THE EFFECTS OF POMEGRANATE (*Punica granatum)* PEEL EXTRACT AS ANTIBACTERIAL AGENT AGAINST *Staphylococcus aureus* IN VITRO

FINAL ASSIGNMENT

To Fulfill the Requirement and Obtain

The Title of Bachelor of Medicine

By:

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MEDICAL PROGRAM

FACULTY OF MEDICINE

UNIVERSITAS BRAWIJAYA

MALANG

2019

APPROVAL PAGE

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ACKNOWLEDGEMENT

I would like to give thanks and praise to the Holy Trinity, the Almighty God, my Gurunathar Chithramuthu Adigalaar for the strength and blessings given throughout my studies in the medical faculty of Universitas Brawijaya and for the completion of this dissertation entitled "**THE EFFECTS OF POMEGRANATE (Punica granatum) PEEL EXTRACT AS ANTIBACTERIAL AGENT AGAINST *Staphylococcus aureus* IN VITRO".**

With this opportunity, I would also like to express my greatest gratitude to:

1. **Dr. dr. Sri Andarini, M.Kes.**, as the Dean of the Faculty of Medicine of Universitas Brawijaya.
2. **dr. Triwahju Astuti, M.Kes.,Sp.P(K).,** as the Head of the Study Program for the Bachelor of Medicine of Universitas Brawijaya.
3. **Prof.Dr.dr.RespatiSuryanto,Sp.OT(K).,** as my first examiner for assessing and correcting this research while encouraging me for better performance in future.
4. **Prof.Dr.dr.Sumarno DMM Sp.M(Kes).**, as my first supervisor and second examiner for spending his time to consult and aid me throughout this dissertation and seeing it through until completion.
5. **Dr.dr.Sri Poeranto Sp.Par.K.Mes.**, as my second supervisor and third examiner for giving his input and sharing information to help in writing this dissertation.
6. **Pak Ali**, as the lab analyst who has helped me throughout the dissertation in Microbiology Laboratory.

7. To my beloved parents, **Mr. Kaliappan Subramaniam** & **Mrs. Parameswari Subramaniam** on their support and prayers up till this very moment.
8. My sibings**, Bavithra Kaliappan,** and **Navinkumar Kaliappan** for their financial assistance and moral support.
9. My friend **Kovaimalar munusamy** for her help and guidance throughout the thesis.
10. All my family members, friends and other involving parties who has aided me in the completion of this dissertation and not been mentioned above.

On the endnote, I realized that this dissertation is still far from perfection, thus, any criticism and recommendations are welcomed. Hopefully this dissertation can be accepted and be useful to whom may need it.

Malang, 24 September 2019

ABSTRACT

Kaliappan, Gayathiri. 2019. The Effects of Pomegranate (*Punica granatum)* Peel Extract as Antibacterial Agent Against *Staphylococcus aureus* In Vitro. Final assignment, Medical Study Program, Faculty of Medicine, Universitas Brawijaya, Malang. Supervisors: (1) Prof.Dr.dr.Sumarno DMM Sp.Mk(k). (2) Dr.dr.Sri Poeranto Sp.Par.K,M.Kes

Staphylococcus aureus is an important cause of nosocomial infections and is acquired in the community. In addition, it is important to determine the relevant isolates obtained during the experiment. So, it is also one of the organisms that is often found in human infections. *S. aureus* is a major case in hospital. The active compound in pomegranate skin *(Punica granatum)* contains flavonoids, tannis, pyrogallol, cinnamic acid, benzoic acid, chlorogenic acid, campferol, genistein, coumaric acid, and quercetin which help antibiotics against *Staphylococcus aureus.* The purpose of this study is to prove the antimicrobial effect of pomegranate skin extract on the growth of *Staphylococcus aureus* bacteria in vitro using agar diffusion method. Pomegranate extract with concentrations of 3%, 6%, 9%, 12%, 15%, 18% and 21% was used in three different isolates of Staphylococcus aureus. Antibiotics such as Gentamicin and Fosfomycin were used in this experiment. Thus, to determine the antimicrobial effect of pomegranate peel extract (Punica granatum) was measured by the zone of inhibition formed around the well in this experiment. Furthermore, for data analysis, one-way ANOVA was used in this study and it was verified that the concentration of pomegranate rind extract and growth of *S. aureus* was $p < 0.05$. In addition, to strengthen the analysis of the data above, the Spearmen correlation results also showed a dominant correlation between extract concentration and zone inhibition by describing p values <005 and $r = 0.843$. In conclusion, the results of the study prove that pomegranate skin extract has an antimicrobial effect against *Staphylococcus aureus* in vitro using the well diffusion method.

Keywords: Antibacterial; *Staphylococcus aureus*; Pomegranate peel; Well diffusion.

ABSTRACT

Kaliappan, Gayathiri. 2019. The Effects of Pomegranate (*Punica granatum)* Peel Extract as Antibacterial Agent Against *Staphylococcus aureus* In Vitro. Final assignment, Medical Study Program, Faculty of Medicine, Universitas Brawijaya, Malang. Supervisors: (1) Prof.Dr.dr.Sumarno DMM Sp.Mk(k) (2) Dr.dr.Sri Poeranto Sp.Par.K,M.Kes

Staphylococcus aureus adalah penyebab penting infeksi nosokomial dan didapat di masyarakat. Selain itu, penting untuk menentukan isolat yang relevan yang diperoleh selama percobaan. Jadi, itu juga salah satu organisme yang sering ditemukan pada infeksi manusia. S. aureus adalah kasus utama di rumah sakit. Senyawa aktif dalam kulit delima (Punica granatum) mengandung flavonoid, tannis, pyrogallol, asam sinamat, asam benzoat, asam klorogenat, campferol, genistein, asam coumaric, dan quercetin yang membantu antibiotik melawan Staphylococcus aureus. Tujuan dari penelitian ini adalah untuk membuktikan efek antimikroba dari ekstrak kulit buah delima terhadap pertumbuhan bakteri *Staphylococcus aureus* secara in vitro menggunakan metode difusi agar. Ekstrak kulit delima dengan konsentrasi 3%, 6%, 9%, 12%, 15%, 18% dan 21% digunakan dalam tiga isolat *Staphylococcus aureus* yang berbeda. Antibiotik seperti Gentamicin dan Fosfomycin digunakan dalam percobaan ini. Dengan demikian, untuk menentukan efek antimikroba dari ekstrak kulit buah delima (Punica granatum) diukur dengan zona hambatan yang terbentuk di sekitar sumur dalam percobaan ini. Selanjutnya untuk analisis data, ANOVA satu arah digunakan dalam penelitian ini dan diverifikasi bahwa konsentrasi ekstrak kulit buah delima dan pertumbuhan *S. aureus* adalah p <0,05. Selain itu, untuk memperkuat analisis data di atas, hasil korelasi Spearmen juga menunjukkan korelasi yang dominan antara konsentrasi ekstrak dan penghambatan zona dengan menggambarkan nilai p <005 dan r = 0,843. Kesimpulannya, hasil penelitian membuktikan bahwa ekstrak kulit buah delima memiliki efek antimikroba terhadap *Staphylococcus aureus* secara in vitro menggunakan metode difusi sumur.

Keywords: Antibacterial, *Staphylococcus aureus* ;Pomegranate peel,Well diffusion.

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LIST OF ABBREVIATIONS

AIDS	:	Acquired immune deficiency syndrome
ANOVA	:	Analysis of Variance
CDC	:	Centers for Disease Control and Prevention
CFA	:	Cyclopropane fatty acids
CFU	:	Colony-forming Unit
DNA	:	Deoxyribonucleic Acid
MSA	:	Mannito Salt Agar
Hep-2	:	Human epithelial type 2
HUS	:	Haemolytic Uremic Syndrome
KIA	:	Kligler Iron Agar
LT	:	heat-labile toxin
MBC	:	Minimum Bactericidal Concentration
MCA	:	MacConkey Agar
MIC	:	Minimum Inhibitory Concentration
MIO	:	Motility Indole Ornithine
mRNA	:	Messenger Ribonucleic Acid
NAP	:	Nutrient Agar Plate
NO_2	:	Nitrate ion
NO_3	:	Nitrogen dioxide
OD	:	Optical Density
PABA	:	Para-Aminobenzoic acid
RLQ	:	Right lower quadrant
SD	:	Standard Deviation

SPSS	:	Statistical Product of Service Solution
ST	:	heat-stable toxin
SSS	:	Semi solid sucrose medium
tRNA	:	Transfer Ribonucleic Acid
Tukey HSD	:	Tukey's Honest Significant Difference
UTI	:	Urinary tract infection
VT	:	Verotoxin
v/v	:	volume over volume ratio

CHAPTER I

INTRODUCTION

1.1 Background

Staphylococcus aureus (S.aureus) is one of the gram positive bacteria originating from the Staphylococcaceae family. *S.aureus* is a member of the normal flora of human body that normally found in part of nose ,respiratory tract,and also on skin (Jawetzet al.,2008). Althrough *S.aureus* as a normal flora but still can cause some common skin infections like abscesses, food poisoning, respiratory infections such as sinusitis. It is also shown that pathogenic strains of *S.aureus* bacteria are promote infections by producing virulence factors such as potent protein toxins, and the expression of a cell-surface protein that binds and inactivates antibodies (Tenaillion et al.,2010).

These bacteria are also found in the air and environment around. *S.aureus* the pathogens are invasive, causing hemolysis, forming coagulase. Some infectious diseases caused by *S.aureus* is boils, pimples, impetigo, and wound infections..*S.aureus* also a major cause of nosocomial infections,food poisoning,and toxic shock syndrome (Kaper et al.,2004)

S. aureus is a bacterium that causes nosocomial infections happens a lot in Indonesia (Morkey et al.,2005). According to WHO in 55 hospitals in 14 countries around the world, shows 8.7% of hospital patients suffered from infection during hospital treatment. Whereas in developing countries more than 40% of patients have an infection nosocomial. The most common bacteria found in cases of infection are *S.aureus* (Who,1999). Worldwide, 10% of hospitalized patients experience new infections during treatment, as many as 1.4 million infections each year (Oyofo et al.,2002).

