EFFECT OF *CYPERUS ROTUNDUS* LINN. AND *CAPSICUM FRUTESCENS* LINN. CRUDE EXTRACTS ON ANTIBIOTIC RESISTANT BACTERIA

Prairadda Cheypratub

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

Rungrudee Srisawat
(Asst. Prof. Dr. Rungrudee Srisawat)
Chairperson

G. Eumkeb.
(Assoc. Prof. Dr. Griangsak Eumkeb)
Member (Thesis Advisor)

(Asst. Prof. Dr. Wilairat Leeanansaksiri)
Member

(Dr. Pongrit Krubphachaya)
Member

(Assoc. Prof. Dr. Mullika Chomnawang)
Member

(Prof. Dr. Santi Maensiri)
Vice Rector for Academic Affairs and Internationalization

(Assist. Prof. Dr. Worawat Meevasana)
Dean of Institute of Science

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รากและหัวของหญ้าแห้วหมู รวมถึงผลของพริกขี้หนูนั้นถูกสกัดโดยการสกัดแบบซอกเลตโดยใช้ เอทานอลเพื่อให้ได้สารสกัดหยาบ การทดสอบความไวของการต้านแบคทีเรียสำหรับ สารสกัดหยาบจากพริกขี้หนู (ซีเอฟอี) และ สารสกัดหยาบจากหญ้าแห้วหมู (ซีอาร์อี) ต่อเชื้อดื้อยาเมทิซิลลิน สแตปฟิโลคอคคัส ออเรียส (เอ็มอาร์เอสเอ) และ เชื้อดื้อยาเซพตาซิดีม อีโคไล (ซีอาร์อีซี) นั้นถูกทดสอบขั้นแรกโดยเทคนิคอาร์การ์ดิสดิฟฟิวชัน และค่าความเข้มข้นยับยั้งต่ำสุด (เอ็มไอซี) โดยอาร์การ์ไดลูชันเทคนิค เช็คเกอร์บอร์ดเอสเส ของสารผสมซีอาร์อีผสมแอมพิซิลลินแสดงการเสริมฤทธิ์กันในการต้านเชื้อเอ็มอาร์เอสเอ ในทางตรงกันข้ามสารผสมซีอาร์อีผสมอะมอกซีซิลลินสำหรับต้านเชื้อซีอาร์อีซีแสดงการไม่เสริมฤทธิ์กัน นอกจากนี้ ไทม์คิลลิงเอสเสยังยืนยันการลดลงอย่างมากของจำนวนเซลล์ที่มีชีวิตอยู่ของเชื้อเอ็มอาร์เอสเอ หลังจากใส่สารผสมด้วยค่าที่ต่ำกว่าครึ่งของเอ็มไอซี จำนวนเซลล์ที่มีชีวิตอยู่ของเชื้อเอ็มอาร์เอสเอที่ใส่สารผสมซีอาร์อี-แอมพิซิลลินที่ 24 ชั่วโมงนั้นลดลงอย่างมาก มากกว่า 2 ล็อก 10 ซีเอฟยูต่อมิลลิลิตร เทียบกับสารเดี่ยวที่ออกฤทธิ์มากที่สุด ยืนยันการเสริมฤทธิ์กันของสารผสม นอกจากนี้ การทดสอบหากลไกเบื้องต้นของซีอาร์อีต้านเชื้อเอ็มอาร์เอสเอได้ถูกศึกษา การศึกษาการเลือกผ่านของเยื่อหุ้มไซโตพลาสมิคเผยให้เห็นถึงการเลือกผ่านของเยื่อหุ้มไซโตพลาสมิคที่เพิ่มขึ้นในช่วงล็อกเฟส 2-6 ชั่วโมง จากผลการศึกษาด้วยการส่องด้วยกล้องจุลทรรศน์อิเล็กตรอน (ทีอีเอ็ม) ความเสียหายของผนังเซลที่รุนแรงที่สุดนั้นสามารถมองเห็นได้ในเชื้อเอ็มอาร์เอสเอที่ได้รับสารผสมซีอาร์อีผสมแอมพิซิลลินที่ช่วงกลางล็อกเฟสที่ 4 ชั่วโมง เบตาแลคทาเมสเอนไซม์เอสเสแสดงให้เห็นว่าเบ็นซิลเพนนิซิลลินที่ผสมกับสารผสมของซีอาร์อีและแอมพิซิลลินเผยให้เห็นถึงการการทำลายเบ็นซิลเพนนิซิลลินที่น้อยลงเปรียบเทียบกับกลุ่มควบคุม นี่เป็นหลักฐานสำคัญที่แสดงถึงความสามารถในการยับยั้งเอนไซม์เบตาแลคทาเมสของสารในซีอาร์อีซึ่งสามารถเพิ่มประสิทธิภาพให้แก่ยาปฏิชีวนะเบตาแลคแตมในการทำลายผนังเซลของแบคทีเรีย นอกจากนี้ ซีอาร์อียังมีความสามารถในการต้านแบคทีเรียได้ด้วยตัวเองเช่นที่แสดงให้เห็นผ่านค่าเอ็มไอซี ผลการวิจัยนี้บ่งชี้ให้เห็นว่าซีอาร์อีไม่เพียงแต่มีสารยับยั้งเอนไซม์ที่ใช้ในการยับยั้งเอนไซม์สำหรับการดื้อยาแต่ยังมีความสามารถในการยับยั้งกลไกการดื้อยาอื่น ๆ อีกด้วย จากข้อมูลทั้งหมดแสดงให้เห็นว่า ซีอาร์อีไม่เพียงแต่มีสารยับยั้งเอนไซม์เบตาแลคทาเมสซึ่งอาจเป็น

ชนิดที่มีหรือไม่มีโครงสร้างของเบตาแลคแทมอยู่ด้วย แต่ยังมีความสามารถในทำลายเชื้อดื้อยาที่ดื้อต่อยาปฏิชีวนะเบตาแลคแทมด้วยกลไกการดื้อยาอื่น ๆ อีกด้วยซึ่งจะต้องทำการวิจัยเชิงลึกต่อไป ดังนั้นซีอาร์อีจึงแสดงความสามารถในการเป็นสารต้านการดื้อยาผ่านกลไกการดื้อยามากกว่า 1 กลไกขึ้นไป เพราะเหตุนี้ ซีอาร์อีอาจมีศักยภาพในการนำมาใช้บำบัดเชื้อเอ็มอาร์เอสเอซึ่งกำลังดื้อต่อยาปฏิชีวนะต่าง ๆ ที่ใช้กันอยู่ในปัจจุบัน โดยสรุป สารผสมซีอาร์อีกับแอมพิซิลลินแสดงศักยภาพเสริมฤทธิ์กันโดยช่วยให้แอมพิซิลลินสามารถทำลายเชื้อเอ็มอาร์เอสเอได้อีกครั้ง และยังอาจสามารถที่จะพัฒนาไปเป็นสารยับยั้งเอนไซม์เบตาแลคทาเมสตัวใหม่ซึ่งอาจเป็นชนิดที่มีโครงสร้างของเบตาแลคแทมอยู่ด้วยหรืออาจเป็นชนิดที่ไม่มีก็ได้ เป็นที่ชัดเจนว่า การค้นพบนี้เป็นรายงานแรกที่แสดงให้เห็นถึงกลไกพื้นฐานของสารผสมซีอาร์อีต่อเอ็มอาร์เอสเอ

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

PRAIRADDA CHEYPRATUB : EFFECT OF *CYPERUS ROTUNDUS* LINN. AND *CAPSICUM FRUTESCENS* LINN. CRUDE EXTRACTS ON ANTIBIOTIC RESISTANT BACTERIA. THESIS ADVISOR : ASSOC. PROF. GRAINGSAK EUMKEB, Ph.D. 166 PP.

C. ROTUNDUS/*C. FRUTESCENS*/MRSA/ANTIBIOTIC/*β*-LACTAMASE

The *C. rotundus* rhizomes and tubers including *C. frutescens* fruits were Soxhlet extracted with ethanol to get crude extracts. The antibacterial susceptibility test for *C. frutescens* crude extract (CFE) and *C. rotundus* crude extract (CRE) against methicillin-resistant *Staphylococcus aureus* (MRSA) and ceftazidime-resistant *Escherichia coli* (CREC) were primary screening by agar disc diffusion and minimum inhibitory concentration (MIC) by the agar dilution. The checkerboard assay of the combination of CRE plus ampicillin exhibited synergistic activity against MRSA. In contrast, the combination of CRE with amoxicillin against CREC showed no interaction. Furthermore, Time killing assay was confirmed a dramatic reduction in the viable count of MRSA after treated with the sub-MIC of this combination. The viable cell count of the CRE-ampicillin treated MRSA at 24 h was dramatically reduced greater than $2\log_{10}$ cfu/ml in comparison to the most active single agent confirming synergistic activity. Moreover, the investigation of elementary mechanisms of action for CRE against MRSA was performed. The study on the cytoplasmic membrane (CM) permeability revealed the increased CM permeability in log phase 2-6 h. According to transmission electronmicroscopy (TEM) result, the most virulent cell wall damage was viewed in the treatment of MRSA with CRE

plus ampicillin in the mid-log phase, 4 h. The β-lactamase enzyme assay demonstrated that the benzylpenicillin treated with the combination of CRE plus ampicillin appeared the less amount of benzylpenicillin hydrolyzation as compared to control. This is the strongest evidence for the presence of the β-lactamase inhibitor in CRE that could enhance the β-lactam antibiotic to damage the bacterial cell wall. Furthermore, CRE also had the antibacterial effect of its own to against MRSA as shown in MIC determination. This indicated that CRE not only contained the inhibitor to destroy enzyme for antibiotic resistance, but also possessed the ability to disrupt other mechanisms of antibiotic resistance. Take-in all data together, CRE not only contained a β-lactamase inhibitor, which may either be with or without β-lactam structure, but also possessed the ability to conquer the β-lactam antibiotic-resistant bacteria by other mechanisms which must be further investigated. Therefore, CRE exerts its antiresistant activity through multiple mechanisms. Because of this, it can be employed for the potential treatment of MRSA, which almost resistant to practically β-lactam antibiotics. In conclusion, the combination of CRE and ampicillin drug demonstrated the potential to be a novel adjuvant phytopharmaceuticals for ampicillin to treat MRSA and to be considered for development of new natural β-lactamase inhibitor which may either be with or without β-lactam structure. Obviously, this is the first report demonstrating the preliminary mechanisms of actions of CRE on MRSA.

School of Preclinic Student's Signature P. Chuyprattub

Academic Year 2014 Advisor's Signature G. Eumkeb.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
°C	Degree Celsius
cfu	Colony forming unit
CLSI	Clinical And Laboratory Standards Institute
CM	Cytoplasmic Membrane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
CRE	Crude extract of *Cyperus rotundus*
CREC	Ceftazidime-Resistant *Escherichia coli*
DMST	Department of Medical Sciences Thailand
ESI	Electrospray ionization
FIC	Fractional Inhibitory Concentration
g	Gram
GAE	Gallic Acid Equivalent
h	Hour
HCL	Hydrochloric acid
H_2SO_4	Sulfuric acid
HEPES	N-2-Hydroxyethyl Piperazine-N'-Ethanesulphonic Acid

LIST OF ABBREVIATIONS (Continued)

HPLC	High-Performance Liquid Chromatography
HSD	Honestly Significant Difference
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
MBC	Minimum Bactericidal Concentration
MHA	Mueller Hueller-Hinton Agar
MHB	Mueller Hueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
mg	Milligram
ml	Millilitre
µg	Microgram
µl	Microlitre
mM	Millimolar
mm	Millimetre
MRM	Multiple Reaction Monitoring
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MW	Molecular Weight
N_2	Nitrogen gas
NaCl	Sodium Chloride
m	Mass, Weight of dry extract
min	Minute
m/z	Mass-to-charge ratio

LIST OF ABBREVIATIONS (Continued)

nm	Nanometer
nm^2	Nano square meters
OD	Optical Density
OsO_4	Osmium Tetroxide
PMX	Polymyxin B sulfate
Psi	Pounds per square inch
QE	Quercetin Equivalent
RE	Rotundine Equivalent
RNA	Ribonucleic Acid
rpm	Rounds per minute
R^2	R-squared
SEM	Standard Error of the Mean
TEM	Transmission Electron Microscopy
V	Volume, Voltage

CHAPTER I

INTRODUCTION

1.1 Introduction

Antibiotic resistance has become a serious problem threatening to public health and medical implications. The 60% of hospital-acquired infections are caused by multidrug-resistant microorganisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) which is no longer confined in the hospital wards but also infiltrates the community level as well (Mahady et al., 2008; Mahady, 2009) while ceftazidime-resistant *Escherichia coli* (CREC) broadly disseminates between distinct health facilities including hospitals, community health centers, and long-term care centers (Oteo et al., 2006). In Thailand, the emergence of antibacterial resistance has also been considerably documented in many areas including in various sections of the Maharat Nakhon Ratchasima Hospital (Chokejindachai, 2007; Maharat Nakhon Ratchasima Hospital, 2012).

Among the bacteria, *Staphylococcus aureus* is one of the most important species in clinical science and public health. It secretes staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1) with superantigen activity causing food poisoning and toxic shock syndrome, respectively. Moreover, it also causes various infectious diseases such as abscesses, skin infection, wound infection, myositis, osteomyelitis, sepsis and bacteremia (Kurlenda and Grinholc, 2012). Since the first resistance to penicillin was found in 1940 (Davies, J., and Davies, D., 2010),

the first report of methicillin- resistant *Staphylococcus aureus* (MRSA) had been published in 1961 (Chambers and DeLeo, 2009; Jevons, 1961; Barber, 1961). Nowadays, there is widespread of MRSA. The MRSA is especially troublesome in hospitals for patients with open wounds, invasive devices and weakened immune systems (Schultz, 2009). The MRSA causes many medical-therapeutic complications since *S. aureus* can pass nearly all the barriers of the host defences (Kurlenda and Grinholc, 2012; Raygada and Levine, 2009). In fact, MRSA not only resists to methicillin and β-Lactam group, but also to aminoglycosides, fluoroquinolones, macrolides and tetracyclines (Kaur and Chate, 2015). Vancomycin is the only drug which still can be used to inhibit MRSA (Pletz et al., 2010). However, in 1997, the sensitivity of MRSA to vancomycin began to decrease (Loomba et al., 2010; Thati et al., 2011). Moreover, certain bacteria develop resistance to ceftazidime including *Escherichia coli.* Virulent strains of *E. coli* are the causes of several bacterial infections, including gastroenteritis, urinary tract infections, and neonatal meningitis (Galoyan, 2012). Rarely, virulent strains can be responsible for the hemolytic-uremic syndrome, peritonitis, mastitis, septicemia and Gram-negative pneumonia (Galoyan, 2012; Kumar et al., 2013b). Antibacterial resistant *E. coli* not only resists to ceftazidime and cefotaxime (Oteo et al., 2006), but also ampicillin, cotrimoxazole, ciprofloxacin, gentamicin (Oteo et al., 2002) including amoxicillin (Kibret and Abera, 2011).

These indicate that antibiotic drugs may not be effective to cure the bacterial infections. Therefore, the research and development of new therapies for bacterial infection treatments are urgently needed (Mahady et al., 2008). Nowadays, pharmaceutical companies have become more interested in the potential use of plant-

based medicines as the antimicrobial agents (Mahady et al., 2008).

Susceptibility tests of natural products against several bacteria include: *Bacillus cereus*, *Bacillus subtillis*, *Chlamydia pneumonia*, *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), ceftazidime-resistant *Escherichia coli* (CREC), *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, vancomycin resistant *Enterococcus*, *Pseudomonas aeruginosa* and *Helicobacter pylori* (Mahady et al., 2008; Eumkeb et al., 2010).

Therefore, many scientists try to find the new strategies such as the use of medicinal plants to substitute for those antibiotics. One of the advantages of medicinal plants is: it creates minimal side effects (George, 2011) and least or no toxicities comparing to synthetic drugs (Ahmad and Beg, 2001). Crude extracts of medicinal plants are much more efficient than the only pure active compound alone (Dupont et al., 2006; Pistelli et al., 2000; Rasoanaivo et al., 2011). The medicinal plant crude extracts contain a variety of different bioactive compounds (Sasidharan et al., 2011). The natural combinations of all these bioactive compounds can exhibit synergistic activities and result in the greater antibacterial activities than those of individual pure compounds (Dupont et al., 2006). Furthermore, the loss of some bioactive compounds during purification can result in the loss of synergistic effects leading to the loss of or the decrease of antibacterial activities (El Sawi et al., 2010). Because of these, many types of research are carried on a crude extract of medicinal plants. In this research, we not only try to find out which crude extracts from which medicinal plants such as chilli fruits and nutgrass contain antibacterial substances to MRSA and CREC but also their mechanisms of actions. Therefore, this will be the alternative approach to inhibit resistant bacteria using natural antibiotics instead of synthetic antibiotics.

1.2 Research objectives

1.2.1 To investigate the effect of chilli and nutgrass crude extracts on antibacterial resistant bacteria such as *S. aureus* and *E. coli* when used singly.

1.2.2 To investigate the effect of these medicinal plant extracts in combination with antibacterial drugs against these antibiotic resistant bacteria.

1.2.3 To investigate the effect of the underlying mechanism of action of these combinations against resistant bacteria by cytoplasmic membrane (CM) permeability, transmission electron microscopy (TEM) and β-lactamase enzyme assay for β-lactamase enzyme inhibitor.

1.3 Research hypothesis

1.3.1 The bioactive compounds present in medicinal crude extracts of these following plants, chilli and nutgrass, can show antibacterial activity against antibiotic resistant bacteria.

1.3.2 These medicinal plant crude extracts in combination with antibiotics can show antibacterial activity against antibiotic resistant bacteria.

1.3.3 The underlying mechanism of action of these combinations against antibiotic resistant bacteria may be elucidated.

1.4 Scope and limitation of the study

1.4.1 The medicinal plants, chilli fruits, and tubers and rhizomes of Nutgrass were purchased from a local market and herb market in Nakhon Ratchasima, Thailand. They were extracted by soxhlet extractor followed by evaporation of the

solvents by rotary evaporator.

1.4.2 The following antibiotic resistant bacteria, *S. aureus* and *E. coli* were obtained from the Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand. The positive control, ATCC strains were obtained from ATCC, USA.

1.4.3 Ampicillin and amoxicillin were obtained from Sigma-Aldrich.

1.4.4 Checkerboard assays of the combinations that show the lowest MIC were selected for further investigations such as cytoplasmic membrane (CM) permeability, transmission electron microscopy (TEM) and β-lactamase enzyme assay for β-lactamase enzyme inhibitor.

1.5 Expected results

1.5.1 To contribute additional scientific data on the synergism antimicrobial activity of the combination between medicinal plant crude extracts and antibiotic drugs.

1.5.2 To contribute the novel knowledge for further investigations, such as the mechanism of action of medicinal plant crude extracts and antibiotic drugs on antibiotic resistant bacteria.

1.5.3 To contribute the useful results for the development of new drug combination against resistant bacteria.

1.5.4 To contribute the benefit to physicians and patients in case of tackling most dangerous resistant bacteria by using new antibacterial combination drugs.

CHAPTER II

LITERATURE REVIEW

2.1 Bacterial structure

The bacterium is a prokaryotic cell that does not have a membrane-bound nucleus (Todar, 2012). A prokaryotic cell comprises of essential structural components, including cell wall (murien or murien sacculus), cell membrane (plasma membrane or cytoplasmic membrane), a nucleoid (chromosomal DNA) and ribosomes (Todar, 2012).

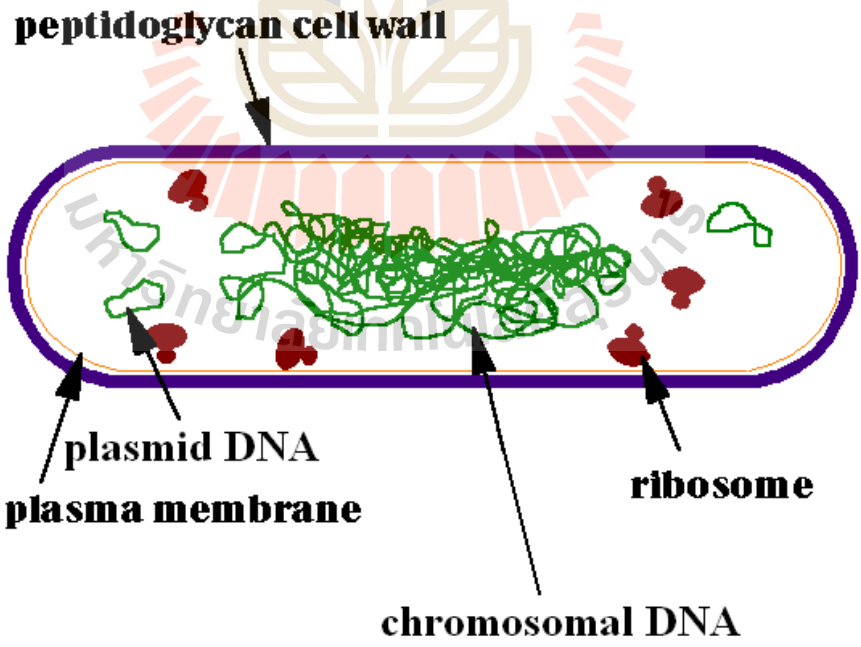

Figure 2.1 Bacterial basic structures (modified from http://www.ucmp.berkeley.edu/bacteria/bacteriamm.html).

2.1.1 Bacterial cell wall

The primary function of bacterial cell wall is to maintain the cell shape (Scheffers and Pinho, 2005). Bacteria are divided into Gram-positive and Gram-negative bacteria groups, depending on their staining characteristics with Gram stain. Gram-negative bacteria are more resistant than Gram-positive bacteria may be due to the differences in the cell wall composition (Oskay et al., 2009; Ahmad and Beg, 2001). The peptidoglycan layer of Gram-negative bacteria is thinner than that of Gram-positive bacteria but surrounded and protected by an outer membrane, whereas Gram-positive bacteria lack the outer membrane (Silhavy et al., 2010). This outer membrane serves as a protective barrier for peptidoglycan layer from the harsh environment including antibacterial agent (Silhavy et al., 2010). The vulnerability of Gram-positive bacteria comparing to Gram-negative bacteria is a consequence of the absence of its outer membrane (Silhavy et al., 2010). The peptidoglycan chain is composed of alternating *N*-acetyl-muramic acid (NAM) with short amino acid side chain and *N*-acetylglucosamine (NAG) units linking together by β-(1,4)-glycosidic bond (Drawz and Bonomo, 2010; Srivastava and Srivastava, 2003). The cross-linking of each peptidoglycan chain form a rigid bacterial cell wall (Drawz and Bonomo, 2010). This cross-linking occurs between D-alanine and L-lysine (the third amino acid) (for Gram-positive bacteria); D-alanine and L-lysine or L-diaminopimelic acid (the third amino acid) (for Gram-negative bacteria) in amino acid side chains of NAMs (Srivastava and Srivastava, 2003). The linkage is catalysed by the transpeptidase enzyme contained in penicillin binding protein (PBP) (Drawz and Bonomo, 2010). In Gram-positive bacteria, there is a pentapeptide (pentaglycine) bridge attached between NAM units of two peptidoglycan chains (Drawz and

Bonomo, 2010; Srivastava and Srivastava, 2003). On the other hand, Gram-negative bacteria contain no peptide bridge. Hence the cross-linking between NAM units of two peptidoglycan chains occurs from the direct binding of the short amino acid side chains between two NAM units (Srivastava and Srivastava, 2003).

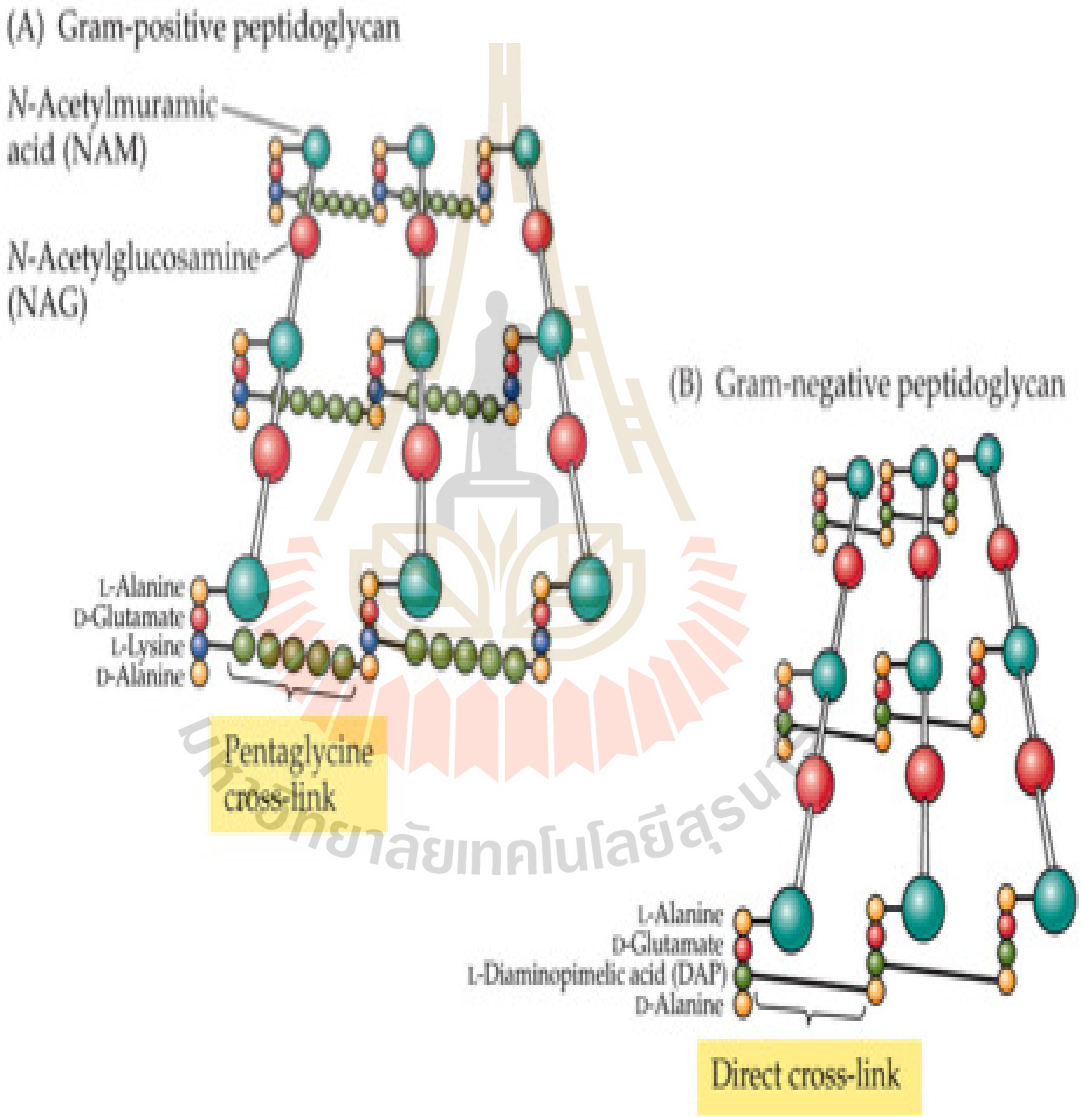

Figure 2.2 Peptidoglycan structures of Gram-positive and Gram-negative bacteria (https://www.studyblue.com/notes/note/n/morphology-pt-ii-111-172/deck/21278).

