EVALUATION OF ANTIBACTERIAL SYNERGY OF *Polyalthia longifolia* (Sonn.) LEAF ETHYL ACETATE FRACTION (PLEAF) WITH AMPICILLIN AGAINST METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA)

KIRUBAKARI A/P BALASUPRAMANIAM

UNIVERSITI SAINS MALAYSIA

2019

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by

KIRUBAKARI A/P BALASUPRAMANIAM

Thesis submitted in fulfilment of the requirements

for the degree of

Master of Science

March 2019

ACKNOWLEDGEMENT

First and foremost, I would like to thank God Almighty for giving me the strength, knowledge, ability and opportunity to undertake this research study and to persevere and complete it satisfactorily. Without His blessings, this achievement would not have been possible. I also would like to thank my main supervisor Assoc. Prof. Dr. Sasidharan a/l Sreenivasan for providing me an opportunity to further my studies in this field of Molecular Medicine. His guidance, patience and support throughout my research initiative provided me with positive thoughts and nurtured my self-confidence. I appreciate his willingness to help me throughout this journey in accomplishing my Master studies.

Besides that, I do not forget my co-supervisor Dr Lai Ngit Shin who never fails to assist me in times of need to ensure a better outcome of my progress in this field of research. Also, I would like to express my gratitude to Universiti Sains Malaysia (USM), Penang for providing me the opportunity to pursue my further studies in this field. I would like to thank all the Laboratory Officers and Assistants of the Institute for Research in Molecular Medicine (INFORMM) and Biological School who aided in times of need in the laboratory and at the institute.

Moreover, I have no other words to express my gratitude and appreciation to my parents and siblings who never failed or fed up with me but provided moral support, financial aid, transport and encouragement. Thank you for being with me while going through the good and bad moments. They have also added humour and

light moments during my worst part of the stressful days. Finally, I need to say that I wish that I will receive the same support from all of them. This will enable me to continue my earnest contribution to the development and enhancement of Science and Technology and enlighten my future prospect in this field.

KIRUBAKARI A/P BALASUPRAMANIAM

Institute for Research in Molecular Medicine

Universiti Sains Malaysia

March 2019

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
CFU/mL	Colony-forming units per milliliter
CO_2	Carbon dioxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
FIC	Fractional inhibitory concentration
g	Relative centrifugal force
LB	Luria Bertani
MBC	Minimum bactericidal concentration
McFarland	MacFarland
mg/mL	milligram per milliliter
MIC	Minimum inhibitory concentration
MLC	Minimum lethal concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NA	Nutrient Agar
Na_2CO_3	Sodium carbonate
OD	Optical density
PBS	Phosphate buffered saline
PLEAF	*Polyalthia longifolia* ethyl acetate fraction
SNP	Sodium Nitroprusside
v/v	volume per volume
µg/mL	microgram per milliliter
°C	Degree Celsius

PENILAIAN KESAN SINERGI AKTIVITI ANTIBAKTERIA DIANTARA FRAKSI ETIL ASETAT DAUN *Polyalthia longifolia* (Sonn.) (PLEAF) DENGAN AMPISILIN TERHADAP *Staphylococcus aureus* RINTANG METHICILIN (MRSA)

ABSTRAK

Polyalthia longifolia yang berasal dari India kaya dengan pelbagai fitokimia berguna yang berharga untuk kesihatan manusia. Tujuan kajian ini dijalankan adalah untuk menilai sama ada kombinasi fraksi etil asetat *Polyalthia longifolia* (PLEAF) dengan ampisilin mempunyai aktiviti antimikrob yang sinergistik untuk menentang isolat MRSA dan juga untuk menilai kapasiti antioksidannya dengan tahap sitotoksisitinya. Penilaian aktiviti sinergi diantara fraksi PLEAF dan ampisilin terhadap isolat MRSA tempatan telah dijalankan dengan pelbagai kaedah antimikrobial iaitu kaedah peresapan cakera, kepekatan perencatan minimum (MIC), kepekatan bakterisidal minimum (MBC), kepekatan perencatan fraksi (FIC), asai waktu-membunuh dan kajian pengimbasan mikroskop elektron (SEM). Kajian kesan gabungan fraksi PLEAF dan ampisilin mempamerkan aktiviti antibakteria yang ketara terhadap MRSA. Ini terbukti apabila nilai MIC fraksi PLEAF (62.5 μg/mL) dan ampisilin (5000 μg /mL) didapati menurun kepada 15.63 μg/mL untuk fraksi PLEAF dan 2500 μg/mL untuk ampisilin masing-masing dalam kajian FIC terhadap bakteria MRSA. Kajian hapus-sisa radikal bebas 2,2-difenil-1-pikrilhidrazil (DPPH) dan nitrik oksida (NO) menunjukkan bahawa PLEAF memiliki aktiviti antioksidan yang tinggi dan kombinasi fraksi PLEAF dan ampisilin mempamerkan aktiviti antioksidan sederhana. Penggunaan fraksi PLEAF dengan aktiviti antioksidan yang tinggi adalah penting untuk mencegah kesan buruk daripada penghasilan berlebihan radikal bebas semasa jangkitan mikrob. Kandungan jumlah fenolik (TPC) fraksi PLEAF adalah