. Scientifically,to overcome the problem of bacterial infection is best to used antimicrobial drugs. Microbes such as bacteria, viruses, fungi and parasites are living things that continue to evolve over time.Microbes also adapt to environmental changes as a way to survive. If there is anything potentially disruptive to growth, eg antibiotics, gene mutations can occur to form a defense mechanism. This defense mechanism is called resistance. In addition, antimicrobial resistance may also occur as a result of errors in the use of antibiotics itself (Subekti,2003).

However,nature has provided an alternative treatment for various diseases through the natural wealth. Moreover, it contai the spices and natural medicine ingredients. Otherside, because it is not practical, although more secure, natural medicine was initially replaced by synthetic drugs. But with the widespread side effects of synthetic drugs, natural remedies are now getting sought after by the community(Brooks et al.,2005).

Furthermore,antibacterial substances contained in this natural remedy act as a bacterial killer (bactericidal) or inhibit the growth of bacteria (bacteriostatic). Along with the trend back to nature, various types of alternative medicinal plants re-examined as an antibacterial drug against infectious diseases in order to be utilized by the community without spending a high cost(Qadri et al.,2005)

One plant that can be used as a traditional medicine is pomegranate *(Punica granatum)*. The peel of pomegranates contains chemicals such as tannins, flavonoids, and alkaloids. Research of P.S.Negi et al. (2010) showed pomegranate skin extracts containing flavonoids, sterols, triterpenes, phenols, and tannins. Pomegranate peel extracts contains the components of flavonoids, sterols, triterpenes, phenols and tannins which act as antibacterials. Flavonoids have activities that inhibit the synthesis or damage the nucleic acid of bacterial cells. Sterols and triterpenes have destructive activity of bacterial cell membranes. Phenols and tannins have activity inhibiting the synthesis of bacterial cell verotoxins.

Based on the existing problems in the community and the benefits of pomegrate peel as mentioned above, the researcher was interested to conduct research on the antimicrobial effects of pomegranate peel extract *(Punica granatum)* to the growth of *Staphylococcus aureus* bacteria.

1.2 Problem Formulation

Does pomegranate peel extract (*Punica granatum*) has the ability to inhibit *Staphylococcus aureus* growth in vitro?

1.3 Research Objectives

1.3.1 General purpose

a. To prove the effect of pomegranate peel extract *(Punica granatum)* to the growth of *Staphylococcus aureus* bacteria in vitro.

1.3.2 Special purpose

a. To analyze the correlation between of different concentration of pomegranate peel extract *(Punica granatum)* to *Staphylococcus aureus* in vitro.

1.4 Research Benefits

1.4.1 Academic Benefits

a. Science development, especially about alternative materials that can be used as antimicrobial agents.

b. Gives preliminary information to the next researcher about the effect of pomegranate peel extract (*Punica granatum)* to inhibit the growth of *S.aureus.*

1.4.2 Practical Benefits

a. Provide additional knowledge to the community about the benefits of pomegranate peel extract (*Punica granatum)* as an antimicrobial agent against *Staphylococcus aureus* bacteria.

b. Explore natural resources in medicine as a natural, cheap and easy alternative treatment.

CHAPTER II

REVIEW OF RELATED LITERATURE

2.1 Pomegranate (*Punica granatum*)

2.1.1 Introduction and Taxonomy

Scientific classification of *Punica granatum*

Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida
Subclass	: Rosidae
Order	: Myrtales
Family	: Punicaceae
Genus	: Punica L.
Species	: *Punica granatum L.*

2.1.2 Morphology and Identification

Punica granatum is shrub plants or molt shrubs which can grow with high reach 5- 8 meters. Pomegranate plants spread from the subtropical region to tropics, from the lowlands to altitude below 1000 m dpl. This plant very suitable for planting on the loose ground and not submerged by water ,and ground water a not deep .Pomegranate flowers are white, red or orange, depending on the type. Pomegranate is round and hanging in bunches. Young fruit green to green reddish, but after the old turned into a yellowish green or reddish green almost brown, depending on the species. Pomegranates can flower throughout the year, single flowers with stalksshort, and out on the tip of the top twig or armpit. Flower pomegranate is usually 1

-5 florets are at the end of twigs, waxy, long and the width of each 5 cm, leaves and petals are the same-same 2-3cm in length .The flesh is a thickened and densely packed leather. This fruit meat is consumed with the seeds (Lansky &Newman ,2007)

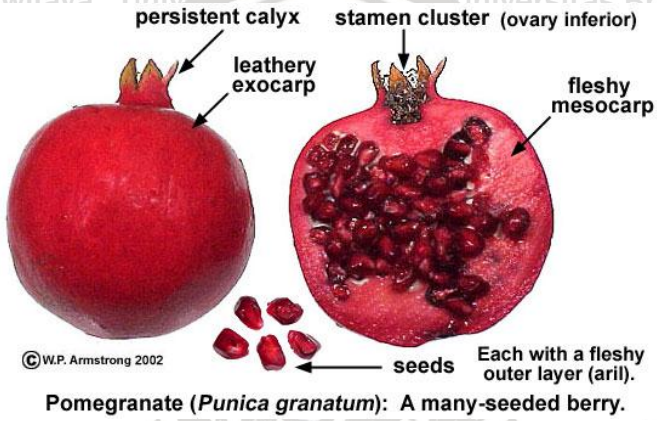

Figure 2.1: Parts of Pomegranate fruit (New direction aromatic, 2017)

2.1.3 Geographic Distribution

The economic importance of the pomegranate in Spain is remarkable because it is one of the major producers of pomegranate in the world, whose fruits besides supplying the domestic market; are exported to Central Europe. Spain is Europe's largest producer and exporter of pomegranate. Currently, pomegranate cultivars in Spain exceeds to 2,500 ha, with a production close to 20,000 t. This production is concentrated mainly in the province of Alicante. It is an alternative fruit tree to many areas, especially where poor soil conditions or the poor quality of irrigation water prevents the profitable operation of other fruit trees; this does not mean that if it is cultivated in better conditions the results are not good. However, these features should not lead to confusion since in

reality pomegranate growing has a specific problem that must be considered in order to achieve quality fruits and abundant crops. In Spain, it is a common practice that pomegranate is combined with other fruit trees such as fig tree and date palm, occupying most of the cases the worst terrain. The marketing of pomegranate as a fourth class product and its use in making jams, jellies, juices, etc., are becoming more important.(Aviram and Dornfeld,2001)

2.1.4 Historical and Traditional uses

Pomegranate has a long and exceptionally colourful history, having been embraced by a number of different cultures, while at the same time it had been a minor horticultural fruit crop in different countries. But, despite its wide geographic distribution across several continents, very little information is available pertaining to its genetic origin and centers of diversity (Still 2006).As befits a fruit with many seeds, the pomegranate is the traditional representation of fertility, and seems to have its origins everywhere. Indian royalty began their banquets with pomegranate, grape, and jujube. We see the pomegranate again in ancient Greece and Rome. In the verses of the Odyssey, Homer mentions it as part of the gardens of Alcinous (probably in Sicily). In different regions of the natural habitat of wild pomegranate, the period of time between the first appearance of the modern type of humans and the tran-sition of their different populations to agricultural activities is anywhere between 2000 and 6000 BP.The derivation of the word pomegranate comes from the Middle French pome garnete (seeded apple), but Europeans were slow to adopt the pomegranate. The pome-granate or date palm was the tree of life portrayed in vari-ous archaeological artifacts from Mesopotamia, the Levant and India. An analysis from a botanical archaeologist con-cluded the tree of creation or immortality that recurs in many archaeological materials

from the first through third millennium BP is actually the Egyptian locust tree,(Madera, 2013).

2.1.5 Chemical composition

The composition of the fruits differs depending on the cultivar, growing region, maturity, cultivation practice, climate, and storage. Nearly 50% of the total fruit weight corresponds to the pomegranate skin, plays main source of bioactive compounds such as phenolics, flavonoids, ellagitannins, and proanthocyanidin compounds, minerals, mainly potassium, nitrogen, calcium, phosphorus, magnesium, and sodium, and complex polysaccharides. The part of the pomegranate fruit (50%) consists of 40% arils and 10% seeds. Arils contain 85% water, 10% total sugars, fructose and glucose, and 1.5% pectin, organic acid, (Lansky and Newman,2007). The seed cover of the fruit contains delphinidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3,5-diglucoside, cyanidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, and pelargonidin-3-glucoside with delphinidin-3,5-diglucoside being the main anthocyanin in pomegranate juice. Therefore,12–20% of total seed weight of pomegranate comprises seed oil and is self-possessed with more than 70% of the conjugated linolenic acids. Well,the fatty acid component of pomegranate seed oil comprises over 95% of the oil, of which 99% is triacylglycerols. Phenolic compounds, together with flavonoids, anthocyanins, and tannins, are the main group of antioxidant phytochemicals that are important due to their biological and free radical scavenging activities. Phenolic acids, flavonoids, and tannins are present in different parts of pomegranate fruit and this is reasons why alot of the studies demonstrated that combinations of pomegranate extracts from different parts of the fruit were more effective than a single extract. In a analysis, anthocyanins from

pomegranate fruit were found to possess higher antioxidant activity than vitamin-E (α-tocopherol), β-carotene, and ascorbic acid (Malik et al;2008).

2.1.6 Antimicrobial properties of Pomegranate peel extract

Pomegranate peel extract contains chemical components such as flavonoids, sterols, triterpenes, phenols, tannins, and alkaloids. The active components in pomegranate peel that can be antibacterial are sterols, triterpenes, phenols,tannins and flavonoids.

Sterols and triterpenes have destructive activity of bacterial cell membranes. Phenols and tannins have activity inhibiting the synthesis of bacterial cell verotoxins (Voravuthikunchai et al, 2005).