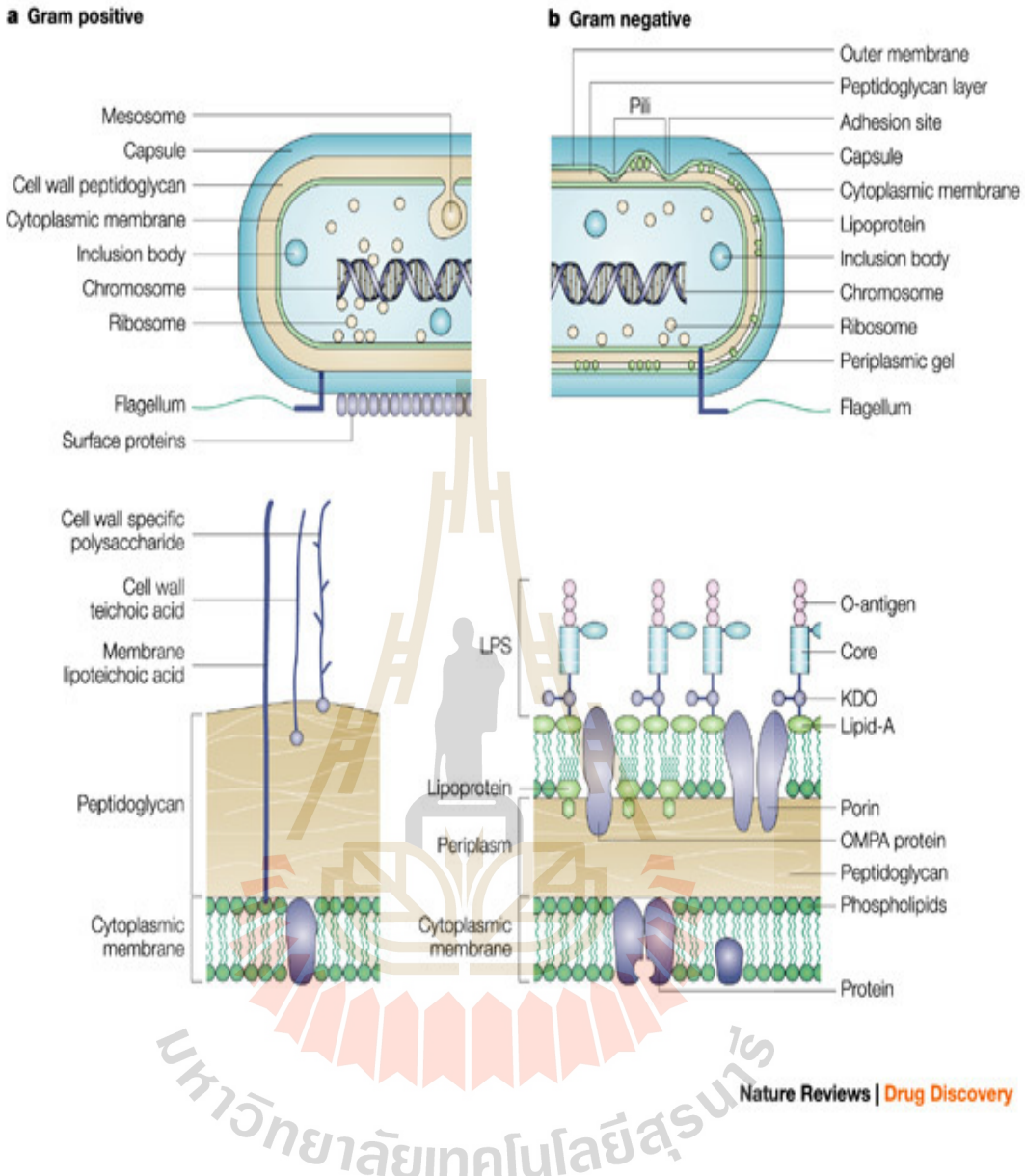

Figure 2.3 Bacterial structures of Gram-positive and Gram-negative bacteria (http://www.nature.com/nrd/journal/v2/n8/fig_tab/nrd1153_F1.html).

2.1.2 Bacterial cell membrane

The bacterial cell membrane is composed of phospholipids and proteins. The functions of cell membrane included permeability barrier, transport of solutes, and location of numerous enzyme systems (Todar, 2012).

2.1.3 Bacterial nucleoid

The bacterial nucleoid is chromosomal DNA which functions as the genetic material of the cell (Todar, 2012).

2.1.4 Bacterial ribosome

The bacterial ribosome functions as a site for protein translation (Todar, 2012).

2.2 Resistant bacteria

2.2.1 *Staphylococcus aureus*

Staphylococcus aureus is Gram-positive coccal bacterium causing a range of sickness from the infection of minor skin, such as pimples, impetigo, boils (furuncles), cellulitis folliculitis, carbuncles, scalded skin syndrome, wound infections and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteraemia, and sepsis (Kurlenda and Grinholc, 2012; Kumar et al., 2013a). It is one of the five most common causes of nosocomial infections, often causing postsurgical wound infections (Vijayalakshmi, 2015). Nowadays, *S. aureus* has been becoming resistant to many commonly used antibiotics, for example penicillin, methicillin, fluoroquinolone and vancomycin.

2.2.2 *Escherichia coli*

Escherichia coli is one of Gram-negative rod-shaped bacteria found as a normal flora in the lower intestine of warm-blooded organisms (Kumar et al., 2013b). Normally, the bacteria benefit the hosts by producing vitamin K and preventing the establishment of pathogenic bacteria within the intestine (Kumar et al., 2013b). Most

of *E. coli* strains are harmless, but some serotypes cause serious food poisoning (Kumar et al., 2013b). Moreover, it can cause many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), traveller's diarrhoea, neonatal meningitis and pneumonia (Khan et al., 2013). In rare cases, virulent strains are responsible for the haemolytic-uremic syndrome (HUS), peritonitis, mastitis, septicemia and Gram-negative pneumonia (Kumar et al., 2013b). At present, *E. coli* has been reported to be resistant to amoxicillin/clavulanic acid, ciprofloxacin, trimethoprim/sulfamethoxazole and gentamycin (Tadesse et al., 2012).

2.3 Antibacterial drugs (emphasise on *β*-lactam and macrolide drugs)

2.3.1 *β*-lactam drugs

β-lactam drugs are antibiotics containing *β*-lactam rings in their chemical structures (Brunton et al., 2008). The *β*-lactam drugs include penicillins, cephalosporins, monobactams and carbapenems (Rang et al., 2012).

2.3.1.1 Penicillins

Penicillins are composed of a thiazolidine ring connecting to a *β*-lactam ring which is attached to a side chain (Brunton et al., 2008). The *β*-lactam ring can be degraded by the *β*-lactamase enzyme to produce penicilloic acid lacking the antibacterial property (Shendurnikar and Pandya, 1996). Penicillins can also be divided into 4 groups (Rang et al., 2012).

1. Benzylpenicillin and its congeners such as penicillin G (benzylpenicillin) and penicillin V (phenoxymethylpenicillin) (Rang et al., 2012) inactivate sensitive strains of Gram-positive cocci (Brunton et al., 2008).

2. *β*-lactamase resistant penicillins for example, methicillin,

flucloxacillin and temocillin, can resist to a β-lactamase (Rang et al., 2012). However, methicillin drug is discontinued in the United States (Brunton et al., 2008).

3. Broad spectrum penicillins such as ampicillin and amoxicillin (Rang et al., 2012) whose antibacterial activity is extended to include some Gram-negative bacteria such as *Haemophilus influenzae*, *Escherichia coli*, and *Proteus mirabilis* (Brunton et al., 2008).

4. Extended spectrum penicillins such as ticarcillin and piperacillin (Rang et al., 2012) whose antibacterial activity is similar to board spectrum penicillins but with the coverage for *Pseudomonas* (Brunton et al., 2008).

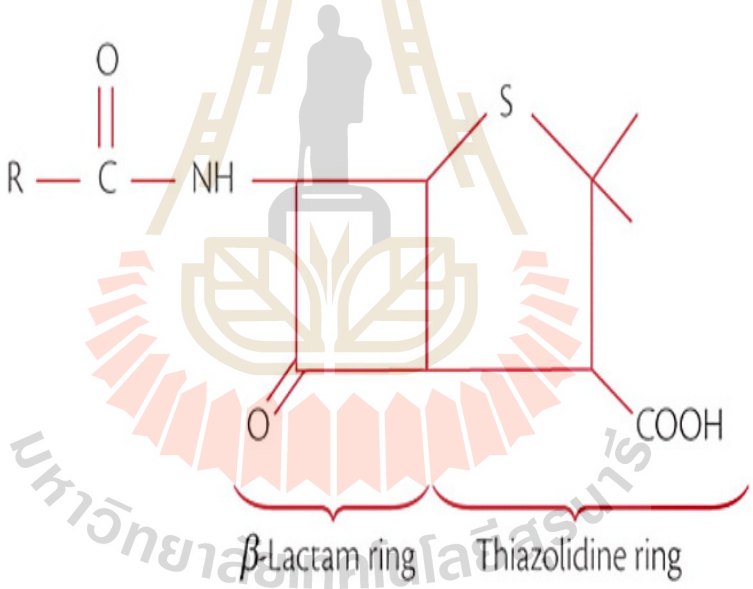

Figure 2.4 Penicillin structure indicating the β-lactam and thiazolidine rings (http://www.bmb.leeds.ac.uk).

2.3.1.2 Cephalosporins

Cephalosporins are quite similar to penicillins regarding the mechanism (Brunton et al., 2008) but more stable to several bacterial β-lactamases.

Thus, they have a broader spectrum of activity (Deck and Winster, 2012a). Cephalosporins can be divided into 4 generations, according to the spectrum of antimicrobial activity (Marino, 2007).

1. First generation cephalosporins such as cefalexin, cefradine and cefadroxil (Rang et al., 2012) can inhibit aerobic Gram-positive cocci but not *Staphylococcus epidermidis* and methicillin-resistant strains of *Staphylococcus aureus* (Marino, 2007).

2. Second generation cephalosporins such as cefaclor and cefuroxime (Rang et al., 2012) can inhibit Gram-negative aerobic and anaerobic bacilli of enteric origin (Marino, 2007).

3. Third generation cephalosporins for example, cefotaxime, ceftriaxone, cefixime, cefpodoxime and ceftazidime (Rang et al., 2012) can inhibit Gram-negative aerobic bacilli, *Pseudomonas aeruginosa* and *Haemophilus influenzae* (Marino, 2007).

4. Fourth generation cephalosporins can inhibit Gram-negative bacteria and some Gram-positive coverage. The only cephalosporin in this generation is cefepime (Marino, 2007).

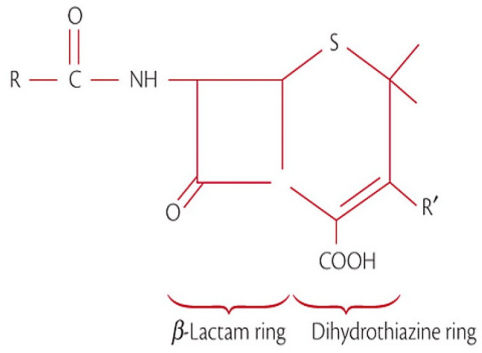

Figure 2.5 Cephalosporin structure (http://www.bmb.leeds.ac.uk).

2.3.1.3 Monobactams

Monobactams including aztreonam can resist to a β-lactamase enzyme (Rang et al., 2012).

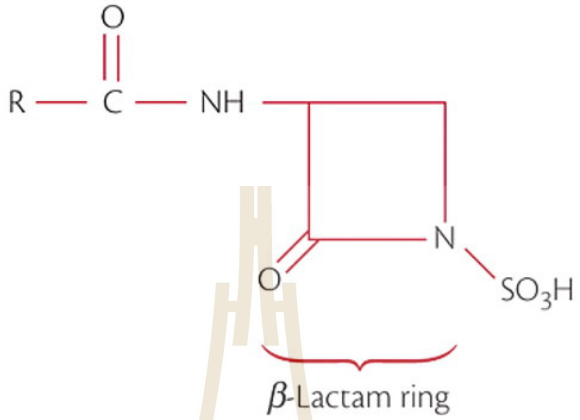

Figure 2.6 Monobactam structure (http://www.bmb.leeds.ac.uk).

2.3.1.4 Carbapenems

Carbapenems have a broader spectrum of activity than that of other β-lactam drugs. Furthermore, some of the carbapenems such as imipenem are resistant to a β-lactamase enzyme (Brunton et al., 2008).

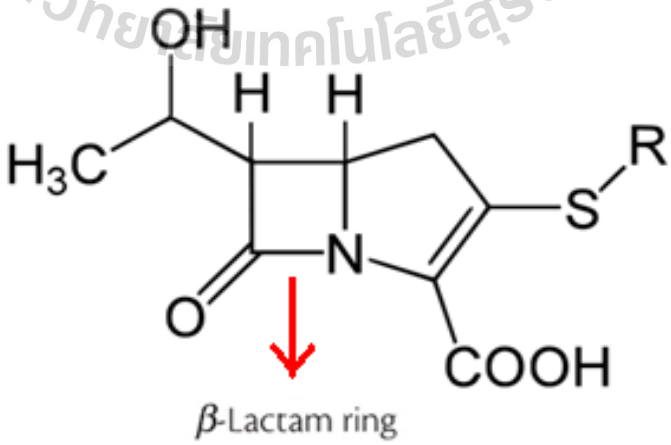

Figure 2.7 Carbapenem structure (http://www.bmb.leeds.ac.uk).

2.3.2 Macrolide drugs

Macrolide drugs are a group of antibiotics whose activity stems from the presence of a macrocyclic lactone rings to which one or more deoxy sugars are attached (Rang et al., 2012; Brunton et al., 2008; Deck and Winster, 2012b). They can interfere with bacterial protein synthesis by inhibiting the translocations of tRNAs from A-sites to P-sites. The drugs bind to 50S subunits of ribosomal RNAs of the bacteria (Rang et al., 2012; Deck and Winster, 2012b; Brunton et al., 2008). The examples of macrolide members are as follows:

1. Erythromycin inactivates most of the aerobic enteric Gram-negative bacilli (Brunton et al., 2008).

2. Clarithromycin can inactivate sensitive strains of *Staphylococci* and *Streptococci* (Brunton et al., 2008).

3. Azithromycin highly inactivates *Moraxella catarrhalis*, *Pasteurella multocida*, *Chlamydia* spp., *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Borrelia burgdorferi*, *Fusobacterium* spp. and *Neisseria gonorrhoeae* (Brunton et al., 2008).

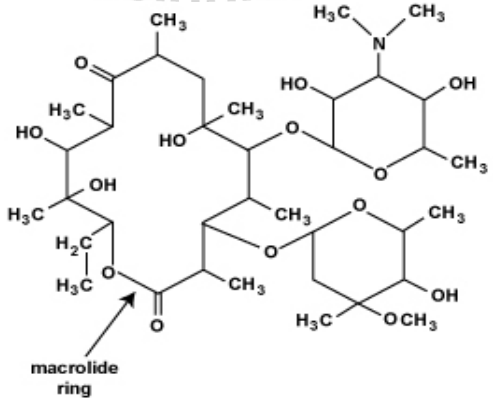

Figure 2.8 Macrolide structure (http://archive.microbelibrary.org).

2.4 Antibiotics and their mechanisms of actions

Antibiotics are one of the most frequently prescribed clinical medications. Antibiotics cure diseases by killing or injuring bacteria. The first antibiotic, penicillin from a mould culture, was founded by Alexander Fleming in 1928 (Wright, 2005; Al-Haroni, 2008; Davies, J., and Davies, D., 2010). The discovery of the *β*-lactam antibiotics is the turning point against pathogenic bacteria (Andersson et al., 2001). Nowadays, over 100 different antibiotics are available for modern medicine (Gould, 2005). Types of antibiotics, according to their mode of mechanisms are as follows: (1) interfere with bacterial cell wall synthesis such as penicillin, (2) interfere with the function of bacterial cell membrane, (3) interfere with bacterial protein synthesis, (4) interfere with the function of bacterial nucleic acid and (5) interfere with a metabolic pathway of bacteria (Tenover, 2006).

2.4.1 Interfere with bacterial cell wall synthesis

The *β*-lactam antibiotic drugs such as penicillins, cephalosporins, monobactams and carbapenems contain *β*-lactam rings in their chemical structures (Brunton et al., 2008; Rang et al., 2012) which can inhibit the cross-linking of peptidoglycan chains (Lancini and Parenti, 1982). The cross-linkage between two peptidoglycan chains for bacterial cell wall synthesis is completed by a transpeptidase enzyme in penicillin-binding protein (PBP). The *β*-lactam ring covalently binds to the active site of PBP on bacteria, thus, inhibit the transpeptidation reaction which performs as the last step of bacterial cell wall synthesis (Brunton et al., 2008; Deck and Winster, 2012a). The binding of the *β*-lactam ring to PBP results in the acylation of the PBP rendering the transpeptidase enzyme incapable to catalyse the transpeptidation reaction (Drawz and Bonomo, 2010). As the rate of cell wall

synthesis decreases to a halt, the rate of constitutive peptidoglycan autolysis continues. Therefore, the breakdown of peptidoglycan subsequently leads to cell wall compromise, inability to maintain cell shape and increased permeability causing bacterial cell lysis (Drawz and Bonomo, 2010). The *β*-lactam antibiotics inactivate bacterial cells only when the bacteria are actively growing and synthesising cell walls (Deck and Winster, 2012a). These can be obviously demonstrated in log phase.

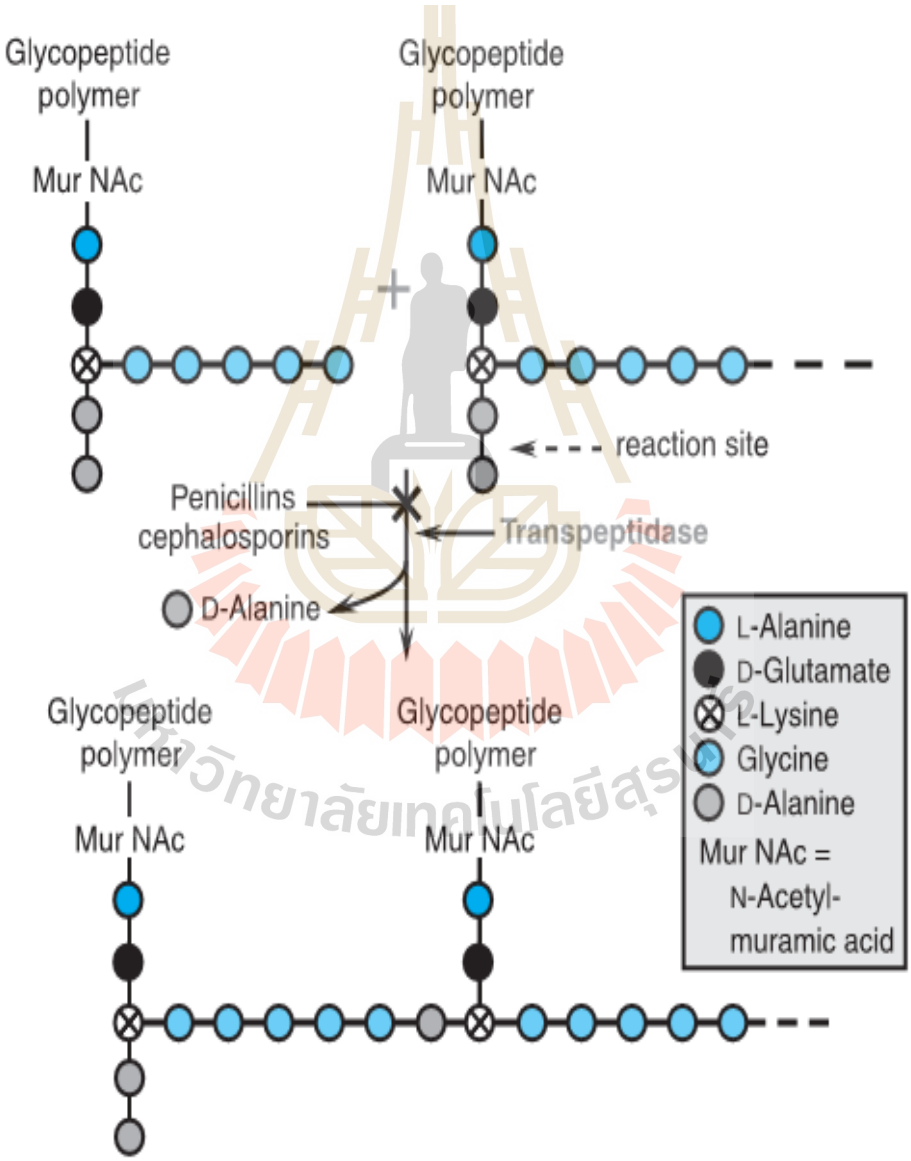

Figure 2.9 Mechanism of action for *β*-lactam antibiotics in *Staphylococcus aureus* (Brunton et al., 2008).

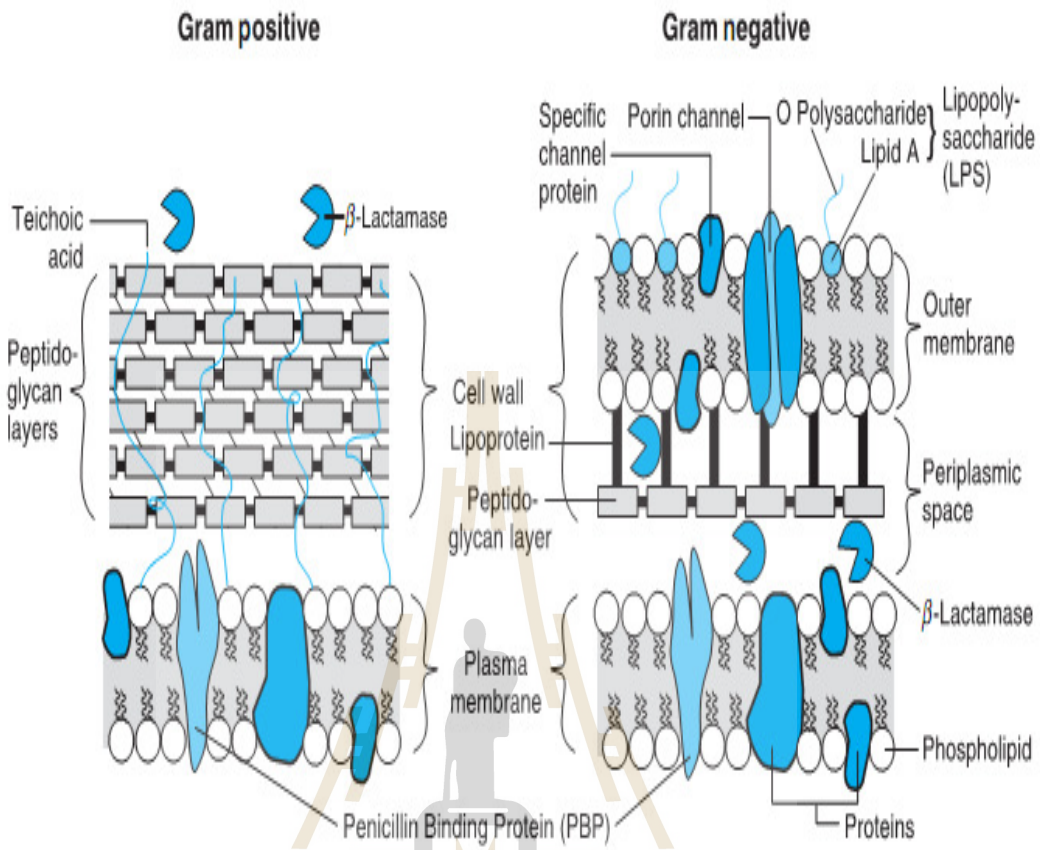

Figure 2.10 The position of of the *β*-lactamase enzyme in Gram-positive and Gram-negative bacteria (Brunton et al., 2008).

Another example such as cycloserine which can inhibit the formation of peptidoglycan building blocks (Lancini and Parenti, 1982). The cycloserine inhibits alanine racemase enzyme that alters the L-alanine to D-alanine leading to the inhibition of uridine diphosphate (UDP)-*N*-acetylmuramyl-pentapeptide, the precursor of peptidoglycan (Lambert and Neuhaus, 1972; Neuhaus, 1967). Furthermore, cycloserine also inhibits the activity of D-alanine:D-alanine ligase or alanylalanine synthetase that can combine the two molecules of D-alanines (Lambert and Neuhaus, 1972). Ristocetin and vancomycin inhibit the growing of peptidoglycan chains. They

interfere with the transfer of muramyl-pentapeptide-acetylglucosamine from the lipid carrier to the peptidoglycan being formed (Lancini and Parenti, 1982), thus, inhibit a late stage of bacterial cell wall synthesis (Cesur and Demiroz, 2013; Tenover, 2006).

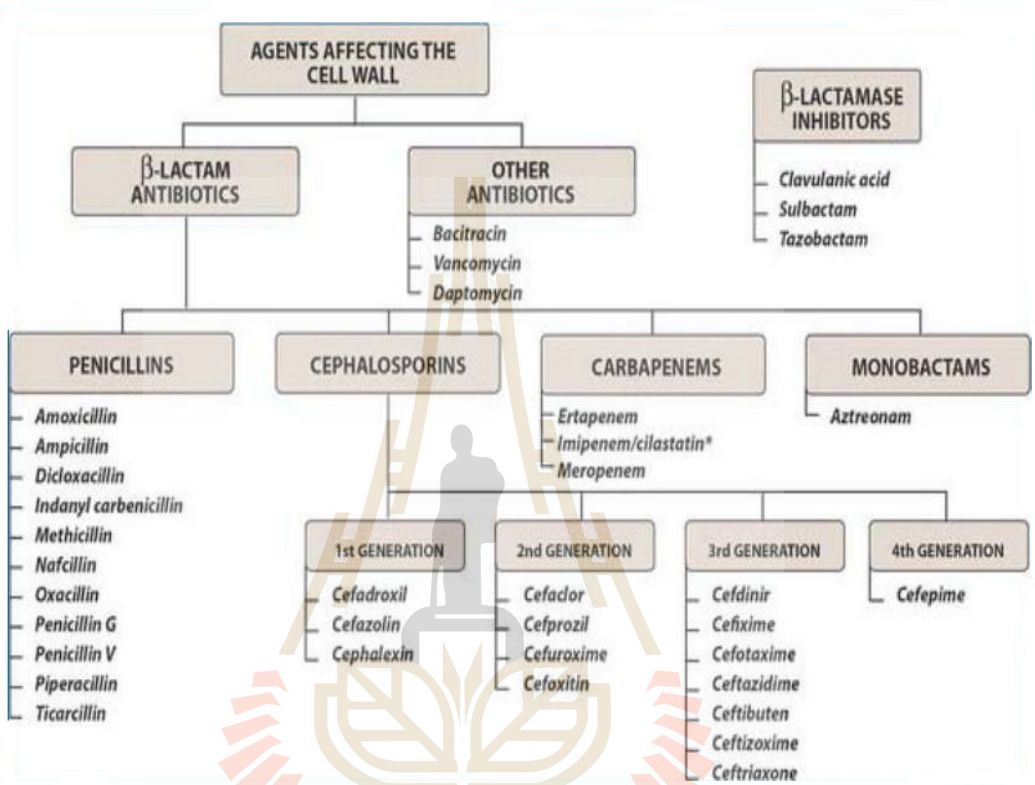

Figure 2.11 Summary of antimicrobial agents that inhibit cell wall synthesis (Harvey et al., 2009).

2.4.2 Interfere with the function of bacterial cell membrane

The molecules of polymyxin and colistin, amphipathic agents, contain both lipophilic and lipophobic parts. These antibiotics can bind to bacterial cell membrane resulting in permeability change (De Gaudio et al., 2005). Consequently, the increased cell membrane permeability causes the leakage of bacterial cell contents, including nucleotides, inorganic ions and proteins from the cell membrane (Scientific

Committee on Emerging and Newly Identified Health Risks, 2009; Tenover, 2006). The cyclic lipopeptide daptomycin induces depolarisation of the outer membrane, leading to subsequent cell death by inserting its lipid tail into the cell membrane of bacteria (Scientific Committee on Emerging and Newly Identified Health Risks, 2009; Tenover, 2006). Gramicidin is a membrane-active antibiotic which can produce aqueous pores in the membrane (Neu and Gootz, 1996). It causes the travel of inorganic monovalent cations such as Na^+, K^+ and H^+ through the cell membrane unrestricted (Steller et al., 2012), therefore, destroying the balance of ion gradient between cytoplasm and extracellular environment (Quigley and Gross, 1994). Finally, this circumstance is followed by osmotic swelling, and cell lysis (David et al., 2013).

2.4.3 Interfere with bacterial protein synthesis

Macrolidesand chloramphenicol can bind to 50S subunit of the ribosome (Tenover, 2006), thus, inactivate the interaction between peptidyl transferase and amino acid resulting in no formation of of the peptide bond (Brenner and Stevens, 2013). Therefore, these drugs inhibit protein synthesis of the bacteria. Tetracycline can bind to 30S subunit of the ribosome (Tenover, 2006) to interrupt the binding between aminoacyl-tRNA and bacterial ribosome (Chopra and Roberts, 2001) resulting in no formation of peptide strand and hence inhibit the growing peptide chain (Brenner and Stevens, 2013). Furthermore, streptomycin can bind to the 70S ribosome. Therefore, this 70S ribosome cannot be separated into 30S and 50S subunits leading to the destruction of the ribosome (Dax, 1997). Moreover, mupirocin binds to isoleucyl-tRNA synthetase of bacteria (Tenover, 2006). The structure of the bacterial ribosome is different from that of the eukaryotic ribosome including the human ribosome. Bacterial ribosome (70S complex) comprises 50S and 30S subunits

while the eukaryotic ribosome including the human ribosome (80S complex) is composed of 60S and 40S subunits. Antibiotic drugs take advantage of this difference to selectively inhibit only the bacterial growth (Tenover, 2006). However, there are some reports indicate the cytotoxic effects of 70S ribosome antibiotic inhibitors on eukaryotic cells. The effect of streptomycin on chloroplasts in *Chlamydomonas* is well documented while erythromycin shows a similar inhibitory effect on chloroplasts in *Euglena* (Weisblum and Davies, 1968). In fact, eukaryotic ribosome is 80S complex, but mitochondrial and chloroplast ribosomes are 70S complexes, therefore, at least in part, these drugs may exert their actions to inhibit 70S complexes in mitochondria and chloroplast (Weisblum and Davies, 1968). In addition, streptomycin has been reported to produce antibodies with altered serological activity (Weisblum and Davies, 1968). It is still not clear whether the effect comes from a direct action on eukaryotic protein synthesis or the formation of a streptomycin-antigen complex (Weisblum and Davies, 1968).

2.4.4 Interfere with the function of bacterial nucleic acid

Novobiocin inhibits DNA synthesis by inactivating DNA gyrase, also known as topoisomerase II (Burlison et al., 2006). Sulfonamides and trimethoprim block the pathway of folic acid synthesis ultimately inhibit DNA synthesis (Tenover, 2006). In addition, actinomycin D inhibits both DNA and RNA synthesis, whereas rifampicin inactivates the activity of RNA polymerase resulting in inhibition of transcription (Jaiswal and Agrawal, 2010).