sebanyak 16.822 ± 0.004 μg GAE/g fraksi PLEAF. Sebatian fenolik mungkin bertanggungjawab untuk aktiviti antioksidan dan antimikrob yang dipamerkan oleh fraksi PLEAF. Selain itu, ujian sitotoksik 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromida (MTT) fraksi PLEAF terhadap sel Vero telah membuktikan sebagai bukan toksik (98.14% sel viabel) dan rawatan kombinasi fraksi PLEAF dan ampisilin terhadap sel Vero menunjukkan peningkatan dalam sel hidup (52.44%) berbanding dengan sel yang dirawat dengan ampisilin sahaja. Kesimpulannya, fraksi PLEAF yang berkombinasi dengan ampisilin berfungsi dengan baik untuk membunuh bakteria MRSA rintang tempatan. Fraksi PLEAF juga menunjukkan aktiviti antioksidan yang menggalakkan dan meningkatkan jumlah sel hidup Vero semasa berkombinasi dengan ampisilin yang merupakan ciri yang penting bagi fraksi PLEAF yang akan digunakan dalam terapi kombinasi pada masa depan. Justeru kajian masa depan dicadangkan untuk menilai keupayaan fraksi PLEAF untuk menindas gen *mecA* dan menilai keupayaan kombinasi fraksi PLEAF dan ampisilin sebagai agen antimikrobial secara *in vivo* dengan menggunakan model haiwan.

EVALUATION OF ANTIBACTERIAL SYNERGY OF *Polyalthia longifolia* (Sonn.) LEAF ETHYL ACETATE FRACTION (PLEAF) WITH AMPICILLIN AGAINST METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA)

ABSTRACT

Polyalthia longifolia which originates from India is rich with various useful phytochemicals which are valuable for human health. The aim of the study is to evaluate whether the combination of *Polyalthia longifolia* ethyl acetate fraction (PLEAF) and ampicillin exhibits synergistic antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria followed by their antioxidant capacity and their cytotoxicity level. The evaluation of synergistic activity of PLEAF fraction and ampicillin against MRSA local isolate was conducted with various antimicrobial assays namely disc diffusion method, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), fractional inhibitory concentration (FIC), time-kill study and scanning electron microscopy (SEM). The combinational effect of PLEAF fraction and ampicillin exhibited significant antibacterial activity against MRSA. This is proven when the MIC values of PLEAF fraction (62.5 µg/mL) and ampicillin (5000 µg/mL) were found to decrease to 15.63 µg/mL for PLEAF and 2500 µg/mL for ampicillin respectively in the FIC assay against the MRSA bacteria. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide free radical scavenging activities showed that PLEAF fraction possessed high antioxidant activity and the combinational of PLEAF fraction and ampicillin exhibited moderate antioxidant activity. The usage of PLEAF fraction with high antioxidants activity is important to prevent the adverse effect of the production of an excessive amount of free radicals during microbial infection. The total phenolic content (TPC) of PLEAF

was 16.822 ± 0.004 µg GAE/g of PLEAF fraction. Phenolic compounds are responsible for the antioxidant and antimicrobial activity of PLEAF fraction. In addition, in *3*-(*4,5*-dimethylthiazol-*2*-yl)-*2,5*-diphenyltetrazolium bromide (MTT) cytotoxicity test against Vero cells, the PLEAF fraction was proven to be non-toxic (98.14% of cell viability). Meanwhile, the combination of PLEAF fraction and ampicillin treatment against the Vero cells showed an improved cell viability (52.44%) as compared with the ampicillin-treated group. In conclusion, the PLEAF fraction works well in combination with ampicillin to kill the MRSA strain. PLEAF fraction also showed favourable antioxidant activity and improved Vero cell viability in the presence of ampicillin which is an important attribute of PLEAF fraction to be used in the future combinational therapy. Further study is recommended to evaluate the ability of PLEAF fraction to suppress the *mecA* gene and to evaluate the *in vivo* antimicrobial efficiency of the combination of PLEAF fraction and ampicillin in an animal model.

CHAPTER 1: INTRODUCTION

1.1 Overview

Nowadays, the number of bacteria that are becoming resistant to a variety of antimicrobial drugs is inclining dramatically and causing a serious threat to human health across the world. These bacteria are termed as multidrug resistant (MDR) bacteria. However, natural plants such as *Polyalthia longifolia* were known to be contented with valuable phytochemicals which are responsible for the antimicrobial activities to combat resistance in microorganisms (Dixit *et al*., 2014). Multidrug resistance (MDR) is defined as the resistance of microorganisms to the given antimicrobial drugs (Tanwar *et al*., 2014). Prolonged treatment using the same antibiotic contributes to the development of resistance in microorganisms that were initially sensitive to the given antimicrobial drugs (Giedraitiene *et al*., 2011). Factors such as poor hygiene and sanitation, poor infection control in health care settings, patients are not completing the entire antibiotic course which contributes to the antibiotic resistance in microorganisms (Andrew, 2015). The resistant microorganisms such as bacteria, viruses, and fungi become resistant to the antimicrobial drugs which cause ineffective treatment which eventually leads to lethality effect among patients. Examples of bacteria which show resistance are *Escherichia coli* against cephalosporin and fluoroquinolones, *Klebsiella pneumonia* against cephalosporin and carbapenems and *Mycobacterium tuberculosis* against rifampicin, isoniazid, and fluoroquinolone (Tanwar *et al*., 2014).