Triterpenes are naturally occurring alkenes of vegetable, animal and also fungal origin, classified among an extensive and structurally diverse group of natural substances, referred to as triterpenoids. Their structure includes 30 elements of carbon and they are constituted by isoprene units. Taking into consideration the structure, triterpenes may be divided into linear ones, mainly derivatives of squalene, tetracyclic and pentacyclic, containing respectively four and five cycles, as well as two- and tricyclic ones. Representatives of those show anti-cancer properties, as well as anti-inflammatory, anti-oxidative, anti-viral, anti-bacterial and anti-fungal ones (Malwina Chudik, 2015).

Sterols is water insoluble and readily partition into membranes. Membrane sterols can be thought of as having three distinct parts, each with a different function. Sterols is anchored to the aqueous interface via a polar –OH group. The rest of the molecule is hydrophobic. The four rigid sterol rings are responsible for the sterols'

major function, controlling membrane fluidity and packing free volume (breathing space) for protein function. It has been well documented that plant sterols, particularly β-sitosterol, do compete with cholesterol uptake, thus providing a potentially beneficial role of reducing human cholesterol levels (William Stillwell, 2016).

Flavonoids are soluble in ethanol 96% and very sensitive to high temperature. Flavonoids have antioxidant properties which helps to neutralize free radicals and prevent their effects on our body. They have tonic effect on the heart such as strengthening the heart muscle and improving circulation which known as cardiotonic effect (Camelia Maier, 2017). Flavonoids activities that inhibit the synthesis or damage the nucleic acid of bacterial cells (Ulanowska et al, 2007).

2.2 *Staphylococcus aureus* (*S.aureus*)

2.2.1 Taxonomy

Scientific classification of *Staphylococcus aureus*(S.aureus)

Domain	: Bacteria
Kingdom	: Eubacteria
Phylum	: Firmicutes
Class	: Bacilli
Order	: Bacillales
Family	: Staphylococcaceae
Genus	: Staphylococcus
Species	: *Staphylococcus aureus*

(Migula, 1895)

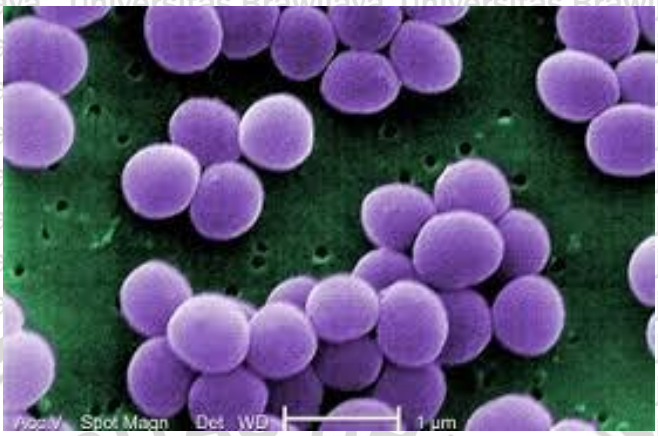

Figure 2.2 : *Staphylococcus aureus* bacteria in very high magnification of 20 000x under scanning electron microscope(SEM) with Gram staining of *Staphylococcus aureus* taken from a vancomycin intermediate resistance culture(VISA)
(Jawetz et al.,2010)

2.2.2 Morphology and Identification

Staphylococci are Gram-positive cocci about 0.5 – 1.0 µm in diameter. They grow in clusters, pairs and occasionally in short chains. The clusters arise because staphylococci divide in two planes. The configuration of the cocci helps to distinguish micrococci and staphylococci from streptococci, which usually grow in chains. Observations must be made on cultures grown in broth, because streptococci grown on solid medium may appear as clumps. Several fields should be examined before deciding whether clumps or chains are present(Todar, 2011). The presence of staphylococci in a lesion might first be suspected after examination of a direct Gram stain. However, small numbers of bacteria in blood preclude microscopic examination and require culturing first. (Brooks et al., 2010). The organism is isolated by streaking material from the clinical specimen (or from a blood culture) onto solid media such as blood agar, tryptic soy agar or heart infusion agar. Specimens likely to be contaminated with other

microorganisms can be plated on mannitol salt agar containing 7.5% sodium chloride, which allows the halo-tolerant staphylococci to grow. Ideally a Gram stain of the colony should be performed and tests made for catalase and coagulase production, allowing the coagulase-positive *S.aureus* to be identified quickly. Another very useful test for *S.aureus* is the production of thermostable deoxyribonuclease.

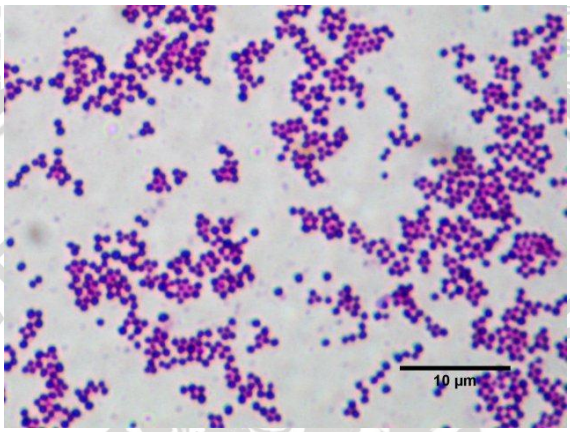

Figure 2.2.2 : *S.aureus* on Gram positive staining under 10x10 maginification

(Jawetz et al.,2010)

S.aureus can be confirmed by testing colonies for agglutination with latex particles coated with immunoglobulin G and fibrinogen which bind protein A and the clumping factor, respectively, on the bacterial cell surface. These are available from commercial suppliers (e.g., Staphaurex). The most recent latex test (Pastaurex) incorporates monoclonal antibodies to serotype 5 and 8 capsular polysaccharide in order to reduce the number of false negatives. (Some recent clinical isolates of *S.aureus* lack production of coagulase and/or clumping factor, which can make identification difficult (Nuraeni, Wibisono, 2008).

2.2.3 Epidemiology

S.aureus is a major cause of nosocomial and community-acquired infections, it is necessary to determine the relatedness of isolates collected during the investigation of an outbreak. Typing systems must be reproducible, discriminatory, and easy to interpret and to use. The traditional method for typing *S.aureus* is phage-typing. This method is based on a phenotypic marker with poor reproducibility. Also, it does not type many isolates (20% in a recent survey at the Center for Disease Control and Prevention), and it requires maintenance of a large number of phage stocks and propagating strains and consequently can be performed only by specialist reference laboratories. (Madappa, 2011). Many molecular typing methods have been applied to the epidemiological analysis of *S.aureus*, in particular, of methicillin-resistant strains (MRSA). Plasmid analysis has been used extensively with success, but suffers the disadvantage that plasmids can easily be lost and acquired and are thus inherently unreliable. (Clak, 2009).Methods designed to recognize restriction fragment length polymorphisms (RFLP) using a variety of gene probes, including rRNA genes (ribotyping), have had limited success in the epidemiology of MRSA. In this technique the choice of restriction enzyme used to cleave the genomic DNA, as well as the probes, is crucial. (Madappa, 2011). Random primer PCR offers potential for discriminating between strains but a suitable primer has yet to be identified for *S.aureus*. The method currently regarded as the most reliable is pulsed field gel electrophoresis, where genomic DNA is cut with a restriction enzyme that generates large fragments of 50-700 kb. (Dzen et al., 2003).

2.2.4 Growth Characteristics

S.aureus is bacteria in the genus Staphylococcus are pathogens of man and other mammals. Traditionally they were divided into two groups on the basis of their ability to clot blood plasma (the coagulase reaction). The coagulase-positive staphylococci constitute the most pathogenic species *S.aureus*. The coagulase-negative staphylococci (CNS) are now known to comprise over 30 other species. Staphylococci are Gram-positive cocci about 0.5 – 1.0 µm in diameter. They grow in clusters, pairs and occasionally in short chains. The clusters arise because staphylococci divide in two planes. The configuration of the cocci helps to distinguish micrococci and staphylococci from streptococci, which usually grow in chains (Dzen et al., 2003).

a. Blood agar plate

S.aureus is a facultative anaerobic, Gram-positive coccal (round) bacterium also known as "golden staph" and "oro staphira". *S.aureus* is nonmotile and does not form spores In medical literature, the bacterium is often referred to as *S.aureus*, Staph aureus *S.aureus* appears as staphylococci (grape-like clusters) when viewed through a microscope, and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates. *S.aureus* reproduces asexually by binary fission. Complete separation of the daughter cells is mediated by *S.aureus* autolysin, and in its absence or targeted inhibition, the daughter cells remain attached to one another and appear as clusters. *S.aureus* is catalase-positive (meaning it can produce the enzyme catalase). Catalase converts hydrogen peroxide (H2O2) to water and oxygen. Catalase-activity tests are sometimes used to distinguish staphylococci from enterococci and streptococci. Previously, *S.aureus*

was differentiated from other staphylococci by the coagulase test. However, not all *S.aureus* strains are coagulase-positive and incorrect species identification can impact effective treatment and control measure Red blood cells on an agar plate are used to diagnose infection. On the left is a positive *Staphylococcus* infection, on the right a positive Streptococcus culture. (Dzen et al, 2010).

b. Manitol Salt Agar

Mannitol salt agar or MSA is a commonly used selective and differential growth medium in microbiology. It encourages the growth of a group of certain bacteria while inhibiting the growth of others. This medium is important in medical laboratories as one method of distinguishing pathogenic microbes in a short period of time. It contains a high concentration (about 7.5%-10%) of salt (NaCl), making it selective for Gram-positive bacteria (Staphylococcus and Micrococcaceae) since this level of salt is inhibitory to most other bacteria. It is also a differential medium for mannitol-fermenting staphylococci, containing carbohydrate mannitol and the indicator phenol red, a pH indicator for detecting acid produced by mannitol-fermenting staphylococci. *Staphylococcus aureus* produces yellow colonies with yellow zones, whereas other coagulase-negative staphylococci produce small pink or red colonies with no colour change to the medium. If an organism can ferment mannitol, an acidic by product is formed that causes the phenol red in the agar to turn yellow.It is used for the selective isolation of presumptive pathogenic (pp) Staphylococcus species (Dzen et al., 2003).

c. McConkey Agar

MacConkey agar is an indicator, a selective and differential culture medium for bacteria designed to selectively isolate Gram-negative and enteric (normally found in the intestinal tract) bacilli and differentiate them based on lactose fermentation. The crystal violet and bile salts inhibit the growth of gram-positive organisms which allows for the selection and isolation of gram-negative bacteria. Enteric bacteria that have the ability to ferment lactose can be detected using the carbohydrate lactose, and the pH indicator neutral red. It contains bile salts (to inhibit most Gram-positive bacteria), crystal violet dye (which also inhibits certain Gram-positive bacteria), neutral red dye (which turns pink if the microbes are fermenting lactose).