2.4.5 Interfere with a metabolic pathway of bacteria

Folic acid analogues and sulfonamides inactivate the enzymatic pathway for bacterial folate synthesis, which acts as a co-enzyme in many biosynthetic

reactions, therefore, inhibit a metabolic pathway (Tenover, 2006).

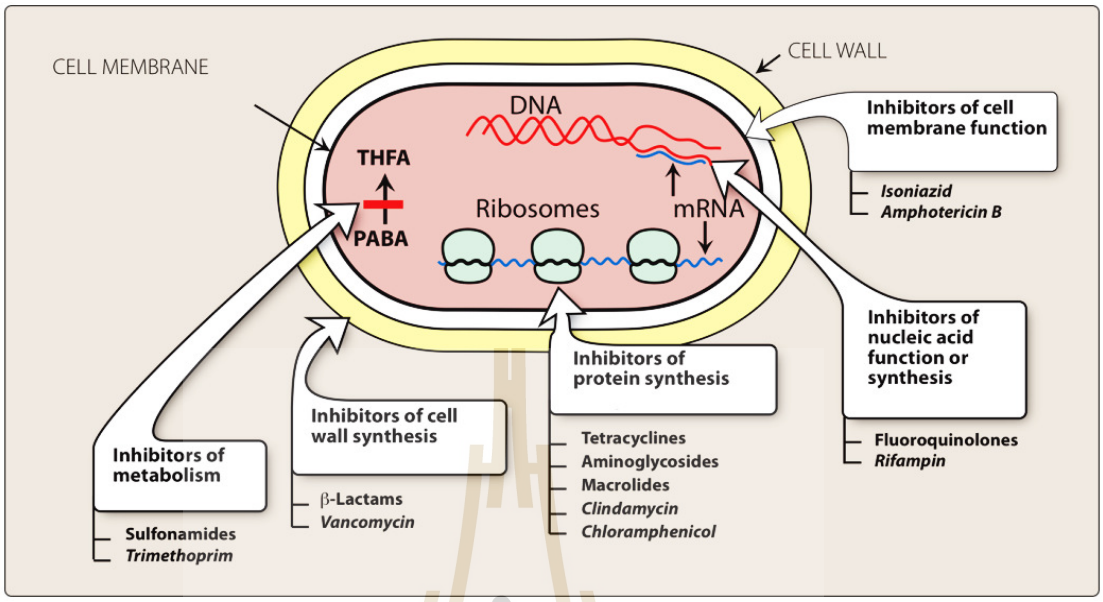

Figure 2.12 Mechanisms of action of antibiotic drugs (Modified from http://microbiology.blogspot.com/2013/04/control-of-mos-by-antibiotics-other_3.html).

Table 2.1 Examples of antibiotics and their mechanisms.

Interfere with bacterial cell wall synthesis	Interfere with the function of bacterial cell membrane	Interfere with bacterial protein synthesis	Interfere with the function of bacterial nucleic acid	Interfere with a metabolic pathway of bacteria
Penicillin	Colistin	Macrolides	Novobiocin	Sulfonamides
Bacitracin	Polymyxin	Chloramphenicol	Sulfonamides	Folic acid analogues
Vancomycin	Gramicidin	Streptomycin	Trimethoprim	
Oxacillin	Daptomycin	Mupirocin	Rifampicin	
Cephalexin		Tetracycline		
Cycloserine				
Ristocetin				

2.5 Antibiotic resistance

Upon the discovery and the clinical use of antibiotics, the resistance to these antibiotics has been observed. Since the introduction in 1937 of the first effective antibiotic, sulfonamides, the bacteria have educated to resist this antibiotic and plagued their therapeutic use (Davies and Davies, 2010). The antibiotic drug resistance in pathogens is caused from drug overuse (Ventola, 2015). Frequently, when bacteria acquire resistance to a certain antibiotic from a particular class (e.g. penicillins), the bacteria also acquire resistance to all other antibiotics in that class. Bacteria can develop resistance to the new drugs as fast as they are introduced. Many effects of antibiotics on bacterial physiology and ecology indicate how difficult it can be to predict the return of bacterial susceptibility (Heinemann et al., 2000). Antibiotic resistance is a major problem for the treatment of infections in hospitals and communities (Levy, 2002). Antibiotic resistance of bacteria is increasing while the production of new antibiotics is slow (Russell, 2002). For example, it is widespread to hear about methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), multidrug resistance in *Mycobacterium tuberculosis* (MDRTB) strains and multi-drug-resistant (MDR) Gram-negative bacteria including penicillin resistance in *Neisseria gonorrhoeae* (Russell, 2002). Therefore, many antibiotics can no longer be used for the treatment of infections caused by antibiotic resistant bacteria (Russell, 2002). Antibiotic resistance usually interferes with binding of antibiotics and bacteria. Antibiotic resistance occurs through four mechanisms (Poole, 2002): (1) target alteration to prevent the interaction of antibiotic with target by modification of the antibiotic target (Wright, 2005; Poole, 2002), (2) impermeability (Poole, 2002), (3) enzymatic destruction or modification: direct

destruction or modification of the antibiotic by synthesis of bacterial modifying enzyme that selectively target and destroy antibiotic activity (Wright, 2005; Poole, 2002) and (4) efflux of the antibiotic from the bacterial cell through a collection of membrane-associated pumping proteins (Poole, 2002; Wright, 2005). All these mechanisms require new genetic programming by the bacterial cell in response to the presence of antibiotics (Wright, 2005).

2.5.1 Target alteration

Spontaneous mutation of a bacterial gene on the chromosome and the selection in the presence of antibiotic confer target changes (Lambert, 2005). Changes in drug targets within the microorganism can interfere with the interaction between antibiotics and bacteria, thus, promote antibiotic resistance (Poole, 2002). For example, the acquisition of high-level resistance to methicillin and other β-lactam antibiotics in MRSA arises from the expression of *mecA* gene. The *mecA* gene is carried on a large genetic element known as the staphylococcal cassette chromosome mec (SCCmec) that can be integrated into the chromosome of MRSA near the origin of replication (Fitzgerald and Musser, 2003; Peacock and Paterson, 2015). This gene regulates the alteration of transpeptidase by encoding penicillin-binding protein 2a (PBP2a) (Peacock and Paterson, 2015). The production of this low-affinity penicillin-binding protein PBP2a leads to the unstable binding between β-Lactam ring and PBP2a, thus, confers resistance to the bacteria (Poole, 2002). The example of β-Lactam resistance bacteria associated with PBP changes has been reported in *Staphylococcus aureus*, *Streptococcus pneumonia* and *Acinetobacter baumanii* (Beceiro et al., 2013). Moreover, modification of the target site on ribosome such as methylation of an adenine residue in domain V of 23S rRNA resulting in macrolides

resistance (Poole, 2002). Bacterial resistance to fluoroquinolones has occurred through the mutations which affect the fluoroquinolone targets, DNA gyrase and topoisomerase (e.g. in *gyrA* and *parC*) (Poole, 2002).

2.5.2 Impermeability

To exert the activities of antibiotics on bacteria, the antibiotics must cross the outer membrane, a permeability barrier to access the intracellular targets (Poole, 2002). The intrinsic resistance of several Gram-negative bacteria to macrolides occurs by reducing the bacterial permeability such as porin of the outer membrane to macrolides (Poole, 2002; Bockstael and Aerschot, 2009). The antibiotic resistance by the bacterial outer membrane barrier is only significant in the context of other resistance mechanisms, such as efflux and β-lactamases that work synergistically to promote the antibiotic resistance (Poole, 2002). In addition, the limitation of antibiotic access to intracellular targets probably, in part, contributes to the biofilm antibiotic resistance (Poole, 2002).

2.5.3 Enzymatic destruction or modification

2.5.3.1 Hydrolysis

Many antibiotics contain chemical bonds, such as ester and amide, which are hydrolytically susceptible. They become the targets for antibiotic resistance to cleave these vulnerable bonds by the bacterial enzymes, thus, result in destroying antibiotic activities (Wright, 2005). Moreover, these enzymes require only water as a co-substrate and can frequently be excreted by the bacteria, therefore inhibiting the antibiotics before they reach the bacteria (Wright, 2005). One of the most well-known examples is β-lactamase. The β-lactamase is an enzyme that inactivates β-lactam antibiotic by catalysing the hydrolysis of the β-lactam ring of the molecule (Poole,

2002). There are four molecular classes of β-lactamases as follows: Class A penicillinases, Class B metallo-β-lactamases, Class C cephalosporinases and Class D oxacillinases (Poole, 2002). Classes A, C, and D are serine enzymes while class B is zinc metalloenzymes (Drawz and Bonomo, 2010). The class B β-lactamases are the most dangerous one since they can inactivate nearly all β-lactam drugs, including carbapenem antibiotics (Al-Haroni, 2008). Gram-positive bacteria usually excrete the β-lactamase in large amounts, thus, in the mixed infections, they may also protect other microbes presenting on the infection site (Al-Haroni, 2008). The β-lactamases employ two main mechanisms to hydrolytically cleave the β-lactam rings of penicillins and cephalosporins through an active site Ser nucleophile or an activation of water via Zn^{2+} centre (Wright, 2005). While β-lactamases inactivate β-lactam activity, macrolide esterases also inhibit the activity of macrolide antibiotics such as erythromycin (Wright, 2005). These resistances occur from the mutation of chromosomal genes or the acquisition of extrachromosomal genetic elements such as plasmids or transposons carrying the resistant genes (Poole, 2002).

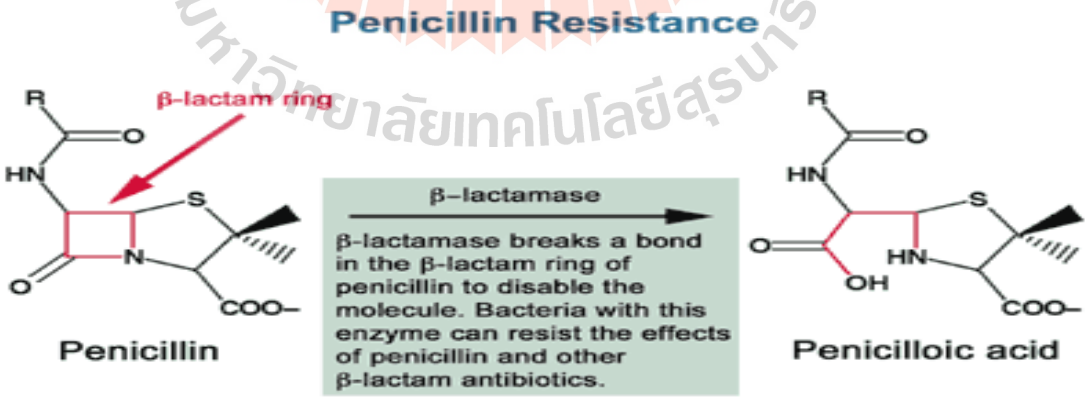

Figure 2.13 β-lactamase activates the hydrolytic opening or the breaking of β–lactam ring of antibiotic (http://www.wiley.com/college/pratt/0471393878/student/activities/bacterial_drug_resistance/index.html).

2.5.3.2 Group transfer

The group of transferase enzymes is the most diverse and the largest family of resistance enzymes. These include acyltransferase, phosphotransferase, glycosyltransferase, nucleotidyltransferase (Wright, 2005), ADP-ribosyltransferase, and Glycosyltransferase. These enzymes can covalently modify antibiotics, leading to structural alteration that weaken the target binding (Wright, 2005). Moreover, these enzymes are only active in the cytosol, since they require a co-substrate for their activity, including ATP, acetyl-Co A, NAD or UDP glucose (Wright, 2005).

2.5.4 Efflux

Efflux is the drug exclusion of bacteria to limit drug accumulation. Antibiotics can be pumped out of the bacterial cells. Most of these are agent specific that provide resistance to a single or a class of antibiotics. However, there are examples of efflux systems that accommodate and provide resistance to a broad range of structure unrelated antibiotics, known as multidrug efflux systems (Poole, 2002).

The efflux is most commonly associated with the tetracyclines such as TetA, TetB and TetK pumps (Chopra and Roberts, 2001; Poole, 2002) and the fluoroquinolones (Poole, 2002). The *tet* genes are typically encoded by plasmid or transposon. Therefore, the resistance arises from the acquisition of the extrachromosomal genetic elements from external sources (Poole, 2002). Many efflux systems provide the resistance to macrolides, *β*-lactams, aminoglycosides and tetracycline (Poole, 2002). Furthermore, the increase of macrolide resistance in *Streptococcus pneumoniae* is due to efflux (Poole, 2002). In addition, it has been reported that one of the resistant mechanisms to macrolide antibiotics of staphylococci is macrolide-specific efflux mechanism by the *msr(A)* gene which

mediates ABC-transporter-mediated efflux located on pMS97 plasmid present in *S. aureus* (Matsuoka et al., 2003).

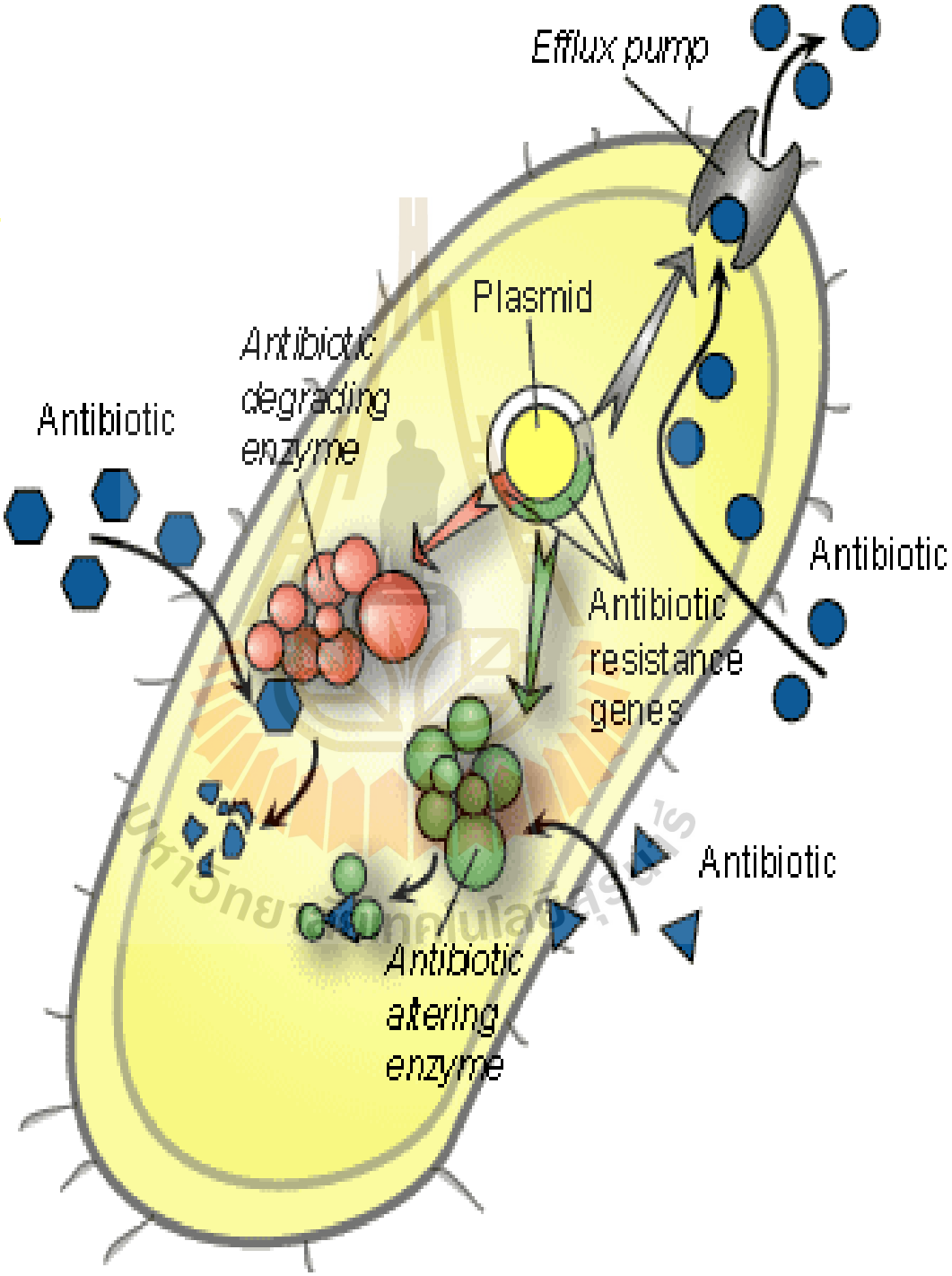

Figure 2.14 Mechanisms of antibiotic resistance (Roe, 2008).

Table 2.2 Antibiotic resistance and their mode of mechanisms.

Target alteration	Impermeability	Enzymatic modification or destruction	Efflux
Macrolide resistant bacteria	Macrolide Gram-negative bacteria	*β*-lactam resistant bacteria	Tetracycline resistant bacteria
		Macrolide resistant bacteria	Fluoroquinolone resistant bacteria
Methicillin resistant *Staphylococcus aureus* (MRSA)			Macrolide resistant bacteria
			β-lactam resistant bacteria
Fluoroquinolone resistant bacteria			Aminoglycoside resistant bacteria
			Macrolide resistant bacteria *Streptococcus pneumoniae*

Table 2.3 Enzymatic mechanism and group transfer of antibiotics resistance (Wright, 2005).

Mechanisms	Types	Antibiotics affected
Hydrolysis		*β*-lactam
		Macrolide
Group transfer	Acyl	Aminoglycoside
		Chloramphenicol
		Type A streptogramin
	Phosphoryl	Aminoglycoside
		Macrolide
		Rifamycin
	Thiol	Fosfomycin
	Nucleotidyl	Aminoglycosinde
		Lincosamide
	ADP-ribosyl	Rifamycin
	Glycosyl	Macrolide
		Rifamycin

2.6 The development of bacterial resistance

Bacterial resistance to antibiotics can be either natural (inherent, intrinsic) or acquired.

2.6.1 Natural (inherent, intrinsic) resistance

Natural (inherent, intrinsic) resistance is not sensitive to some antibiotics due to the lacking of certain molecular structures that serve as the target molecules for antibiotics or the lacking of metabolic processes essential for activation of the antibiotics (Al-Haroni, 2008). For example, bacteria without a cell wall, *Mycoplasma*

species are naturally resistant to antibiotics such as β-lactam which its activity against bacterial cell wall (Al-Haroni, 2008). Other examples, *Actinomyces* species, *Streptococcus* species, and *Aggregatibacter* lack the enzyme nitroreductase which is not only necessary to convert metronidazole to its active metabolites but also is not affected by the drug at normal therapeutic concentrations (Al-Haroni, 2008).

2.6.2 Acquired resistance

Acquired resistance in bacteria evolves with two genetic mechanisms: by chromosomal mutation or, by horizontal gene transfer between bacteria (Al-Haroni, 2008) (conjugation, transduction and transformation), both within and outside the species (Al-Haroni, 2008; Tenover, 2006). Horizontal gene transfer is the most frequent pathway for spreading of antibiotic resistant genes (Al-Haroni, 2008). The antibiotic resistance gene can be inserted into a transferable genetic element, such as plasmid, transposon, and integron, and can also be linked to other resistant genes that contained in the element (Al-Haroni, 2008). Transposons and integrons are mobile DNA elements that can integrate into bacterial chromosomes or plasmids (Al-Haroni, 2008). The integrons are composed of an integrase gene, two promoters and an array of other genes, including antibiotic resistance genes but they do not possess a site-specific recombination and cannot randomly excise or insert into DNA regions (Al-Haroni, 2008). Antibiotic resistant genes presenting on some transposons and plasmids may be the result of integron insertion (Al-Haroni, 2008). The introduction of transferable genetic elements carrying antibiotic resistant genes into bacteria can occur via three mechanisms; transformation, transduction, and conjugation (Al-Haroni, 2008). In transformation, exogenous segments of DNA carrying resistant genes are acquired by the bacteria from the environment (Al-Haroni, 2008).

Transformation occurs in bacteria that are naturally competent, such as Pneumococci, Haemophilus, and some oral Streptococci (Al-Haroni, 2008). Transduction is the process that the exogenous bacterial DNA is transferred from one bacterium to another by phage particle whereas conjugation is the phenomenon that resulted from direct contact between the two bacterial strains, which facilitated the transfer of plasmid DNA from a donor to a recipient bacterium (Al-Haroni, 2008). Many plasmids are resistant to a variety of antibiotics (Al-Haroni, 2008).

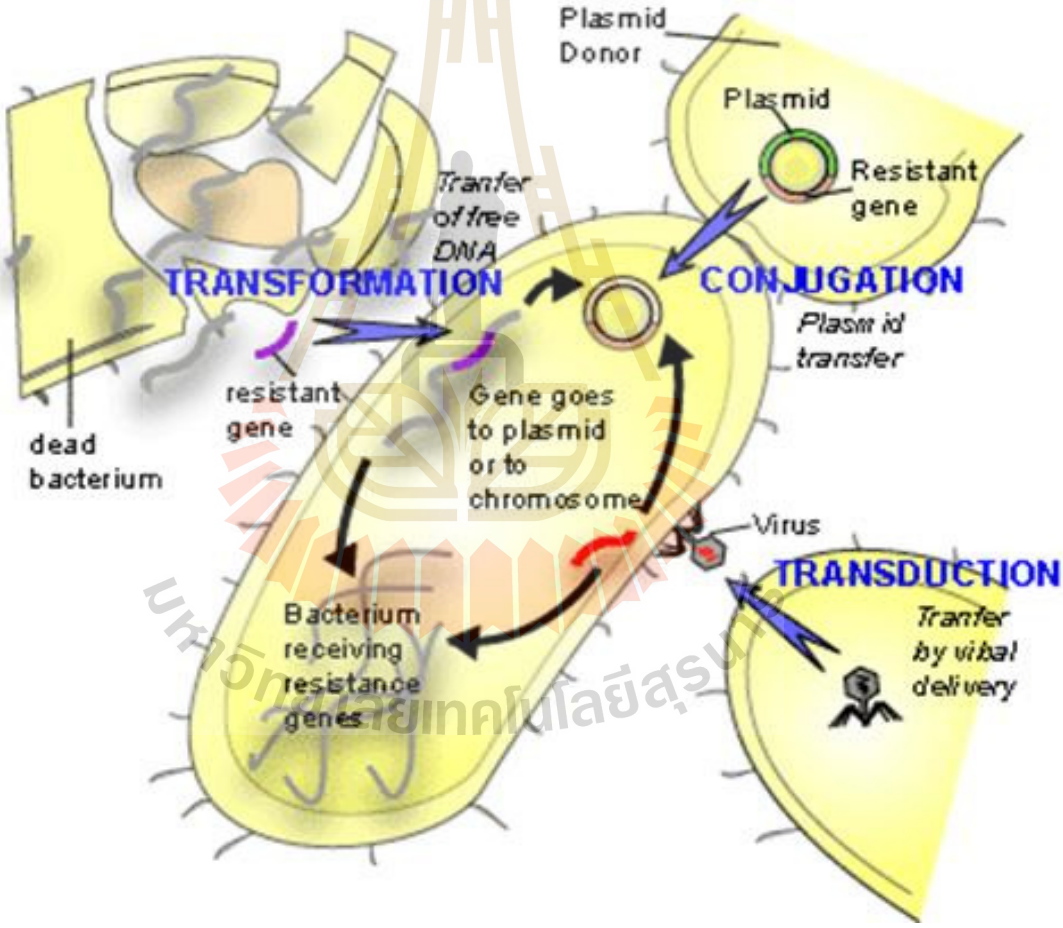

Figure 2.15 Transferring of genetic elements carrying antibiotic resistant genes into bacteria via three mechanisms: transformation, transduction and conjugation (http://biofilmbook.hypertextbookshop.com/public_version/contents/chapters/chapter002/section006/blue/page003.html).

Table 2.4 Main antibiotics used in dentistry, mechanisms of action, their spectra, and main bacterial resistance mechanisms involved (Al-Haroni, 2008).

Drug	Mechanism	Spectrum	Main resistance mechanism (s)
Phenoxymethylpenicillin	Inhibition of cell wall synthesis	Aerobic G+, anaerobic G+ anaerobic G- (narrow-spectrum)	Enzymatic (*β*-lactamases), alteration of the target site (mosaic PBP)
Amoxicillin, Ampicillin	Inhibition of cell wall synthesis	As above plus *Haemophilus* spp. (broad-spectrum)	As above
Metronidazole	Inhibition of RNA synthesis	Strict anaerobic bacteria, some facultative anaerobes	Enzymatic (5-nitroimidazole reductase)
Erythromycin	Inhibition of protein synthesis	Mainly G+	Target site modification, enzymatic inactivation, and active efflux
Clindamycin	Inhibition of protein synthesis	As above plus additional activity on anaerobes	As above
Tetracycline	Inhibition of protein synthesis	Main G+ and G-	Active efflux, enzymatic inactivation, ribosomal protection proteins

2.7 The relationship between antibiotic use and antibiotic resistance

One of the important for clinical failure is antibiotic resistance, especially by mutations selected during therapy. Antibiotic resistance in pathogens is the reflex of

overuse in medicine and agriculture (Heinemann et al., 2000). Many effects of antibiotics on microbial physiology and ecology give the picture of how difficult it can be to predict the return of susceptibility (Heinemann et al., 2000). An increased use of antibiotics is directly associated with an increase of antibiotic resistance (Guillemot, 1999). The control of methicillin-resistant *Staphylococcus aureus* (MRSA) is still a major healthcare burden in most healthcare institutions (Guillemot, 1999). The antibiotic resistance tends to develop both in pathogenic bacteria and normal bacterial flora (Guillemot, 1999). Resistant bacteria normally survive in an environment with the presence of various antibiotics leading to horizontal transfer of resistant genes either within or between bacterial species. Even with the optimal doses of antibiotic use, antibiotic resistances will probably not quickly decline (Guillemot, 2001). Therefore, the diffusion of existing antibiotic resistance must be limited in the population and avoided the rise of new strains of resistant bacteria (Guillemot, 2001).

2.8 Molecular strategies to overcome antibiotic resistance

2.8.1 Development of inhibitors for resistance enzymes

These inhibitors can serve as co-drugs with the antibiotics to block the resistance and to rescue the antibiotic activity of the drugs (Wright, 2005). The inhibitors contain the ability to avoid bacterial resistance may be due to the structural similarities between the substrates and the inhibitors (Buynak, 2006). This application has been highly successful to overcome the resistance to penicillinases (type 2 class of β-lactamases) for example, amoxicillin/clavulanate and ampicillin/sulbactam combinations (Wright, 2005).

2.8.2 Improvement of the delivery or accessibility of antibiotics to their target sites

For example, liposome preparations of hydrophobic antibiotics such as ethambutol for the treatment of mycobacterial infections (Wright, 2005).

2.8.3 Linkage of two different classes of antibiotics

For example, β-lactams and quinolones, or β-lactams and oxazolidinones; these combinations of antibiotics are tethered together by stable or labile linking bonds (Wright, 2005).

2.8.4 Screening of new chemical entities for known targets

One of the highly promising new chemicals is antimicrobial peptides (AMPs). Antimicrobial peptides (AMPs) are molecules (20–50 amino acid residues) that widely spread in all life-forms, from multi-cellular organisms to bacterial cells employed to interfere with microbial growth (Tavares et al., 2013). The AMPs can interact with bacterial cell membranes. Many AMPs have been shown to be effective against multi-drug resistant bacteria and tend to develop low resistance due to their distinguished mode of action (Tavares et al., 2013; Seo et al., 2012). Most of the AMPs are positively charged, which can interact with negatively charged surfaces of bacterial membranes, hence, inactivate the bacteria via a non-receptor mediated mechanism (Guralp et al., 2013). With this characteristic mode of action, distinct from that of conventional antibiotics, empowers them to avoid the common resistance mechanisms against classic antibiotics (Seo et al., 2012). However, the AMPs may also effect on several other cell components and act as metabolic inhibitors of cellular processes of bacteria, including cell wall biosynthesis, nucleic-acid and proteins (Tavares et al., 2013). In these cases, the cell death can be caused by

the multiple inhibitory effects (Tavares et al., 2013). Moreover, they are active at very low concentrations (nanomolar to micromolar level) (Guralp et al., 2013).

2.8.5 Identification of new targets with less mutable (Tan et al., 2000)

2.8.6 Use of virulence factors as targets

This approach expands the bacterial targets and exerts less selective pressure, leading to the decrease in antibiotic resistance (Tan et al., 2000; Clatworthy et al., 2007).

2.8.7 Phenotypic conversion of antibiotic resistance to antibiotic sensitivity (Tan et al., 2000)

2.8.8 Identification of various therapeutic antibodies

This strategy either directly targets the bacterial cells at a site of infection, or alternatively neutralises the bacterial toxins (Tan et al., 2000).

