DEVELOPMENT OF FLUORESCING METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) AS A TOOL FOR THE SCREENING OF ANTIBACTERIAL PROPERTIES OF *MIMUSOPS ELENGI* LINN.

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by

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This thesis is dedicated to my family especially my husband, my kids, my parents and also all of my siblings.

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In the name of Allah, the most Merciful and Compassionate.

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Agr	Accessory gene regulator
Amp	Ampicillin
Amp^R	Resistant gene to Ampicillin
Ap	Ammonium per sulphate
ATCC	American Type Culture Collection
BA	Blood agar
bp	base pair
BORSA	Borderline Oxacillin Resistant *Staphylococcus aureus*
º C	degree Celcius
Ca^{2+}	Calcium cation
$CaCl_2$	Calcium chloride
CAT	Chloramphenicol acetyl trasnferase
CFU	Colony Forming Unit
dH_2O	distilled water
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DMSO	Dimethyl sulphoxide
ECFP	Enhanced cyan fluorescent protein
EDTA	Ethylenediamine tetra acetic acid
EGFP	Enhanced green fluorescent protein
EtBr	Ethidium bromide
ET	Exfoliative toxin
EYFP	Enhanced yellow fluorescent protein
g	Gram
GFP	Green fluorescent protein
GFPuv	Ultra violet-optimized GFP
GFPuvk	Ultra violet-optimized GFP resistant to kanamycin
GFPmut1	GFP mutant 1
GISA	Glycopeptide intermediate *Staphylococcus aureus*

GLC	Gas liquid chromatography
HCl	Hydrogen chloride
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
Kan^R	Resistant gene to Kanamycin
kb	Kilobase
KCl	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium di-hydrogen phosphate
L	Liter
LB	Luria Bertani
M	Molar
mA	miliAmpere
mM	miliMolar
min	Minute
mg	Miligram
ml	Mililiter
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
$MgCl_2$	Magnesium chloride
MH	Mueller Hinton
MODSA	Moderately Resistant *Staphylococcus aureus*
MSSA	Methicillin Sensitive *Staphylococcus aureus*
MRSA	Methicillin Resistant *Staphylococcus aureus*
MW	Molecular Weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaH_2PO_4	Sodium dihydrogen phosphate
NCCLS	National Committee for Clinical Laboratory Standards

ng	nanogram
NEB	New England Biolab
OD	Optical density
PBP 2a/PBP 2’	Penicillin binding protein 2a
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PC	Paper chromatography
plac	lac promoter
PMN	Polymorphonuclear leukocytes
Pfu DNA polymerase	*Pyrococcus furiousus* DNA polymerase
RE	Restriction endonuclease
R_f	Retention factor
RNA	Ribonucleic acid
rpm	Rotation per minute
sarA	Staphylococcal accessory regulator A
SCC	Staphylococcal cassette chromosome
SEA-G	Staphylococcal enterotoxin A-G
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	Tris acetate EDTA
Taq DNA	*Thermus aquaticus* DNA
TE	Tris-EDTA
TLC	Thin layer chromatography
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
Tm	melting temperature
U	unit
UTI	Urinary tract infection
v	volt
VISA	Vancomycin intermediate *Staphylococcus aureus*
wtGFP	wild type GFP

w / v	weight / volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosylpyranoside
μ	micro
α	alpha
β	beta
TM	Trade mark
®	Registered
%	percentage

PEMBANGUNAN METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) BERPENDAFLUOR SEBAGAI SATU ALAT UNTUK PENYARINGAN CIRI-CIRI ANTIBAKTERIA *MIMUSOPS ELENGI* LINN.

ABSTRAK

Staphylococcus aureus adalah sejenis bakteria Gram positif yang menyebabkan abses, beberapa jangkitan pyogenik (contohnya 'endocarditis' dan 'osteomyelitis'), keracunan makanan dan sindrom kejutan toksik. Ianya juga merupakan salah satu daripada penyebab jangkitan nosokomial (radang paru-paru, keracunan darah dan jangkitan luka pembedahan). "Methicillin Resistant *Staphylococcus aureus*" (MRSA) adalah salah satu strain yang menunjukkan kerintangan terhadap antibiotik β-laktam disebabkan oleh perubahan ke atas protein pengikat penicillin (penicillin binding protein) di dalam membran selnya. Secara lazimnya, semua antibiotik yang mempunyai ikatan β-laktam (β-lactam ring) seperti kumpulan penicillin dan 'cephalosporin' tidak berupaya untuk merencat aktiviti organisma ini.

Green fluorescent protein, GFP (protein berpendafluor hijau) adalah sejenis protein yang tidak diketahui fungsinya asalnya dan dijumpai di dalam sejenis obor-obor laut iaitu *Aequorea victoria*. GFP ini adalah sejenis protein yang boleh bergabung di dalam sitoplasma dengan menunjukkan tanda pola. Kajian ini akan memberi fokus ke atas pembangunan MRSA berpendafluor dengan membina MRSA yang membawa vektor gen GFP dengan bertujuan untuk menentukan samada MRSA berpendafluor boleh digunakan sebagai alat untuk penyaringan segera ke atas ciri-ciri antibakteria bagi sumber semulajadi. Sumber semulajadi yang digunakan adalah

ekstrak akuas dan ekstrak beberapa pelarut organik daripada batang tumbuhan *Mimusops elengi* Linn. atau dikenali sebagai pokok Bunga Tanjung. Beberapa kajian terdahulu menunjukkan pokok ini ada mempunyai kandungan elemen antibakteria yang mujarab. Dalam kajian ini, penyelidikan fitokimia ke atas ekstrak akuas, dietil eter dan etil asetat daripada batang tumbuhan telah menunjukkan kehadiran komponen alkaloid, flavonoid dan tannin. Manakala, ekstrak dietil eter dan petroleum eter pula cuma menunjukkan kehadiran komponen alkaloid sahaja dan tidak menunjukkan kehadiran komponen flavonoid dan tanin. Kesemua metabolit sekunder ini diketahui disintesis sebagai kesan daripada tindakbalas terhadap infeksi mikrob.