2.2.5 Serological Classification

The PCR product was generated when in vitro-cultured *S. aureus* was used to prepare simulated clinical specimens of blood, urine, cerebrospinal fluid, or synovial fluid. No PCR product was generated when the sterile body fluids were tested. However, the sensitivity of the PCR was reduced when *S. aureus* in blood or urine was tested in comparison with that when bacteria in saline were tested. With the bacteria in blood, the detection limit of the PCR was 10(3) CFU. A positive PCR result was recorded when a limited number of clinical samples from wounds verified to be infected with *S. aureus* were tested, while the PCR product was not detected in materials from infections caused by other bacteria. Generation of PCR products was not affected by exposure of *S. aureus* to bactericidal agents, including cloxacillin and gentamicin, prior to testing, but was affected by exposure to UV radiation.The antigenic properties of *Staphylococcus*

aureus have been considered difficult to explore. Methods which were used with success on related bacteria, failed with staphylococci. There has been a general feeling that the antigenic structure of this organism is so complex that a systematic classification can hardly be achieved. Consequently most work has been done on thecultural and biochemical behavior of the organism, whereas there has been little interest serologic and immunologic properties. Staphylococci are extremely widespread and *S.aureus* is one of the organisms most frequently found in human infections. At present staphylococcal hospital infections are a world-wide problem of major importance. Knowledge of the antigens of *S.aureus* and their activities is not merely of interest for epidemiologic typing, but may also give possibilities for successful research in the field of immunology and infection. Extensive reviews have recently been written on *S.aureus* in general and on its pathogenicity . As there is today an increasing interest in the antigenic properties of *S.aureus*, a review may be of help to those working or planning to work in this field. This review has been limited to the bacterial antigens of *S.aureus* and serologic typing, whereas toxins, pathogenicity, and immunity in man are not covered. (Dzen et al, 2010).

2.2.6 Determinants of Pathogenicity

S.aureus expresses many cell surface-associated and extracellular proteins that are potential virulence factors. For the majority of diseases caused by this organism, pathogenesis is multifactorial. Thus it is difficult to determine precisely the role of any given factor. This also reflects the inadequacies of many animal models for staphylococcal diseases.However, there are correlations between strains isolated from particular diseases and expression of particular factors, which suggests their importance in pathogenesis. With some toxins, symptoms of a human disease can be reproduced in animals with pure proteins. The application of molecular biology has led to recent

advances in the understanding of pathogenesis of staphylococcal diseases. Genes encoding potential virulence factors have been cloned and sequenced and proteins purified. This has facilitated studies at the molecular level on their modes of action, both in in vitro and in model systems.

a. Enzymes

S.aureus produces various enzymes such as coagulase (bound and free coagulases) which clots plasma and coats the bacterial cell, probably to prevent phagocytosis. Hyaluronidase (also known as spreading factor) breaks down hyaluronic acid and helps in spreading it. *S.aureus* also produces deoxyribonuclease, which breaks down the DNA, lipase to digest lipids, staphylokinase to dissolve fibrin and aid in spread, and beta-lactamase for drug resistance. (Amansyah, 2010).

b. Superantigens

Antigens known as superantigens can induce toxic shock syndrome (TSS). This group includes the toxins TSST-1, and enterotoxin type B, which causes TSS associated with tampon use. Toxic shock syndrome is characterized by fever, erythematous rash, low blood pressure, shock, multiple organ failure, and skin peeling. Lack of antibody to TSST-1 plays a part in the pathogenesis of TSS. Other strains of *S. aureus* can produce an enterotoxin that is the causative agent of a type of gastroenteritis. This form of gastroenteritis is self-limiting, characterized by vomiting and diarrhea 1-6 hours after ingestion of the toxin, with recovery in 8 to 24 hours. Symptoms include nausea, vomiting, diarrhea, and major abdominal pain (Dzen et al, 2010).

c. Exfoliative toxins

Exfoliative toxins are exotoxins implicated in the disease staphylococcal scalded skin syndrome (SSSS), which occurs most commonly in infants and young children. It also may occur as epidemics in hospital nurseries. The protease activity of the exfoliative toxins causes peeling of the skin observed with SSSS. (Dzen et al, 2010).

2.2.7 Clinical Manifestation

S.aureus bacteria that have infected humans can cause a variety of symptoms and complaints. Some of the most common manifestations of *S.aureus* infections are:

a.Skin infections

Skin infections are the most common form of *S.aureus* infection. This can manifest in various ways, including small benign boils, folliculitis, impetigo, cellulitis, and more severe, invasive soft-tissue infections. *S.aureus* is extremely prevalent in persons with atopic dermatitis, more commonly known as eczema. It is mostly found in fertile, active places, including the armpits, hair, and scalp. Large pimples that appear in those areas may exacerbate the infection if lacerated. This can lead to staphylococcal scalded skin syndrome, a severe form of which can be seen in newborns. The presence of *S.aureus* in persons with atopic dermatitis is not an indication to treat with oral antibiotics, as evidence has not shown this to give benefit to the patient. However,

topical antibiotics combined with corticosteroids have been found to improve the condition. Colonization of *S.aureus* drives inflammation of atopic dermatitis.

b. **Food poisoning**

S. aureus is also responsible for food poisoning. It is capable of generating toxins that produce food poisoning in the human body.Its incubation period lasts one to six hours, with the illness itself lasting from 30 minutes to 3 days.Preventive measures one can take to help prevent the spread of the disease include washing hands thoroughly with soap and water before preparing food. Stay away from any food if ill, and wear gloves if any open wounds occur on hands or wrists while preparing food. If storing food for longer than 2 hours, keep the food above 140 or below 40 °F.(Zakia Bakri, 2015).

c. Bone and joint infections

S.aureus is the bacterium commonly responsible for all major bone and joint infections. This manifests in one of three forms: osteomyelitis, septic arthritis, and infection from a replacement joint surgery (Dzen et al, 2010).

d. Bacteremia

S.aureus is a leading cause of bloodstream infections throughout much of the industrialized world. Infection is generally associated with breaks in the skin or mucosal membranes due to surgery, injury, or use of intravascular devices such as catheters, hemodialysis machines, or injected drugs. Once the bacteria have entered the bloodstream, they can infect various organs, causing infective endocarditis, septic arthritis, and osteomyelitis. This disease is particularly prevalent and severe in the very young and very old. Without antibiotic treatment, *S.aureus* bacteremia has a

case fatality rate around 80%.With antibiotic treatment, case fatality rates range from 15% to 50% depending on the age and health of the patient, as well as the antibiotic resistance of the *S.aureus* strain (Dzen et al, 2010).

2.3 Antibacteria

2.3.1 Definition

Antibacterial main role is destroying or suppressing the growth or reproduction of bacteria and an agent having such properties. Antibacterial is a material or a chemical component that has the ability to inhibit or kill harmful microorganisms (Amirah et al, 2010). Antibacterials are referred as bacteriostatic (inhibit bacterial growth) or bactericidal (killing bacteria) (Katzung, 2010).

2.3.2 Antibacterial Mechanism of action

a. **Inhibits Cell Wall Synthesis**

Penicillin, cephalosporin, and carbapenems blocks the peptide bond formation catalysed by transpeptidases (PBPs), thus inhibiting the cross-linking of peptidoglycan units. Besides that, vancomycin inhibits the synthesis of peptidoglycan by binding themselves to peptidoglycan units, as well as blocking transglycosylase and transpeptidase activity. . Examples of this type of antibacterial is, β-lactam (penicillin and cephalosporin) (Ahmad Muhlisin, 2016).

b. **Inhibits Cell Membrane Function**

Cell membrane is a membrane barrier for free diffusion between internal and external environments. Disturbance in the integrity of the cell membrane can lead to leakage and cell death, affect the concentration of metabolites and nutrients in the cell, inhibit respiratory processes and certain biosynthetic activities that affect the whole life of the bacterial cell itself (Ahmad Muhlisin, 2016).

c. Inhibits Protein Synthesis

Protein synthesis is the result of two main processes of transcription and translation. This synthesis occurs in the ribosome. Streptomycin can bind to the 30S ribosome causing code in mRNA to be read by tRNAs and abnormal and non-functional proteins for bacterial cells (Ahmad Muhlisin, 2016).

d. **Inhibits Synthesis of Nucleic Acid**

Synthesis of nucleic acid is closely related to the process of duplication and transcription. Any substances that interfere with this synthesis will affect all phases of growth and bacterial cell metabolism. Rifampicin may baffle with the Polymerase-RNA enzyme thus inhibiting RNA and DNA synthesis by the enzyme (Ahmad Muhlisin, 2016).

a.Inhibits Bacterial Cell Metabolism

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2.3.3 Bacterial Resistance

There are several mechanisms that cause a bacterial population to be resistant to antibacterial drugs:

a. Resistance due to Global cell adaption process

b. Alter the permeability of the cell membrane by bacteria

c. Target structure to the drug change by bacteria

d. Develop new metabolic pathways by bacteria

e. Bacteria develops an enzyme that remains functioning for its metabolism, but not drug-induced

f. Prevention to be antibiotic target by decreasing penetration.