2.9 Antibacterial activities of natural products

There is growing evidence that the extraction of specific plant species can be used for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) (Mahady et al., 2008). For example, various *Garcinia* species that had powerful activity against MRSA were discovered in 2005 (Mahady et al., 2008). Moreover, the crude methanol extract of the twigs of *Garcinia nigrolineata,* along with xanthones isolated from twigs and stem bark contained antibacterial activity to MRSA (Mahady et al., 2008). A 50% ethanol extract of dried fruits of *Terminalia chebula* Retz. can inhibit MRSA growth, with a minimum inhibitory concentration (MIC) of 31.3 mg/ml (Mahady et al., 2008). In addition, *Hypericum perforatum* L. known as St. John's wort, is another common plant appears to have some activity against MRSA (Mahady

et al., 2008). The lipophilic phloroglucin-derivative hyperforin from *Hypericum perforatum* L. has antibacterial effects and has been demonstrated to inhibit the growth of MRSA of the concentration of 1 µg/ml (Mahady et al., 2008).

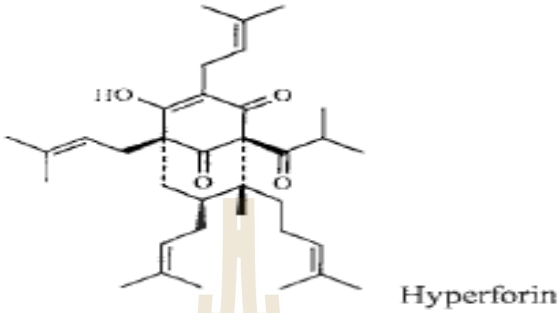

Figure 2.16 Chemical structure of hyperforin from *Hypericum perforatum* L. (Mahady et al., 2008).

Flavones chrysosplenol-D and chrysoplenetin, from *Artemisia annua*, enhance the antibacterial activity of berberine against *Staphylococcus aureus* (Mahady et al., 2008). This potentiation appears to be caused by the inhibition of *S. aureus* multidrug resistance (MDR) pump (Mahady et al., 2008).

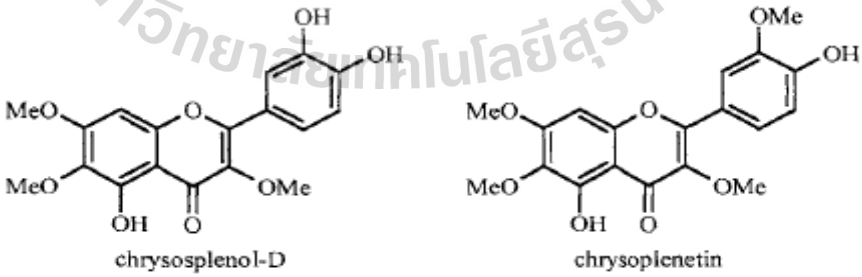

Figure 2.17 The chemical structures of the flavones chrysosplenol-D and chrysoplenetin, isolated from *Artemisia annua* that enhance the antibacterial activity of berberine by inhibiting the MDR pump (Mahady et al., 2008).

Staphylococcus aureus was tested with ethanol extracts of bitter kola *(Garcinia kola)* (Mahady et al., 2008). The minimum inhibitory concentration (MIC) of the extracts is 0.08 µg/ml to 1.8 mg/ml, whearas the minimum bactericidal concentration (MBC) is 0.135 mg/ml to 4.2 mg/ml. These indicate the extracts of *Garcinia kola* were strongly active against *S. aureus* (Mahady et al., 2008). In addition, many medicinal plants, including some common food and spice plants exerted antibacterial activity against various strains of *Escherichia coli* (Mahady et al., 2008). For examples, ethanol extracts of *Z. officinale*, *P. granatum* and acetone extract of *T. chebula* showed inhibition zone of 14 mm. against *E. coli* (Sharma et al., 2009) whereas essential oil extracted from oregano showed inhibition zone of > or = 70 to 80 mm. with MIC ≈ 8 ppm against *E. coli* (Mahady et al., 2008).

2.9.1 Chili (*Capsicum frutescens* L.)

Chili (*Capsicum frutescens* L.) or *Capsicum spp.* belongs to a genus of flowering plants in the Solanaceae family with 1.0 m. in height (Iwu, 1993) and 2-3 cm. length of pungent fruits (McKenna et al., 2002). Some of these members are used as spices, vegetables, and medicines (Reddy and Sasikala, 2013). *Capsicum* fruits have been called as a variety of names such as chilli pepper, red or green pepper, or sweet pepper (Reddy and Sasikala, 2013).

Figure 2.18 Super hot bird chillies (http://web.tradekorea.com).

2.9.1.1 Phytochemical composition of the *Capsicum* species

Phytochemical composition of the *Capsicum* species includes a fixed oil, pungent principles, volatile oil, and carotenoids, mostly capsanthin pigments (Shruthi Hegde et al., 2009). Furthermore, one of the most important active compounds in capsicum fruits is capsaicin. Capsaicin is a main pungent alkaloid among capsaicinoids produced only in the fruits of *Capsicum spp*. (Prasad et al., 2006; Simonsen, 2009) with the level up to 1% by dry weight (w/w) (Proudlock et al., 2004). It is produced as the secondary metabolites, probably as deterrents against certain herbivores and fungi (Simonsen, 2009). The capsaicin is biosynthesised by capsaicin synthase (CS) through both the phenylpropanoid and the branched chain fatty acid pathways in placental tissues (Prasad et al., 2006; Arora et al., 2011).

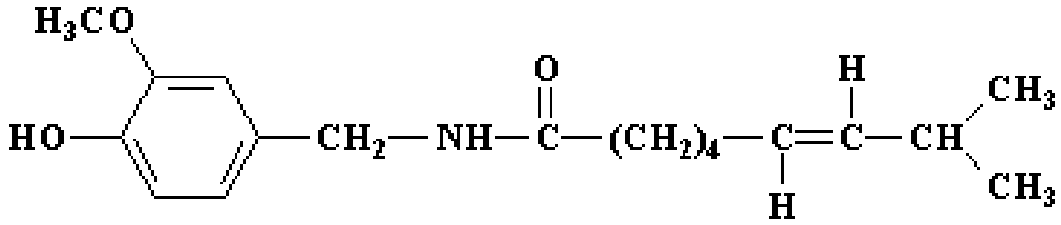

Figure 2.19 Chemical structure of capsaicin (IUPAC name 8-methyl-n-vanillyl-trans-6-nonenamide) Capsaicin contains a benzene ring and long hydrophobic carbon tail with a polar amide group (http://www.clinicalpharmacology.com).

Furthermore, it is a fat soluble, odourless and pungent taste, with a melting point of 62–65°C and 305.4 kDa molecular weight (Hayman and Kam, 2008). The capsaicin is not water soluble. Thus, alcohols and other organic solvents are used to solubilize capsaicin (Hayman and Kam, 2008). Pure capsaicin is not only hydrophobic, colourless, odourless but also crystalline to waxy compound (Patil et al., 2015) which is rapidly absorbed through the skin (O'Neill et al., 2012).

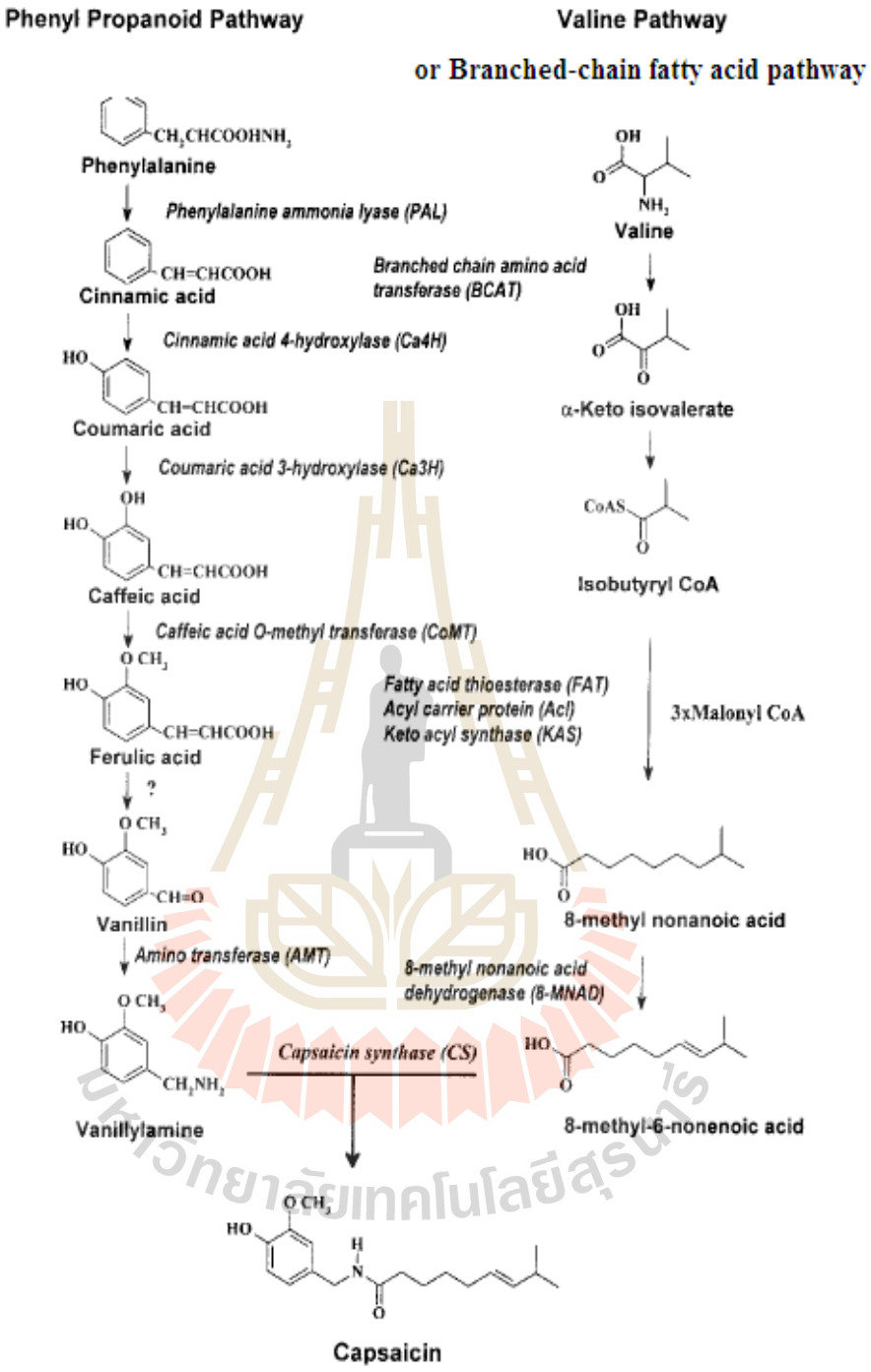

Figure 2.20 Capsaicin biosynthetic pathway. PAL, phenylalanine ammonia lyase; Ca4H, cinnamic acid 4 hydroxylase; Ca3H, coumaric acid 3 hydroxylase; CoMT, caffeic acid *O* methyltransferase; pAMT, putative amino transferase; CS, capsaicin synthase; KAS, keto acyl synthase (Prasad et al., 2006).

2.9.1.2 Capsaicin and pharmacological activities

Capsaicin has shown a wide range of pharmacological properties such as antigenotoxic, antimutagenic, and anticarcinogenic effects (Singh et al., 2001) including induced apoptosis in human gastric cancer cells through an effect on the fragmentation of cellular DNA (Singh et al., 2001). It has also demonstrated as a high degree of biological activities affecting on nervous, cardiovascular and digestive systems (Omolo et al., 2014). Therefore capsaicin preparations can be used as a counterirritant in lumbago, neuralgia and rheumatic disorders (Rao and Ravishankar, 2002). Furthermore, capsaicin and its interaction with the TRVP1 receptor for use in pain management have been studied (Capsaicin Technical Fact Sheet). Two commercial creams, viz. Zostrix and Axsain, are formulated with purified capsaicin to relieve arthritis pain (Rao and Ravishankar, 2002). Moreover, capsaicin can be served as a bacteriostatic or fungistatic compound (Rao and Ravishankar, 2002).

Table 2.5 Summary of clinically significant uses of capsaicin (Hayman and Kam, 2008).

Indication	Efficacy	Level of evidence	Tolerability
Neuropathic pain	NNT = 5.7 over 8 weeks Modest efficacy	ll a	Limited by burning upon application
Postherpetic neuralgia	NNT = 3.26 (2.26-5.85)	ll a (Systematic review-two small studies, *N* = 175; Hempenstall)	
Musculoskeletal Chronic pain	NNT = 8.1	ll b	
Neurogenic bladder Hyperreactivity	Increase bladder capacity and reduce incontinence	l b (Three RCTs with consistent results)	Intravesical Resiniferatoxin better tolerated than capsaicin
Gastroprotection in NSAID and ethanol use	Gastroprotective effect	l b (One RCT)	
Postoperative nausea and vomiting	Capsaicin applied at acupressure points, comparable to ondansetron	ll b (Wide confidence intervals)	
Pruritus	Reduced pruritus in renal dialysis patients and in pruritus ani	ll b (Small RCTs)	
A postoperative sore throat	Reduction in a sore throat at 24 h after capsaicin applied at the acupressure point	ll b (Small RCTs)	

2.9.1.3 Capsaicin and microbial relations

Researchers have shown the antibacterial activity of capsaicin to some foodborne pathogens (Dorantes et al., 2000). However, in some cases, capsaicin cannot show an inhibitory effect on the growth of some bacteria (Dorantes et al., 2000), even be utilised as a nutrient to stimulate some bacterial growth such as *Variovorax paradoxus* and *Pseudomonas putida* (Flagan and Leadbetter, 2006). In addition, good growth was achieved when these two isolates, were cultured together. (Flagan and Leadbetter, 2006).

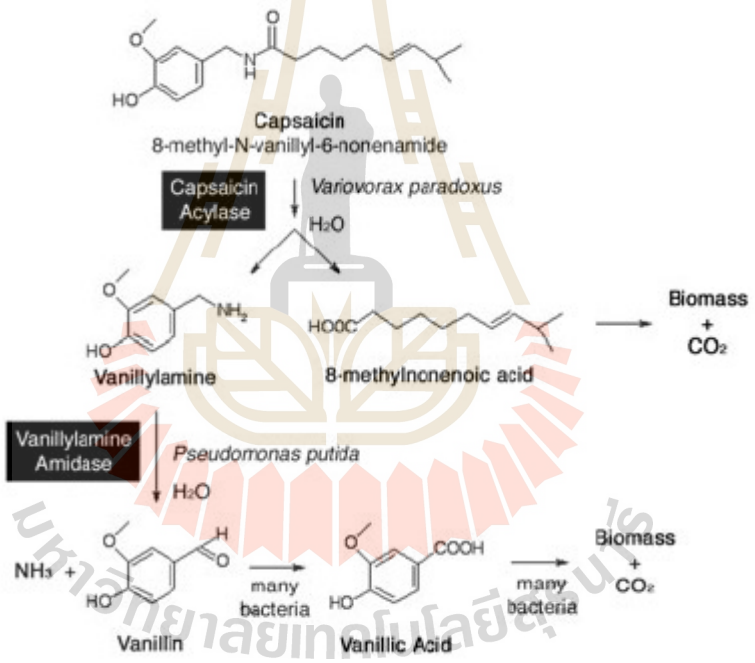

Figure 2.21 Hypothesized pathways of capsaicin degradation by *Capsicum* associated bacterial consortia (Flagan and Leadbetter, 2006).

The ethanol extracts of three *Capsicum spp*. (*Capsicum frutescens* L. (chilli pepper), *C. annuum* L. var. longum (curly pepper), and *C. annuum* L. var. longum (sweet pepper)) showed similar potencies for antimicrobial activities against Gram-

positive and Gram-negative bacteria including fungi as well (Soetarno et al., 1997). According to bioautographic tests, it demonstrated that capsaicin was the major compound for antimicrobial activity against bacteria (Soetarno et al., 1997).

Table 2.6 Botanical description of the three capsicum fruits, antimicrobial activity and bioautographic data of their ethanol extracts (Soetarno et al., 1997).

Criteria evaluated	Fruit-1	Fruit-2	Fruit-3
Species	*C. frutescens* (Chilli pepper)	*C. annuum* (Curly pepper)	*C.annuum* (Sweet pepper)
Local name	cabe rawit	cabe keriting	cabe besar
Length/diameter (cm)	3.5-5.0/1.0-1.2	6.5-10.0/0.5-1.0	13.0-15.0/2.0-2.5
Color	red (mature) green (raw)	red (mature) green (raw)	red (mature) green (raw)
Shape	stright	curly	stright
Pungency and capsaicin content	very hot 8.49	hot 4.28	moderately hot 2.18
In extract			
MIC {% (µg/well)}			
S. aureus	0.40 (80)	0.40 (80)	0.40 (80)
E. coli	0.06 (12)	0.08 (16)	0.06 (12)
P. aeruginosa	5.00 (1.000)	-	5.00 (1.000)
B. subtilis	0.40 (80)	0.20 (40)	0.60 (120)
S. lutea	0.10 (20)	0.10 (20)	0.08 (16)
C. albicans	0.06 (12)	0.04 (8)	0.06 (12)
HRx of active bioautographic spots to *B. subtilis* and *S. aureus*	100 (capsaicin) 123.3	100 (capsaicin)	100 (capsaicin) 153.8 165.4

Capsaicin with high concentrations only retarded the growth of *E. coli* and *P. solanacearum*, while the growth of *B. subtilis* was strongly inhibited (Molina-Torres et al., 1999). Interestingly, *S. cerevisiae* was initially enhanced (Molina-Torres et al., 1999). However, the information about the antimicrobial activity of capsaicin is insufficient, and the available information is obscured by the use of crude extracts that contain different amounts of many active components (Cichewicz and Thorpe, 1996; Molina-Torres et al., 1999).

2.9.1.4 Capsaicin and microbial toxicity

Capsaicin has been reported as the potential inhibitor of cholera toxin (CT) production in *Vibrio cholera*, a Gram-negative aquatic bacterium (Chatterjee et al., 2010). Interestingly, the methanol extract of red chilli could inhibit cholera toxin production in *V. cholerae* O1 El Tor variant strains without affecting their viability (Chatterjee et al., 2010). Furthermore, capsaicin also drastically inhibited cholera toxin production in *V. cholera* strains belonging to various serogroups, including variants (Chatterjee et al., 2010).

Transcription of *ctxA*, *tcpA* and *toxT* genes were repressed by capsaicin but not *toxR* and *toxS* genes (Chatterjee et al., 2010). On the contrary, the transcription of *hns* gene was enhanced by capsaicin (Chatterjee et al., 2010). The *hns* gene product is known to regulate the transcription of *ctxAB*, *tcpA* and *toxT* genes negatively mention above (Chatterjee et al., 2010). These results indicated that capsaicin might act as a potent repressor for cholera toxin production probably by enhancing the transcription of *hns* gene (Chatterjee et al., 2010). However, *V. cholerae* strains have become resistant to multiple antibiotics through mutations and horizontal gene transfer (Mwansa et al., 2007; Chatterjee et al., 2010). It was

concluded that capsaicin inhibited the mutagenicity of vinyl carbamate and diethylnitrosamine toward *Salmonella typhimurium* TA100 (Proudlock et al., 2004).

2.9.1.5 Toxicity from capsaicin

Capsaicin stimulates the release of the neuropeptide P from the sensory nerve fibres of the C type (Capsaicin Technical Fact Sheet). In mammals, capsaicin binds to the TRPV1 vanilloid receptor resulting in the release of sensory neuropeptides that trigger a neurogenic inflammatory response (Capsaicin Technical Fact Sheet). Moreover, capsaicin has shown to be a tumour promoter and potential mutagen, and a carcinogen (Singh et al., 2001). It can also directly generate hydroxyl radicals in the presence of Cu(II) (Singh et al., 2001).

Table 2.7 Toxicity classification of capsaicin (Capsaicin Technical Fact Sheet).

	High Toxicity	Moderate Toxicity	Low Toxicity	Very low Toxicity
Acute Oral LD_{50}	Up to and including 50 mg/kg (≤ 50 mg/kg)	Greater than 50 through 500 mg/kg (> 50-500 mg/kg)	Greater than 500 through 5000 mg/kg (> 500-5000 mg/kg)	Great than 5000 mg/kg (> 5000 mg/kg)
Inhalation LC_{50}	Up to and including 0.05 mg/L (≤ 0.05 mg/L)	Greater than 0.05 through 0.5 mg/L (> 0.05-0.5 mg/L)	Greater than 0.5 through 2.0 mg/L (> 0.5-2.0 mg/L)	Greater than 2.0 mg/L (> 2.0 mg/L)
Dermal LD_{50}	Up to and including 200 mg/Kg (≤ 200 mg/kg)	Greater than 200 through 2000 mg/kg (> 200-2000 mg/kg)	Greater than 2000 through 5000 mg/kg (> 2000-5000 mg/kg)	Greater than 5000 mg/kg (> 5000 mg/kg)
Primary Eye Irritation	Corrosive (irreversible destruction of ocular tissue) or corneal involvement or irritation persisting for more than 21 days	Corneal involvement or other eye irritation clearing in 8-21 days	Corneal involvement or other eye irritation clearing in 7 days or less	Minimal effects clearing in less than 24 hours
Primary Skin Irritation	Corrosive (tissue destruction into the dermis and/or scarring)	Severe irritation at 72 hours (severe erythema or oedema)	Moderate irritation at 72 hours (moderate erythema)	Mild or slight irritation at 72 hours (no irritation or erythema)

Capsaicin causes protein synthesis inhibition and microtubule disassembly through TRPV1 activities both on the plasma membrane and intracellular membranes (Han et

al., 2007). It activates TRPV1, a non-selective cationic channel; then the sustained TRPV1 channel activation causes severe cytotoxicity leading to cell death (Han et al., 2007).

Table 2.8 Morphological changes of TRPV1 expressing cells upon capsaicin treatment (Han et al., 2007).

	Increment of cell size (%) (n = 7)	Shrinkage of cell processes	Microtubule structure
200 nM capsaicin	25.2 ± 4.4	29.4% (20 out of 68 cells)	Depolymerized
200 nM capsaicin + 10 μM A-425619	0.27 ± 1.1	1.7% (1 out of 60 cells)	Intact
200 nM capsaicin + 5 mM EGTA	2.6 ± 0.7	23.7% (18 out of 76 cells)	Depolymerized
200 nM capsaicin + 5 mM EGTA + 10 μM A-425619	−0.64 ± 0.76	6.3% (3 out of 48 cells)	Intact

Pure capsaicin is not active in the standard battery of genotoxicity assays recommended by the International Conference on Harmonisation (Proudlock et al., 2004). However, *in vitro* genotoxic activity is probably associated with mutagenic impurities in commercial grades of the material (Proudlock et al., 2004). The genotoxic potential of pure *trans*-capsaicin (Although there are two geometric isomers of capsaicin; *trans*-capsaicin and *cis*-capsaicin, only *trans* capsaicin occurs naturally) is very low (Chanda et al., 2004). Moreover, the purity and source of capsaicin are an important consideration for toxicological evaluations (Chanda et al., 2004).

2.9.2 Nutgrass (*Cyperus rotundus* L.)

Nutgrass (*Cyperus rotundus*) usually grows up to 50 cm high with dark green basal leaves vary in number from 6-14 (Akobundu and Agyakwa, 1998). It has an extensive underground system of rhizomes and tubers (Ampong-Nyarko, and De Datta, 1991). Tubers are 1-3 cm. long, blackish with a characteristic odour (Richa and Suneet, 2014). This medicinal plant is also known as purple nutsedge, motha and musta (Akobundu and Agyakwa, 1998; Singh et al., 2012b; Nagarajan et al., 2015).

Figure 2.22 Nutgrass (https://en.wikipedia.org/wiki/Cyperus_rotundus and http://www.pathtoayurveda.com/musta-cyperus-rotundus/).

2.9.2.1 Phytochemical composition of the *Cyperus rotundus*

Phytochemical studies on rhizomes and tubers of *Cyperus rotundus* show the presence of following phytochemical composition; alkaloids, flavonoids, tannins, starch, glycosides, furochromones, monoterpenes, sesquiterpenes, sitosterolan, saponins, phenolic compounds, essential oils and fatty oil containing a

neutral waxy substance, glycerol, linolenic, myristic and stearic acids (Vetha et al., 2015; Ghannadi et al., 2012; Jeyasheela et al., 2014; Nagarajan et al., 2015). Among these chemical constituents, the main active compound is sesquiterpenes, which is the major metabolite of this plant (Shin et al., 2015). Furthermore, sesquiterpene has been reported as an anti-MRSA in vitro (Datta et al., 2007). Furthermore, the essential oil providing for the characteristic odour and taste of *C. rotundus* comprises mostly sesquiterpene hydrocarbons, epoxides, ketones, monoterpenes and aliphatic alcohols (Sivapalan, 2013). These sesquiterpenes include selinene, isocurcumenol, nootkatone, aristolone, isorotundene, cypera-2,4(15)-diene, norrotundene and sesquiterpene alkaloids; rotundines A-C (Sivapalan, 2013). The sesquiterpene alkaloids, rotundines A-C was the first report of alkaloids from this herbal plant (Jeong et al., 2000; Nagarajan et al., 2015). *C. rotundus* has also been shown to contain sugars and minerals (Sivapalan, 2013; Vetha et al., 2015).

2.9.2.2 *Cyperus rotundus* and pharmacological activities

Cyperus rotundus acquires many medicinal values and is recommended for use in several clinical conditions such as dyspepsia, thirst, fever, blood diseases, biliousness, dysentery, pruritis, pain, vomiting, epilepsy, ophthalmia, erysipelas, etc (Singh et al., 2012b). It exhibits many pharmacological activities including anticonvulsant, antiemetic, gastroprotective, wound healing activities as well as anti-inflammatory and antidiarrhoeal activities (Sivapalan, 2013; Singh et al., 2012a; Singh et al., 2012b). In addition, *C. rotundus* not only demonstrates an effective antioxidant potential but also shows a potential source of natural antioxidants (Bashir et al., 2012; Krishnamoorthy and Surendran, 2014). Therefore, this medicinal plant can be used as a therapeutic agent for combating oxidative stress

related diseases through its antioxidant activity (Kumar et al., 2014). In addition, *C. rotundus* is widely used as analgesic, sedative, antispasmodic and antimalarial (Sivapalan, 2013). For antimalaria, *C. rotundus* extract contains the antimalarlial compounds such as alpha-cyperone, autoxidation products of beta-selinene and novel endoperoxide sesquiterpene, 10, 12-peroxycalamenene (Singh et al., 2012b). The 70% ethanolic extract of the rhizomes of *C. rotundus* reveals several constituents, including the sesquiterpene derivatives (Jin et al., 2011). These sesquiterpenes, contribute the anti-allergic activity of this plant (Jin et al., 2011). The anticancer activity in *C. rotundus* has been demonstrated. The study indicated that its essential oil is highly effective against L1210 leukaemia cells line with dramatically increased apoptotic DNA fragmentation (Singh et al., 2012b). Moreover, *C. rotundus* extract exerts antimicrobial activity against both Gram-positive and Gram-negative bacteria, including fungi (Bisht et al., 2011; Balpande and Cherian, 2013; Kakarla et al., 2014; Kilani et al., 2008).

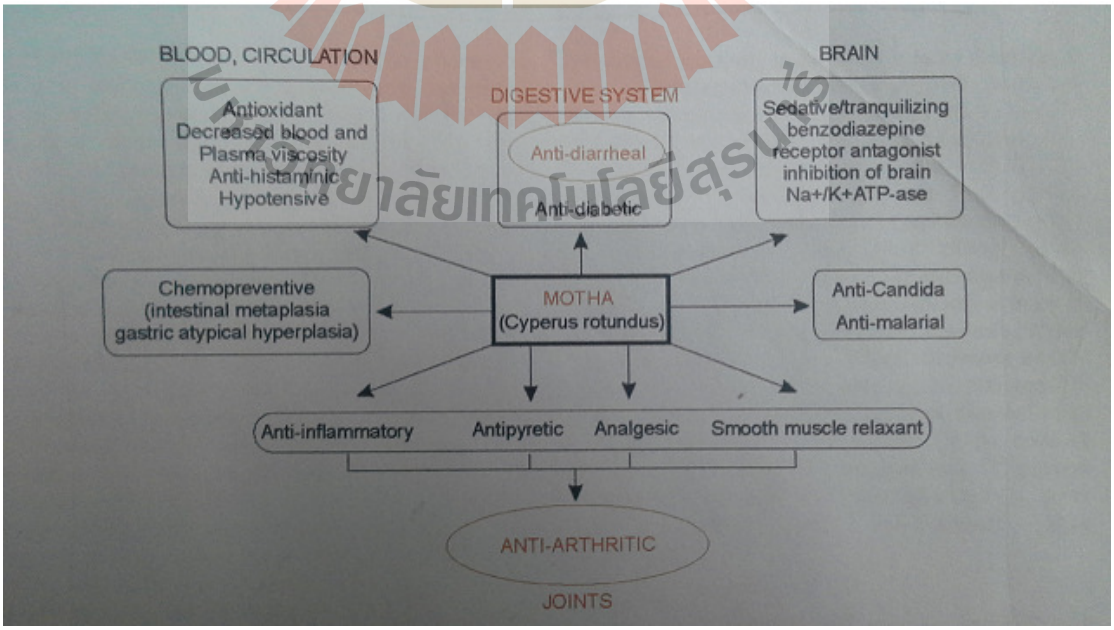

Figure 2.23 Pharmacological activities of *Cyperus rotundus* L. (Singh et al., 2012b).