According to O'Neill (2014), it was estimated that the mortality number of patients due to MDR infection will increase up to 10 million people by the year 2050

around the world. The MDR bacteria were discovered in different regions of the world such as Africa, some parts of America, Eastern Mediterranean Region, Europe, South-East Asia and Western Pacific Region (Tanwar *et al*., 2014). The drastic development of pathogen resistance to antibiotics may cause an impact to the different aspects of the medical field such as transplantation, care of the critical illness, chemotherapy for cancer, premature infant care and surgery (Worthington & Melander, 2013). This dramatically increases the death incidence among immune compromised patients caused by the infection of MDR bacteria makes these resistant pathogens to be named as 'super-bugs' (De Lima *et al*, 2006). This raises the attention of worldwide researcher to discover a new antimicrobial drug to reduce the level of MDR microorganism's activity.

There is a necessity to invent a new antimicrobial drug as the old antibiotics are not effective against the resistant microorganisms to prevent MDR infections. But, there is a doubt that even a new antimicrobial invention is not efficient as there are cases where newly discovered drugs have a short life span due to the resistance development against the newly discovered antimicrobial to compare against MDR bacterial infection (Sibanda & Okoh, 2007). Due to the high resistance of bacteria towards antibiotics, bioengineering of existing antibiotics should be conducted to overcome the bacterial resistance issues.

Therefore, combination therapies between drugs should be performed against resistant microorganisms. Various combinations can be performed such as antibiotic with an antibiotic, antibiotic with a non-antibiotic adjuvant molecule, and antibiotic with a natural product (Sasidharan *et al*., 2014). In combination therapy, the combined

second substance can enhance the efficiency of the antibiotic by blocking the mechanism of resistance of the pathogens to the antibiotics. Consequently, the level of resistance of the pathogens against the antibiotic will be decreased and the resistant microbe can be easily treated. Hence, by using combinational drug therapy, the resistant mechanism in microorganisms can be reduced as the drugs are able to alter different signalling pathways in the infected cells. Their therapeutic effects are maximised compared to that of using a single drug therapy (Greco & Vicent, 2009). One such effort has been made by a study which reported the synergistic effect of *Curcuma longa* with cephalosporin antibiotics against bacteria that causes diarrhoea (Sasidharan *et al*., 2014).

Medicinal plants are the richest bioresource of phytochemicals where these compounds were discovered to possess good antibacterial activity in combination with antibiotics against the resistant microorganisms. One such effort has been made by a study which reported the synergistic effect of *Curcuma longa* with cephalosporin antibiotics against bacteria that causes diarrhoea (Sasidharan *et al*., 2014). However, the cytotoxicity of the combination should be tested against Vero cells which are widely used as an initial screening test for toxicity effect. The Vero cells have been used in the test as it aids the use of colorimetric testing to assess cell viability (Chan *et al*., 2015).

1.2 Plants as Antimicrobial Medicine

Plants with various healing properties used in ancient medicine are now attracting the attention of worldwide scientists to be used in the development of antimicrobial drug

against multidrug-resistant (MDR) bacteria. According to World Health Organization (WHO), it has been reported that about 80% of the world's population is currently using herbal medicine for treatment of various diseases (Ahmad *et al*., 2013). Plants rich with various phytochemicals are an important resource to be used as chemical substances in the synthesis of drugs, pharmaceuticals, folk medicines, food supplements, nutraceuticals and modern medicines (Das *et al*., 2010).

Medicinal plants contain a vast number of bioactive compounds which are useful to produce antimicrobial drug due to their antimicrobial properties (Vijayarathna *et al*., 2017). Due to an increase in the number of bacterial resistance to the antimicrobial drugs, scientist nowadays targets medicinal plant phytochemicals to produce an antimicrobial drug (Moussaoui & Alaoui, 2016). These compounds are the secondary metabolites produced by the plants such as phenols, flavonoids, tannins, steroids, and alkaloids which exhibit medicinal properties of the plant (Ciocan & Bara, 2007).

The secondary metabolites known as the phytochemicals possess numerous beneficial activities such as anti-inflammatory, antibacterial, anti-mutagenic, anti-viral and antioxidant properties (Bazzaz *et al*., 2013). Phytochemicals such as phenols and flavonoids are found in medicinal plants in a large amount compared to other compounds (Bazzaz *et al*., 2013), which are an important source to treat various diseases including the infections caused by the MDR bacteria.

Hence, bioengineering of antimicrobial drugs such as common antibiotics combined with phytochemicals from the plant products would impose an efficient result against the MDR bacteria leading to effective treatment for healthy living of patients infected with the resistant bacteria.

1.3 Problem statement

Resistance to commonly used antibiotics is a rising increasing problem that in a few years could make infections unable to be treated and bring the state of medical care back to the pre-antibiotic era that existed as early as the beginning of the last century. During the early days, it has been discovered that microorganisms became resistant to single antibiotics only (Alekshun & Levy, 2007). As years pass by, the resistance level attained by the microorganisms has increased to multidrug resistance due to the increase in the frequency of selective pressure of different drugs given to the same microorganism (Alekshun & Levy, 2007). As a result of that, the infected bacteria in patients can develop resistance as it encounters the same antibiotic frequently (Rai *et al*., 2012). On the other hand, antibiotic-resistant organisms are thought to result in higher morbidity and mortality rates compared to antibiotic-susceptible strains (Cosgrove *et al*., 2003).