(Blair et al, 2015)

2.3.4 Sensitivity Test On Antimicrobial In Vitro

The bacterial susceptibility test against antimicrobials in vitro main aims to determine the antimicrobial tested can be used to treat the bacterial infection. The susceptibility test can basically be carried out by dilution method or disc diffusion method.

a. **Tube Dilution Method**

Tube Dilution method normally used to determine minimal inhibitory (MIC) and minimum bactericidal concentration (MBC) of an antimicrobial. This method consists of two stages, the first stage to determine the inhibitory concentration (MIC) by using liquid media and the second stage on solid media to determine the minimum kills (MBC). Minimal inhibitory concentration (MIC) is the concentration of ethanol extract of that can still inhibit the growth of certain organisms such as *S.aureus* bacteria. This procedure is used to determine the concentration of the extract of pomegranate skin that is still effective to prevent *S.aureus*. Inoculum of standardized microorganisms is added to the tube containing an acid extract of pomegranate peel and its growth is observed in the presence of turbidity. In this way, MIC from the extract of pomegranate peel, which can be used to prevent the growth of in vitro microorganisms is determined. Minimum bactericidal concentration (MBC) is the lowest concentration of pomegranate

peel extract that can give marks of colonies of clean bacteria in hatchery media. With MBC examination can be determined the concentration of pomegranate peel extract is bakterisidal to *S.aureus*. MBC is a quantitative assessment because it based on observations of the number of colonies remaining. MIC is qualitative because its judgment is based on the clarity level of the tube or media used.

a. Disc Diffusion Method

Disc Diffusion method is used by saturating antimicrobials into paper discs. These paper discs will be planted on solid seed mediums that have been mixed with the bacteria tested. Observations were made on clear zones around paper discs showing no microbial growth. Evaluation of the results of this test can be done by Kirby Bauer using standard table by NMLLS or by Joan-Stokes measured using controlled medicines that are planted together in one plate in order and have been known sensitivity (Dzen et al, 2010).

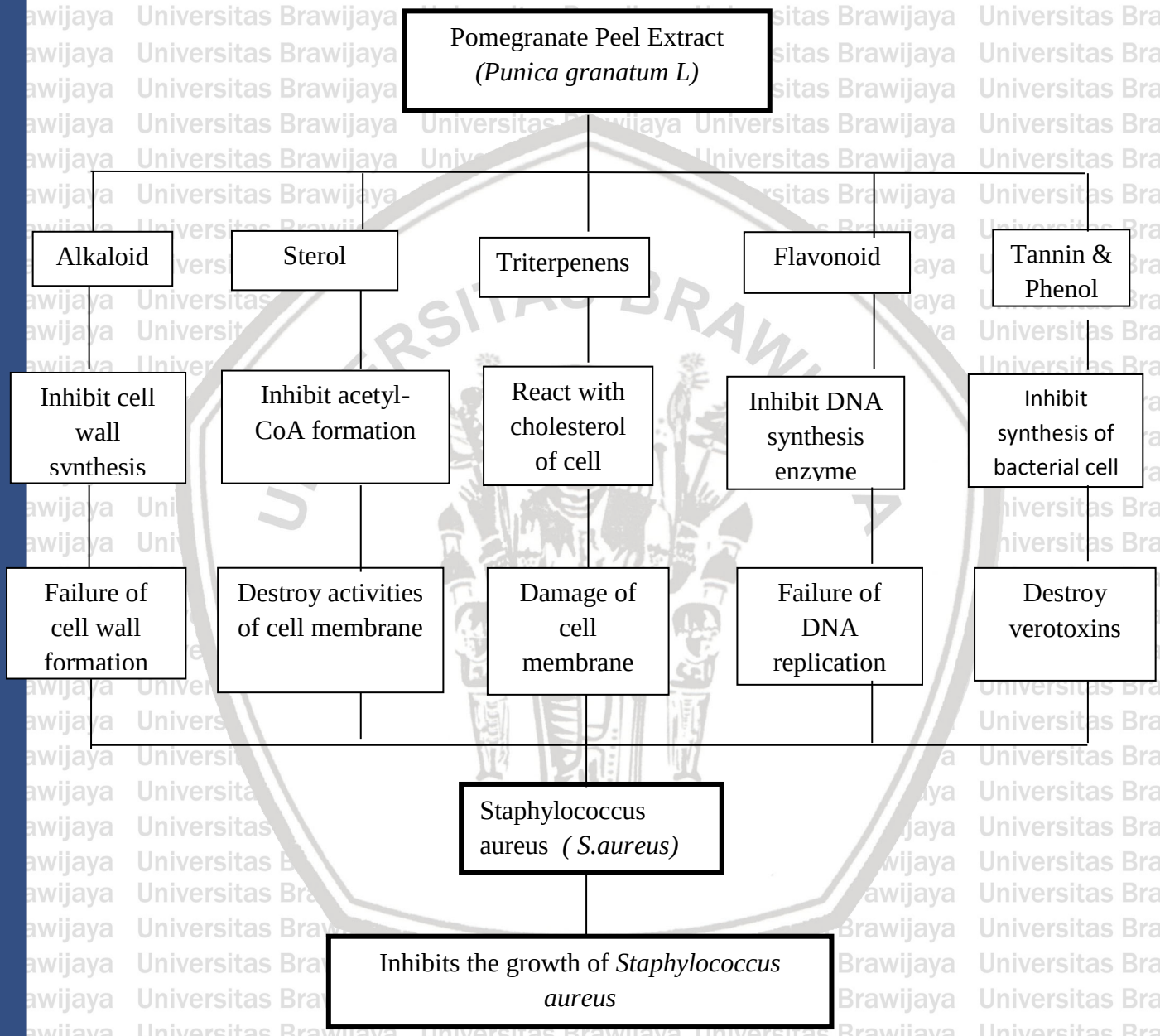
b. Well Diffusion Method

Inoculum containing 10^6 CFU/ml of each bacterial culture to be tested was spread on nutrient agar plates with a sterile swab moistened with the bacterial suspension. The agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole punched and a volume (20–100 µL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested (Parekh and Chanda, 2007).

Chapter III

Conceptual Framework and Hypothesis

3.1 Conceptual Framework



The pomegranate peel contains flavonoid and tannin which gives antibacterial properties. Flavonoid forms a complex with the extracellular protein by forming hydrogen bond, covalent bond and hydrophobic effects. This will lead to the destruction of the cytoplasmic membrane of the hydrogen ion from the phenol compound. The phospholipid molecule will unravel to form glycerol, carboxylic acid, and phosphate acid, thus, unable to maintain the shape of the cytoplasm membrane causing the membrane to leak. Hence, the cell wall is destroyed. Besides that, flavonoid also inhibits the synthesis of DNA and RNA, inhibits the cytoplasmic membrane function and the bacteria energy metabolism. Flavonoid also inhibits the microbial adhesin. (Prajitno, 2017)

Tannin acts as an antibacterial by destroying the cell wall and the cytoplasm, thus causing a rapid structural destruction. It also inhibits the microbial adhesin, hydrolytic enzyme, such as proteases and carbohydrolases and cell envelope transport proteins. Tannin also binds to the proline rich proteins, causing interference to the protein synthesis. (Sung et al., 2012)

3.2 Hypothesis

Pomegranate peel extract (*Punica granatum* L) take over antimicrobial effect against the bacteria *Staphylococcus aureus*..

CHAPTER 4

RESEARCH METHOD

4.1 Research Design

The research design used was experimental research with a post test control group design. This antimicrobial test was done to know if different concentration of pomegranate peel extract affects the growth of *Staphyloccocus aureus* bacterial growth in vitro using Well diffusion method.

4.2 Location and Period of Research

This research was conducted at the Laboratory of Microbiology, Faculty of Medicine, Brawijaya University in the period of February 2019 until July 2019.

4.3 Research Sample

This study uses *S.aureus* bacterial isolates, which is owned by the Laboratory Microbiology FKUB. The number of samples obtained using the calculation formula: **P(n-1) ≥ 15** (Solimun, 2011). This study uses seven different concentrations (3% 6%, 9%, 12%, 15%, 18% and 21%) so the number of samples required in accordance with the following calculation:

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

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

$$7n - 7 \geq 15$$

$$7n \geq 15 + 7$$

$$n \geq 3{,}1 = 4$$

Descripton : p = the amount of concentration used

n = the amount of sample needed

Thus, to meet the requirements necessary, repetition tests were done 3 times using 3 different isolates of *S.aureus..*

4.4 Variable Identification

4.4.1 Independent Variable

The independent variable in this study is the concentrations of pomegranate peel used. The variables were determined by preliminary research results using 3% 6%, 9%, 12%, 15%, 18% and 21% extract concentrations.

4.4.2 Dependant Variable

The dependent variable in this study is the diameter of zone of inhibition formed on agar plate caused by different concentation of pomegranate peel extract on growth of *Staphylococcus aureus..*

4.4.3 Confounding Variable

The confounding variable in this study was the working process, example the sterilization technique between repetitions and the time interval between the production of the extract and the treatment of the extract.

4.5 Operational Definition

1. Pomegranate skin (Punica granatum) used were dark red colour pomegranate fruits imported from India.
2. Pomegranate skin extract was conducted in UPT Materia Medica Batu, Malang. The pomegranate peel was extracted by maseration method using 96% ethanol on dry pomegranate peels, then filtering and evaporation was done using rotary evaporator.
3. *Staphylococcus aureus* isolates used in this study are obtained from Mikrobiology Laboratory of Faculty of Medicine, Universitas Brawijaya.

4. Well's diffusion method was used to test the effectivity of pomegranate peel extract as antimicrobial.
5. The clear zone formed around the well is zone of inhibition which indicates antimicrobial effect of the extract used was measured in milimeters (mm).

4.6 Instruments and Materials

4.6.1 Instruments

Blade, Bunsen burner, microscope, object glass, mortar and pestle, vortex, inoculating loop, incubating equipment, rotatory evaporator, test tube, calibrated, ruler, matches, forceps, cork borer (4mm), spectrometer, glass jar, pipette, petri dish, incubator.