Oleh yang demikian, kajian ini telah dilakukan untuk menyelidik potensi ekstrak batang *M. elengi* Linn. daripada akuas dan beberapa pelarut organic yang berbeza terhadap MRSA. Aktiviti antibakteria setiap ekstrak diuji untuk menunjukkan kepekatan kerencatan terendah (MICs) dan kepekatan bakterisidal terendah (MBC) yang boleh merencatkan pertumbuhan sehingga 99.9 % koloni bakteria ini.

Ekstrak akuas, dietil eter dan etil asetat telah menunjukkan keputusan MIC (16 µg / ml) dan MBC (32 µg / ml) yang sama. Manakala, ekstrak etanolik dan petroleum menunjukkan keputusan MIC dan MBC yang lebih tinggi iaitu 32 µg / ml dan 64 µg / ml. Hasil kajian menunjukkan bahawa kesemua ekstrak samada daripada akuas atau pelarut-pelarut organik menunjukkan aktiviti antibakteria terhadap MRSA.

DEVELOPMENT OF FLUORESCING METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) AS A TOOL FOR SCREENING OF ANTIBACTERIAL PROPERTIES OF *MIMUSOPS ELENGI* LINN.

ABSTRACT

Staphylococcus aureus is a Gram positive bacterium that can cause abscesses, various pyogenic infections (e.g. endocarditis and osteomyelitis), food poisoning and toxic-shock syndrome. It is also one of the most common causes of nosocomial infection (pneumoniae, septicaemia and surgical-wound infections). The Methicillin Resistant *Staphylococcus aureus* (MRSA) is a strain that is resistant to β-lactam antibiotic by virtue of changes in the penicillin-binding protein within their cell membrane. Consequently, all antibiotics that has β-lactam ring like penicillin group and cephalosporin group are unable to inhibit the growth of this organism.

Green fluorescent protein (GFP) is a protein of unknown function found in the jellyfish, *Aequorea victoria*. This GFP-fusion protein shows a punctuate pattern when localizes in the cytoplasm. The present study focused on the development of a fluorescing MRSA by the construction of MRSA vector carrying GFP gene with the intention of determining whether the fluorescing MRSA strain can be used as a tool for the rapid screening of antibacterial properties of natural product. The natural products that had been used were aqueous and several organic solvents extracts from the bark of plant *Mimusops elengi* Linn. known locally as 'Bunga Tanjung' plant. This plant has been reported to contain a potent antibacterial component. In this study, phytochemical investigation of aqueous, ethanolic and ethyl acetate extracts

of *M. elengi* Linn. revealed the presence of alkaloids, flavonoids and tannin compounds. Whereas, the diethyl ether and petroleum ether extracts revealed the presence of alkaloids only but absence of flavonoids and tannin compounds. These secondary metabolites are known to be synthesized in response to microbial infection.

The present study is, therefore, designed to assess the antibacterial potency of different solvent extracts (aqueous, diethyl ether, ethyl acetate, ethanolic and petroleum ether) of the bark of *M. elengi* Linn. on MRSA. The antibacterial activity of each extract was tested for their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) which can inhibit the growth of at least 99.9 % of the bacterial colonies.

The aqueous, diethyl ether and ethyl acetate extracts had recorded the same results of MIC (16 µg / ml) and MBC (32 µg / ml) against MRSA. Whereas, the ethanolic and petroleum ether extracts had recorded higher results of MIC and MBC i.e. 32 µg / ml and 64 µg / ml against MRSA. These results showed that all aqueous and organic solvents extracts had antibacterial activities against MRSA.

CHAPTER 1

Introduction

1.1 Background of *Staphylococcus aureus*

Staphylococcus aureus literally means "Golden Cluster Seed" and also known as golden staphylococcus, is the most common cause of *Staphylococcus* infections. The name "*Staphylococcus*" originates from the Greek word *staphyle*, meaning a bunch of grapes, and *kokkos*, meaning berry, and that is what *Staphylococcus* looks like under the microscope, like a bunch of grapes or little round berries as shown in Figure 1.1

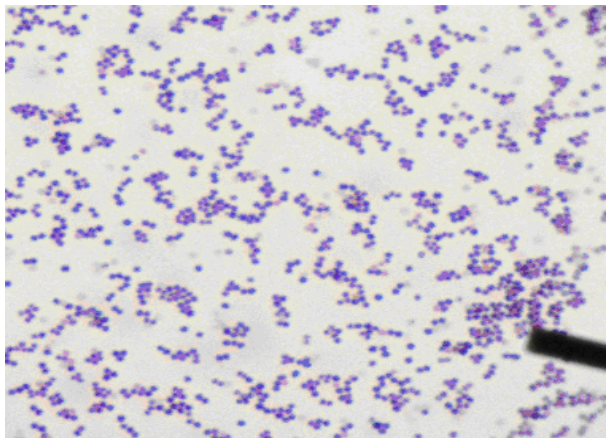

Figure 1.1: Gram positive *S. aureus* under light microscope (100 x oil objective)

It is a spherical bacterium, frequently living on the skin or in the nose of a person. Approximately 20–30% of the general populations are "S*taphylococcus* carriers". *S. aureus* can cause a range of illnesses from minor skin infections, such as pimples, impetigo (may also be caused by *Streptococcus pyogenes*), boils, cellulitis, folliculitis, furuncles, carbuncles, scalded skin syndrome, wound infection and abscesses, to life-threatening diseases, such as pneumonia, meningitis, osteomyelitis, endocarditis, Toxic Shock Syndrome (TSS), urinary tract infection (UTI) and septicemia.