The resistant microorganism normally alters their mechanism and becomes resistant to the given antimicrobial drug which makes treatment difficult leading to death among the patients. It has been claimed that there are 95 000 people who were infected with methicillin-resistant *Staphylococcus aureus* (MRSA) in the United States and almost 19 000 people have died from infection which were caused from Human

Immunodeficiency Virus (HIV) or Acquired Immune Deficiency Syndrome (AIDS), emphysema and Parkinson's disease (Worthington and Melander, 2013). In Malaysia, the MRSA infection has increased from 17% in 1986 to 44.1% in 2007 (Sit *et al*., 2017). The epidemiological data of MRSA infections are not comparable globally due to different population density. However, the rate of its infection was (>50%) in North and South America, Asia and Malta, 25-50% in China, Australia and Africa and (<50%) in European countries (Stefani *et al*., 2012).

Hence, an alternative way to reduce the effect of multidrug-resistance (MDR) in bacteria combined with the antibacterial synergy of natural products with antibiotics need to be evaluated. This is to determine the combinational antimicrobial activity which leads to the identification of potential combinational therapies. This triggers the researchers and scientists to find an alternative way where a combination of natural products together with antibiotics is found to have a great significance synergistic effect in the treatment of MDR bacterial infections. Due to its rapid and non-specific mechanism of action, these antibacterial synergies of natural products with antibiotics could help to treat resistance pathogens. Hence, the current study was conducted to investigate the synergistic activity of *Polyalthia longifolia* ethyl acetate fraction (PLEAF) with clinically used antibiotic ampicillin against MRSA bacteria.

1.4 Objectives of the Study

The current study was conducted with the following objectives:

i. To investigate the synergistic activity of *Polyalthia longifolia* ethyl acetate fraction (PLEAF) with clinically used antibiotic ampicillin against MRSA,

ii. To determine the antioxidant activity of PLEAF fraction with clinically used antibiotic ampicillin,

iii. To evaluate the cytotoxicity of PLEAF fraction with clinically used antibiotic ampicillin against Vero cells.

CHAPTER 2: LITERATURE REVIEW

2.1 Natural Products and Antimicrobial Activity

Antibiotics are from natural substances produced by fungi but also from certain bacteria to defend against other bacteria (Moussaoui & Alaoui, 2016). The steady rise in the level of resistance of microorganisms given with the commercially available antibiotics in the market is now a serious global health problem. Due to the high peak cases involving MDR in bacteria, antimicrobial natural products have gained attention in the treatment to these microbes (Moussaoui & Alaoui, 2016). Natural plants are known as the richest bio-resource of chemical entitled for synthetic drugs, pharmaceuticals, folk medicines, food supplements, nutraceuticals, modern medicines and traditional medicines (Das *et al*., 2010).

In the olden days, medicinal plants have been used as a therapeutic source to treat various diseases (Hemaiswarya *et al*., 2009). As it is known in the past, plant-based medicines have provided a good alternative for human health. They tend to exhibit a good source of a novel drug compound (Ciocan & Bara, 2007). It has been predicted that about 80% of the world's population is now depending on traditional medicine for their primary healthcare (Jagtap *et al*., 2006). This is for the reason natural products are cheap, safe and an effective way to be used in the treatment of various diseases.

The therapeutic potential of traditional medicines such as antimicrobial, anticarcinogenic and antioxidant is because they possess secondary metabolites (Jothy

et al., 2013a). The secondary metabolites in plants are deposited in some or sometimes in all parts of the plant. Examples of these are phenol compounds, tannins, steroids and alkaloids (Ciocan & Bara, 2007). The presence of phenolic compounds in plants induces plants to exhibit anti-inflammatory, antibacterial, anti-mutagenic, antioxidant and antiviral properties (Bazzaz *et al*., 2013).

Nowadays, potential plants which provide beneficial biological activity are being used in modern medicines as a proven drug supplement for human health (Ciocan & Bara, 2007). Plant bioactive compounds exert similar target sites as found in human as they act by resembling neurotransmitters, hormones, ligands and endogenous metabolites. Hence, the search for plant's bioactive compounds to combat the rising resistance in microbes is a continuous process as they possess beneficial medicinal properties which have not been discovered (Jothy *et al*., 2013b).

2.2 Antimicrobial evolution

In the past years, as the chemotherapeutic properties of antibiotics have been discovered, it was a big relief for medical practitioners in the application of antibiotics to treat diseases as they are effective in the treatment against microbes. Unfortunately, as years pass by, the effectiveness in the treatment has been reversed as the microbes tend to become resistant to the given antimicrobial agents (Sibanda & Okoh, 2007).

The emerging crisis of resistance to the antimicrobial agent by microorganisms, either gram-positive nor gram-negative bacteria is now becoming a serious public health problem. This is as a result that there are less or no antimicrobial medicines available to treat patients infected with these MDR bacteria (Magiorakos *et*

al., 2012). It was identified that not only bacteria but also viruses, fungi and parasites possess a raised level of MDR in them which tends to increase morbidity and mortality among patients. As a result, they are called 'superbugs' (Tanwar *et al*., 2014).

Since there is an occurrence of prolonged infection in patients due to the high resistance of microorganisms, the cost required for the treatment will also increase. This is because the commercially available drugs are no longer effective against these microorganisms and hence the medical practitioners go for expensive therapies to treat the infected patients (Tanwar *et al*., 2014). Examples of resistant microorganisms are *Klebsiella pneumoniae* exhibiting extended-spectrum β-lactamases (ESBL), vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) (Alekshun & Levy, 2007).

