentration had an average inhibition growth rate of *Salmonella Typhi* in vitro of 1.330 ± 0.050. Meanwhile the isolated *Salmonella Typhi* culture which obtained Hibiscus sabdariffa L. extract with concentration of 25% had an average inhibition rate of 1.550 ± 0.060 and the average inhibition growth spurt of *Salmonella Typhi* after obtaining Hibiscus sabdariffa L. extract with 50% concentration are 1.930 ± 0.100. While isolated *Salmonella Typhi* cultures which obtained Hibiscus sabdariffa L. extract with 100% concentration had an average inhibition growth rate of *Salmonella Typhi* at 2.150 ± 0.060.

5.2 Data Analysis

The study result data was then analyzed using the SPSS (Statistical Package for the Social Sciences).

5.2.1 Data Analysis with Normality Test

Normality test on the effect of *Hibiscus sabdariffa L.* extract on growth inhibition of *Salmonella Typhi* in vitro aims to know the normal or not residual resulting from the effect of *Hibiscus sabdariffa L.* extract on growth inhibition of *Salmonella Typhi* in vitro. Residual normality test was performed using Kolmogorov Smirnov, with criterion if probability value> 0.05 then residual is stated normal. The residual normality effect test of *Hibiscus sabdariffa L.* extract on the growth inhibition of *Salmonella Typhi* in vitro produced results in Kolmogorov Smirnov statistic of 0.161 with probability of 0.062. It can be seen that the residual normality test effect of *Hibiscus sabdariffa L.* extract on growth inhibition of *Salmonella Typhi* in vitro have probability >0.05, so the residual is stated normal.

5.2.2 Data Analysis with Homogeneity of Variance Test

The homogeneity of variance test is used to detect whether the set of data has heterogeneity or not. The residual homogeneity test effect of *Hibiscus sabdariffa L.* extract on inhibition growth of *Salmonella Typhi* in vitro was conducted using *Levene Test*, with criterion if probability value> 0.05 then result is homogeneous. The homogeneity test of effect of *Hibiscus sabdariffa L.* extract on growth inhibition of *Salmonella Typhi* in vitro by Levene statistic is 4.579 with probability of 0.004. It can be concluded that residual testing effect of *Hibiscus sabdariffa L.* extract on growth of *Salmonella Typhi* in vitro have a probability <0.05 so the residual is not homogenous.

5.2.3 Data Analysis with *Kruskal Wallis* method

The test of difference of effect of *Hibiscus sabdariffa L.* extract on growth inhibition of *Salmonella Typhi* in vitro resulted in Chi-Square test statistics of 26,643 with probability of 0.000. It is known that probability <0.05. Therefore, it can be stated that there are significant differences in the effect of *Hibiscus sabdariffa L.* extract on growth inhibition of *Salmonella Typhi.*

To know the effect of *Hibiscus sabdariffa L.* extract on growth inhibition of *Salmonella Typhi* at different significant was determine using Mann Whitney with criterion that if one pair of meeting time yield probability ≤ 0.05 it can be stated that there is difference of effect of *Hibiscus sabdariffa L.* extract against growth inhibition of *Salmonella Typhi* in vitro.

Table 5.2 The result of Mann Whitney's analysis

Variable	Mean	Probability							Notation
		C-	P1	P2	P3	P4	P5	C+	
C-	0.00		0.013	0.013	0.011	0.013	0.013	0.013	A
P1	0.88	0.013		0.019	0.017	0.019	0.019	0.019	B
P2	1.15	0.013	0.019		0.017	0.018	0.019	0.018	C
P3	1.33	0.011	0.017	0.017		0.017	0.017	0.017	D
P4	1.55	0.013	0.019	0.018	0.017		0.019	0.018	E
P5	1.93	0.013	0.019	0.019	0.017	0.019		0.019	F
C+	2.15	0.013	0.019	0.018	0.017	0.018	0.019		G

Explanation: C- = Control negative (0% extract),

P1 = Group 1(3.125% extract)

P2 = Group 2(6.5% extract)

P3 = Group 3(12.5% extract)

P4 = Group 4(25% extract)

P5 = Group 5 (50% extract)

C+ = Control positive (100% extract)

The result of Mann Whitney's analysis informed that:

- The positive control group (C+) resulted in the highest growth inhibition of *Salmonella Typhi*
- The positive control group (C+) had a significantly different with negative control group (C-) that was not given *Hibiscus sabdariffa L.* extract, P1, P2, P3and P4.
- The positive control group (C+) does not have significant difference with P5
- The negative control group (C-) produced the lowest inhibition rate of *Salmonella Typhi*
- The negative control group (C-) is significantly different with all treatment group.