Its incidence is from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the four most common causes of nosocomial infections, often causing postsurgical wound infections. *S. aureus* was discovered in Aberdeen, Scotland in 1880 by the surgeon Sir Alexander Ogston in pus from surgical abscesses (Ogston, 1984).

1.2 Microbiology of *S. aureus*

It is Gram positive cocci in cluster about 1 μm in diameter which appears as grape-like clusters when viewed through a microscope and has large about 6-8 mm in diameter, rounded and smooth, golden-yellow or pale yellow to orange colonies (Figure 1.2), often with β-hemolysis, when grown on blood agar plates (Ryan and Ray, 2004) as shown in Figure 1.3. The golden appearance is the etymological root of the bacteria's name: *aureus* means "golden" in Latin.

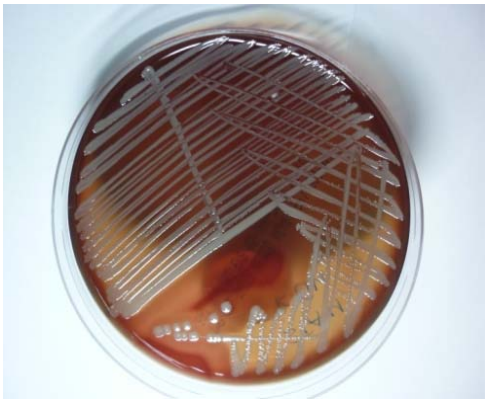

Figure 1.2: *S. aureus* on Blood Agar (BA)

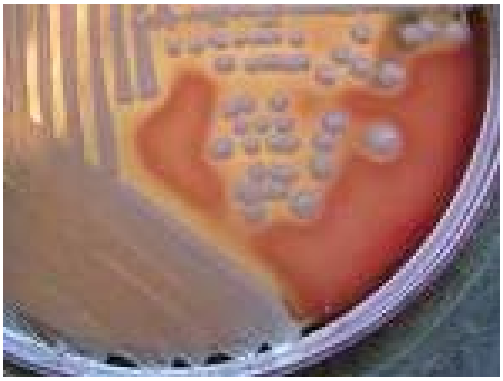

Figure 1.3: *S. aureus* colonies with beta-hemolysis on BA.

It is a facultative anaerobe, it grows in the air, but can also thrive in anaerobic conditions. It is an opportunistic pathogen, it may be present in the body without causing any harm, but in lowered immunity or injury, it may cause a disease. Staphylococci bacteria have no flagella, so they are immobile. They grow in pairs, short chains or clusters (Figure 1.1). *Staphylococcus* is in the Family Staphylococcaceae, which includes three other genera, *Gamella, Macrococcus* and *Salinicoccus. S. aureus* is catalase positive (meaning that it can produce the enzyme "catalase") and able to convert hydrogen peroxide (H_2O_2) to water and oxygen, which makes the catalase test useful to distinguish staphylococci from enterococci and streptococci. A large percentage of *S. aureus* can be differentiated from most other staphylococci by the coagulase test: *S. aureus* is primarily coagulase-positive (meaning that it can produce the enzyme "coagulase" that causes clot formation) while most other *Staphylococcus* species are coagulase-negative (Ryan and Ray, 2004).

However, while the majority of *S. aureus* are coagulase-positive, some may be atypical in that they do not produce coagulase. Incorrect identification of an isolate can impact implementation of effective treatment and or control measures (Matthews *et al*., 1997). It is medically important to identify *S. aureus* correctly as *S. aureus* is much more aggressive and likely to be antibiotic-resistant. *S. aureus* can grow at temperature range of 15 – 45°C and at high salt concentrations as high as 15% and resistant to drying. It is a normal flora of the human and is found on the nasal passages, skin and mucous membranes.

S. aureus express many potential virulence factors such as surface protein that promote colonization of host tissues, invasions that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule, Protein A), biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production), immunological disguises (Protein A, coagulase, clotting factor), membrane-damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin), exotoxins that damage host tissues or otherwise provoke symptoms of disease (SEA-G, TSST, ET) and inherent and acquired resistance to antimicrobial agents.

1.3 The structure of *S. aureus* cell wall

S. aureus cell wall consists of three layers: outer polysaccharide capsule, peptidoglycan (murein) layer, and inner cytoplasmic membrane. Into this structure, proteins and teichoic acid are embedded and protrude from the cell wall on its outer side, forming a "fuzzy coat" (Figure 1.4**).** Capsule is thin but it can still survive outside the body, e.g. on bed lining or computer keyboards from few days to several weeks as has been reported by Hsu *et al.*, (2005). This thin capsule may be seen only under the electron microscope. Sometimes more bacteria share one capsule and form a slime layer or biofilm, mostly found on the inner wall of venous and urinary catheters.