2.3 Microbial Resistance towards Antibiotics

In the past, research conducted in the development of new antibiotics was the most common activity carried out by pharmaceutical companies. The drugs that were invented were potentially efficient in clinical treatments. Later it was discovered that its efficiency has been decreased over time. This is due to the emerging of a long list of bacterial strains finding ways to combat the different structural drug classes. As such they are no longer classified as an effective treatment given. The way MDR bacteria used to attack the antimicrobial drugs are over limiting the invention of therapeutic ailments (Alekshun & Levy, 2007).

2.4 Methicillin-Resistant *Staphylococcus aureus* (MRSA)

In the year 1960, methicillin-resistant *S. aureus* (MRSA) has been discovered after the discovery of penicillin-resistant *S. aureus.* At first, MRSA was detected in hospitals only and thereafter it became to propagate among the community as well (Dereskenski, 2005). MRSA is a nosocomial infection where 52.3% of the infected patients have been treated in the intensive care unit (ICU) and the percentage of this infection among patients has increased to 37% from 1994 to 1998 (Cosgrove *et al*., 2003). Figure 2.1 shows clusters of MRSA bacteria in cocci shape.

The aerobic and anaerobic gram-positive cocci were initially categorized into two families namely *Micrococcaceae* and *Streptococcaceae* according to their activities, aggregation and cell morphology (Patel, 2012). The genetic studies on *S. aureus* show that *S. aureus* is more like the *Bacillus*, *Lactobacillus* and *Streptococcus* group compared to that of *Micrococcus* or *Stomatococcus* clusters. The *S. aureus* genus is comprised of 41 species in the group which are further grouped into coagulase positive (CPS) or coagulase negative (CNS) (Resch *et al*., 2008). Pyogenic infection in humans is caused by CPS *S. aureus.* Meanwhile, CNS *S. aureus* acts as an opportunistic pathogen where it is introduced from medical devices or colonizing exposed wounds (Faria *et al*., 2009). Table 2.1 shows the scientific classification of *S. aureus* in the taxonomical hierarchy.

Table 2.1: Scientific classification of *S. aureus* in the taxonomical hierarchy

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

Staphylococcus aureus involves in various skin and soft infections like hair, impetigo and follicles (Hisata *et al*., 2011). The effects that *S. aureus* causes after being infected were abscess formation, suppuration, pyogenic infections and fatal septicaemia in human beings (Kim *et al*., 2005). It also causes invasive infections such as hospital-acquired pneumonia. Meningitis is also caused by *S. aureus* due to the infection of this pathogen in the blood or as a result of infection after brain surgery (Patel, 2012). The production of penicillin-binding protein (PBP2a) in *S. aureus* is the reason for the tendency of *S. aureus* resistant to methicillin. In addition to being resistant to methicillin and β-lactam antibiotics, MRSA has also become resistant to other antimicrobial agents like macrolide (Kim *et al*., 2005).

The ability of MRSA to alter its penicillin-binding protein (PBP) to penicillin-binding protein 2a (PBP2a) enables it to have less affinity for penicillin and other β-lactam antibiotics and to maintain its transpeptidase activity (Deresinski, 2005).

Hence, the reduced rate for acylation and incline rate for dissociation of PBP2a to β-lactam antibiotics enhances the resistance of MRSA to β-lactam antibiotics.

The staphylococcal cassette chromosome *mec* (SCC*mec*) in MRSA genome is where the *mecA* gene complex resides within. The *mecA* gene complex contains the regulator genes, *mecI* and *mecR* together with the *mecA* gene (Deurenberg *et al*., 2007). Among the 2.9 million base pair of *S. aureus* chromosome, S*CCmec* represents about 1%-2% of its genome. The coding of PBP2a occurs in the *mecA* gene complex. As the β-lactam antibiotics bind to the cytoplasmic membrane of the sensor-transducer receptor which was encoded by the *mecR1* gene, a signal will be triggered. This signal will then lead to the proteolytic release of the *mecI* repressor from the operator region of the *mecA* gene. The PBP2a will then be expressed (Deresinski, 2005).

2.5 The Mechanism of Antimicrobial Drug Inhibition on Microbes

When a microbe is attacked by an antibiotic, those bacterial strains which are susceptible to the attack will eventually be destroyed. Meanwhile, those that adapt to its structure and modify itself to become resistant to the given antibiotic will survive (Giedraitiene *et al*., 2011). There are two types of antimicrobial therapies namely bactericidal drugs and bacteriostatic drugs (Russell, 2002). More than 99.9% of microbes will be killed using the bactericidal drugs. The bacteriostatic drugs are the drugs which only inhibits the growth of the microbes. There are five mechanisms by which an antibiotic can encounter the invading microbes such as inhibition of cell wall synthesis, cell membrane function, protein synthesis, and nucleic acid synthesis

*(*Kapoor *et al., 2017)*. Figure 2.2 shows the mechanism of action of an antimicrobial drug on microbes.