5.6.2 Materials

S.aures bacteria sample, pomegranate (Punica granatum) peel ethanolic extract, Nutrient Broth (NB), Nutrient Agar (NA), Mannitol Salt Agar, 9% NaCl, crystal violet, iodine, alcohol 96%, safranin, immersion oil and water.

4.7 Research Procedure

4.7.1 Pomegranate Peel Extract Preparation

Pomegranate peels are cut into small pieces and dried and then pulverized in a blender and weigh as much as 1100 grams (dry samples). The extraction process uses maceration. Dry samples were inserted into a glass flask of 1 litter. Then soaked with 96% ethanol by volume 600ml left for one night. After maceration, filter using Whatman filter paper ready to be evaporated. Evaporation is done to separate the products of extraction. Extraction product then inserted into the container while the rotary evaporator flask, chilled water circulation pumping device, and instrument vacuum pump is turned on. The results of the evaporation of ethanol are condensed to a collecting flask. After becoming condensed, evaporation was stopped and collected.

The vapour was placed in a vapour cup and then put into the oven for 2 hours to evaporate the remaining solvent to obtain 100% extract.

4.7.2 Bacterial Preparation and Identification Tests

4.7.2.1 Gram Staining

1. Clean object glasses were heated over the fire to remove fat and let it to cool.

2. Bacterial preparation was created on the object glass with sufficient thickness and allowed to dry in the air, then fixed above the Bunsen lamp.

3. The object glass with bacterial isolate were covered with Crystal Violet and rinsed with water after 1 minute.

4. Then iodine was poued onto the glass and after 1 minute, rinsed with water.

5. Preparations covered with 96% alcohol drops and rinsed using water after 20-30 seconds.

6. Preparations were dried using absorbent paper, then viewed under a microscope with magnification of 1000x. Purple coloures Gram positive rods were observed. The results were recorded.

4.7.2.2 Manitol Salt Agar (MSA) Planting

Planting bacteria on Manitol Salt Agar (MSA) which is a selective medium for the growth of *Staphylococcus aureus.*. Planting bacteria on the media will produce a yellow colonies with yellow zone precipitate on MSA.

4.7.2.3 Preparation of Bacterial Suspension

a. *Staphylococcus aureus* colonies were obtained using inoculating loop.

b. The bacterial samples were deposited into the sterile test tubes containing nutrient broth, then spectrophotometry was done on the test tube with a wavelength of 625nm, to determine the optical density (OD) and the suspension.

To obtain a concentration of 10^8 bacteria/mL equivalent to OD= 0.1, calculations performed as the following formula (Murray et al., 1999) :

$$N_1 \times V_1 = N_2 \times V_2$$

Descriptions:

N_1 = Spectrophotometry results

V_1 = Volume of bacteria to be added to the diluent

N_2 = OD (0,1 equal to 10^8)

V_2 = Suspense Volume of test bacteria(10mL)

c. Bacterial suspension of 10^8 CFU/mL was diluted by adding 9mL of 9% NaCl to obtain 10^7 CFU/mL of bacterial suspension in 10mL.

d. Having obtained a bacterial suspension with a concentration of 10^7 CFU/mL in 10ml, it was further diluted with 1mL of 9% NaCl, so that the concentration of bacteria becomes 10^6CFU/mL. The bacterial suspension is ready to be used for research.

4.7.3 Antimicrobial Effectivity Test

1. Four sterile plates were prepared for each bacterial isolates.
2. Plates were labelled as isolate A,B,and C respectively.
3. The bacterial suspension of 10^6 CFU/ml is centrifuged until it is homogenous with the nutrient agar and placed into the plates.
4. Seven wells were made on each agar plate labelled and filled with the concentrations of 3% 6%, 9%, 12%, 15%, 18% and 21%
5. All plates are the incubated for 18-24 hours at 37^0C.
6. The diameter of zone of inhibition formed around the each wells were measured using a ruler in milimeters(mm) and the data collected were analysed.

4.8 Data Analysis

The data Analysis used is One-way ANOVA test and Spearman correlation test. The One-way ANNOVA test is used to identify the effect of different concentrations of the pomegranate peels extract have against the growth of *S.aureus* colonies and to know the potency of extract compared to Gentamicin and Fosfomycin. Besides, The Spearman correlation statistical analysis is used to prove the consistency between the concentration of the pomegranate peels extract against the growth of *S.aureus*. To analyse the data, the SPSS (Statistic Product of Service Solution) program is used.

CHAPTER 5
RESULTS AND DATA ANALYSIS

5.1 Research Results

5.1.1 Identification of *Staphylococcus aureus*

Staphylococcus aureus bacteria in this research was used from Microbiology laboratorium of Medical faculty of Universitas Brawijaya. Several types of identification tests were done on each isolate to manifest that bacteria used in this experiment were *Staphylococcus aureus* .As identification test macroscopic and microscopic tests were done to prove that bacteria used in this experiment was *Staphylococcus aureus.* Therefore, macroscopic test was done by growing *Staphylococcus aureus* bacteria in its selective media which is Mannitol Salt Agar while microscopic test was done by using gram staining method.

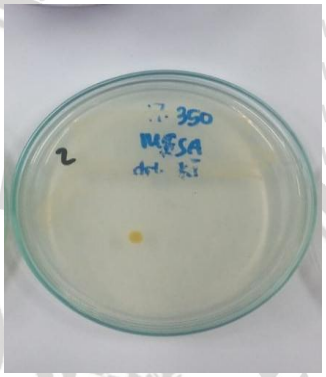
The result from macroscopic test of *Staphylococcus aureus* bacteria on selective media Mannitol Salt Agar is yellow colonies with yellow zone were found.

Table 5.1: Identification test results of *Staphylococcus aureus*

S.aureus strains	**Isolate A**	**Isolate B**	**Isolate C**
Gram staining	Gram positive rods	Gram positive rods	Gram positive rods
MSA Agar culture	Yellow colonies	Yellow colonies	Yellow colonies

. Mannitol Salt Agar contains a high concentration (about 7.5% -10%) of salt (NaCl), making it selective for Gram-positive bacteria since this level of salt is inhibitory to most other bacteria (David P Kateete,2010).

Isolate A

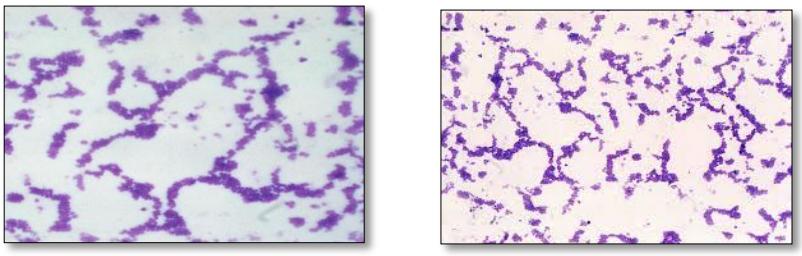


Isolate B

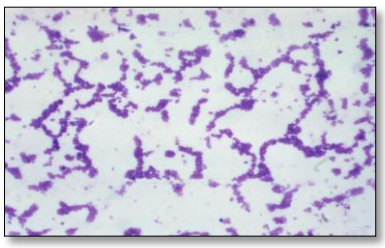
Isolate C

Figure 5.1 Colonies of *Staphylococcus aureus* isolates on Mannitol Salt Agar with yellow colonies with yellow zone.

Staphylococcus aureus bacteria was tested microscopically using Gram staining method. Purple coloured cocci were found which showed it is Gram positive bacteria. The results were shown in (Figure 5.2).



Isolate A Isolate B

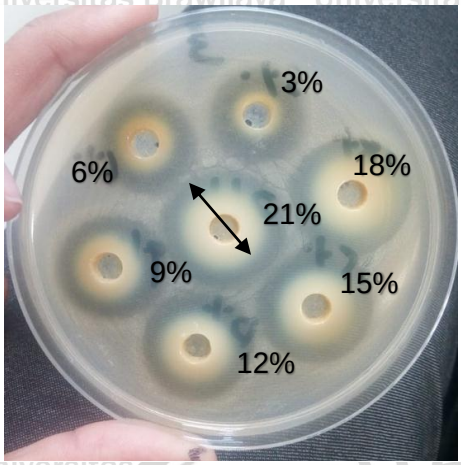


Isolate C

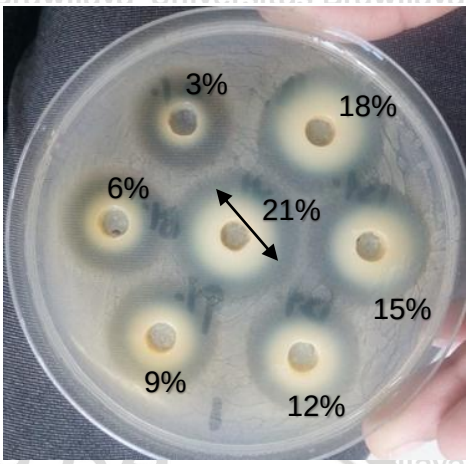
Figure 5.2 Gram staining showing purple colored cocci shaped bacteria found under

5.1.2 Antimicrobial Sensitivity Test Results

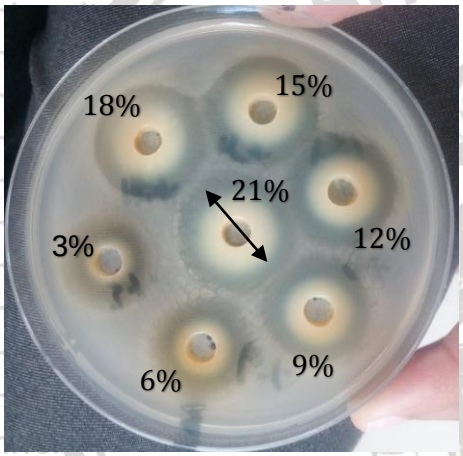
Antimicrobial sensitivity test on *Staphylococcus aureus* with pomegranate peel extract was done using concentrations such as 3%, 6%, 9%, 12%, 15%, 18% and 21% with negative control which is 0% done without pomegranate peel extract. This is to show whether zone of inhibition was formed or not around the well diffusion hole. For this experiment, three petri dishes were used. Each petri dish filled with 10ml Nutrient Agar mixed with *Staphylococcus aureus* specimen and seven holes were made with 5mm diameter than in each petri dish pomegranate peel extract were filled with different concentrations. Continuosly, each petri dishes were placed in incubator for 24 hours at temperature of 37°C. Quantifiable observations were done to see whether there is an inhibition in bacterial growth with measuring the diameter of zone of inhibition formed around the wells using ruler in millimeters(mm).