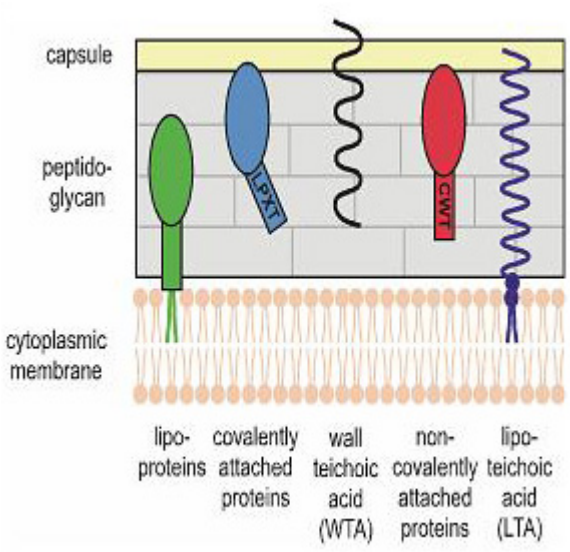

Figure 1.4: *S. aureus* cell wall structure
Adapted from www.healthhype.com/staphylococcus-aureus.html
(Cited on 10 / 09 / 2009)

1.4 Epidemiology of *S. aureus*

The main primary reservoir for *S. aureus,* including MRSA, is the human. *Staphylococcus* is also common in cows, poultry and pets such as cats and dogs. In healthy people, especially among *S. aureus* carriers, *Staphylococcus* lives in the mucosa of the nose, less commonly in the throat, vagina, intestines and lungs, on the skin mainly in the armpits, under the breasts and in the groin. In infected people, *Staphylococcus* is common in skin lesions, blood, stool, urine, respiratory mucosa, genitals and abscesses of internal organs. It can be spread from one skin location to another, person to person spread via skin-on skin contact or with sharing toys, towel, sport equipment, public showers, sauna or swimming. Infection may also be spread by healthy staphylococci carriers especially by health personnel. Infection by milking a cow

with staphylococci mastitis is possible and staphylococci food poisoning may occur after ingesting food contaminated with staphylococci, released from an infected food worker.

Entry points of staphylococci infection are skin and respiratory tract. Examples of skin infection are acne, eczema, skin injury, burns, injection, vascular and urinary catheters and surgical wounds. For respiratory tract infection, staphylococci may cause staphylococcal pneumonia due to complication of influenza and aspiration. Incubation period of *S. aureus* infection may vary a lot, but it is commonly between 4 to 10 days. For staphylococci food poisoning, the incubation period is 30 minutes to 8 hours.

1.5 Diagnosis of *S. aureus* infection

Diagnosis of *S. aureus* infection is dependent on the type of infection that has occurred and that an appropriate specimen is obtained accordingly and send to the laboratory for definitive identification by using biochemical or enzyme-based tests. In most clinical microbiology laboratory, examination and identification is done by conventional culture, identification by biochemical identification and susceptibility test to detect the presence of MRSA. A Gram stain is first performed to show the presence of typical gram-positive bacteria and cocci in clusters.

Secondly, the organism should be cultured onto mannitol salt agar which is a selective medium with 7 – 9% NaCl that allows *S. aureus* to grow producing yellow-colored colonies as a result of mannitol fermentation. For differentiation at the species

level, the enzyme-based tests are to be performed. The tests are catalase (positive for all *Staphylococcus* species), coagulase (fibrin clot formation, positive for *S. aureus*), DNAse (zone of clearance on nutrient agar), lipase (a yellow colour and rancid odour smell) and phosphatase (a pink colour). Other tests are rapid diagnosis with polymerase chain reaction (PCR) and *S. aureus* detection kit such as the Oxoid penicillin-Binding Protein Latex Agglutination Test.

A number of studies have examined the use of molecular methods for direct detection of MRSA in blood culture positive in order to facilitate rapid diagnosis of MRSA and enable appropriate therapeutic decisions to be made in a timely manner. Such methods are gel-based and real-time PCR, DNA probes and peptide nucleic acid probes. Rapid and precise identification of MRSA is a prerequisite for control of hospital infections. Molecular methods require much less time than conventional microbiological methods, therefore, it can provide not only a considerable advantage with respect to reliability but also in speed.

1.6 Treatment of *S. aureus* infection

The choice of treatment for *S. aureus* is penicillin, but in most countries, penicillin - resistance is extremely common and first-line therapy is most commonly a penicillinase - resistant penicillin (for example, oxacillin or flucloxacillin). Combination therapy with gentamicin may be used to treat serious infections like endocarditis (Bayer *et al.*, 1998) but its use is controversial because of the high risk of damage to the kidneys (Cosgrove *et al*., 2009).

Staphylococcal colonization may be treated with mupirocin nasal gel and daily Hibiclens skin cleanser baths. Local skin infections are treated with antibacterial ointments. In more extensive infections, *S. aur*eus has to be tested for antibiotic sensitivity and then usually oral antibiotics are used. In systemic infection, hospitalization and intravenous antibiotics are needed. Severe staphylococcal infections require treatment with parenteral penicillinase-resistant penicillin like nafcillin and oxacillin or cephalosporin of 1st or 2nd generation (e.g. cephalexin, cefuroxime) plus clindamycin. Vancomycin is reserved for MRSA and clindamycin resistant strains or for life-threatening infections. Staphylococcal food poisoning usually heals on its own.

1.7 *Staphylococcus* antibiotic resistance

Antibiotic resistance in *S. aureus* was almost unknown when penicillin was first introduced in 1943. Indeed, the original petri dish on which Alexander Fleming of Imperial College London observed the antibacterial activity of the penicillium mould also showed growth of a culture of *S. aureus*. By 1950, 40% of hospital's *S. aureus* isolates were penicillin resistant, and by 1960, this had risen to 80 % (Chambers, 2001).

Hospital strains of *S. aureus* are usually resistant to a variety of different antibiotics. In 1945, only two years after the introduction of penicillin in infection treatment, staphylococcus resistance to penicillin was recognized in hospitals. In 1960, methicillin (later replaced by oxacillin) was used to treat penicillin resistant *Staphylococcus* leading to the emerging of MRSA strains to the worldwide. In 1961, the first MRSA case was reported. Over 90% of staphylococci now contain the enzyme

penicillase (an enzyme in a form of β-lactamase which breaks down the β-lactam ring of the penicillin molecule) so staphylococci have to be treated with penicillinase-resistant penicillins, cephalosporins or other antibiotics.