2.8.2.3 *Cyperus rotundus* and microbial relations

C. rotundus extract shows considerable activity against both Gram-positive and Gram-negative bacteria. The rhizome extract of *C. rotundus* exhibits antibacterial activity to *Staphylococcus epidermidis*, *Bacillus cereus*, and *Pseudomonas aeruginosa* (Sharma and Singh, 2011). Furthermore, *Staphylococcus aureus*, *Salmonella enteritidis*, and *Enterococcus faecalis* are remarkably inhibited by the tuber extract of *C. rotundus* (Kilani et al., 2008). The antibacterial activity of essential oil from the tubers of *C. rotundus* is effective against several microorganisms such as *Staphylococcus aureus*, *Bacillus subtilis*, *Eschirichia coli*, *Pseudomonas aeruginosa*, *Candida parapsilosis*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Fusarium oxysporum* (Bisht et al., 2011; Kakarla et al., 2014). In addition, there is an important report for 75% ethanolic crude extract of *C. rotundus* against MRSA with 2% inhibition, published in 2015. Still no evidence of strong information presents regarding minimum inhibitory concentrations (MIC) including investigation of mechanisms of actions (Gawad et al., 2015).

2.9.2.4 Toxicity from *Cyperus rotundus*

A single oral administration of 95% ethanolic extract from the rhizomes of *C. rotundus* for acute toxicity test at the dose of 5,000 mg/kg rat does not exhibit signs of toxicity and mortality (Sivapalan, 2013). While, the result of subacute toxicity with the administration of the ethanolic extract from the rhizomes of *C. rotundus* at the dose of 1,000 mg/kg rat, daily over 14 days does not cause any mortality or behavioural changes (Sivapalan, 2013). In addition, the kidney and liver functions also do not show any change even after long term exposure (Sivapalan, 2013).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant species

Super hot bird chillies (*Capsicum frutescens* L. or *C. frutescens*) and Nutgrass was known as *Motha* or *Cyperus rotundus* L. (*C. rotundus*) were purchased from a local market and herb market in Nakhon Ratchasima, Thailand, respectively. The specimens of these plants were authenticated with voucher specimens at the Forest Herbarium, Bangkok, Thailand. Thailand. Dried and powdered ripe fruits of *C. frutescens* including dried and powdered tubers and rhizomes of *C. rotundus* were extracted to obtain crude extracts. The extraction method was mentioned in this chapter.

3.1.2 Test organisms

3.1.2.1 Bacterial strains

Clinical isolates of *Staphylococcus aureus* ATCC 29213 (*S. aureus*), clinical isolates of methicillin-resistant *Staphylococcus aureus* DMST 20651 (MRSA), clinical isolates of *Escherichia coli* ATCC 25922 (*E. coli*), clinical isolates of ceftazidime-resistant *Escherichia coli* DMST 20662 (CREC) were obtained from the Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand and the American Type Culture Collection (ATCC), USA.

3.1.2.2 Preparation and maintenance of stock cultures

The clinical isolates of bacteria were inoculated on nutrient agar slants and incubated overnight at 37°C. These cultures were stored in a refrigerator at 4°C. Fresh slant cultures were refreshed every 2-3 weeks.

3.1.3 *β*-lactam antibiotics

Ampicillin, amoxicillin and nisin were obtained from Sigma, Bristol-Myers.

3.1.4 Culture media

Nutrient agar, Mueller-Hinton broth and agar were obtained from Oxoid. Approximate formulae per litre of each medium were detailed in the appendix.

3.1.5 Chemicals

All chemicals used were detailed in the appendix.

3.1.6 Equipments

All equipments used were detailed in the appendix.

3.2 Methods

3.2.1 Plant extraction

The freshly ripe fruits of *C. frutescens* including tubers and rhizomes of *C. rotundus* were washed 2-3 times under running tap water and distilled water, then completely dried in hot air oven at 55-60°C for 30-60 h. Both dried plant materials were grounded into a fine powder. Capsaicin is highly soluble in alcohol (Arora et al., 2011). Hence, ethanol has been used for capsaicin extractions from *Capsicum* fruits, for food and pharmaceutical grade (Liljana et al., 2013) since it is less toxic and lower risk to human health (Food and Drug Administration). In addition, the ethanolic

extractions of *C. rotundus* has been found to be the most active against several bacterial strains comparing to the other solvents including aqueous extraction (Parekh and Chanda, 2006; Sharma and Singh, 2011; Ahmad and Beg, 2001). Therefore, the dried powder of *C. frutescens* ripe fruits, including the dried powder of *C. rotundus* tubers and rhizomes were extracted by 95% ethanolic Soxhlet extraction at 70°C for 8 h (Bullangpoti et al., 2002; Sia Su et al., 2013; Shivakumar et al., 2009; Pal, 2009) to obtain crude extracts followed by filtration through Whatman No 1. filter paper. Evaporations were operated under reduced pressure in a rota evaporator at 40°C (Shivakumar et al., 2009) by slowly reducing the pressure down to 110-70 mbar with rotation rate 30 rpm to expel the solvent and kept in -80°C for 4-5 days before freeze-drying under vacuum for 4-5 days to remove the trace amount of ethanol. The crude extracts were stored in a refrigerator at -20°C. The percent yields of crude extracts were calculated as:

$$\% \text{ Yield of crude extract} = \frac{\text{Weight of freeze-dried plant parts} \times 100}{\text{Weight of dried plant parts}}$$

Dried powder of *C. rotundus* tubers, rhizomes and dried powder of *C. frutescens* fruits were always kept in dry and dark place until used.

3.2.2 Phytochemical screening test

3.2.2.1 Qualitative phytochemical screening

Primary qualitative phytochemical screening of *C. rotundus* and *C. frutescens* crude extracts were analysed for ubiquitous bioactive compounds such as alkaloids, saponins, flavonoids, tannins, glycosides and polyphenols. All reagents used were detailed in the appendix. The test procedures are as follows:

1. Test for alkaloids

1.1 Mayer's test

Each 1 ml of 1% HCl was added into each 1 ml of 100 mg/ml of crude extract in ethanol, then heated gently followed by a few drops of Mayer's reagent. The presence of cream or pale yellow precipitate was an evidence of alkaloids (Mamta and Jyoti, 2012; Kodangala et al., 2010; Musa et al., 2009).

1.2 Wagner's test

A few drops of Wagner's reagent were added into each 1 ml of 100 mg/ml of crude extract in ethanol. The presence of brown or reddish brown precipitate was an evidence of alkaloids (Mamta and Jyoti, 2012; Musa et al., 2009).

2. Test for saponins

2.1 Froth test

A pinch of each dried powdered crude extract was added to 2-3 ml of distilled water then shaken vigorously. The formation of stable honey comb froth presenting at least for 30 min indicated the presence of saponins. (Mamta and Jyoti, 2012; Jamilu et al., 2015).

3. Test for flavonoids

3.1 Shinoda's test

A few fragments of magnesium ribbon were added to each 1 ml of 100 mg/ml of crude extract in ethanol followed by a few drops of concentrated HCl. The presence of pink, crimson red or occasionally green to blue was an evidence of flavonoids (Mamta and Jyoti, 2012; Musa et al., 2009).

3.2 Lead acetate test

A few drops of 10% lead acetate solution were added to each 1 ml of 100

mg/ml of crude extract in ethanol. The formation of white or yellow precipitate indicated the presence of flavonoids. (Deepa and Padmaja, 2014; Roopalatha and Nair, 2013).

4. Test for tannins

4.1 Gelatin test

The 4-5 drops of 1% gelatin solution containing 10% NaCl were added into each 1 ml of 100 mg/ml of crude extract in ethanol. The development of white precipitate confirmed the presence of tannins. (Roopalatha and Nair, 2013; Saklani et al., 2012).

5. Test for glycosides

5.1 Liebermann's test

The 2 ml of chloroform and 2 ml of glacial acetic acid were added into each 1 ml of 100 mg/ml of crude extract in ethanol followed by cooling in ice and carefully added concentrated H_2SO_4. A change in colour from violet to blue to green was an evidence of aglycone potion of glycosides (Prabha et al., 2013).

5.2 Keller-Killiani's test

The 1 ml of glacial acetic acid and 1 ml of 5% ferric chloride solution were added into each 1 ml of 100 mg/ml of crude extract in ethanol then mixed. The 2 ml of concentrated H_2SO_4 was added carefully on the side of the test tube. The reddish brown colour appeared at the junction of two layers was an evidence of 2-deoxy sugar of glycosides (Roopalatha and Nair, 2013; Saklani et al., 2012).

6. Test for polyphenols

The 1 ml of 1% ferric chloride and 1 ml of 1% potassium ferricyanide were added to each 1 ml of 100 mg/ml of crude extract in ethanol. The fresh radish blue

colour indicated the presence of polyphenols (Farhan et al., 2012; Zeidan et al., 2014).

3.2.2.2 Quantitative phytochemical determination

C. rotundus crude extract (CRE) was analysed to determine for total alkaloid, flavonoid and polyphenol contents by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Rotundine, quercetin and gallic acid were employed as standard alkaloid, flavonoid and polyphenol, respectively. All mobile phases were filtered through sterile nylon membrane filter 0.22 µm while standard chemicals and crude extracts in methanol were filtered through a syringe filter with sterile cellulose regenerated acetate membrane filter 0.22 µm.

1. Determination of total alkaloid content in *C. rotundus* crude extract (CRE) by LC-MS/MS

Rotundine in methanol was employed as the standard for the quantification of total alkaloid content. The 0.07 mg of rotundine was dissolved in 1ml of methanol then diluted to 0.28, 0.14, 0.07, 0.014, 0.007 µg/ml for standard curve preparation: Y axis was peak area and X axis was a rotundine concentration in µg/ml. A concentration of 100 mg/ml of CRE in methanol was used as a stock solution for determination of total alkaloid content. The 0.1% formic acid and 5 mM ammonium formate in water, and methanol were used as mobile phase A and B, respectively (Xiao et al., 2014). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Agilent technologies 6490 Triple Quad LC/MS coupled with Agilent Technologies 1290 Infinity) was operated with the following setting:

Liquid chromatography: injection volume 2 µl, column zorbax sb-C18 RRHD (1.8 µm, 2.1 x 150 mm) (Agilent Technologies), column temperature 30°C, the ratio of gradient elution changed are as follows; 0-5 min, 22-30% B, 5-10 min, 30-40% B, 10-

15 min, 40-60% B, 15-20 min, 60-40% B, 20-25 min, 40-22% B, flow rate 0.2 ml/min Electrospray ionisation (ESI): capillary voltage 3000 V, drying gas (N_2) flow rate 16 l/min, drying gas temperature 300°C, nebuliser 45 psi

Mass spectrometry: Multiple reaction monitoring (MRM)

Product ion scans (m/z 192) in positive mode (Xiao et al., 2014).

The total alkaloid content of CRE was calculated as:

Total alkaloid content = RE x V/m

Where; RE is a rotundine equivalence (mg/ml)

V is the volume of extract (ml)

m is the weight of dry extract (g)

2. Determination of total flavonoid content in *C. rotundus* crude extract (CRE) by LC-MS/MS

Quercetin in methanol was employed as the standard for the quantification of total flavonoid content. The 100 mg of quercetin was dissolved in 1ml of methanol then diluted to 1, 0.5, 0.2, 0.1, 0.05 µg/ml for standard curve preparation: Y axis was peak area, and X axis was the quercetin concentration in µg/ml. A concentration of 10 mg/ml of CRE in methanol was used as a stock solution for determination of total flavonoid content. The 0.1% formic acid in water and 0.1% formic acid in acetonitrile were used as mobile phase A and B, respectively (Sanchez-Rabaneda et al., 2003). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Agilent Technologies 6490 Triple Quad LC/MS coupled with Agilent Technologies 1290 Infinity) was operated with the following setting:

Liquid chromatography: injection volume 1 µl, column zorbax sb-C18 RRHD (1.8

µm, 2.1 x 150 mm) (Agilent technologies), column temperature 40°C, the ratio of gradient elution changed are as follows; 0-8 min, 10% B, 8-14 min, 90% B, flow rate 0.3 ml/min

Electrospray ionisation (ESI): capillary voltage 3000 V, drying gas (N_2) flow rate 14 l/min, drying gas temperature 200°C, nebuliser 45 psi

Mass spectrometry: Multiple reaction monitoring (MRM)

Product ion scans (m/z 151) in negative mode (Sanchez-Rabaneda et al., 2003).

The total flavonoid content of CRE was calculated (Engida et al., 2013) as:

Total flavonoid content = QE x V/m

Where; QE is a quercetin equivalence (mg/ml)

V is the volume of extract (ml)

m is the weight of dry extract (g)

3. Determination of total polyphenol content in *C. rotundus* crude extract (CRE) by LC-MS/MS

Gallic acid in methanol was employed as the standard for the quantification of total polyphenol content. The 100 mg of gallic acid was dissolved in 1ml of methanol then diluted to 2.5, 1, 0.5, 0.2, 0.1 µg/ml for standard curve preparation: Y axis was peak area and X axis was the gallic acid concentration in µg/ml. A concentration of 10 mg/ml of CRE in methanol was used as a stock solution for determination of total polyphenol content. The mobile phase A and B including liquid chromatography and electrospray ionisation setting were the same as mentioned in the determination of total flavonoid content (Sanchez-Rabaneda et al., 2003). Mass spectrometry setting for gallic acid was multiple reaction monitoring (MRM), product ion scan (m/z 125)

in negative mode (Sanchez-Rabaneda et al., 2003).

The total polyphenol content of CRE was calculated (Basma et al., 2011) as:

Total polyphenol content = GAE x V/m

Where; GAE is gallic acid equivalence (mg/ml)

V is the volume of extract (ml)

m is the weight of dry extract (g)

3.2.3 Bacterial suspension standard curve

A separate loopful of each bacterium was inoculated with 50 ml of Mueller-Hinton Broth (MHB) for 18 h. Each bacterium was separately transferred to each 50 ml conical centrifuge tube followed by harvesting at 4000 rpm, 25°C for 10 min and washing three times with 10 ml of 0.85% NaCl. The bacterial cells were resuspended in 10 ml of 0.85% NaCl, then diluted to optical density (O.D) of 0.05, 0.10, 0.15, 0.20 and 0.25 at 500 nm wavelength using 0.85% NaCl as a blank. The spread plate technique was employed for the triplicate viable plate count on overdried Mueller-Hinton Agar (MHA). Standard curves of each bacterial strain mentioned above (in 3.1.2.1) were plotted to obtain 10^8 cfu/ml, (widely used in laboratory and have been accepted by the Clinical and Laboratory Standards Institute or CLSI) when each Y axis was bacterial log cfu/ml, and each X axis was O.D at 500 nm (Eumkeb, 1999).

3.2.4 Agar disc diffusion screening

The agar disc diffusion technique was applied for primary sensitivity screen test. One loopful of each bacterium was inoculated in 50 ml of MHB for 18 h. Then the 18 h cultures of each bacterium in MHB were prepared to achieve 10^8 cfu/ml by using bacterial suspension standard curves as shown in Figure D-G detailed in appendix. Each bacterium with 10^8 cfu/ml was thoroughly swabbed on MHA plates.

Each 10 µl of *C. rotundus* and *C. frutescens* crude extracts at 250 mg/ml in DMSO was separately dropped onto each paper disc to obtain 2.5 mg/disc. Moreover, 10 µl of 1 mg/ml of ampicillin in distilled water was employed as a positive control for *S. aureus* and MRSA while 10 µl of 2 mg/ml of amoxicillin in distilled water was also employed as a positive control for *E. coli* and CREC. Each positive control was separately dropped onto each Ø 0.5 mm, sterile paper disc to acquire 10 µg/disc of ampicillin and 20 µg/disc of amoxicillin, respectively whereas 10 µl of 10% DMSO was used as a negative control. All discs were put on the overdried surface of Mueller-Hinton agar plates and were incubated at 37°C for 18 h. Finally, diameters of inhibition zone were measured and compared to positive and negative controls (Ortez, 2005; Voravuthikunchai and Kitpipit, 2005).

Note: All soluble antibiotics in distilled water and crude extracts in DMSO used in method 3.2.4-3.2.10 were filtered through sterile cellulose acetate membrane filter 0.22 µm.

3.2.5 Minimum inhibitory concentration (MIC) determination by agar dilution technique with some modifications

MIC is defined as the lowest concentration of an antimicrobial agent that can inhibit the visible growth of a microorganism after overnight incubation. The 18 h of bacterial cultures were tested on MHA containing various concentrations of CRE, ampicillin and amoxicillin. The twofold serial dilutions were started from 0.0625 to 32 mg/ml for CRE and 0.00195312 to 1024 µg/ml of ampicillin and amoxicillin. Briefly, the twofold serial dilution stock of CRE in DMSO was started from 320 to 0.625 mg/ml while the twofold serial dilution stocks of ampicillin for *S. aureus* and

MRSA, and amoxicillin for *E. coli* and CREC in distilled water were started from 10240 to 0.0195312 µg/ml. Each 0.2 ml of each twofold serial dilution stock was added into 1.8 ml of melted MHA in sterile 6 well-plates then mixed thoroughly and allowed them to solidify (can be kept in the dark at 4°C for 3 days). This made the final concentrations of CRE in MHA range from 0.0625 to 32 mg/ml when the final concentrations of ampicillin and amoxicillin in the MHA range from 0.00195312 to 1024 µg/ml. The 0.2 ml of DMSO without CRE in 1.8 ml MHA was subjected as a positive control for the use of CRE. The 0.2 ml of distilled water without ampicillin in 1.8 ml MHA was used as a positive control for *S. aureus* and MRSA whereas 0.2 ml of distilled water without amoxicillin in 1.8 ml MHA was used as a positive control for *E. coli* and CREC. The 18 h of MHB cultures for each bacterium were prepared to reach 10^8 cfu/ml according to bacterial suspension standard curves and diluted to 10^7 cfu/ml with 0.85% NaCl. Finally, each 1 µl containing 10^7 cfu/ml of each bacterium was separately spotted onto overdried MHA surface with various concentrations of CRE, ampicillin and amoxicillin as mentioned above. The final concentration of bacteria per spot was 10^4 cfu/spot. All dilutions and spots were performed in triplicates. Then all 6 well-plates were incubated at 37°C for 18 h. The lowest concentration of CRE, ampicillin and amoxicillin with no bacterial growth was defined as MIC (Rankin, 2005; Clinical and Laboratory Standards Institute, 2006a; Wiegand et al., 2008; European Committee for Antimicrobial Susceptibility Testing, 2000).

3.2.6 Checkerboard assay

Checkerboard assay is the technique that has been used to assess the activity of antibacterial combinations *in vitro*. The twofold serial dilution stocks of

CRE in DMSO for MRSA and CREC were started from 5 to 0.078125 mg/ml and 10 to 0.078125 mg/ml, respectively. The twofold serial dilution stock of ampicillin in distilled water was started from 640 to 10 µg/ml for MRSA when the twofold serial dilution stock of amoxicillin in distilled water was started from 1280 to 10 µg/ml for CREC. The 0.2 ml of each stock were combined and added in 1.6 ml of melted MHA while each 0.2 ml of each stock was added in 1.8 ml of melted MHA for use singly. This made the final concentrations of CRE in MHA range from 0.0078125 to 0.5 mg/ml (for MRSA) and 0.0078125 to 1 mg/ml (for CREC). The final concentrations of ampicillin in MHA ranged from 1 to 64 µg/ml while the final concentrations of amoxicillin in MHA ranged from 1 to 128 µg/ml. The 18 h of each bacterium culture in MHB was harvested, washed and prepared to obtain 10^8 cfu/ml using bacterial suspension standard curve as references and diluted to 10^7 cfu/ml with 0.85% NaCl. Finally, each 1 µl containing 10^7 cfu/ml of each bacterium was separately spotted onto overdried MHA surface containing various concentrations of CRE, ampicillin and amoxicillin ranging from 0-0.5 mg/ml of CRE for MRSA, 0-1 mg/ml of CRE for CREC, 0-64 µg/ml of ampicillin for MRSA and 0-128 µg/ml of amoxicillin for CREC. The 0.2 ml of DMSO without CRE in 1.8 ml MHA was used as a positive control for the use of CRE. The final concentration of each bacterium per spot was 10^4 cfu/spot. All dilutions and bacterial spots were performed in triplicates. Then all 6 well-plates were incubated at 37°C for 18 h. The checkerboard assay and the isobologram were plotted: each Y axis was CRE concentration in mg/ml, while each X axis was ampicillin or amoxicillin concentration in µg/ml (Eumkeb, 1999). Fraction inhibitory concentration (FIC) index for the antibacterial combination was calculated by the following a formula to determine synergistic activity (Jayaraman et al., 2010;

Odds, 2003).

$FIC_{index} = FIC_a + FIC_b$

$$= \frac{\text{Concentration of A in MIC of A+B}}{\text{MIC of A alone}} + \frac{\text{Concentration of B in MIC of A + B}}{\text{MIC of B alone}}$$

FIC index ≤ 0.5 was defined as Synergism.

FIC index > 0.5-4.0 was defined as No interaction or Indifference.

FIC index > 4.0 was defined as Antagonism.

3.2.7 Time killing assay

Time killing assay can be employed to evaluate the antibacterial activity of CRE either alone or in combination with antibiotics. Furthermore, the assay can also be used to confirm checkerboard assay. Shortly, the concentration stocks of ampicillin in distilled water, CRE in DMSO and the combination of CRE with ampicillin were 320 µg/ml, 2.5 mg/ml, and 1.25 mg/ml (CRE in DMSO) with 10 µg/ml (ampicillin in distilled water), respectively. Each 5 ml of each ampicillin and CRE stock was added in 40 ml of MHB while 5 ml of 1.25 mg/ml CRE and 5 ml of 10 µg/ml ampicillin were added in 35 ml of MHB. The MRSA was cultured in 50 ml of MHB for 18 h then 1 ml of the culture was transferred to 49 ml of MHB and incubated at 37°C for 4 h to obtain the mid-log phase culture. The mid-log phase MRSA was diluted with 0.85% NaCl to obtain the concentration of 5 x 10^6 cfu/ml by using bacterial suspension standard curve. Then, each 5 ml of 5 x 10^6 cfu/ml of MRSA test sample was subjected to each 45 ml of MHB with different concentrations described above. This made the final concentrations of ampicillin, CRE and the combination of CRE with ampicillin in 50 ml MHB become as follows: 32 µg/ml of ampicillin (1/2 MIC), 0.25 mg/ml of CRE (1/2 MIC), including the concentration which determined the

synergistic activity in checkerboard assay: the combination of 0.125 mg/ml of CRE mixing with 1 µg/ml of ampicillin. The 5 ml of 5 x 10^6 cfu/ml MRSA test sample in 45 ml of MHB without antibacterial substance was used as a control. The final concentration of each bacterium per test was 5 x 10^5cfu/ml. All test samples and control were incubated at 37°C. The 1 ml of each test was taken every 1 h interval ranging from 0 to 6 h and at 24 h for viable plate counts. The 0.1 ml of each taken sample was dropped and spread on overdried MHA. All viable plate counts were performed in triplicates. Time killing assay curve was plotted: Y axis was bacterial cfu/ml and X axis was time in an hour (Eumkeb, 1999; Eumkeb and Chukrathok, 2013; Belley et al., 2008; Clinical and Laboratory Standards Institute, 1999).

Viable cell count decrease $\geq 2\log_{10}$ cfu/ml was defined as Synergistic activity comparable to the most active single agent at 24 h.

Viable cell count increase $\geq 2\log_{10}$ cfu/ml was defined as Antagonistic activity comparable to the most active single agent at 24 h.

Viable cell count decrease $\geq 3\log_{10}$ cfu/ml was defined as Bactericidal activity comparable to the initial inoculum at 24h.

Viable cell count decrease $< 3\log_{10}$ cfu/ml was defined as Bacteriostatic activity comparable to the initial inoculum at 24h.

(Jacqueline et al., 2003; Belley et al., 2008; Messick et al., 1999).

3.2.8 Cytoplasmic membrane (CM) permeability assay

The alteration in the cytoplasmic membrane (CM) permeability can be determined by the leakage of materials absorbing at 260 nm (OD), mostly DNA and RNA. The concentration stocks of ampicillin in distilled water, CRE in DMSO and the combination of CRE with ampicillin were 320 µg/ml, 2.5 mg/ml, and 0.625 mg/ml

(CRE in DMSO) with 5 µg/ml (ampicillin in distilled water), respectively. Each 20 ml of each ampicillin and CRE stock was added in 160 ml of 2.5 mM sodium HEPES buffer pH 7.0 with 100 mM glucose while 20 ml of 0.625 mg/ml CRE and 20 ml of 5 µg/ml ampicillin were added in 140 ml of 2.5 mM sodium HEPES buffer pH 7.0 with 100 mM glucose. The mid-log phase MRSA with the concentration of 5 x 10^6 cfu/ml was prepared as detailed in time killing assay. Then, each 20 ml of 5 x 10^6 cfu/ml of MRSA test sample was subjected to each 180 ml of 2.5 mM sodium HEPES buffer pH 7.0 with 100 mM glucose containing different concentrations described above. This made the final concentrations of ampicillin, CRE and the combination of CRE with ampicillin in 2.5 mM sodium HEPES buffer pH 7.0 with 100 mM glucose become as follows: 32 µg/ml of ampicillin (1/2 MIC), 0.25 mg/ml of CRE (1/2 MIC), including half of the concentration which determined the synergistic activity in checkerboard assay: the combination of 0.0625 mg/ml of CRE mixing with 0.5 µg/ml of ampicillin. The 20 ml of 5 x 10^6 cfu/ml MRSA test sample in 180 ml of 2.5 mM sodium HEPES buffer pH 7.0 supplemented with 100 mM glucose without antibacterial substance was used as a negative control. Moreover, 20 ml of 160 µg/ml of nisin in distilled water was added in 160 ml of 2.5 mM sodium HEPES buffer pH 7.0 with 100 mM glucose. Then 20 ml of 5 x 10^6 cfu/ml MRSA test sample in 180 ml sodium HEPES buffer pH 7.0 supplemented with 100 mM glucose containing nisin (16 µg/ml as final concentration) was used as a positive control. The final concentration of each bacterium per test was 5 x 10^5cfu/ml. All test samples and controls were incubated at 37°C.

The 5 ml of each test were taken every 1 h interval, ranging from 0 to 6 h. The taken samples of each interval were immediately filtered through sterile cellulose

acetate membrane filter 0.22 µm. The 200 µl of each filtrate was transferred to 96-well microplates for UV visible range then measured the O.D at 260 nm. The 200 µl pretreatment filtrate (before adding MRSA suspension) of each test was used as a blank for each corresponding test sample. All O.D measurements were performed in triplicates. Cytoplasmic membrane (CM) permeability curve was plotted: Y axis was O.D at 260 nm and X axis was time in an hour (Carson et al., 2002; Shen et al., 2012; Zhou et al., 2008).

3.2.9 Transmission electronmicroscopy (TEM)

The concentration stocks of ampicillin in distilled water, CRE in DMSO and the combination of CRE with ampicillin were 6400 µg/ml, 50 mg/ml, and 12.5 mg/ml (CRE in DMSO) with 100 µg/ml (ampicillin in distilled water), respectively. Each 1 ml of each ampicillin and CRE stock was added in 198 ml of MHB whereas 1 ml of 12.5 mg/ml CRE and 1 ml of 100 µg/ml ampicillin were added in 197 ml of MHB. One loopful of MRSA was inoculated in 50 ml of MHB for 18 h then prepared to achieve 10^8 cfu/ml by using bacterial suspension standard curves. Each 1 ml of 10^8 cfu/ml MRSA test sample was subjected to each 199 ml of MHB with different concentrations described above. This made the final concentrations of ampicillin, CRE and the combination of CRE with ampicillin in MHB become as follows: 32 µg/ml of ampicillin (1/2 MIC), 0.25 mg/ml of CRE (1/2 MIC), including half of the concentration which determined the synergistic activity in checkerboard assay: the combination of 0.0625 mg/ml of CRE mixing with 0.5 µg/ml of ampicillin. The 1 ml of 10^8 cfu/ml MRSA test sample in 199 ml of MHB without antibacterial substance was used as a control. The final concentration of each bacterium per test was 5 x 10^5cfu/ml. All test samples and control were prepared triplicately then incubated at

37°C for 4 h. Each bacterium sample was separately transferred to each 50 ml conical centrifuge tube followed by harvesting at 8000 rpm, 4°C for 15 min. Each bacterial pellet was re-suspended in 1 ml of 0.1 M phosphate buffer pH 7.2 to wash the cells and transferred to 1.5 ml eppendorf tube, then centrifuged at 8000 rpm, 4°C for 15 min to remove the supernatants.