5.3 Determining the Relationship of *Hibiscus sabdariffa L.* Extract Concentration with Growth Inhibition of *Salmonella Typhi*

5.3.1 Analysis of Relationship between Concentration of *Hibiscus sabdariffa L.* extract with Growth inhibition of *Salmonella Typhi*

The results show that the correlation test of *Hibiscus sabdariffa L.* extract concentration with growth inhibition of *Salmonella Typhi* has a probability of 0.000. It is known that the probability is <0.05. Therefore, it can be stated that there is a significant correlation of *Hibiscus sabdariffa L.* extract concentration with growth inhibition of *Salmonella Typhi*.

The correlation coefficient of 0.962 indicates that there is a positive (direct) and very strong relationship. This means that the higher concentration of *Hibiscus sabdariffa L.* extract the growth inhibition of *Salmonella Typhi* are also higher, and thus the lower the concentration of the extract, the lower growth inhibition of *Salmonella Typhi*.

CHAPTER 6

DISCUSSION

Based on the results of the study, the hypothesis is proven. It can be concluded that the rosella extract significantly inhibits *Salmonella Typhi* growth. Well's diffusion method was used in order to accommodate seven concentrations in a single petri dish. The data obtained was analyzed using the *Kruskal Wallis* method which showed that there was a significant difference ($p = 0.000$) in the application of rosella extract at each concentration on the growth inhibition of *Salmonella Typhi.* Following the variance analysis, *Mann Whitney* tests observed that 0% and 100% concentrations had significant effect in increasing the diameter of clear zone .Finally, *Pearson* correlation demonstrated that the application of rosella extract with increasing concentrations can further reduce the diameter of growth of *Salmonella Typhi* as evidenced by the r value of 1.0. Furthermore, there was a positive correlation between the extract concentrations and its inhibitory effect on *Salmonella Typhi*. The higher the concentration of rosella extract, the higher the growth inhibition of *Salmonella Typhi* is seen.

Rosella contains flavonoid, when mixed with ethanol, increases the total flavonoid content. Ethanol has the ability to attract flavonoids. The function of flavonoid is to inhibit DNA synthesis. Without the ability to replicate, bacterial cells cannot undergo division and growth and hence, inhibition of growth occurs. The antimicrobial effect of rosella defers from that of chloramphenicol, which inhibits protein synthesis. This is an alternative especially for multiple drug-resistant strains. From previous studies conducted, 2.60 µg/ml of chloramphenicol was the minimum inhibitory concentration used against *Salmonella Typhi*. Since the clear zone formed in this experiment was as low as 3.125%, the amount of rosella extract used was as high as 12.5 µg/ml.

Although a higher concentration of rosella is used compared to chloramphenicol, the adverse side effects of chloramphenicol such as hematological toxicity is a higher risk

compared to rosella's side effects of mild bowel discomforts and reduced blood sugar and blood pressure levels.

From previous research conducted in Aceh in 2012 by Zinatul and Winda, the effective growth inhibitory concentration for the rosella extract could be identified as low as 12.5%. Hence the percentage concentrations tested in this study were halved twice, to 6.25% and 3.125%, and also doubled to 25% and 50%. The reason the concentrations were halved twice was to prove that rosella at a much smaller concentration has the potential to inhibit bacterial growth. The doubled concentration values were used to prove the specific objective that the concentration of extract directly influences the rate of bacterial inhibition.

CHAPTER 7

CONCLUSION

7.1 Summary

From this study, the general conclusion is that:

1. *Hibiscus sabdariffa L.* extract exhibits antibacterial effects on the growth of *Salmonella Typhi* in vitro.

From this study, the specific conclusion is that:

1. A difference in concentrations of *Hibiscus sabdariffa L.* extract caused a direct change in the growth inhibition of *Salmonella Typhi*.
2. The Minimum Inhibitory Concentration (MIC) of *Hibiscus sabdariffa L.* extract on *Salmonella Typhi* was 3.125%.
3. The higher the concentration of *Hibiscus sabdariffa L.* extract, the higher the degree of growth inhibition of *Salmonella Typhi* is observed.

7.2 Suggestions

Suggestions for this study are as follows:

- Other methods to determine the growth inhibition should be done such as the tube dilution, disc diffusion or the agar dilution method.
- The effectiveness of rosella using other methods or forms besides extract should be explored.
- Further study is required to explore the safety and effectiveness of the rosella extract in vivo (in trial animals and by clinical trials) before it is used as an alternative treatment for typhoid fever.

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