MRSA is resistant to most antibiotics derived from penicillin, including methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin and flucloxacillin. Staphylococci have two mechanisms for resistance to beta-lactam antibiotics. One is the production of beta-lactamases, enzymes that hydrolytically destroy the beta-lactam ring. The other is the expression of an exogenous gene, *mec*A that encodes an additional penicillin-binding protein 2a (PBP 2a or PBP 2'), which is not susceptible to inhibition by beta-lactam antibiotics. This *mecA* gene is carried by a mobile genetic element, designated Staphylococcal Cassette Chromosome *mec* (SCC *mec*), inserted near the chromosomal origin of replication of staphylococcus which confers resistance to all currently available beta-lactam agents antibiotic such as penicillins, cephalosporins and carbepenems and this resistance obviates their clinical use during MRSA infections. Methicillin-resistant isolates with alteration to existing PBPs have been described. These isolates have been termed 'moderately resistant *S. aureus'* (MODSA) and 'borderline oxacillin-resistant *S. aureus*' (BORSA), which produced large amounts of penicillinase (a form of β-lactamase). In United Kingdom, only 2% of all *S. aureus* isolates are sensitive to penicillin with a similar picture in the rest of the world, due to penicillinase.

Today, *S. aureus* has become resistant to many commonly used antibiotics and are susceptible only to glycopeptides antibiotics such as vancomycin and investigational drugs. However, with the worldwide emergence of glycopeptides intermediate *S. aureus* (GISA) or also known as vancomycin-intermediate *S. aureus* (VISA) strains with

vancomycin MICs of 8 or 16 µg / ml (Chui and Hiramatsu, 2003; Hiramatsu, 1997), the likelihood of this resistance reaching global magnitude causes great concern about the possibility of losing the last treatment option for multidrug resistant staphylococcus infection (Cui *et al*., 2005). MRSA is now a problem in hospitals worldwide and is increasingly recovered from nursing homes and the community.

In Malaysia, even though the MRSA incidence rate is still less than 0.5% (according to the National Nosocomial Infection and Infection Control Report for the year of 2008 by Ministry of Health) but shows an increasing trend over five years period. This has fostered a sense of urgency with regards to need of acquiring new drugs.

1.8 Infection control of *S. aureus*

Spread of *S. aureus* including MRSA is through human-to-human contact although recently some veterinarians have discovered that the infection can be spread through pets. Emphasis on basic hand washing techniques are therefore effective in preventing the transmission of *S. aureus*. The use of disposable aprons and gloves by staff reduces skin-to skin contact and therefore further reduces the risk of transmission.

S. aureus has had facilitated transportation in medical facilities mainly because of insufficient healthcare worker hygiene. Therefore, all the surfaces of equipments in hospitals should be cleaned with alcohol, quaternary ammonium or iodine compounds, which are effective against methicillin sensitive *S. aureus* (MSSA) and MRSA. Effective

sprays for air disinfection also exist. In March 2007, the BBC reported that a vaporizer spraying some essential oils (including tea tree oil) into the atmosphere reduced airborne bacterial counts by 90% and kept MRSA infections at bay and may hold promise in MRSA infection control.

S. aureus is a major cause of Hospital Acquired Infection (Nosocomial Infection) of surgical wounds and infections associated with indwelling medical devices. The reservoir of MRSA is in infected and colonized patients and the major mode of transmission from patient to patient is on the contaminated hands of healthcare workers. Laboratory diagnosis and susceptibility testing are crucial steps in treating, controlling and preventing MRSA infection. MRSA can be life-threatening to patients with deep wounds on intravenous catheters or other foreign-body instrumentation, or as a secondary infection in patients with compromised immune systems.

1.9 New drug discovery from natural product.

Modern medicine is engaged in an eager quest for new antibiotics, which can treat the infections caused by constantly emerging antibiotic resistant bacteria (Rangaman *et al*., 2007). The limited life span of antimicrobial is due to resistance developing by indiscriminate use which necessitates the continuous search for alternatives. Awareness for misuse of antibiotics and also the potential risk of using synthetic form of phytochemicals have been reported (Borris, 1996).

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many

based on their use in traditional medicine. The use of traditional plant extracts as well as other alternative forms of medical treatments have been gathering momentum since 1990s (Cowan, 1999). Recent research has identified plants as a promising source of natural antibiotics against various pathogenic microorganisms (Rangaman *et al*., 2007). In this respect, plants used in traditional medicinal systems have been proven to be a reliable source. Being secondary metabolites, the production of these antibiotic compounds by plants are affected by various stress conditions experienced by the plants. Traditionally common people use crude extracts of plant parts as curative agents (Mendoza *et al*., 1997; Sanches *et al*., 2005).

Malaysia is a country that is rich in natural products which have great potential in possessing antimicrobial properties. Some of these natural products might have antimicrobial properties against MRSA. However, high-throughput screening of new antimicrobial agents require the development of rapid, inexpensive screening system such as the use of fluorescent recombinant bacteria expressing green fluorescent protein (GFP). High-throughtput screening is defined as a process for rapid assessment of activity in samples, usually derived from a combinatorial library or other compound collection. It allows drug screening test in a short period of time through a combination of modern robotic, data processing and control software, liquid handling devices and sensitive detectors.