Glutaraldehyde was used as a fixative reagent to maintain and stabilise the cellular biomass and prevent the autolysis. The 500 µl of 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 was added to each sample and mixed by rotator for 5-10 min prior to incubating overnight at 4°C then centrifuged at 14000 rpm 4°C for 5 min to discard the supernatant and washed three times with 1000 µl of 0.1 M phosphate buffer pH 7.2. The 500 µl of 1% osmium tetroxide in 0.1 M phosphate buffer pH 7.2 (obtained from TEM unit, Suranaree University of Technology) was added to each bacterial sample for fixing and staining the lipid in membranous structure then mixed in the rotator for 1 h. After removing the supernate by centrifugation at 5000 rpm 4°C for 5 min and washing three times with 500 µl of distilled water, a graded series of increasing acetone concentrations was employed to dehydrate the bacterial cells. The 1000 µl of 20%, 40%, 60%, 80%, 100% and 100% acetone were subsequently added to each sample, then centrifuged at 5000 rpm 4°C for 5 min to remove the supernatant. A series of acetone : resin was applied to infiltrate the bacterial cells. The 1000 µl of acetone : resin (2:1) and (1:1) were subsequently added to each bacterial sample and incubated at room temperature for 3 and 12 h, respectively, then centrifuged at 5000 rpm 4°C for 5 min to remove the supernatant. The pure resin was added to each sample and incubated at room temperature for 3 h followed by gently removing the upper part of the resin. Embedding, the 500 µl of pure resin was

additionally added to each sample and stood to allow the upper and lower parts of the resin to become homogeneous for 2 h then incubated at 70°C for 8 h. Each embedding sample was removed from the eppendorf tube followed by sectioning using a diamond knife on an ultramicrotome to obtain the thin sections with 80 nm thickness. Each sample was triplicately transferred onto the grids, then stained with uranyl acetate and lead citrate (obtained from TEM unit, Mahasarakham University), in sequence followed by washing with boiled distilled water (absence of CO_2). Finally, bacterial cells on the grids were examined with a transmission electron microscope JEM-1230 (JEOL) (Eumkeb, 1999; Richards et al., 1993; Richards et al., 1995). Furthermore, the bacterial cell area of each MRSA treatment in electronmicrographs was calculated from bacterial cell width (nm) x bacterial cell length (nm) to assert the effect of CRE either used singly or in combination with ampicillin on MRSA cell size.

3.2.10 β-lactamase enzyme assay for β-lactamase enzyme inhibitor

Enzyme activity of *β*-lactamase type IV from *Enterobacter cloacae* was adjusted to the sufficient concentration to hydrolyse 50-60% of the substrate, benzylpenicillin (penicillin G sodium salt), within 5 min. The 400 µg/ml benzylpenicillin was dissolved in mobile phase (10 mM ammonium acetate (pH 4.5 acetic acid) : acetonitrile (75:25)) then diluted to 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 µg/ml for standard curve preparation: Y axis was peak area and X axis was benzylpenicillin concentration in µg/ml. The 10 ml of 12,500 µg/ml *β*-lactamase enzyme in 50 mM phosphate buffer (pH 7.0) and 10 ml of 1,000 µg/ml benzylpenicillin in mobile phase were employed as stock solutions for enzyme and substrate, respectively. The concentration stocks of ampicillin in distilled water, CRE in DMSO and the combination of CRE with ampicillin were 320 µg/ml, 2.5 mg/ml,

and 0.625 mg/ml (CRE in DMSO) with 5 µg/ml (ampicillin in distilled water), respectively. The 1 ml of mobile phase without any antibacterial agent was used as a control. Each 1 ml of sample stock including the control was pre-incubated with 1 ml of the enzyme stock solution for 5 min at 37°C before adding 7 ml of mobile phase and 1 ml of the benzylpenicillin stock solution followed by incubating at 37°C for 20 min. This made the final concentrations of ampicillin, CRE and the combination of CRE with ampicillin in the test become as follows: 32 µg/ml of ampicillin (1/2 MIC), 0.25 mg/ml of CRE (1/2 MIC), including half of the concentration which determined the synergistic activity in checkerboard assay: the combination of 0.0625 mg/ml of CRE mixing with 0.5 µg/ml of ampicillin. The final concentrations of *β*-lactamase enzyme and benzylpenicillin in the test become 1250 µg/ml and 100 µg/ml, respectively. For every 5 min interval (ranging from 0 to 20 min), each 0.5 ml of the sample assay was immediately transferred into each eppendorf tube containing 0.5 ml of stopping agent, methanol : acetic acid (100:1).

Finally, each 20 µl of hydrolysed benzylpenicillin was subjected to high performance liquid chromatography (HPLC) Water 600, using 10 mM ammonium acetate (pH 4.5 acetic acid) : acetonitrile (75:25) as a mobile phase for measuring the remaining substrate. The HPLC was operated with the following setting: injection volume 20 µl, column C18 (5 µm, 250 x 4.6 mm) (Bio-sil), column temperature 35°C, flow rate 1ml/min, UV detector at 200 nm, run time 5 min. The mobile phase was filtered through sterile nylon membrane filter 0.22 µm while the other chemical solutions were filtered through a syringe filter with sterile cellulose acetate membrane filter 0.22 µm. The remaining benzylpenicillin substrates were calculated by comparing the peak area from chromatographic curve, and standard curve as shown in

Figure H detailed in appendix (Eumkeb et al., 2010; Richards et al., 1995; Teethaisong et al., 2014).

3.2.11 Statistical analysis

CM permeability assay, β-lactamase enzyme assay and TEM were carried out in triplicate. The data were expressed as a mean ± standard error of the mean (SEM). Significant differences between these groups were examined using one-way ANOVA and Tukey's HSD post-hoc test, $p<0.01$ were considered to be a statistically significant difference.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Plant extraction

The 95% ethanolic crude extract of *C. frutescens* gave the percent yield of 10.625% w/w, while the 95% ethanolic crude extract of *Cyperus rotundus* (CRE) gave the percent yield of 11.34% w/w after evaporation and freeze-drying. The dried crude extracts of *C. frutescens* and CRE were kept at -20°C for the study of a complete research. The calculation of percent yields was shown below.

$$\% \text{ Yield of } C.\ frutescens \text{ crude extract} = \frac{212.5\ (g) \times 100}{2000\ (g)} = 10.625\%$$

$$\% \text{ Yield of } C.\ rotundus \text{ crude extract} = \frac{226.8\ (g) \times 100}{2000\ (g)} = 11.34\%$$

According to the results, the yield of ethanolic *C. frutescens* crude extract (10.625% w/w) in this study was not significantly different from the other studies such as the phytochemical study and chemical tests of *C. frutescens* crude extract (8.77% w/w) (Freire et al., 2015). Moreover, it was higher than the other solvent extractions e.g. methanolic crude extract (5.50% w/w), chloroform extract (0.99% w/w), hexane extract (0.09% w/w) and ethyl acetate extract (0.06% w/w) (Emran et al., 2015). The ethanolic *C. rotundus* crude extract gave the percent yield at 11.34% w/w which was also not significantly different from the other publications including anticonvulsant effect of *C. rotundus* rhizome crude extract in rats (12% w/w) (Shivak

umar et al., 2009) and the determination of brain biogenic amines in *Cynodon dactylon* (*C. dactylon*), and roots and rhizomes of *C. rotundus* treated mice (12.3% w/w) (Pal, 2009). Furthermore, the percent yield of ethanolic extraction of *C. rotundus* was higher than the other solvent extractions such as methanolic extract (6.88% w/w), chloroform extract (2.92% w/w), and ethyl acetate extract (1.75% w/w) (Kumar et al., 2010).

Therefore, these indicated that the ethanolic extraction for *C. rotundus* crude extracts contributed the highest yield comparing to the other solvents. Moreover, the ethanolic extractions of medicinal plants were the most active against several bacterial strains comparing to the other solvents including aqueous extraction (Parekh and Chanda, 2006; Sharma and Singh, 2011; Ahmad and Beg, 2001). This is due to plant bioactive principles for medicinal importance such as antibacterial activity can be most effectively extracted by ethanol (Ogbulie et al., 2007; Shobowale et al., 2013).

4.2 Phytochemical screening test

4.2.1 Qualitative phytochemical screening

As shown in Table 4.1 was the result of the preliminary phytochemical screening test, the results indicated the presence of alkaloids, flavonoids, tannins, and polyphenols in *C. frutescens* and *C. rotundus* extracts, whereas saponins and glycosides were absent in all extracts on the methods used. The methods for the phytochemical screening test are shown in Table 4.1, Figure 4.1 and 4.2.

Table 4.1 Phytochemical analysis of ethanolic crude extracts of *C. frutescens* fruits and *C. rotundus* tubers and rhizomes.

Name of the Test	Procedure	Observation	*C. frutescens*	*C. rotundus*
Alkaloids	Mayer's test	White precipitate	+	+
	Hager's test	Reddish brown precipitate	+	+
Saponins	Froth test	No stable foam	-	-
Flavonoids	Shinoda's test	Crimson red	+	+
	Lead acetate test	White precipitate	+	+
Tannins	Gelatin test	White precipitate	+	+
Glycosides	Libermann's Test	No change in color from violet to blue to green	-	-
	Keller Killiant's Test	No reddish brown color appears at the junction of two layers and no upper layer appears bluish green	-	-
Polyphenols	Test for polyphenol	Reddish blue color	+	+

(+) Presence, (-) Absence

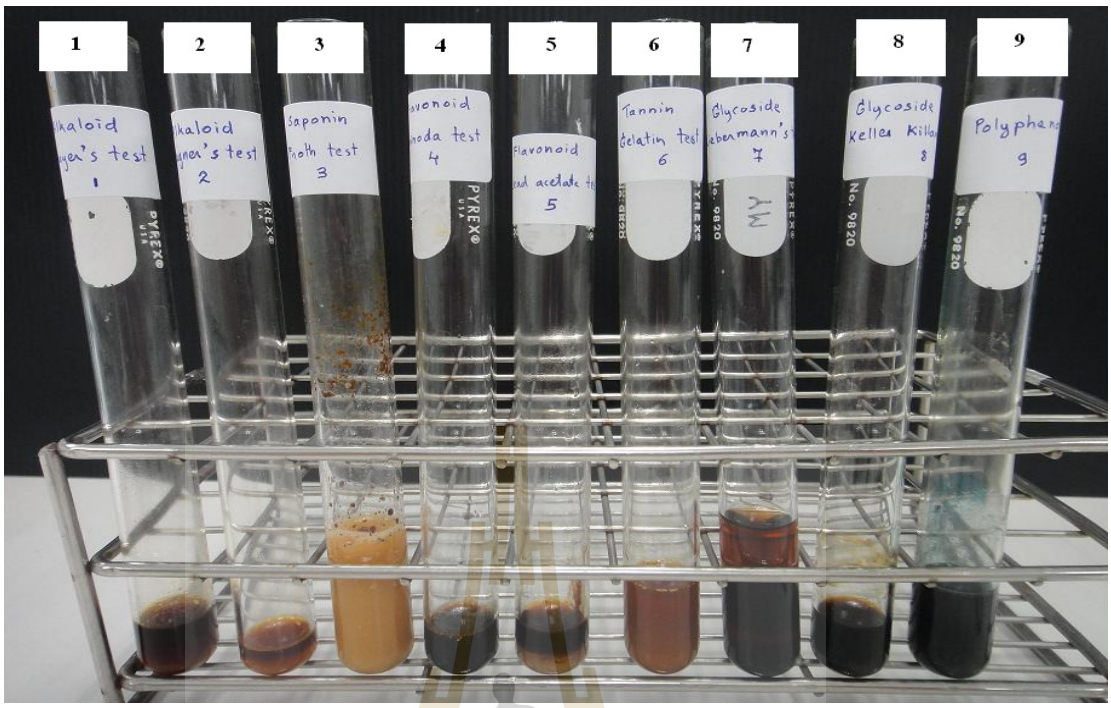

Figure 4.1 Phytochemical analysis of ethanolic crude extract of *C. frutescens* fruits: 1= Mayer's test, 2 = Wagner's test, 3 = Froth test, 4 = Shinoda's test, 5 = Lead acetate test, 6 = Gelatin test, 7 = Liebermann's test, 8 = Keller Killiani's test and 9 = Test for polyphenols.

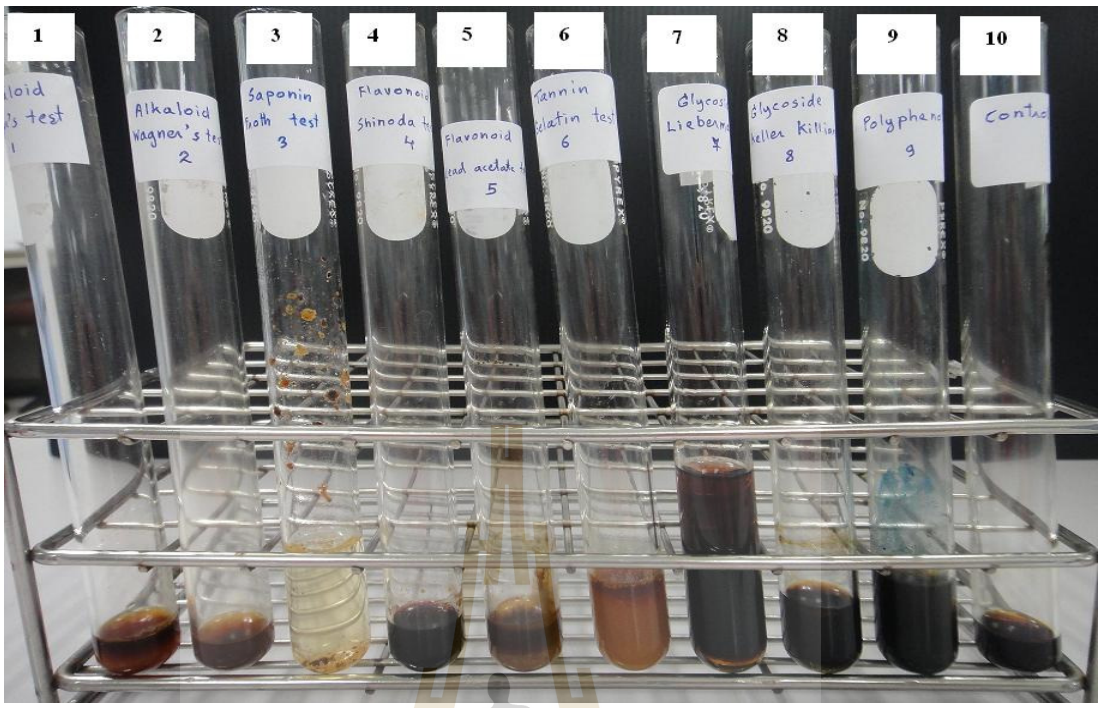

Figure 4.2 Phytochemical analysis of ethanolic crude extract of *C. rotundus* tubers and rhizomes: 1= Mayer's test, 2 = Wagner's test, 3 = Froth test, 4 = Shinoda's test, 5 = Lead acetate test, 6 = Gelatin test, 7 = Liebermann's test, 8 = Keller Killiani's test, 9 = Test for polyphenols and 10 = Control.

This phytochemical screening test revealed the presence of alkaloids, flavonoids, tannins, and polyphenols in *C. frutescens* as same as in many reports such as phytochemical screening and antimicrobial activity of ethanolic *C. frutescens* fruit crude extract on microorganisms (Sia Su et al., 2013), comparative morphological, anatomical, cytological and phytochemical studies on *C. frutescens* and *Capsicum annuum* (*C. annuum*) (Wahua et al., 2013), changes in phytochemical and antioxidant activity of *Capsicum* species as influenced by maturity (Howard et al., 2000) and antibacterial activity of two bell pepper extracts (Koffi-Nevry et al., 2012). Alkaloids, flavonoids, tannins, and polyphenols were present in *C. rotundus* crude extract in this

study which was consistent with several studies including phytochemical analysis of ethanolic *C. rotundus* tuber extract and its effect on ethanol treated rats (Jeyasheela et al., 2011), phytochemical and pesticidal properties of *C. rotundus* (Banez and Castor, 2011), the anticonvulsant effect of *C. rotundus* crude extract in rats (Shivakumar et al., 2009), Chemical composition and antioxidant potential of polyphenol compounds of *C. rotundus* rhizomes (Al-Jumaily and Al-Isawi, 2014) and physio-chemical and phytochemical study of rhizome of *C. rotundus* (Sivapalan and Prince, 2012).

There were many evidences revealing antibacterial activities of alkaloids including sesquiterpene alkaloids (Jawad et al., 1988; Konate et al., 2012; Zoraghi et al., 2011; Williams et al., 2010), flavonoids (Alcaraz et al., 2000; Wang et al., 2014; Tim and Lamb, 2005) and polyphenols (Karou et al., 2005; Taguri et al., 2004; Daglia, 2012) against several Gram-positive and Gram-negative bacteria, and MRSA. Therefore, the presence of these bioactive compounds with antibacterial activity against several Gram-positive, Gram-negative bacteria and MRSA indicated that *C. rotundus* might contain antibacterial activity, either for antibiotic sensitive bacteria or resistant bacteria, through these compounds.

Interestingly, the presence of polyphenols in *C. rotundus* crude extract suggested that this medicinal plant could be a potential source of natural antioxidant.

4.2.2 Quantitative phytochemical determination

The total of alkaloid, flavonoid and polyphenol contents were determined only for CRE extract since the preliminary screening of antibacterial activity had not been found in *C. frutescens* extract. Rotundines A-C were the only alkaloid (sesquiterpene alkaloid) found in rhizomes of *C. rotundus* (Nagarajan et al., 2015; Jeong et al., 2000), while quercetin and gallic acid were ubiquitously present in many

plants including rhizomes and tubers of *C. rotundus* (Krishna and Renu, 2013; Jahan et al., 2013; Jayashree et al., 2015) and their ethanolic extracts (Seema et al., 2011; Al-Jumaily and Al-Isawi, 2014). Therefore, rotundine, quercetin and gallic acid were used as standards for the determination of total alkaloid, flavonoid and polyphenol contents in rhizomes of *C. rotundus*, respectively. The calculation for total contents of these bioactive compounds using standard curves in Figure A-C detailed in the appendix was shown below. The total alkaloid, flavonoid and polyphenol contents were expressed in terms of mg RE/g extract, mg QE/g extract and mg GAE/g extract, respectively as demonstrated in Table 4.2.

$$\text{Total alkaloid content} = \text{RE} \times \text{V/m} = \frac{0.00000703 \times 1}{0.1}$$

= 0.0000703 mg of rotundine equivalent per g dry weight of CRE

$$\text{Total flavonoid content} = \text{QE} \times \text{V/m} = \frac{0.00077825 \times 1}{0.01}$$

= 0.077825 mg of quercetin equivalent per g dry weight of CRE

$$\text{Total polyphenol content} = \text{GAE} \times \text{V/m} = \frac{0.00110772 \times 1}{0.01}$$

= 0. 110772 mg of gallic acid equivalent per g dry weight of CRE

Table 4.2 Total alkaloid, flavonoid and polyphenol contents in the ethanolic crude extract of *C. rotundus* tubers and rhizomes.

Medicinal plant	Total alkaloid content (mg of rotundine equivalent per g dry weight of CRE)	Total flavonoid content (mg of quercetin equivalent per g dry weight of CRE)	Total polyphenol content (mg of gallic acid equivalent per g dry weight of CRE)
C. rotundus	0.0000703	0.077825	0.110772

The results showed lower total alkaloid content (0.0000703 mg of rotundine equivalent/g dry weight of CRE or 0.0703 ppm) in tubers and rhizomes of *C. rotundus* comparing to the study of rotundine A, B and C (0.7-1.1 ppm) (Nagarajan et al., 2015; Jeong et al., 2000).

The total flavonoid content (0.077825 mg of quercetin equivalent/g dry weight of CRE) from this study was less than that of in the study on isolation and identification of flavonoids from *C. rotundus* (0.09 mg of quercetin equivalent/g dry weight of CRE) (Krishna and Renu, 2013), and the comparative study on antioxidant potential and anticataract activity of *C. rotundus* (83.17 mg/100g dry weight) (Seema et al., 2011).

Furthermore, the total polyphenol content (0.110772 mg of gallic acid equivalent/g dry weight of CRE) in this study was less than the total phenolic compound content (30.23 mg of gallic acid equivalent/g dry weight of CRE) in the study of investigation on the antioxidant activity of Dheela grass (Bashir et al., 2012) including the total phenolic content (300 mg of gallic acid equivalent/g dry weight of

CRE) in the evaluation of antioxidant activity of *C. rotundus* (Jain and Aggarwal, 2012).

The amount of each total bioactive compounds presenting in *C. rotundus* may suggest the dominant type of the therapeutic property of this medicinal plant. According to the results, the total polyphenol content was highest compared to the total flavonoid and alkaloid contents. Therefore, this may indicate its antioxidant activity. However, polyphenols, flavonoids and alkaloids also can also demonstrate the antibacterial activity against both sensitive and resistant strains (Jawad et al., 1988; Konate et al., 2012; Zoraghi et al., 2011; Williams et al., 2010; Alcaraz et al., 2000; Wang et al., 2014; Tim and Lamb, 2005; Karou et al., 2005; Taguri et al., 2004; Daglia, 2012). Thus, the presence of various types of these compounds may be in charge of the highest antibacterial activity of *C. rotundus* crude extract. They may work together as a natural combination of various bioactive compounds rather than as a sole compound, to inhibit bacteria. This was consistent with some studies showing the crude extracts from medicinal plants were much more efficient than the only pure active compound, alone (Dupont et al., 2006; Pistelli et al., 2000; Rasoanaivo et al., 2011).

In addition, the presence and total contents of these bioactive compounds, alkaloids, flavonoids and polyphenols, in tubers and rhizomes of *C. rotundus* crude extract were varied by several parameters including growing conditions, e.g., season, temperature, soil quality, moisture, etc.; time of harvest, i.e., age; as well as processing procedures e.x., the solvents used for extraction and extraction methods.

Moreover, some sesquiterpene alkaloids demonstrated antibacterial activity (Williams et al., 2010), because of this, rotundine as the only sesquiterpene alkaloid

presenting in rhizomes of *C. rotundus* (Nagarajan et al., 2015; Jeong et al., 2000) may play the important role as a bioactive compound for antibacterial activity.

Note: the HPLC determination of total bioactive compound content is more precise and accurate compared to the spectrophotometric method (Olszewska et al., 2001). The spectrophotometric method creates significant error due to the presence of other compounds in the test sample, which can react with a reagent for spectrophotometric method (Olszewska et al., 2001). Furthermore, the advantage of established LC-MS/MS method for determination of total bioactive compound content is more specific and sensitive than HPLC method (Chang et al., 2014), since the pattern of ion fragmentation is highly specific. The HPLC method also gives the error because the compounds which are dissolved in the solvent and adsorbed in the column at the same degree must be eluted from the column at the same retention time showing the same peak as a combination of the compounds.

4.3 Bacterial suspension standard curves

As shown in Figure D and E detailed in the appendix, the 10^8 cfu/ml of *S. aureus* sensitive strain (ATCC 29213) and *S. aureus* DMST 20651 (MRSA) suspensions found O.D equal to 0.08 must be taken. To obtain 10^8 cfu/ml of *E.coli* sensitive strain (ATCC 25922) and *E.coli* resistant strain CREC (DMST 20662), the bacterial suspensions with O.D 0.10 and 0.13 must be taken, respectively (Figure F and G detailed in the appendix).

4.4 Agar disc diffusion screening

Agar disc diffusion is the method for primary screening of compounds or antibiotics against the selected bacteria. Antibacterial susceptibility of medicinal plants by agar disc diffusion was recorded in Table 4.3 and 4.4. CRE at 250 mg/ml gave the highest inhibition zone for both *S. aureus* sensitive strain and MRSA at 12 mm and 15 mm, respectively. Furthermore, 10 mm of ampicillin inhibition zone was shown for *S. aureus* sensitive strain (Table 4.3 and Figure 4.3A) whereas there was no inhibition zone for MRSA treated with ampicillin (Table 4.3 and Figure 4.3B). Even though the inhibition zone of MRSA treated with CRE contributed wider diameter (15 mm) comparing to that of *S. aureus* sensitive strain (12 mm), the inhibition zone showed less clear area with more bacteria (Figure 4.3A and B). In contrast, CRE contributed no inhibition zone for both *E. coli* sensitive and CREC (Table 4.4; Figure 4.4A and B).

The crude extract from *C. frutescens* at 250 mg/ml showed no inhibition zone for *S. aureus* sensitive and MRSA including both *E. coli* strains (Table 4.3 and 4.4). Therefore, the concentration higher than 250 mg/ml (>250 mg/ml) may contribute the inhibition zone for both sensitive and resistant strains of *S. aureus* and *E. coli*. Moreover, 12 mm of amoxicillin inhibition zone was shown for *E. coli* sensitive strain (Table 4.4 and Figure 4.4A) whereas there was no inhibition zone for CREC treated with amoxicillin (Table 4.4 and Figure 4.4B).

Table 4.3 Antibacterial susceptibility of medicinal plants by agar disc diffusion method for *S. aureus* ATCC 29213 and MRSA.

Pathogenic bacteria	Inhibition zone of medicinal plants (mm.)			
	Capsicum frutescens Super hot (250 mg/ml.)	*Cyperus rotundus* (250 mg/ml.)	Ampicillin 10 µg/disc (Positive control)	10% DMSO (Negative control)
S. aureus ATCC 29213	0 mm.	12 mm.	10 mm.	0 mm.
MRSA	0 mm.	15 mm.	0 mm.	0 mm.

Table 4.4 Antibacterial susceptibility of medicinal plants by agar disc diffusion method for *E. coli* ATCC 25922 and CREC.

Pathogenic bacteria	Inhibition zone of medicinal plants (mm.)			
	Capsicum frutescens Super hot (250 mg/ml.)	*Cyperus rotundus* (250 mg/ml.)	Amoxicillin 20 µg/disc (Positive control)	10% DMSO (Negative control)
E. coli ATCC 25922	0 mm.	0 mm.	12 mm.	0 mm.
CREC	0 mm.	0 mm.	0 mm.	0 mm.

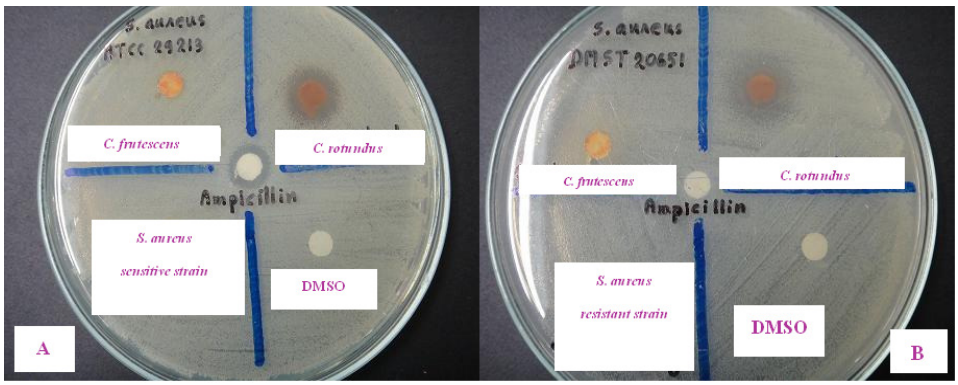

Figure 4.3 Antibacterial susceptibility of medicinal plants by agar disc diffusion method (A) *S. aureus* sensitive strain, (B) MRSA.

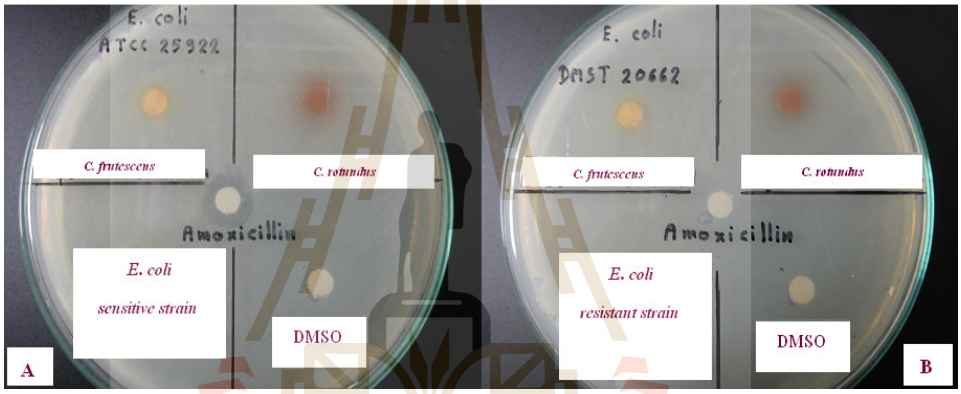

Figure 4.4 Antibacterial susceptibility of medicinal plants by agar disc diffusion method (A) *E. coli* sensitive strain, (B) CREC.

Obviously, CRE inhibited both Gram-positive *S. aureus* and MRSA but did not inhibit both Gram-negative *E. coli* and CREC as shown in Table 4.3 and 4.4; Figure 4.3A and B; Figure 4.4A and B. These demonstrated the Gram-negative bacteria were more resistant than Gram-positive bacteria. The results from this study were similar to the study on activity of some plant extracts against multi-drug resistant human pathogens (Oskay et al., 2009) and antimicrobial studies on Indian medicinal plants against multi-drug resistant pathogens (Ahmad and Beg, 2001). This may be due to the differences in the cell wall composition of Gram-positive bacteria and Gram-

negative bacteria (Oskay et al., 2009; Ahmad and Beg, 2001). The peptidoglycan layer of Gram-negative bacteria is thinner than that of Gram-positive bacteria but surrounded and protected by an outer membrane, whereas Gram-positive bacteria lack the outer membrane (Silhavy et al., 2010). This outer membrane serves as a protective barrier for peptidoglycan layer from the harsh environment including antibacterial agent (Silhavy et al., 2010). The vulnerability of Gram-positive bacteria comparing to Gram-negative bacteria is a consequence of the absence of its outer membrane (Silhavy et al., 2010). This makes Gram-negative bacteria more resistant to the antibacterial agent than the Gram-positive bacteria.