2.5.1 Inhibition of cell wall synthesis

There are two ways by which a bacterial cell wall synthesis can be inhibited by an antibiotic which is lytic cell death and non-lytic cell death (Kohanski *et al*., 2010). In the lytic cell death, β-lactams and glycopeptides are the classes of antibiotics that disrupt cell wall biosynthesis in bacteria. The cell wall of bacteria is enclosed with a layer of peptidoglycan (PG) which is a covalently cross-linked polymer matrix made up of peptide-linked β-(1,4)-N-acetyl hexosamine 54. Transglycosylase and transpeptidase enzymes add disaccharide pentapeptides to extend glycan strands of the PG molecules to maintain the layer (Heijenoort, 2001). The mechanical strength provided by the PG layer enables the bacteria to survive adverse environmental conditions. The antibiotics given combat the bacteria by blocking the cross-linking of PG units by constraining the peptide bond formation reaction catalysed by transpeptidases called penicillin-binding protein (PBP). This indirectly reduces the cellular mechanical strength and structural integrity as the antibiotics are capable of incorporate themselves into the membrane and induce depolarization. Moreover, as the depletion of the PG layer occurs, cell death occurs as a result of the build-up of internal pressure which results in cell wall expansion and finally leading to cell death.

It has been proven that lytic cell death involves many cellular processes and is also a more complex mechanism compared to that from non-lytic cell death. Treatment with using a lytic cell death enhancer such as a β-lactam to *Streptococcus pneumonia* of which lack of amidase activity known as murein hydrolase or autolysin activity will

either grow or undergo fatal condition. The treatment effect on the antibiotic is called as antibiotic tolerance 64. Autolysins are membrane-associated enzymes that degrade bonds within the PG strands (Novak *et al*., 2000). Hence, it was well understood that autolysins contribute to cell death lysis with the inhibition of PG layer synthesis by a β-lactam antibiotic and this lysis process is called lysis 64.

On the other hand, bacterial strains like *S. pneumoniae* which have quite less, or no autolysin activity can still be destroyed with a β-lactam antibiotic. This is known as the non-lytic cell death mechanism where the killing process occurs but at a slower rate compared to that of lytic cell death mechanism. There are two component systems in bacteria that regulate the non-lytic mechanism. In *S. aureus*, the LytSR two-component system regulates the autolysin activity 71 causing an effect to cell lysis (Groicher *et al*., 2000). The LytSR obstruct autolysin activity causing antibiotic tolerance 73. Meanwhile, LrgA regulates the entry of autolysins to the peptidoglycan layer. With these activities, holing-like system namely cidAB in *S. aureus* activates the autolysins enabling *S. aureus* to be effectively killed by β-lactams (Kohanski *et al*., 2007). Figure 2.3 below elucidates the mechanism of cell wall synthesis inhibition.

2.5.2 Inhibition of cell membrane

The peptides which contain more than 20 amino acid residues tend to form α-helices to span a lipid membrane of microbes. With more peptides, the transmembrane orientation may destabilise the membrane or they may also oligomerise into either barrel-stave or toroidal-pores (Park *et al*., 2011). So, ions, toxins and metabolites will eventually flow through the pores and this prevents maintenance of homeostasis

leading to the death of the bacteria. In the barrel-stave mechanism, the peptides attach themselves to the hydrophobic core region of the membrane. Here, they oligomerise and arrange themselves in alignment to expose their hydrophobic surfaces facing towards the interior of the lipid bilayer and the hydrophilic region facing away from the lipid bilayer to form the interior lining of the pore (Park *et al*., 2011). Whereas, in the toroidal-pore mechanism, the peptides initiate the membrane curvature in the microbe until the inner and outer surfaces extend continuously as the peptides enter the microbes. This leads to a channel formed through the membrane with the lumen of the pore being lined by the hydrophilic surface of the peptide interspersed with the phospholipid head groups (Brogden, 2005).

Whereas, peptides act through a carpet mechanism if they are comprised of less than 20 amino acids as they are not capable to span a membrane. These short peptides settle on the surface of the microbe's membrane until a critical threshold concentration is reached. At this point, they will act as a detergent by lysing or solubilising the membrane (Fernandez *et al*., 2009). Figure 2.4 illustrates the different mechanisms involved in the inhibition of cell membrane of microbes.

2.5.3 Inhibition of protein synthesis

There are two types of drugs inhibitors namely 50S ribosome and 30S ribosome inhibitors which blocks the synthesis of proteins. Examples of 50S inhibitors are macrolide, lincosamide, streptogramin, amphenicol and oxazolidinone. Meanwhile, 30S inhibitors are tetracycline and aminocyclitol which is comprised of spectinomycin and aminoglycoside family of antibiotics. The 50S ribosome inhibitors inhibit the

translocation of peptidyl-tRNAs and the initiation of protein translation. This indirectly contributes to the hindrance of peptidyltransferase reaction which causes elongation of the peptide chain (Kohanski, 2010). It has been identified that macrolide, lincosamide and streptogramin drugs restrict the flow of peptidyl-tRNAs to the ribosome causing blockage of peptidyltransferase elongation reaction by steric inhibition and finally leading to segregation of the peptidyl-tRNA.

The restriction of the flow of aminoacyl-tRNAs to the ribosome92 is carried out by tetracyclines (Connell *et al.*, 2003). Meanwhile, the aminocyclitol binds the 16S rRNA component of the 30S ribosome subunit. Besides that, spectinomycin is not a factor inducing protein mistranslation 93-95, but they hinder elongation factor-catalysed translocation which causes instability in the binding of peptidyl-tRNA to the ribosome. On the other hand, the interaction between aminoglycosides and the 16S rRNA leads to a change in the structure of the complex formed between an mRNA codon and its cognitive pair from aminoacyl-tRNA during translation process causing mistranslation of protein. Figure 2.5 elucidates the mechanism of inhibition of protein synthesis in pathogen by antibiotic to reduce resistance level in the pathogen.