The present study will focus on the development of a fluorescing MRSA by the construction of MRSA carrying green fluorescent protein with the intention of determining whether the MRSA strain can be used as a tool for rapid screening of antibacterial properties of natural product. Rapid screening of antibacterial properties

from natural product allows drug screening in a short period of time and may reduce the misuse and resistance of the antibiotics in population.

1.10 *Mimusops elengi* Linn.

M. elengi Linn. belongs to the family Sapotaceae. It is an evergreen tree, 5 – 8 metre tall (Figure 1.5). It is cultivated throughout as an ornamental tree. Medicinal properties of *M. elengi* Linn., a common tree species in Malaysia, is not very well known. Phytochemical investigation of the plants reveals that the bark decoction, flowers (Figure 1.6) and fruits (Figure 1.7) are used as teeth cleaner and tender twigs are used as tootbrush (Dymock *et al*., 1981) and the extract of the seed had showed good antimicrobial activity (Hazra *et al*., 2007) and are used in constipation (Nair & Chanda, 2007).

In India, the bark (Figure 1.8) is used as gargle for odontopathy, ulitis and ulemorrhagia (Abbas *et al.*, 2008). The fruits are used as astringent, coolant and antihelmintic whereas the tender stems are used as tooth brushes and in cystorrhea, diarrhea and dysentery (Abbas *et al.,* 2008). The flowers' lotion is used for wounds and ulcers (Nair and Chanda, 2007). In Indonesia, the *M. elengi* Linn. leaves are often used as traditional medicine for asthma, mouth and throat infections (Susan *et al*., 2006). Due to the fact that the plant *M. elengi* Linn. is very useful, as found by above mentioned reports and the fact that little information cited in the literature (Nair and Chanda, 2007; Hazra *et al*., 2007; Mohamed *et al*., 1996 and Sahu *et al*., 2001) is available on the biological activities, there is a need to find out more about the potentiality of this plant as an antimicrobial agent.

The present study is, therefore, designed to assess the potency of different solvent extracts of *M. elengi* Linn. on MRSA. The antibacterial activity of each extract was tested for their minimum inhibitory concentrations (MICs) using the MIC broth macrodilution method (Jorgensen & Turnidge, 2005; Andrews, 2001) and minimum bactericidal concentrations (MBCs) to determine the concentration of drug that inhibits at least 99.9 % of the bacterial colonies.

Figure 1.5: *M. elengi* Linn. plant (pokok Bunga Tanjung).

Figure 1.6: The flowers of *M. elengi* Linn.

Figure 1.7: The fruits of *M. elengi* Linn.

Figure 1.8: The bark of *M. elengi* Linn.

1.11 Green fluorescent protein (GFP)

The green fluorescent protein (GFP) is a protein composed of 238 amino acids (26.9kDa), which exhibits bright green fluorescence when exposed to blue light. Although many other marine organisms have similar green fluorescent proteins, GFP traditionally refers to the protein first isolated from the jellyfish *Aequorea victoria*.

GFP was first discovered in 1961 by Osamu Shimomura *et al*. (Tsein, 1998). GFP produces an intense and stable non-catalying green fluorescence by absorbing blue light maximally at 395 nm and emitting green light with peak at 509 nm (Chalfie *et al*., 1994). This process takes place when the protein aequorin, also produced by *A. victoria*, interacts with Ca^{2+} ions thus emitting a blue glow (Shimomura *et al*., 1962; Morin & Hasting, 1971; Ward *et al*., 1980).

GFP folds into a unique, compact structure known as β-can that is strongly resistant to chemical denaturation and found to be stable in most cells (Corish and Tyle-Smith, 1999). The β-can structure encloses an α-helix containing the chromophore (Figure 1.9). This structure is required to provide a proper environment for the chromophore to fluoresce as shown by the fact that nascent GFP do not fluoresce (Clontech, 2001). GFP fluorescence occurs without any cofactors and this property allows GFP fluorescence to be utilized as a reporter in non-native organism.

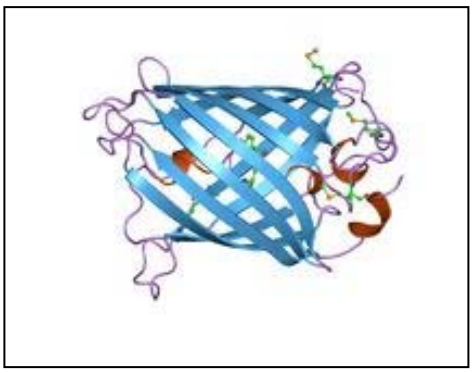

Figure 1.9: Structure of the *A. victoria* green fluorescent protein. Image from Yang *et al*. (1996).

The fluorescence is derived from post-translatinonal cyclization of a serine-tyrosine-glycine tripeptide of the GFP protein, followed by dehydrogenation of the tyrosine. It is generated by sequential activation of two photon proteins, aequorin and GFP. Upon calcium binding, aequorin emits blue light which in turn excites GFP to fluoresce. GFP produces green fluorescence which requires only the presence of oxygen to maturate with no external compounds needed (Andersen *et al*., 1998; Chalfie *et al.*, 1994).

The cloning of the wild-type GFP gene (Prasher *et al*., 1992; Inouye & Tsuji, 1994) and its subsequent expression in heterologous systems (Chalfie *et al*., 1994) established GFP as a novel genetic reporter system. Its adaptation for microbial studies led to the development of optimised, more soluble, brighter, blue or red-shifted mutants extending potential applications (Cormack *et al*., 1996; Crameri *et al.*, 1996; Heim *et al*., 1994).