Isolate A



Isolate B



Isolate C

Figure 5.3 Well diffusion of pomegranate peel extract in different concentrations against *Staphylococcus aureus* and the arrows showing diameter of inhibition zones.

The antibiotics such as Gentamycin and Fosfomycin were used to compare the effects with pomegranate peel extract against *Staphylococcus aureus* bacterial growth. The concentrations of both antibiotics used are 50% v/v. The results of using Gentamycin and Fosfomycin against the growth of *Staphylococcus aureus* are shown in (Figure 5.4) below.

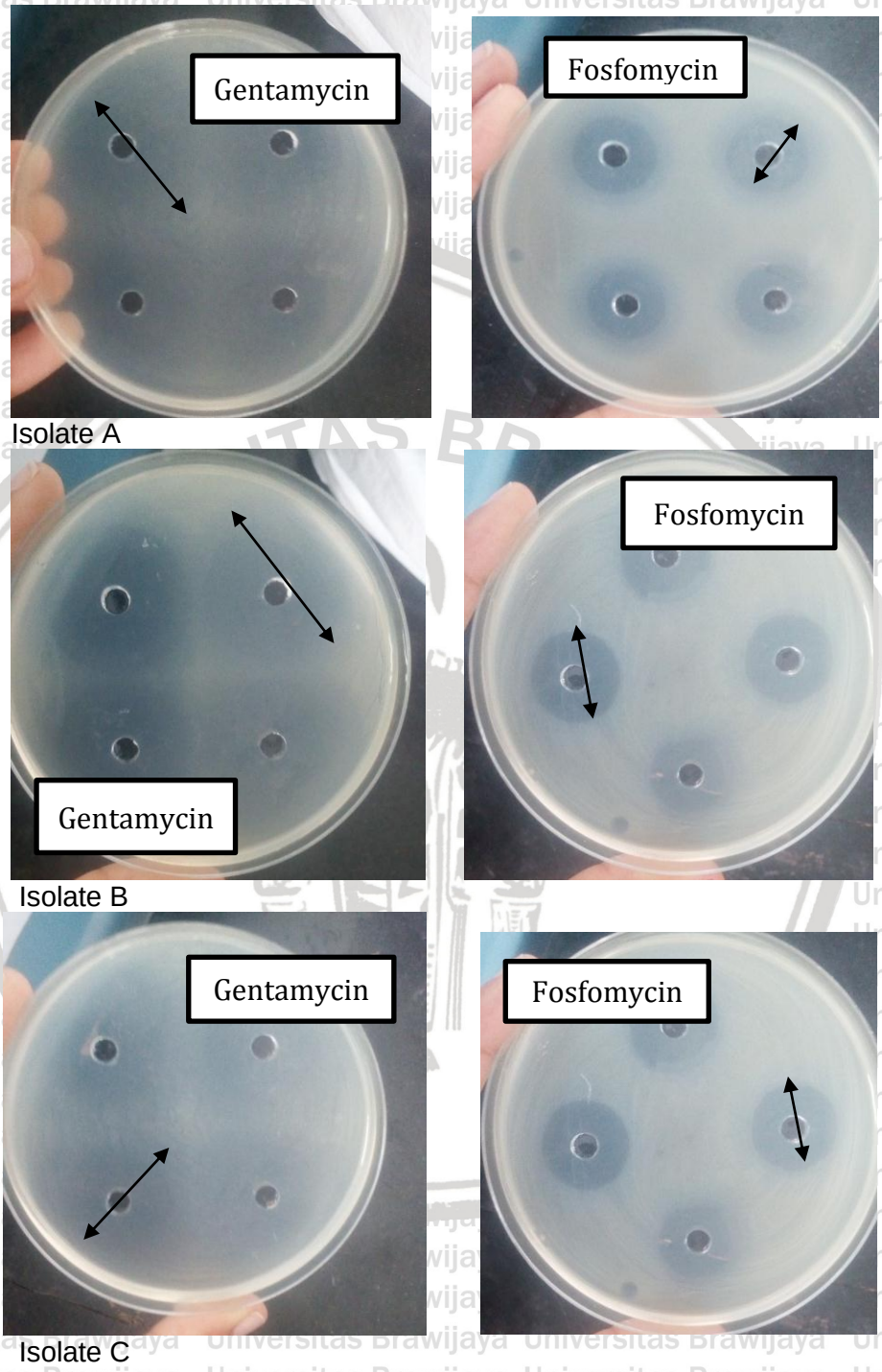


Figure 5.4 Well diffusion of antibiotics (Gentamycin and Fosfomycin) against growth of *Staphylococcus aureus* with the arrows showing diameter of inhibition zones.

Table 5.2 Concentrations of *Punica granatum* and inhibition zones on each isolate.

Concentration	Inhibitory zone (mm)			
	Isolate A	Isolate B	Isolate C	Average
0%	0	0	0	0
3%	11	15	18	14.67
6%	14	18	21	17.67
9%	19	22	24	21.67
12%	23	23	25	23.67
15%	23	23	26	24.00
18%	23	23	28	24.67
21%	26	26	28	26.67
(Gentamycin)	20	20	24	21.33
(Fosfomycin)	10	11	12	11.00

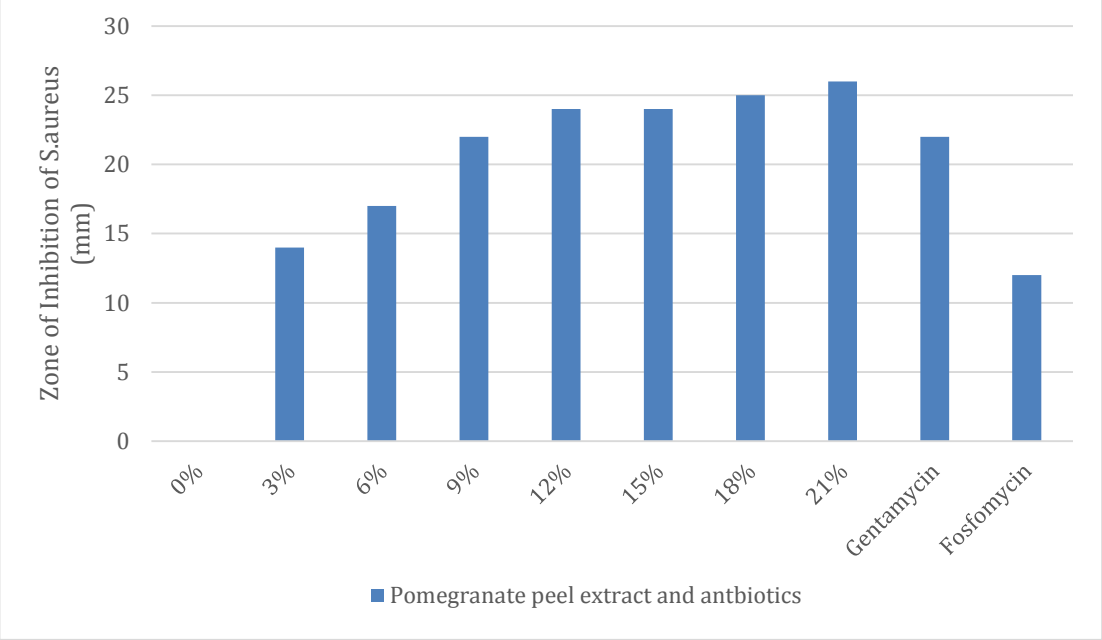


Figure 5.5 shows the concentration of pomegranate peel extract and antibiotics with the average zone of inhibition and error bars representing standard deviation.

5.2 Data Analysis

5.2.1 Homogeneity and Normality Test

One-way ANOVA test was conducted under two terms. The terms that needed are normality test and homogeneity test. One-way ANOVA is a parametric test that requires the data with the characteristics of normal distribution and homogeneous. Normality tests must also be carried out. The purpose of this normality test is carried out to assess the distribution of data in a group of data or variables whether the distribution of the data is normal or not by using kolmogorov - smirov and shapiro-wilk test. Data is said to be homogeneous if the value of p> 0.05. Normality test is performed with shapiro-wilk since the sample data is less than 50 and is considered to be non-significant and is normally more than 0.05.Appendix 1 shows that the p-value in shapiro-wilk test is 0.153 which is more than the α value..

Homogeneity test must be carried out from data obtained from research.The purpose of this homogenesis test is to find out whether the data in variable x (concentration of pomegranate peel extract) and Y (number of bacterial colonies on NAP media) are homogeneous or not using the homegenity of varience test (levene test). Data is said to be homogeneous if the value of p> 0.05. The test of Homogeneity shows the value of p is 0.112 which ratify that the observation data is homogenous. Since the confidence interval in this statistics is 95%, the α value is 0.05. This corroborates that the data is normally distributed. Considering that the statistics are homogenous and normally distributed, the terms of parametric test were fulfilled. Therefore, the suitable parametric test for data analysis in this experiment is One-way ANOVA Test.

5.2.2 One-Way ANOVA Test

One –Way ANOVA Test was conducted to study the difference between concentrations of the pomegranate peel extract on *Staphylococcus aureus* bacterial growth. The result from statistics was that there are no significant differences in antimicrobial activity of pomegranate peel extract on the growth of *S.aureus* bacteria.