2.5.4 Inhibition of DNA synthesis

DNA synthesis, mRNA transcription and cell division acquire the supercoiling of chromosome through topoisomerase-catalysed strand breakage and re-joining reactions. Quinolones such as fluoroquinolones attack the DNA topoisomerase and prohibit DNA synthesis (Fournier *et al*., 2000). The quinolones disrupt the topology of the chromosome by attacking the DNA gyrase which is the topoisomerase II and IV respectively. The quinolones prevent the DNA strands from re-joining by trapping the

topoisomerase enzymes at the DNA cleavage stage. But, this DNA topoimerase varies with different bacterial species which makes it difficult for the quinolone to be susceptible to these targets.

The stable interaction complex formed between quinolone and DNA topoisomerase enables the bacteria to be killed by the quinolone antibiotic (Drlica & Malik, 2003). After the exposure of quinolone, the double-stranded DNA detach which were initially covalently bounded by topoisomerases as quinolones attach themselves to these enzymes. Due to the formation of quinolone-topoisomerase-DNA complex formation, DNA replication machinery becomes arrested at blocked replication forks. This contributes to the restriction of DNA synthesis which leads to bacteriostatic and causes bacterial cell death (Kohanski, 2010). Figure 2.6 shows the inhibition mechanism of DNA synthesis in microbes by antibiotic leading to the destruction of the microbes.

2.6 Combinational therapy

Simultaneous action of two or more pharmacologically active agents or the combination of different types of therapy is called combination therapy (Greco & Vicent, 2009). This means that bacteria used to fight against introduced commercial antibiotics are inclining day by day and getting beyond the intervention of therapeutic treatment itself (Alekshun & Levy, 2007). Also, the raised in the number of bacteria being resistant to antimicrobial agents are increasing around the world. This reduces the drug's effectiveness provided for the treatment leading to treatment failure (Sibanda & Okoh, 2007). An introduced antimicrobial agent to a bacterium which at

first has a significant effect with the bacteriostatic or bactericidal action on the bacteria does not appear to be effective to the bacteria after a period of months to years (Worthington & Melander, 2013).

Hence, researchers intend to find an alternative way by using a newfound combinational therapy of using drug combinations to combat against the MDR in bacteria effectively (Chanda & Rakholiya, 2011). The combinational therapy can be performed by using a combination of a pairing of antibiotic with an antibiotic, antibiotic with a non-antibiotic adjuvant molecule or antibiotic with a natural compound against the resistance in bacteria (Sasidharan *et al*., 2014). These types of combinational therapy are believed to reduce resistance in bacteria effectively (Alekshun & Levy, 2007).

Combinational therapy is being used for most cancer treatments, HIV infections and also artemisinin-based combination treatments are the most effective treatment for malaria. Moreover, combination therapy is also found to be effective to treat bacterial infections. These can be proven with the combinational therapy treatment performed on Mycobacterium tuberculosis infection by using a mixture of four types of drugs (Worthington & Melander, 2013).

The combinational therapy involving antibiotic with antibiotic pairing combination can be divided into three categories namely inhibition of targets in different pathways, inhibition of different targets in the same pathway and inhibition of the same target in different ways (Worthington & Melander, 2013). The

combination of an adjuvant molecule to the antibiotic increases the activity of the antibiotic such as by blocking the mechanism of resistance to the antibiotic. This will directly contribute to a decrease in the resistance to the antibiotic given and leads to the death of the microbe (Worthington & Melander, 2013). Meanwhile, a combination of natural product with antibiotic is found to be effective to combat MDR in bacteria. The chemical structures of compounds of natural products provide an infinite figure which serves as lead molecules where their activities can be enhanced by manipulation through combinations with other compounds (Sasidharan *et al*., 2014). Moreover, herbal drugs are the best modern synthetic drug that can be invented which has less or no side effects and considered safe to be consumed. Herbal drugs such as carvacrol, eugenol, and cinnamaldehyde are found to be effective against bacteria like *S. aureus* and *Escherichia coli*. The combination of natural products with antibiotics has also been reported to give synergistic, additive or antagonistic effect against different microbes (Araoka, 2012).

One of the purpose of combinational therapy is the expectation of synergistic effects of the combinational antimicrobial products against the microbes. Another purpose is delaying resistance from occurring in bacteria when exposed to antibiotics (Rakholiya & Chanda, 2012). The antibiotic combinations with another partner have been proven to show synergism effect to many bacterial species. It couldn't be proved whether it is possible to be applied in the clinical settings for better treatment (Sun *et al*., 2016). Examples of such synergism combination that can be found in vitro are aminoglycoside and beta-lactam antibiotics against Gram-negative bacteria, *Pseudomonas aeruginosa, Klebsiella, E. coli* and other Enterobacteriaceae (Araoka,

2012). At the same time, it has been identified that combinational therapy performed *in vivo* is not suitable for these microbes.