GFP has become popular reporter system for use in both prokaryotes and eukaryotes. In eukaryotes, it has been used in numerous applications, including

transcriptional protein fusions to study protein targeting (Cowley and Av-Gay, 2001). In prokaryotes, it has been used primarily as a reporter for promoter activity by creating transcriptional fusions in a wide range of bacterial species, including *S. aureus* (Cui *et al.,* 2005), *Escherichia coli*, *Brucella suis, Salmonella typhimurium* and *Mycobacterium* spp. (Dhandayuthapani *et al*., 1995, Valdivia *et al.,* 1996). In modified form, it has been used to make biosensors. GFP generally does not interfere with the growth of the host and thus it is an excellent choice for non-disruptive studies of bacterial communities or other systems, which require live cells to be studied at the single cell level (Clontech, 2001).

1.12 Recombinant Staphylococcal Expressing GFP

The utilization of GFP has been extended by modification of the GFP structure by mutating and re-constructing the GFP gene. There are several variants of GFP which has been introduced such as UV-optimized GFP (GFPuv), GFP excitation (enhanced green fluorescent protein, EGFP- e.g. GFPmut1) and GFP emission (enhanced yellow fluorescent protein, EYFP and enhanced cyan fluorescent protein, ECFP). These variants are able to fluoresce more intensely compared to the original wild type GFP (wtGFP) (Clontech, 2001).

The GFPuv had been widely used as a reporter for studying the genetic basis for virulence in *S. aureus* (Bateman *et al*., 2001). In another study by Schneider *et al*. (2002), the GFP had been used in differential fluorescence induction (DFI) assay in an effort to identify *S. aureus* gene whose products can be targeted for antimicrobial drug

development. Wamel *et al*. (2002) had used green fluorescent protein reporter gene system to examine the expression and growth regulation of the capsular polysaccharide 5 genes by two major global regulator of *S. aureus* (*agr* and *sarA*) *in vitro* and in a rabbit endocarditis model.

The reporter vector expressing green fluorescent protein under the control of xylose-inducible promoter had been constructed to be used in the comparative studies of antisense effects in *S. aureus* (Nekhotiaeva *et al*., 2004). This antisense approach had been developed to improve understanding of the genetic and environmental requirement for *S. aureus* growth and pathogenesis. It is important to understand the *S. aureus* growth and pathogenesis because of the emergence and spread of vancomycin-resistant *S. aureus* strains and therefore highlight the need for new strategies in therapeutic development.

Frank *et al*. (2005) had used the green fluorescent protein variant (gfpmut3.1 from Clontech) as a reporter gene system in *S. epidermidis*. In a study by Cui *et al*. (2005), the green fluorescent protein had been used as a transcriptional fusion system in identification of gene associated with glycopeptides resistance in *S. aureus*. A study by Grundling and Schneewind (2006) had used purified GFP fused to C-terminal cell wall-targeting domain to reveal species-specific association of the reporter with staphylococci. The recent study by Jamie *et al*. (2009) had used *S. aureus*-expressing cytolisis GFP to provide a novel probe of the fate of *S. aureus* in human polymorphonuclear leukocytes (PMN).

These studies suggest that GFP is a useful reporter of viability in staphylococci and furthermore it has been used for antibacterial screening and survival study of staphylococci towards PMN action. However, the use of pathogenic or even opportunistic staphylococci for screening assays raises safety concerns.

1.13 Rational of study

MRSA is an important nosocomial pathogen that exhibit resistance to a wide range of antibiotic drugs. The spread, survival and prevalence of antibiotic resistant clones of *S. aureus* are immensely important problems for human health. The rapid and accurate identification of the disease causing agent is therefore a prerequisite for disease control as well as for epidemiological surveillance. Thus, there is a need to screen new anti-MRSA compounds that requires the development of a rapid and inexpensive assay method. Therefore, the development of fluorescing MRSA as a tool for rapid screening of antibacterial properties of *M. elengi* Linn. is one of inexpensive screening systems that allows drug screening in a short period of time and may reduce the antibiotics resistance in population.

1.14 Objectives of the study

The main objective of this study is to develop a recombinant plasmid that can be used to express GFP at an optimum level in MRSA. The fluorescent recombinant MRSA strain can then be used in rapid screening system for evaluating the antibacterial properties of natural product such as the bark of *M. elengi* Linn.

To achieve this objective, the following specific aims will be carried out:

1. To construct the *E. coli* - *S. aureus* shuttle vector that can be used for transformation and expression of GFPuv in *E. coli* and *S. aureus.*
2. To investigate the antibacterial properties of different solvent extracts of *M. elengi* Linn. on MRSA.
3. To analyze the phytochemical and antibacterial properties of *M. elengi* Linn. extracts in different solvents.