Therefore, *C. rotundus* crude extract can be used as an antibacterial agent for both MRSA and *S. aureus* sensitive strain, whereas *C. frutescens* has no antibacterial activity against MRSA and *S. aureus* sensitive strain, including CREC and *E. coli* sensitive strain.

4.5 Minimum inhibitory concentration (MIC) determination by agar dilution technique with some modifications

The MIC value was beneficial for further analysis, for example; checkerboard assay determination to identify the synergistic activity, time killing assay and investigation of mechanisms of action for CRE against MRSA. The MIC values were determined only for CRE since the preliminary screening of antibacterial activity (agar disc diffusion) had not been found in *C. frutescens* extract. MICs for both *S. aureus* sensitive strain and MRSA treated with CRE and ampicillin were demonstrated in Table 4.5. MRSA treated with CRE displayed the MIC result at 0.5 mg/ml which was higher than that of *S. aureus* sensitive strain at 0.25 mg/ml (Table

4.5 and Figure 4.5). According to the result, MIC of CRE against the sensitive strain (0.25 mg/ml) was not significantly different from the other reports such as antibacterial activity of CRE against oral pathogens (MIC = 1.5 mg/ml) (Jain et al., 2012) and alternative therapies in *S. aureus* diseases (MIC = 0.5 mg/ml) (Kurlenda and Grinholc, 2012). The bacteria treated with ampicillin also showed the MIC results at 0.25 µg/ml for sensitive strain and 64 µg/ml for MRSA (Table 4.5 and Figure 4.5) correlated to CLSI (≤ 0.25 µg/ml is defined as sensitive strain; ≥ 0.5 µg/ml is defined as resistant strain) proven to be sensitive and resistant strains to ampicillin, respectively (Clinical and Laboratory Standards Institute, 2006b).

There was a report on the study of CRE against MRSA presenting in percentage inhibition (2%) (Gawad et al., 2015) but there is still no MIC report for MRSA inhibition including the investigation of mechanisms of action for CRE against MRSA as well.

In conclusion, *C. rotundus* crude extract can act as an antibacterial agent for both MRSA and *S. aureus* sensitive strain. These confirmed the result from agar disc diffusion.

The MICs for both *E. coli* sensitive strain and CREC treated with CRE and amoxicillin are demonstrated in Table 4.6. The CREC treated with CRE displayed the MIC result at 1 mg/ml which was higher than that of *E. coli* sensitive strain at 0.5 mg/ml (Table 4.6 and Figure 4.6). The MIC of CRE against *E. coli* sensitive strain in this study was not significantly different from the other studies including the antibacterial activity of CRE against oral pathogens (MIC = 2 mg/ml) (Jain et al., 2012). The bacteria treated with amoxicillin showed the MIC results at 64 µg/ml for sensitive strain and 128 µg/ml for CREC (Table 4.6 and Figure 4.6).

Table 4.5 Minimum inhibitory concentration (MIC) of medicinal plant extracts by agar dilution method with some modifications for *S. aureus* ATCC 29213 and MRSA.

Pathogenic bacteria	MIC	
	Cyperus rotundus (mg/ml)	Ampicillin (µg/ml)
S. aureus ATCC 29213	0.25[ND]	0.25[S]
MRSA	0.5 [ND]	64[R]

[S] = sensitive, [R] = resistant strain, [ND] = No data in CLSI

Table 4.6 Minimum inhibitory concentration (MIC) of medicinal plant extracts by agar dilution method with some modifications for *E. coli* ATCC 25922 and CREC.

Pathogenic bacteria	MIC	
	Cyperus rotundus (mg/ml)	Amoxicillin (µg/ml)
E. coli ATCC 25922	0.5 [ND]	64 [ND]
CREC	1 [ND]	128 [ND]

[ND] = No data in CLSI

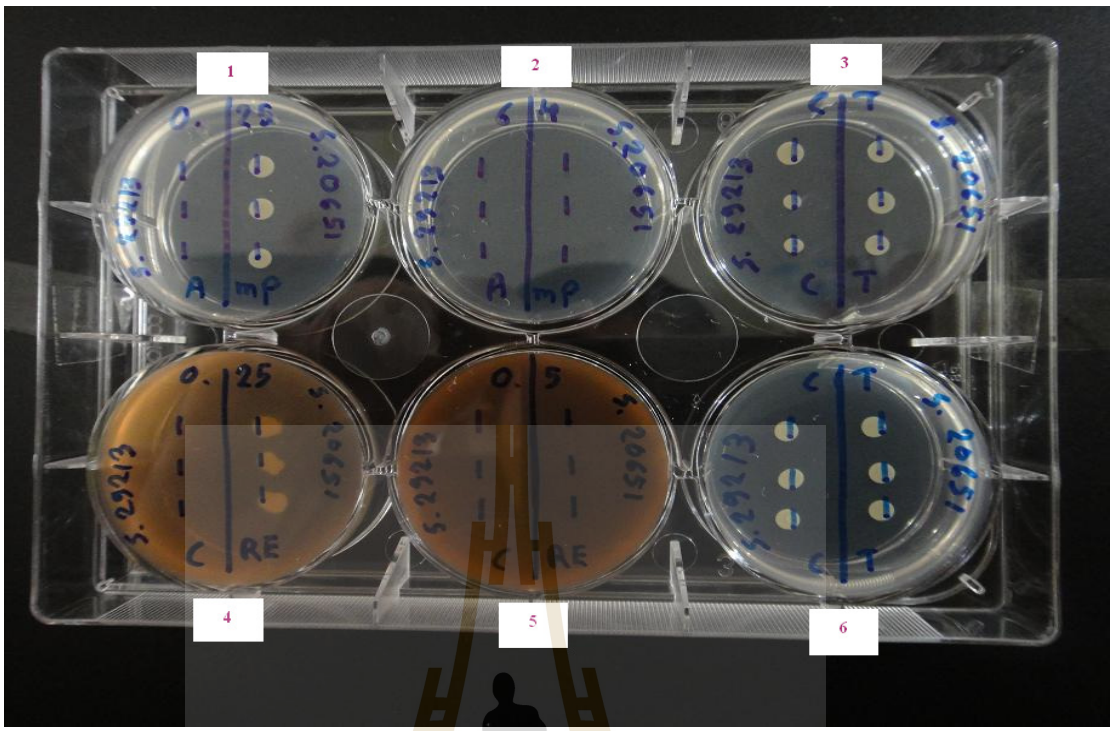

Figure 4.5 Minimum inhibitory concentration (MIC) by agar dilution method with some modifications for *S. aureus* sensitive strain and MRSA: 1 = ampicillin at 0.25 µg/ml, 2 = ampicillin at 64 µg/ml, 3 = Control (Distilled water) for the use of ampicillin whereas, 4 = CRE at 0.25 mg/ml, 5 = CRE at 0.5 mg/ml and 6 = Control (DMSO) for the use of CRE; Left = *S. aureus* sensitive strain and Right = MRSA.

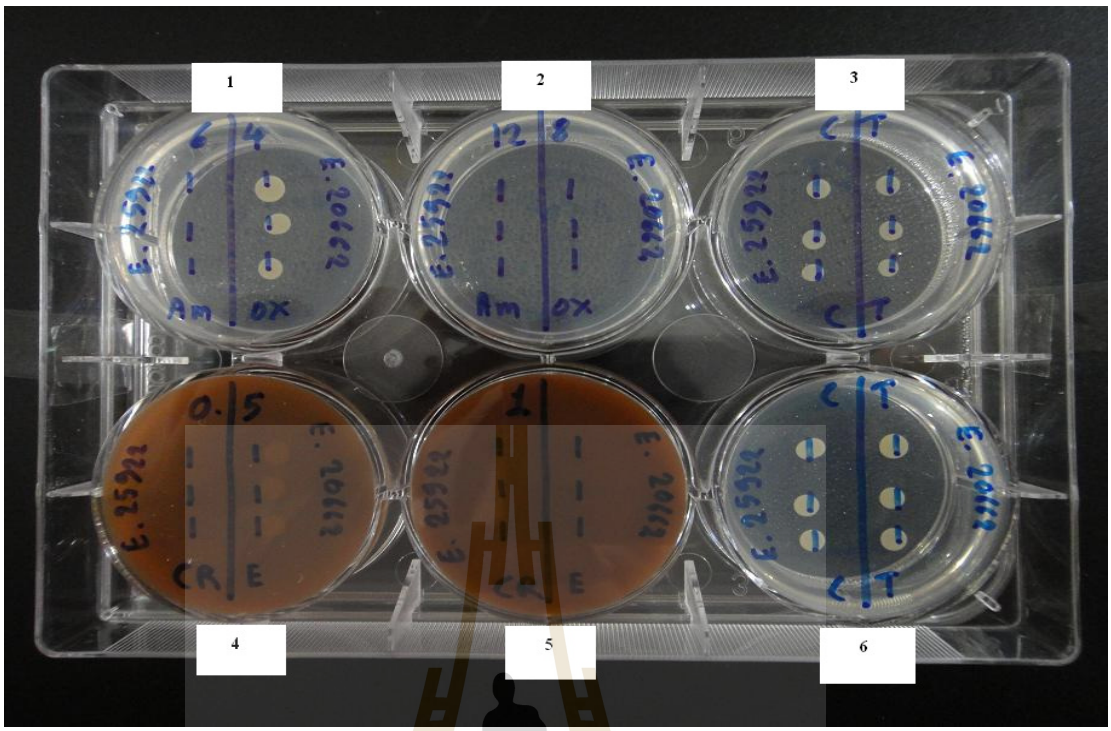

Figure 4.6 Minimum inhibitory concentration (MIC) by agar dilution method with some modifications for *E. coli* sensitive strain and CREC: 1 = amoxicillin at 64 µg/ml, 2 = amoxicillin at 128 µg/ml, 3 = Control (Distilled water) for the use of amoxicillin whereas, 4 = CRE at 0.5 mg/ml, 5 = CRE at 1 mg/ml and 6 = Control (DMSO) for the use of CRE; Left = *E. coli* sensitive strain and Right = CREC.

As mentioned in agar disc diffusion, Gram-negative bacteria are more resistant than Gram-positive bacteria possibly due to the differences in the cell wall composition (Oskay et al., 2009; Ahmad and Beg, 2001). Therefore, the MIC values of Gram-negative *E. coli* either sensitive or resistant strains was higher than those of both Gram-positive sensitive and resistant *S. aureus* strains.

Moreover, the MIC results of CRE treatment for *E. coli* sensitive strain (0.5 mg/ml) and CREC (1 mg/ml) were in contrast to the result from agar disc diffusion (0 mm) due to the higher initial inoculums size, 10^8 cfu/ml for agar disc diffusion and

10^4 cfu/spot for MIC test. Therefore, the concentration greater than 250 mg/ml of CRE must be applied on MHA for agar disc diffusion to obtain the inhibition zone.

Note: The precipitation of CRE in MHB interfered the visibility of bacterial growth. Thus, the agar dilution method for the MIC test was being employed to determine the MIC value. According to OIE Terrestrial Manual, The advantages of agar dilution method include 1.) the ability to detect multiple bacteria on the same MHA plate at the same time with the same condition and 2.) the ability to semi-automate using a replicator which can transfer 32-60 different bacterial inocula onto the same MHA plate (OIE Terrestrial Manual, 2012). However, there are also some disadvantages of this method such as 1.) without replicating apparatus, this method is very laborious including requiring economic and technical resources and 2.) the prepared MHA plates can be stored at 4°C not more than a week or less depending on the antibacterial agent degradation (OIE Terrestrial Manual, 2012).

4.6 Checkerboard assay

According to the result, checkerboard assay with FIC_{index} at 0.27 indicated synergistic activity (Jayaraman et al., 2010; Odds, 2003) for the combination of CRE (0.125 mg/ml) and ampicillin (1 μg/ml) against MRSA as shown in the calculation formula below, Table 4.7 including checkerboard assay (Figure 4.7) and isobologram (Figure 4.8).

This indicated that CRE was able to contribute the synergistic activity with ampicillin antibiotic to inhibit MRSA.

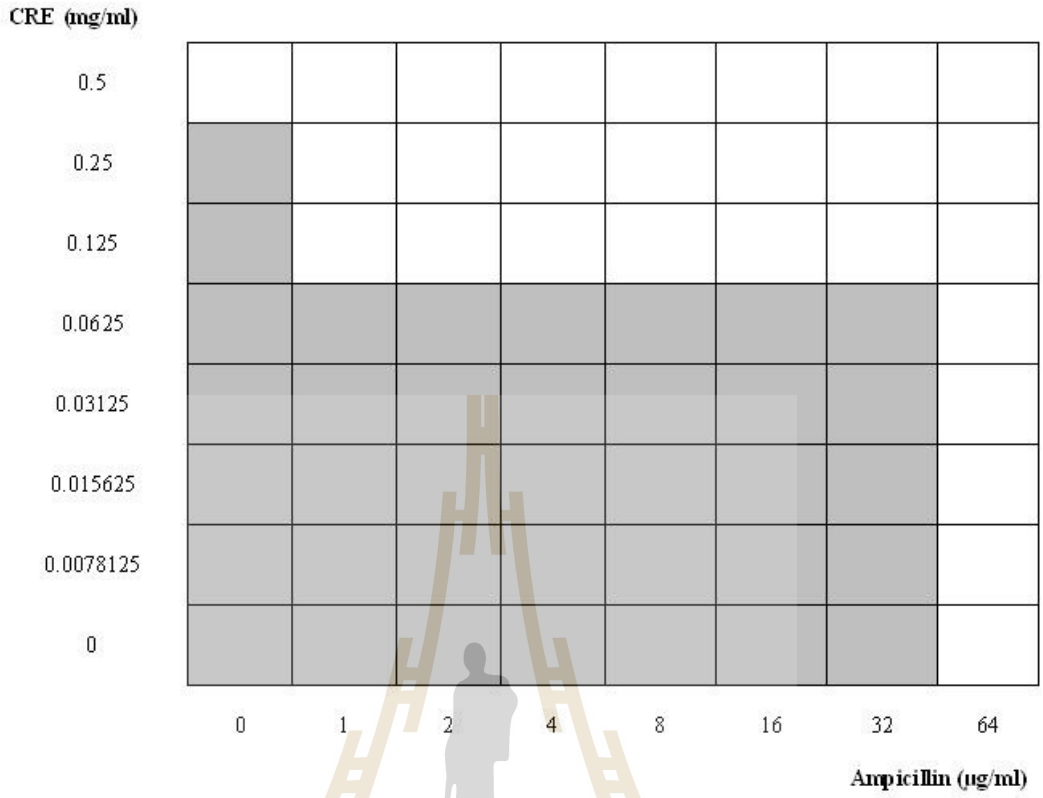

Figure 4.7 Checkerboard assay of the combination of ampicillin and crude extract of *C. rotundus* (CRE) for MRSA: ▨ was MRSA growth, □ was no MRSA growth.

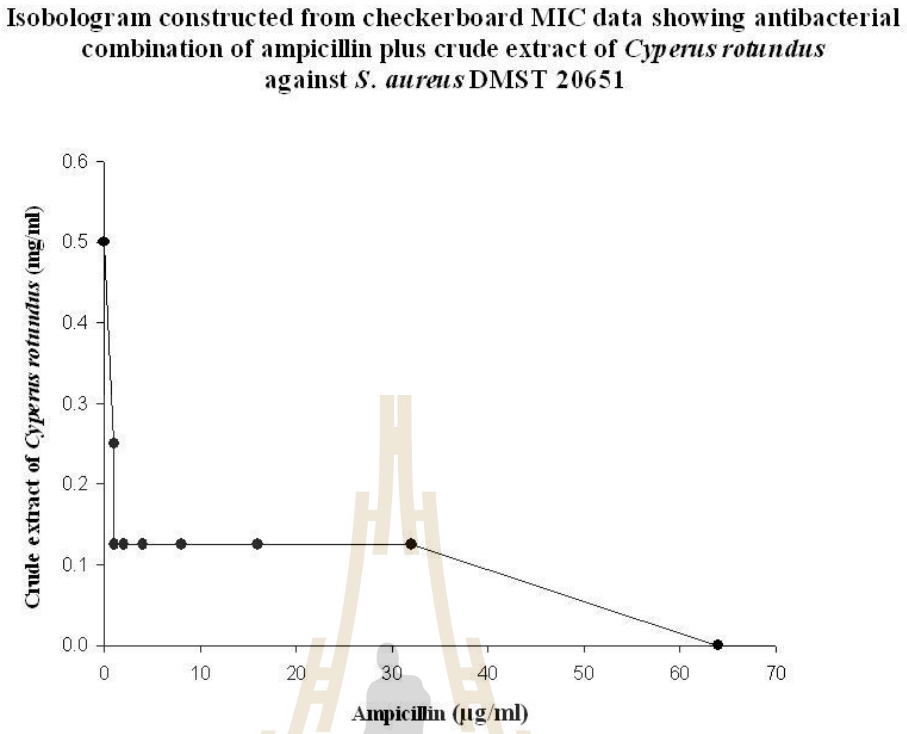

Figure 4.8 Isobologram of the combination of ampicillin and crude extract of *C. rotundus* (CRE) for MRSA.

$FIC_{index} = FIC_a + FIC_b$

$$= \frac{\text{Concentration of A in MIC of A+B}}{\text{MIC of A alone}} + \frac{\text{Concentration of B in MIC of A + B}}{\text{MIC of B alone}}$$

$$= \frac{1}{64} + \frac{0.125}{0.5}$$

= 0.27 (FIC index $\leq$ 0.5 was defined as Synergism)

Table 4.7 Summary the FIC_{index} for checkerboard assay of *Cyperus rotundus* crude extract (CRE) used alone and in combination with ampicillin against drug-resistant bacteria MRSA.

MRSA					
MIC_a (µg/ml)	MIC_b (mg/ml)	FIC_a	FIC_b	FIC_{index}	Type of interaction
64	0.5	0	1	1	no interaction
		0.02	0.5	0.52	no interaction
		0.02	0.25	**0.27**	**synergism**
		0.03	0.25	0.28	synergism
		0.06	0.25	0.31	synergism
		0.13	0.25	0.38	synergism
		0.25	0.25	0.5	synergism
		0.5	0.25	0.75	no interaction
		1	0	1	no interaction

MIC_a = MIC of Ampicillin; MIC_b = MIC of *Cyperus rotundus* crude extract

FIC_a = FIC of Ampicillin; FIC_b = FIC of *Cyperus rotundus* crude extract

There was no synergistic activity found from the combination of CRE and amoxicillin against CREC (FIC_{index} at 1 defined as no interaction) as shown in the calculation below, Table 4.8 including checkerboard assay (Figure 4.9) and isobologram (Figure 4.10).

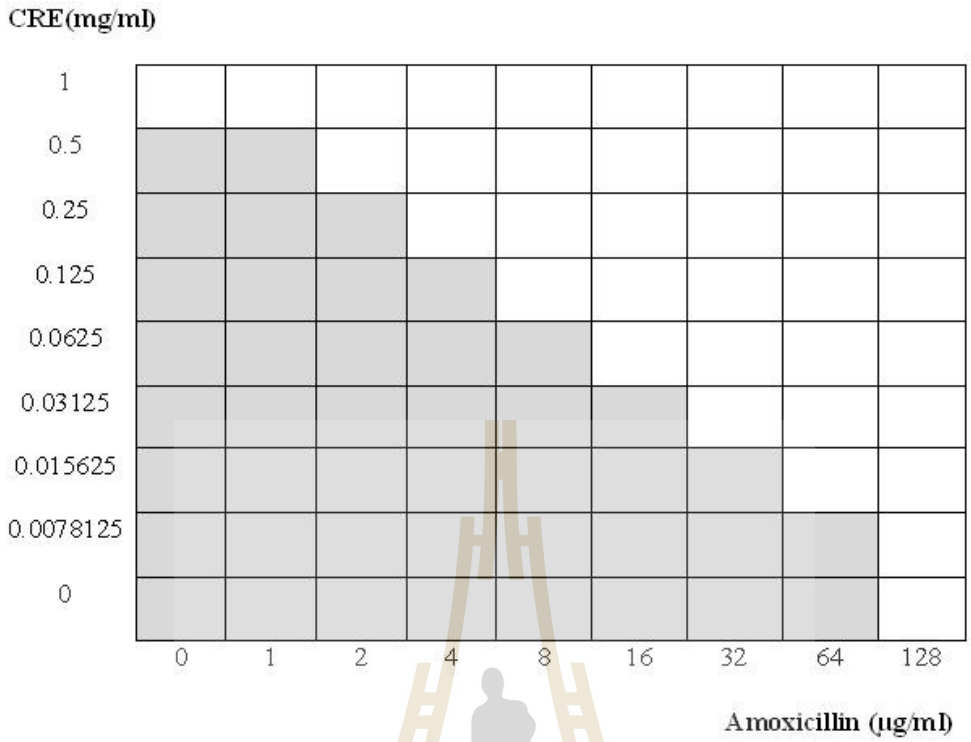

Figure 4.9 Checkerboard assay of the combination of amoxicillin and crude extract of *C. rotundus* (CRE) for CREC: was CREC growth, was no CREC growth.

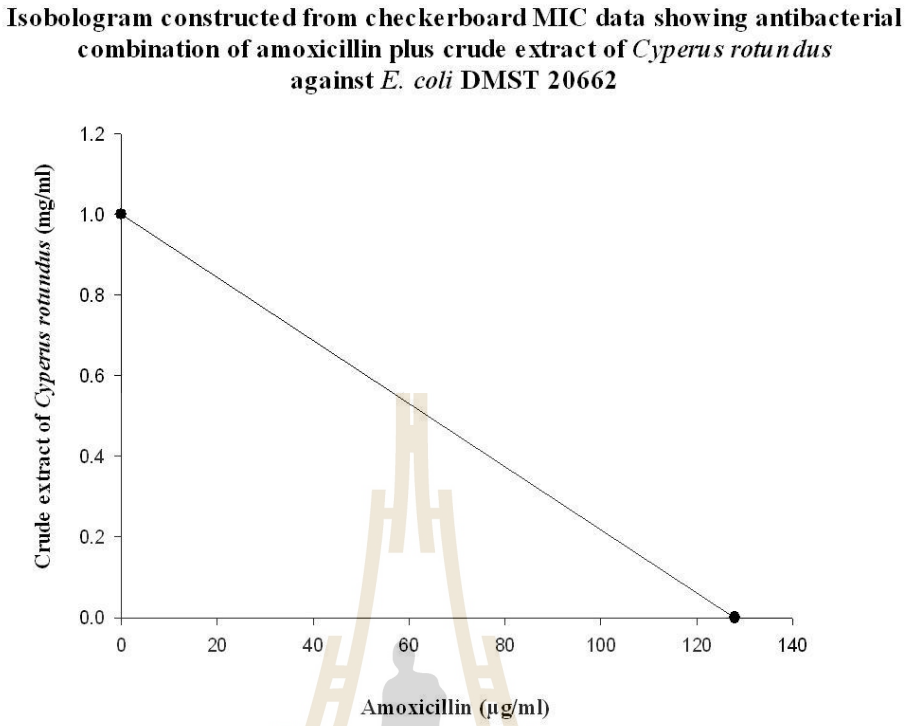

Figure 4.10 Isobologram of the combination of amoxicillin and crude extract of *C. rotundus* (CRE) for CREC.

$FIC_{index} = FIC_a + FIC_b$

$$= \frac{\text{Concentration of A in MIC of A+B}}{\text{MIC of A alone}} + \frac{\text{Concentration of B in MIC of A + B}}{\text{MIC of B alone}}$$

$$= \frac{0}{128} + \frac{1}{1}$$

= 1 (FIC index > 0.5-4.0 was defined as No interaction)

Table 4.8 Summary the FIC_{index} for checkerboard assay of *Cyperus rotundus* crude extract (CRE) used alone and in combination with amoxicillin against drug-resistant bacteria CREC.

CREC					
MIC_a (µg/ml)	MIC_b (mg/ml)	FIC_a	FIC_b	FIC_{index}	Type of interaction
128	1	0	1	1	no interaction
		1	0	1	no interaction

MIC_a = MIC of Amoxicillin; MIC_b = MIC of *Cyperus rotundus* crude extract

FIC_a = FIC of Amoxicillin; FIC_b = FIC of *Cyperus rotundus* crude extract

There were many reports showed antibacterial activities of the combinations of medicinal plants and antibiotics against both Gram-positive and Gram-negative bacteria such as *Trigonella foenum-graecum* L. and chloramphenicol on MRSA; *Thea sinensis* L. and chloramphenicol on *S. aureus* (Darwish et al., 2002), *Scutellaria amoena* (containing baicalin) and cefotaxime on MRSA (Liu et al., 2000) including *Ficus exasperata* and gentamycin on *Escherichia coli* (Odunbaku et al., 2008).

According to the results, these indicated that CRE was able to contribute the synergistic activity with ampicillin to inhibit MRSA.

One approach to treat drug-resistant bacteria is the combination using two or more antibacterial agents (Worthington and Melander, 2013). The advantages of this approach are 1.) decrease the emergence of resistant strains due to the simultaneous use of two or more antibacterial agents against bacteria which develop resistance to each antibacterial agent by each different mechanism, the possibility that the bacterial

colonies will emerge resistant to all of the antibacterial agents employed is very low 2.) decrease dose-related toxicity because of reduced dosage, and 3.) use for treating poly-bacterial infections (Pillai et al., 2005). The mechanisms of drug combinations are as follows; 1.) synergistic multi-target effects, 2.) pharmacokinetic or physicochemical effects which can improve the solubility, resorption rate and enhanced bioavailability, 3.) interactions of agents with resistance mechanisms of bacteria, and 4.) elimination or neutralization of adverse effects originated by one drug through the other agents contained in the drug combination (Wagner and Ulrich-Merzenich, 2009; Wagner, 2011).

4.7 Time killing assay

Time kill assay was determined only for the combination of CRE and ampicillin for MRSA since the combination of CRE and amoxicillin for CREC did not show synergism.

The survival rates of MRSA with various treatments such as ampicillin and CRE, when used singly including the combination of CRE and ampicillin were determined by time killing assay.

Synergistic activity was defined as a decrease of equal to or more than $2\log_{10}$ cfu/ml in comparison to the most active single agent at 24 h (Jacqueline et al., 2003; Belley et al., 2008; Messick et al., 1999) while the interaction was considered for bactericidal activity if there was a decrease of equal to or more than $3\log_{10}$ cfu/ml relative to the initial inoculum at 24 h (Jacqueline et al., 2003; Belley et al., 2008). Bacteriostatic activity was defined as a reduction of less than $3\log_{10}$ cfu/ml comparing to the initial inoculum at 24 h (Belley et al., 2008). In this study, checkerboard assay

determination showed synergistic activity of the combination of CRE at 0.125 mg/ml and ampicillin at 1 µg/ml against MRSA (Figure 4.7 and Figure 4.8). The viable cell count of the CRE-ampicillin treated MRSA at 24 h was dramatically reduced to 130 cfu/ml which was decreased more than $2\log_{10}$ cfu/ml comparing to the most active single agent, CRE at 13,666,667 cfu/ml. The data did not only indicate dramatic synergistic activity (Jacqueline et al., 2003; Belley et al., 2008; Messick et al., 1999) but also indicated bactericidal activity due to the reduction of bacterial cfu/ml more than $3\log_{10}$ cfu/ml comparing to the initial inoculum at 566,666 cfu/ml (Jacqueline et al., 2003; Belley et al., 2008). Moreover, the number of MRSA was not able to recover within 24 h. According to the time killing curve, MRSA treated with 32 µg/ml of ampicillin alone gave the high survival rate because of its highly resistant ability.

According to all data from time killing assay, it had been shown that the combination (synergism) between CRE and ampicillin drug gave greatly high inhibition for MRSA indicating the potential use in the pharmaceutical area.

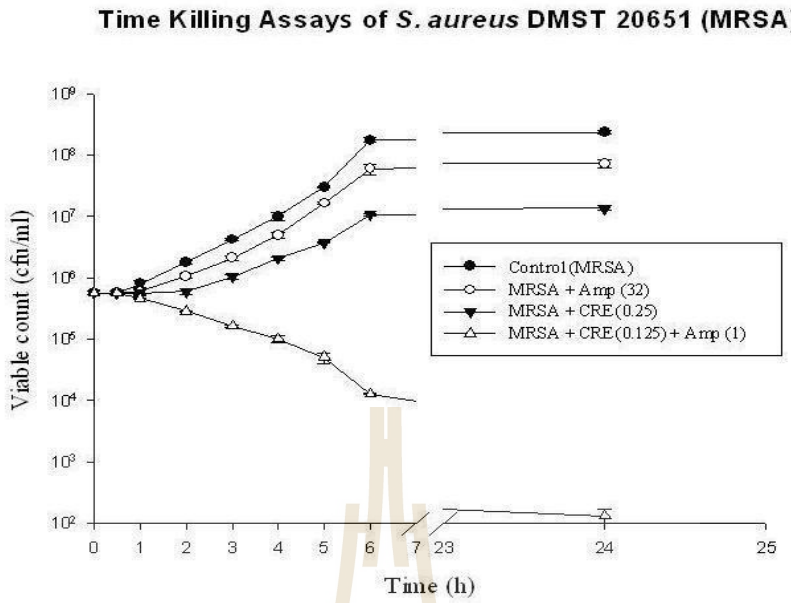

Figure 4.11 Time killing assays of MRSA: CT (control) = MRSA without antibacterial substance, Amp (32) = MRSA with ampicillin concentration at 32 µg/ml, CRE (0.25) = MRSA with crude extract of *C. rotundus* concentration at 0.25 mg/ml, CRE (0.125) + Amp (1) = MRSA with crude extract of *C. rotundus* concentration at 0.125 mg/ml + Ampicillin concentration at 1 µg/ml.