It has been reported that mortality rates induced by MDR bacteria have declined from 57.8% to 13.3% by using combinational therapy as compared to when using monotherapy treatment (Sun *et al*., 2016). Reduction in the development of resistance will occur with the frequent use of combinational therapy. Also, this treatment will help to reverse the predominance of the infection induced by MDR bacteria. It is necessary to consider the two points before choosing clinically useful antibiotic combinations for therapeutic treatment. One of the points is the correct amount of dosage of each antibiotic in the combination needs to be determined. This is important as the precise amount of dosage used will not only reduce the cost and the increase of resistance, but they also reduce the toxicity effect of the drugs used on infected patients. Another point is that it is a challenging task to test all the possible combinations of drugs as there are a mounting number of drug combinations to be tested (Sun *et al*., 2016). This problem can be settled with the integration of low-cost HIGA with the drug combination design. This is based on observing the individual drug concentration below achievable human plasma concentrations, a different mechanism of action and clinical susceptibility breakpoints (Sun *et al*., 2016).

2.7 Plants as a new source of antibiotic

Over the years, traditional medicines have played the role as the main source of primary health care which includes Thai folk medicine (Teanpaisan *et al*., 2017). Traditional medicines use plants as the major component in their treatment which is

the main source of inspiration to defend the body against various diseases (Jothy *et al*., 2013b). Plants have become the base for the development of medicine for many ailments and diseases. They also serve as a phytomedicine for treatment of diseases. It was predicted that 14% - 28% of higher plant species are used for medicinal purposes and about 74% of pharmacologically active plant components were discovered by using plants for ethnomedicinal values (Ncube *et al*., 2008).

The chemical substances found in the ethnomedicinal plants provide the medicinal value that causes physiological action on the human body. The plants contain a wide array of chemical compounds such as alkaloids, glycosides, saponins, resins, oleoresins, and sequiterpene (Ahmad *et al*., 2013). These compounds are called as secondary metabolites which act on their potential target sites by resembling signal transduction molecules, hormones, ligands and endogenous metabolites and they exert a medicinal effect on the human body (Ciocan & Bara, 2007). Hence, it is important to screen plants for active compounds which play a vital role to defend the body against the pathogens.

Researchers are constantly searching for phytochemicals from plant kingdom for the treatment of incurable diseases. This is necessary especially when the issue of resistance among bacteria has developed which needs more effective antimicrobial agents to kill the microbes to defend the body (Ncube *et al*., 2008). The polyphenolic and phenolic compounds in plants serve as the secondary metabolites which exhibit antimicrobial activity (Ncube *et al*., 2008). Natural products have been reported to be beneficial as a source of antimalarial, anti-sickling, anti-helminthic, antimicrobial, anti-convultant, anti-hypertensive and anti-schistosomal (Aiyegoro & Okoh, 2009).

On the other hand, commercial antibiotics in the market have been found to be causing effects of consumption such as bone marrow depression, liver damage, hypertensives, and neurotoxic (Aiyegoro & Okoh, 2009). The possible combination of antibiotic therapy with natural products may produce a synergistic effect in the treatment against pathogens which also delays the emergence of resistance in microorganisms (Chung *et al*., 2011). Hence, efforts should be made to discover more bioactive compounds in plants which have a beneficiary effect to combat against MDR resistance in bacterial for the possible benefit that can be gained through compound (Savoia, 2012).

2.8 *Polyalthia longifolia*

Polyalthia longifolia is an evergreen tree which is commonly used as an ornamental plant as it combats the noise pollution (Ghosh *et al*., 2008). The previous report states that trees with heavier branches deflect or refract the sound waves as those found in *P. longifolia* (Kumar *et al*., 2013). It belongs to the order Magnoliales and is under the family of *Annonaceae*. *P. longifolia* native from Sri Lanka. is a small medium-sized tree with linear-lanceolate leaves of 1 to 1.5 cm broad which is planted along roadsides and gardens for their beautiful appearances (Jothy *et al*., 2012). It is also a stress tolerant plant which contains important phenolic compounds such as flavonoids, alkaloids, terpenoids and saponins (Sampath, 2013).

Indian traditional medicinal uses almost all parts of this plant for a wide range of treatments such as gonorrhoea, helminthiasis, hypertension, diabetes, skin disease, fever and uterine disorders (Jothy *et al*., 2013a). It has been known that the bark of this

plant acts as a febrifuge (Faizi *et al*., 2003). Figure 2.7 pictures the *P. longifolia* tree. Also, Table 2.2 shows the taxonomical hierarchy of the plant *P. longifolia.*

Table 2.2: Classification of the plant *P. longifolia* according to the taxonomical hierarchy

Kingdom:	Plantae
Phylum:	Magnoliophyta
Class:	Magnoliopsida
Order:	Magnoliolales
Family	Annonaceae
Genus:	*Polyalthia*
Species:	*longifolia*

P. longifolia has been used as a traditional medicine in the olden days. Its bark is used to treat helminthiasis, hypertension, diabetes, fever and skin diseases (Manjula *et al*., 2010). Various biological activities such as antioxidant, anti-inflammatory, antidiabetic and antibacterial activities are found in the leaves, stem barks and roots of this plant (Ghosh *et al*., 2008; Jothy *et al*., 2013a; Sampath, 2013). The bark of the tree acts as a source to provide diterpenes, alkaloids, steroid and miscellaneous lactones which involves a wide variety of biological activities such as antioxidant, antifungal, cytotoxicity and antibacterial (Adaramola *et al*., 2017).

Antifeedant properties have also been found in the clerodane diterpenoids isolated from the acetone extract of leaves. Besides that, antibacterial and antifungal activities are found as a result of the isolation of diterpenoids from the hexane extract of *P. longifolia* (Manjula *et al*., 2010). The analgesic activity and the anticancer