1.15 Experimental overview

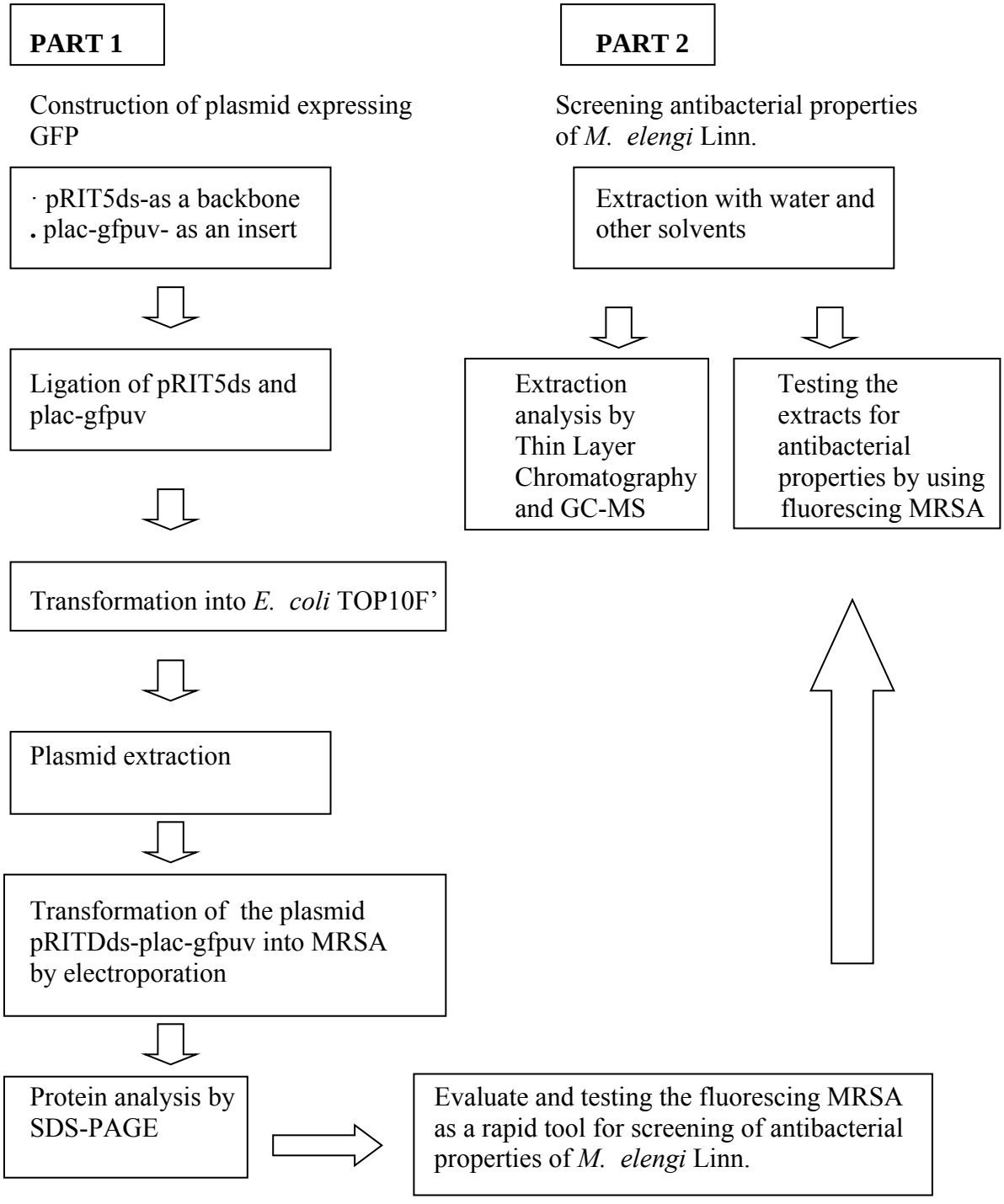

Figure 1.10: Flow chart of experimental overview

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

The list of bacterial strains used in this study is shown in Table 2.1. *E. coli* TOP10F' strains are grown at 37˚C in the Luria-Bertani (LB) medium whereas MRSA are grown at 37˚C in B2 medium (refer page 28). For a longer period of storage, cells were maintained in the form of glycerol stocks and stored at -80˚C.

Table 2.1 List of bacterial strains used in this study

Bacterial species and strains	Source
E. coli TOP10F'	Invitrogen, USA
MRSA	
ATCC 700698	Institute for Medical Research (IMR)
MR 352	Stock culture from clinical sample, Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia
MSSA	
BL 92	Stock culture from clinical sample, Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia

2.1.2 Plasmids

The plasmids used in the study are pGFPuvk supplied by Becton Dickinson Bioscience Clontech and pRIT5ds-gfp was a gift from by Cui *et al*. (2005). The maps of the plasmids are shown in Figure 2.1.

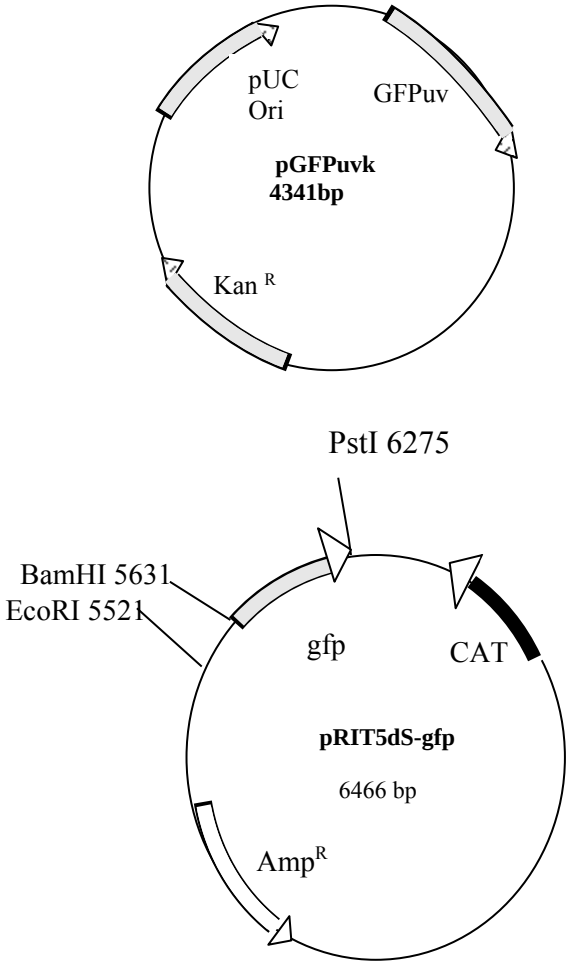

Figure 2.1: Maps of pGFPuvk plasmid and pIT5ds-gfp plasmid