Further, the (p-value) from One-Way ANOVA Test is 0.000 are shown in Appendix 2. Thus, its proves that the value is significant and the antimicrobial activity of pomegranate peel extract on *S.aureus* is very small from alpha ($p < 0.05$). So that it is analyzed that there is antimicrobial activity of pomegranate peel extract on *S.aureus* growth.

5.2.3 Post Hoc Tukey HSD Test

Post Hoc Tukey Test was further conducted when it is found that pomegranate peel extract does have antimicrobial effect on *S.aureus* growth. Post Hoc Tukey test help to observe in which groups does the differences lie if there are significant values*. nh*The p-value is considered definitely significant when it is $p < 0.05$ and non-significant when the $p > 0.05$. Appendix 3 shows result that obtained from this research. According to that, there are non-significant different values when concentrations of pomegranate peel extract between 3% with 6%, 6% with 9%, 9% with 12%, 9% with 15%, 9% with 18%, 12% with 15%, 12% with 18%, 12% with 21%, 15% with 18%, 15% with 21% and 18% with 21% used. There is also non-significant value in antibiotics comparison when 5% with Gentamycin, Fosfomycin with 5%, 20%, 30%, 40%, 50% and 100% used.

5.2.4 Spearman's Correlation Test

Spearman's Correlation test measures the strength and direction of monotonic association between two variables.Monotonicity is less restrictive than that of a linear relationship. In the table Appendix 4, it can be discerned that the p-value is 0.000 ergo the data is significant. The coefficient value of 0.843 which is a positive value and close to the value of 1. This demonstrates the strong correlation between both variables. Consequently, when the concentrations of pomegranate peel extract increase the zone of inhibition formed against *S.aureus* also increases. Hence, the data are monotonically correlated which means when one variable increases the other variable increases too.

CHAPTER 6

DISCUSSION

6.1 Discussion

This research was destined to know if the antimicrobial activity of pomegranate peel extract (*Punica granatum. L*) can inhibit *Staphylococcus aureus* bacterial growth in vitro. This study was conducted in the Microbiology Laboratory of the Faculty of Medicine at Malang University in February 2018 to October 2018.

The method used in this research is well diffusion that helps to observed the antimicrobial activity of pomegranate peel extract against *Staphylococcus aureus* bacteria by measuring the zone of inhibition formed on the *Nutrient agar plate (NAP).*

From the chapter 5 data analysis, can be proven that pomegranate peel extract does have antimicrobial capacities towards *Staphylococcus aureus* in vitro using well diffusion method. Thus, it is also confirmed that there is a strong equivalence between the concentration of pomegranate peel extract used and the diameter of zone of inhibition formed.

The extract concentrations used in the initial experiment are 3.125%, 6.25%, 12.5%, 25%, 50% and 100%. Further,the inhibition zones were formed with the *Pomegranate Peel (Punica granatum.L)* extract concentrations of 6.25% and above. Therefore, concentrations of 3%, 6%, 9%, 12%, 15%, 18% and 21% were used in the second tests to determine the antimicrobial sensitivity.

Besides, there were also few obstacles in this experiment which need ethical and cautious handling such as bacterial contamination towards extracts so the testing process was very detailed and always kept sterile. Further, a micro pipette was used so that the volume of the extract solution and bacterial suspension are appropriate.

Methicillin-resistant *Staphylococcus aureus* (MRSA) refers to a group of Gram-positive bacteria that are genetically distinct from other strains of *Staphylococcus aureus*.. MRSA is any strain of *S. aureus* that has developed, through horizontal gene transfer and natural selection, multiple drug resistance to beta-lactam antibiotics. β-lactam antibiotics are a broad-spectrum group that include some penams (penicillin derivatives such as methicillin and oxacillin) and cephems such as the cephalosporins..Strains unable to resist these antibiotics are classified as methicillin-susceptible *S. aureus*, or MSSA.

MRSA is common in hospitals, prisons, and nursing homes, where people with open wounds, invasive devices such as catheters, and weakened immune systems are at greater risk of hospital-acquired infection.MSSA, is a skin infection that is not resistant to certain antibiotics. MSSA colonizes the skin, causing no symptoms and without causing infection, but then may later lead to infection. The infection spreads via direct skin-to-skin contact and also may spread via contact with contaminated items or surfaces.

According to research, Methicillin-susceptible *Staphylococcus aureus* (MSSA) can arise from methicillin-resistant S. *aureus* (MRSA) following partial or complete excision of staphylococcal cassette

chromosome *mec* (SCC*mec*). Twenty-five multiresistant MSSA isolates recovered between 2002 and 2006 were tested for SCC*mec* DNA by PCR and were genotyped by multilocus sequence typing and *spa* typing. We have previously shown that the MRSA could be divided into two sub-groups on the basis of sensitivity or resistance to aminoglycoside antibiotics. Production of enterotoxins A, B, C and D, and α-, β-, γ- and δ-haemolysins was detected by reverse passive latex agglutination (RPLA) and agar overlay methods respectively: 60% of BC MSSA and a similar proportion of MSSA from other sources produced enterotoxin; 87% of aminoglycoside-sensitive MRSA produced enterotoxin (89% of these produced enterotoxin A alone) whereas only 27% of aminoglycoside-resistant MRSA were enterotoxin-positive, significantly less than either MSSA or aminoglycoside-sensitive MRSA. The proportion of haemolysin-producing isolates did not differ amongst the isolates of MSSA and MRSA; there was no difference in the distributions of haemolysins between aminoglycoside-sensitive and -resistant strains of MRSA. GP MSSA had higher and lower numbers of γ- and δ-haemolysin producers respectively than other *S. aureus* isolates. α-Haemolysin producers were commoner amongst MRSA isolates, which were also more likely than MSSA isolates to produce several haemolysins. Differences in enterotoxin production between aminoglycoside-sensitive and -resistant MRSA isolates reflect subgroups.

According to L.C.Braga experiment, In Brazil, pomegranate (*Punica granatum* L. (Punicaceae)) is widely used as a phytotherapeutic agent. This study evaluates the effect of pomegranate extract on *Staphylococcus aureus* FRI 722 growth and subsequent enterotoxin production. At a low extract concentration (0.01% v/v) bacterial growth was delayed, while a higher

concentration (1% v/v) eliminated bacterial growth. Most interestingly, a 0.05% (v/v) concentration of extract was found to inhibit Staphylococcal enterotoxin (SE) A production. These data further implicate pomegranate extracts as potential antibacterial therapeutics with the added ability to inhibit enterotoxin production. The adhesion assay conducted on *Escherichia coli* with aqueous pomegranate peel extract at MIC exhibits up to 80% of reduction of adhesion index which demonstrate that the large amount of saponins, alkaloids and polyphenols caused the extract to work as anti-adhesive (Zam & Khaddour, 2017).

Additionally, many researchers conducted diverse studies using pomegranate peel due to its active compounds and antibacterial abilities. Microorganisms such as *S. aureus, E. coli, Salmonella enterica, Shigella sonnei, Enterococcus faecalis,* and *Bacillus subtilis* are sensitive towards extracts of *Punica granatum L.* peel was reported in literature data. The peel extracts have higher amount of polyphenols compared to the other part of the pomegranate fruit.

Ferrazzano (2017) investigated the inhibitory effect of hydroalcoholic pomegranate extracts on *R. dentocariosa*, bacteria isolated from dental plaque and demonstrated the antibacterial agents from plant were effective to prevent and contrast oral and periodontal disease and tooth decay.

Besides, the sensitivity of *S.aureus,* the sensitivity pattern of S. aureus to the following antibiotics; Gentamicin, Amoxycillin/clavulanate, Streptomycin, Cloxacillin, Erythromycin, Chloramphenicol, Cotrimoxazole, Tetracycline, Penicillin, Ciprofloxacin, Ofloxacin, Levofloxacin, Ceftriaxone, Amoxycillin and vancomycin were 92.4%, 63.0%, 44.2%, 35.8%, 52.4%, 61.9%, 15.5%, 31.2%,

7.1%, 78.9%, 76.6%, 100%, 71.4%, 30.7% and 100% respectively. Methicillin resistant isolates were sensitive to Levofloxacin 93.7% and Ofloxacin 68.7%.

6.2 Limitations

From this research have some limitations as the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) are not able to be discerned.Thus, the pomegranate peel extract is shady and fuzzy, when the disk diffusion method was carried out.Besides, the MIC and MBC from this method was not determined. Futhermore, the certain active compounds in the pomegranate peel extract were not clarified from the extraction process. Maybe the active ingredients work alone or all the active ingredients work together in inhibiting bacterial growth. Continuously, geographic differences between countries and regions are also limitations in this research. Although, further tests about pharmacodynamics, pharmacokinetic, toxicity and side effects are needed to resolute the full potential of pomegranate peel extract to clinical usage.

CHAPTER 7
CONCLUSION

7.1 Conclusion

From the results and discussion of this research, it can be concluded that:

1. Pomegranate peel extract (*Punica granatum. L)* does have the antibacterial ability to inhibit *Staphylococcus aureus* growth using well diffusion in vitro.
2. The higher the concentration of extract, the larger the zone of inhibition of formed which clarified that there is a strong correlation between concentrations of pomegranate peel extract used and growth of *S.aureus.*
3. Gentamicin (50% v/v) have higher antimicrobial effect than Fosfomycin (50% v/v) but lesser effect than pomegranate peel extract with the same concentration against the growth of *Staphylococcus aureus.*.

7.2 Suggestions

These suggestions are given so that the future studies will be done more precisely and productively about:

1. Tests on pharmacodynamics, pharmacokinetics, toxicity and side effects of pomegranate peel extract (*Punica granatum*) should be done so that in future it can be useful to humans.
2. Experiments to clarified and measure the active compounds in pomegranate peel extract that have antimicrobial abilities.
3. Additional studies on the influence of duration and storage condition of *Punica granatum* peel extract on its antimicrobial effect.

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