Furthermore, the synergistic activity of CRE and ampicillin combination from the checkerboard assay determination was confirmed by time killing assay (viable cell count decrease $\geq 2\log_{10}$ cfu/ml comparing to the most active single agent at 24 h was determined as synergistic activity) (Jacqueline et al., 2003; Belley et al., 2008; Messick et al., 1999).

The results from a phytochemical screening test, agar disc diffusion, MIC, checkerboard assay determination and time killing assay provided evidence that bioactive compounds such as alkaloids, flavonoids and polyphenols in CRE may be responsible for MRSA inhibition. All these bioactive compounds mentioned above,

possessed the antibacterial activities (Jawad et al., 1988; Konate et al.,, 2012; Zoraghi et al., 2011; Williams et al., 2010; Alcaraz et al., 2000; Wang et al., 2014; Tim and Lamb, 2005; Karou et al., 2005; Taguri et al., 2004; Daglia, 2012).

4.8 Cytoplasmic membrane (CM) permeability assay

There are several assays for determination of the alteration in cytoplasmic membrane permeability (to detect bacterial membrane damage) including the BacLight method of molecular probes, leakage of ATPs and a loss of 260 nm absorbing materials (O'Neill et al., 2004; Oliva et al., 2004).

In this study, the loss of 260 nm absorbing materials was employed for the determination of the alteration in CM permeability. The change in CM permeability resulted in the leakage of materials absorbing at 260 nm (OD), mostly DNA and RNA, indicating cell membrane damage (Carson et al., 2002; Shen et al., 2012). This technique was one of the methods employing to investigate mechanisms of actions of CRE alone and in combination with ampicillin to discover how these antibacterial agents effect on MRSA, as shown in Figure 4.12.

There were some publications reported the combinations of antibacterial agents and antibiotics causing an alteration in CM permeability of some Gram-positive and Gram-negative bacteria (Eumkeb and Chukrathok, 2013; Eumkeb et al., 2012).

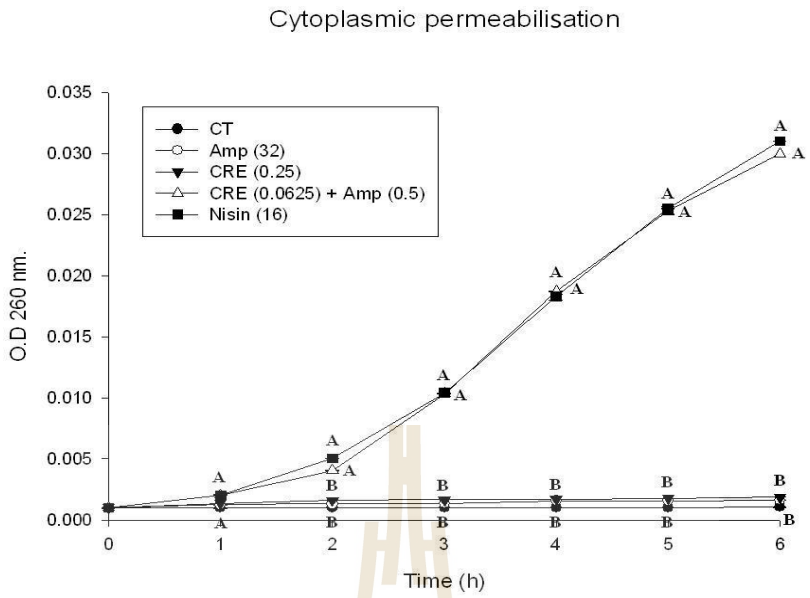

Figure 4.12 Cytoplasmic membrane permeability of MRSA in the presence of either CRE alone or in combination with ampicillin: All data were expressed as mean ± SEM. Each treated group was compared using one-way ANOVA and Tukey's HSD Post-hoc test, $p<0.01$. The A and B (capital alphabets) were presented. The same superscripts were not significantly different from each other. CT (control) = MRSA without antibacterial substance, Amp (32) = MRSA with ampicillin concentration at 32 µg/ml, CRE (0.25) = MRSA with crude extract of *C. rotundus* concentration at 0.25 mg/ml, CRE (0.0625) + Amp (0.5) = MRSA with crude extract of *C. rotundus* concentration at 0.0625 mg/ml + Ampicillin concentration at 0.5 µg/ml, Nisin (16) = Nisin concentration at 16 µg/ml.

The alteration in CM permeability of MRSA treated with ampicillin or CRE, alone was not significantly different from those of each other including control (MRSA without an antibacterial agent). In contrast, the CM permeability of MRSA treated with the combination of CRE and ampicillin was significantly different from

that of control and the others (p<0.01) but not significantly different from that of nisin.

The data from this study indicated that CRE in combination with ampicillin was able to increase CM permeability of MRSA since the CRE may exert its effects through one of the several important mechanisms of action leading to cell lysis. These mechanisms of action may include the damage of the MRSA cell wall during cell division in log phase. As the rate of cell wall synthesis decreases to a halt, the rate of constitutive peptidoglycan autolysis continues, therefore, the breakdown of peptidoglycan leads to cell wall compromise, inability to maintain cell shape resulting in disruption of the CM permeability and increased CM permeability (Drawz and Bonomo, 2010; Holtje, 1998; Scheffers and Pinho, 2005; Chapot-Chartier and Kulakauskas, 2014; Huang et al., 2008; Daly et al., 2011; Mitchell et al., 2013). The increased CM permeability subsequently caused the leakage of intracellular constituents such as β-galactosidase, ATP, DNA and RNA (O'Neill et al., 2004; Oliva et al., 2004; Carson et al., 2002; Shen et al., 2012), and finally, lead to cell lysis.

Therefore, in the presence of CRE in combination with ampicillin, the MRSA cell could not maintain its cell shape leading to disruption of the CM permeability and the leakage of essential molecules such as nucleic acid (DNA and RNA) resulting in cell lysis and death.

In conclusion, the result revealed that the synergistic activity between CRE and ampicillin contributed inhibition highly for MRSA in the log phase, approximately 2-6 h. This result was correlated to the data obtained from time killing assay demonstrating markedly high inhibition at the log phase, 2 to 6 h.

Note: Nisin was used as a positive control for Gram-positive bacteria due to its wide inhibition effect on Gram-positive bacteria (Li et al., 2005; Shen et al., 2012). The nisin inhibits peptidoglycan biosynthesis and forms cellular membrane pores (Wiedemann et al., 2001; Teethaisong et al., 2014), whereas polymyxin B sulphate (PMX) is typically used as a positive control for Gram-negative bacteria (Eumkeb and Chukrathok, 2013).

4.9 Transmission electronmicroscopy (TEM)

Transmission electronmicroscopy was manipulated to view the damage of the cell wall of the MRSA treated by either CRE or the combination between CRE and ampicillin antibiotic as shown in Figure 4.13. The electronmicrographs for MRSA treated with ampicillin, alone exhibited slight damage of the cell wall (30-40%) while the MRSA treated with singly CRE exhibited moderate damage of the cell wall (60-70%), comparing to that of control. In contrast, the most virulent cell wall damage (80-90%) was illustrated in the treatment of MRSA with the combination of CRE and ampicillin at the mid-log phase, 4 h.

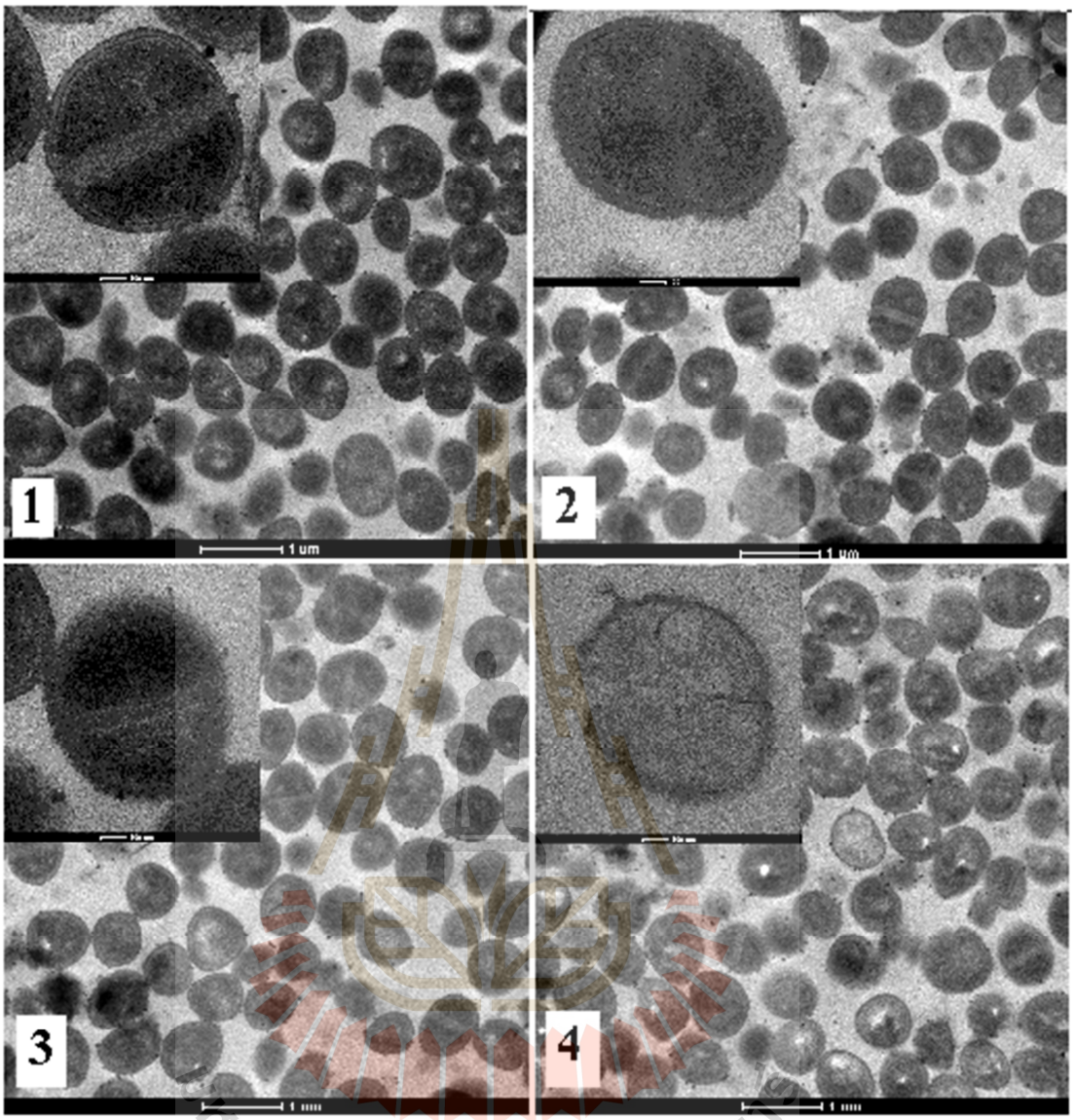

Figure 4.13 Transmission electronmicroscopy of ultrathin sections of MRSA in the presence of either CRE alone or in combination with ampicillin: 1 = Control; MRSA without antibacterial substance, 2 = MRSA with ampicillin concentration at 32 µg/ml, 3 = MRSA with CRE concentration at 0.25 mg/ml, 4 = MRSA with CRE concentration at 0.0625 mg/ml + Ampicillin concentration at 0.5 µg/ml. All electronmicrographs x 43000, bar = 100 nm; inset x 7000, bar = 1 µm.

As described above, this result was correlated with the data from both time killing assay and CM permeability which demonstrated that the damage to the MRSA cell originally occurred from the cell wall damage in the log phase during bacterial cell division, approximately 2-6 h.

Figure 4.14 The comparison of cell size of MRSA growth in the presence of either CRE alone or combination with ampicillin: The mean ± SEM for three, n = 3, treated cells in each group were presented. The graph showed the area of the cell determined by cell width x cell length (nm^2). Each treated group was compared using one-way ANOVA and Tukey's HSD Post-hoc test, $p<0.01$. The A, B and C (capital alphabets) were presented. The same superscripts were not significantly different from each other. CT (control) = MRSA without antibacterial substance, Amp (32) = MRSA with ampicillin concentration at 32 µg/ml, CRE (0.25) = MRSA with crude extract of *C. rotundus* concentration at 0.25 mg/ml, CRE (0.0625) + Amp (0.5) = MRSA with crude extract of *C. rotundus* concentration at 0.0625 mg/ml + Ampicillin concentration at 0.5 µg/ml.

The cell size of MRSA without antibacterial agent, control, (983166.67 ± 9575.72 nm^2) seemed to be larger than those of the other treatments including ampicillin (923466.67 ± 8490.26 nm^2), CRE alone (855453.33 ± 5635.96 nm^2) and the combination of CRE and ampicillin (826106.67 ± 8414.52 nm^2) as shown in Figure 4.14.

The result from the cell size study provided evidence that MRSA treated with the combination of CRE and ampicillin exhibited significantly difference from that of control (MRSA without an antibacterial agent) (p<0.01). The average cell area of MRSA treated with this combination was significantly smaller than that of control. This indicated the dramatic loss of cell wall integrity, leading to an increased MRSA cell size which related to the electronmicrographs (Figure 4.13) showing the most virulent cell wall damage in the treatment of MRSA with the combination of CRE and ampicillin.

There was evidence showed the combinations between antibacterial agents and antibiotics damaging cell wall of both Gram-positive and Gram-negative bacteria (Eumkeb and Chukrathok, 2013; Eumkeb et al., 2012; Teethaisong et al., 2014).

In conclusion, the information from this study revealed the cell wall damage of MRSA correlating to the data from time killing assay and CM membrane permeability which demonstrated markedly high inhibition at the log phase during cell division 2-6 h. This indicated that the damage to the MRSA cell could occur from the cell wall and cytoplasmic membrane disruption during bacterial cell division.

4.10 *β*-lactamase enzyme assay for *β*-lactamase enzyme inhibitor

The *β*-lactamase enzyme assay was applied to test for the presence of a

substance which can act as an inhibitor to inactivate the β-lactamase enzyme. The enzyme activity of β-lactamase was adjusted to the sufficient concentration to hydrolyse 50-60% of the substrate, benzylpenicillin (penicillin G sodium salt), within 5 min. The remaining benzylpenicillin substrates were calculated from the standard curve as shown in Figure H detailed in the appendix. The more β-lactamase inhibitor presence, the more substrate remaining. The results obtained from this study were substantial agreement with the study on the inhibition of the β-lactamase enzyme by galangin in a concentration-dependent manner (Eumkeb et al., 2010).

The assay demonstrated that the benzylpenicillin treated with a β-lactamase enzyme with the combination of CRE and ampicillin contributed the most benzylpenicillin remaining which was significantly different from that of control (MRSA without an antibacterial agent) ($p<0.01$) with the retention time of 2.5 min as shown in Figure 4.15.

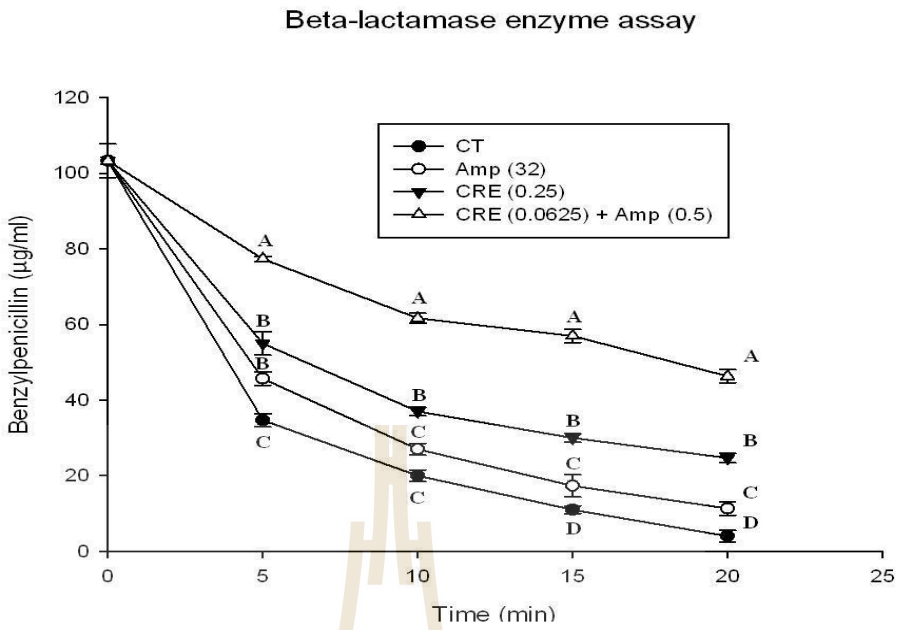

Figure 4.15 The *β*-lactamase enzyme assay of MRSA in the presence of either CRE alone or combination with ampicillin: All data were expressed as mean ± SEM. Each treated group was compared using one-way ANOVA and Tukey's HSD Post-hoc test, $p<0.01$. The A, B, C and D (capital alphabets) were presented. The same superscripts were not significantly different from each other. CT (control) = Benzylpenicillin treated with *β*-lactamase enzyme, Amp = Benzylpenicillin treated with *β*-lactamase enzyme and ampicillin (32 µg/ml), CRE = Benzylpenicillin treated with *β*-lactamase enzyme and crude extract of *C. rotundus* (0.25 mg/ml), CRE + Amp = Benzylpenicillin treated with *β*-lactamase enzyme and the combination of crude extract of *C. rotundus* (0.0625 mg/ml) + Ampicillin (0.5 µg/ml).

The *β*-lactamase plays a major role in the resistance mechanism of bacteria to *β*-lactam antibiotics (Shendurnikar and Pandya, 1996). This study provided strong evidence that the combination of CRE and ampicillin imparted the inhibitory activity of *β*-lactamase implying the presence of *β*-lactamase inhibitor in CRE. The

mechanisms of action of the β-lactamase inhibitor in CRE may occur due to the formation of this combination and β-lactamase complex leading to the inactivation of β-lactamase activity. This may be because of its either similar key structure to the β-lactam antibiotic (Eumkeb et al., 2010) or its ability to act as a non-β-lactam inhibitor. Thus, the β-lactamase enzyme was inactivated by this inhibitor, therefore allowing or enhancing the β-lactam antibiotic to damage the cell wall of MRSA (Bernal et al., 2013). This may be at least one of the major mechanisms of action of this combination against MRSA. Therefore, CRE could reverse the resistance of MRSA to the activity of the primary ampicillin antibiotic through β-lactamase inhibitor. In addition, the synergistic activity of the combinations of plant crude extracts and antibiotics has been proven by several publications to be an interesting approach to combat antibiotic resistant bacteria (Eumkeb and Chukrathok, 2013; Eumkeb et al., 2010; Eumkeb et al., 2012; Darwish et al., 2002).

Moreover, CRE also had the antibacterial effect of its own to against antibiotic resistant bacteria as shown in MIC determination (0.5 mg/ml) similar to the other plant crude extracts reported by some publications (Teethaisong et al., 2014; Adwan et al., 2010; Aiyegoro et al., 2011). This indicated that CRE not only contained the inhibitor to destroy enzyme for antibiotic resistance but also possessed the ability to disrupt other mechanisms of antibiotic resistance including target alteration, impermeability and efflux. On the other hand, CRE might exert mechanisms of actions of typical antibiotic drugs, like other antibiotics, such as (1) interfere with bacterial cell wall synthesis, (2) interfere with the function of bacterial cell membrane, (3) interfere with bacterial protein synthesis, (4) interfere with the function of bacterial nucleic acid and (5) interfere with a metabolic pathway of bacteria.

Recent studies demonstrated that β-lactamase inhibitors, which possessed β-lactam structures caused the induction of β-lactamase expression and hyperproduction of β-lactamase (Tzouvelekis et al., 1997; Stapleton et al., 1995; Eumkeb et al., 2010). Therefore, these currently most available β-lactamase inhibitors can also lose their activities through the same mechanism as the β-lactam antibiotics (Eumkeb et al., 2010). This indicates that the other alternative β-lactamase inhibitors with non-β-lactam structures are urgently needed to overcome the β-lactam antibiotic resistance via multiple mechanisms. The CRE contains bioactive compounds such as alkaloids, flavonoids and polyphenols which may be responsible for MRSA inhibition. Some of these bioactive compounds i.g. galangin, quercetin and baicalein can act as a β-lactamase inhibitor with non-β-lactam structure (Eumkeb et al., 2010). Thus, the bioactive compounds present in CRE may also act as a non-β-lactam β-lactamase inhibitor (non-β-lactam inhibitor) as well. This becomes the great benefit of CRE to overcome the β-lactam antibiotic resistance through multiple mechanisms.

In consonance with, all data, CRE can be employed to defeat β-lactam antibiotic resistance not only via β-lactamase inhibitor, which may either be with or without β-lactam structure, but also via other strategies such as exert the anti-resistant activity by other mechanisms which must be further investigated.

In conclusion, CRE can conquer the β-lactam antibiotic resistant bacteria by multiple mechanisms as mentioned above.

CHAPTER V

CONCLUSIONS

Antibiotic resistance has become a serious problem threatening to public health and medical implications. In Thailand, the emergence of antibacterial resistance has also been considerably documented in many areas including in various sections of the Maharat Nakhon Ratchasima Hospital (Chokejindachai, 2007; Maharat Nakhon Ratchasima hospital, 2012).

The MRSA causes many medical-therapeutic complications (Kurlenda and Grinholc, 2012; Raygada and Levine, 2009). In fact, MRSA not only resists to methicillin and β-Lactam group but also to aminoglycosides, fluoroquinolones, macrolides and tetracyclines (Kaur and Chate, 2015). Furthermore, certain bacteria develop resistance to ceftazidime including *Escherichia coli.* Antibacterial resistant *E. coli* not only resists to ceftazidime and cefotaxime (Oteo et al., 2006) but also ampicillin, cotrimoxazole, ciprofloxacin, gentamicin (Oteo et al., 2002) including amoxicillin (Kibret and Abera, 2011).

These indicate that antibiotic drugs may not be effective to cure the bacterial infections. Therefore, the research and development of new therapies for bacterial infection treatments are urgently needed (Mahady et al., 2008).

An advantage of medicinal plants is: it creates minimal side effects (George, 2011) and least or no toxicities comparing to synthetic drugs (Ahmad and Beg, 2001). Moreover, crude extracts of medicinal plants are much more effective than the only

pure active compound alone (Dupont et al., 2006; Pistelli et al., 2000; Rasoanaivo et al., 2011). Because of these, many scientists are currently trying to use medicinal plants to substitute for those antibiotics.

The crude extract of *C. rotundus* (CRE) can be utilized as an antibacterial agent for both MRSA and *S. aureus* sensitive strain, whereas *C. frutescens* at the concentration used in this study (250 mg/ml) has no antibacterial activity against MRSA and *S. aureus* sensitive strain, including CREC and *E. coli* sensitive strain. The CRE contains bioactive compounds such as alkaloids, flavonoids and polyphenols which may be responsible for MRSA inhibition. MRSA treated with CRE displayed the MIC result at 0.5 mg/ml which was higher than that of *S. aureus* sensitive strain at 0.25 mg/ml. Furthermore, the CREC treated with CRE displayed the MIC result at 1 mg/ml which was higher than that of *E. coli* sensitive strain at 0.5 mg/ml. In addition, CRE was able to contribute the synergistic activity with ampicillin to inhibit MRSA (FIC_{index} = 0.27) whereas no synergistic activity found from the combination of CRE and amoxicillin against CREC (FIC_{index} = 1). Time killing assay was confirmed a dramatic reduction in the viable count of MRSA after treated with the sub-MIC of this combination compared to the most active single agent**.**

Transmission electronmicroscopy (TEM) revealed the cell wall damage of MRSA during cell division at log phase. The breakdown of peptidoglycan leads to cell wall compromise, inability to maintain cell shape, resulting in disruption of the CM permeability and increased CM permeability (Drawz and Bonomo, 2010; Holtje, 1998; Scheffers and Pinho, 2005; Chapot-Chartier and Kulakauskas, 2014; Huang et al., 2008; Daly et al., 2011; Mitchell et al., 2013). The increased CM permeability subsequently caused the leakage of intracellular constituents such as nucleic acid,

DNA and RNA (Carson et al., 2002; Shen et al., 2012), and finally, lead to cell lysis.

The β-lactamase enzyme assay provided strong evidence that the combination of CRE and ampicillin imparted the inhibitory activity of β-lactamase implying the presence of β-lactamase inhibitor in CRE. The mechanisms of action of the β-lactamase inhibitor in CRE may occur due to the formation of this combination and β-lactamase complex leading to the inactivation of β-lactamase activity. This may be because of its either similar key structure to the β-lactam antibiotic (Eumkeb et al., 2010) or its ability to act as a non-β-lactam inhibitor. Thus, the β-lactamase enzyme was inactivated by this inhibitor, therefore allowing or enhancing the β-lactam antibiotic to damage the cell wall of MRSA (Bernal et al., 2013). This may be at least one of the major mechanisms of action of this combination against MRSA. Therefore, CRE could reverse the resistance of MRSA to the activity of the primary ampicillin antibiotic through β-lactamase inhibitor.

Moreover, CRE also had the antibacterial effect of its own to against antibiotic resistant bacteria as shown in MIC determination (0.5 mg/ml). This indicated that CRE not only contained the inhibitor to destroy enzyme for antibiotic resistance but also possessed the ability to disrupt other mechanisms of antibiotic resistance including target alteration, impermeability and efflux. On the other hand, CRE might exert mechanisms of actions of typical antibiotic drugs, like other antibiotics, such as to interfere with the ultrastructures of bacteria or the functions of its organelles, e.g. cell wall synthesis, protein synthesis, a metabolic pathway of bacteria, etc.

Taken all data together, CRE not only contained a β-lactamase inhibitor which may either be with or without β-lactam structure, but also possessed the ability to conquer the β-lactam antibiotic resistant bacteria by other mechanisms which must be

further investigated. Therefore, CRE exerts its anti-resistant activity through multiple mechanisms. Because of this, it can be employed for the potential treatment of MRSA, which almost resistant to practically β-lactam antibiotics.

In conclusion, the combination (synergism) between CRE and ampicillin drug gave greatly high inhibition for MRSA indicating the potential to be a novel adjuvant phytopharmaceuticals for ampicillin to treat MRSA and to be considered for development of new natural β-lactamase inhibitor which may either be with or without β-lactam structure.

Actually, crude extracts of medicinal plants are much more efficient than the only pure active compound alone (Dupont et al., 2006; Pistelli et al., 2000; Rasoanaivo et al., 2011), and creates minimal side effects (George, 2011) and least or no toxicities comparing to synthetic drugs (Ahmad and Beg, 2001). Because of these, CRE becomes more interesting to be use as one of the medicinal plant extracts to substitute for those synthetic antibiotics.

This is the first report demonstrating the mechanisms of action of CRE on MRSA by a β-lactamase enzyme inhibitor. Further investigations should be performed on the active ingredients study, toxicity and the level of synergistic effect on blood and tissue confirmation in animals and humans. However, the oral administration of CRE in rats showed non-acute-toxicity, and no mortality or no behaviour change for subacute toxicity (Sivapalan, 2013).

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APPENDIX

APPENDIX

1. Nutrient agar

HiMedia® nutrient agar was used for the preparation of stock cultures on agar slants.

The formula was:

	g/L
Peptic digests of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	1.5

pH (at 25°C) 7.4 ± 0.2

2. Mueller-Hinton broth (MHB)

Difco® Mueller Hinton broth was the medium used for determining the antimicrobial susceptibility testing.

The formula was:

	g/L
Beef infusion solids	4.0
Casein hydrolysate	17.5
Soluble starch	1.5

pH 7.4 ± 0.2 at 37°C

3. Mueller-Hinton agar (MHA)

Difco® Mueller Hinton agar was the medium used for determining the antimicrobial susceptibility testing.

The formula was:

	g/L
Agar	17.0
Beef heart infusion	2
Casein acid hydrolysate	17.5
Soluble starch	1.5
pH 7.3 ± 0.2 at 25°C	

All culture media were dissolved by water then sterilize in autoclave at 121°C for 15 min. Do not overheat.

Note: The advantages of MHA for antimicrobial susceptibility testing include 1.) acceptable batch-to-batch reproducibility, low sulfonamide, trimethoprim and tetracycline inhibitors, the satisfactory growth of most bacterial pathogens and better diffusion of microbial agents (Clinical Laboratory Standards Institute, 2006a). On the other hand, MHA is not suitable for assay with slow growing microorganisms, anaerobes and capnophiles (Clinical Laboratory Standards Institute, 2006a).

4. Chemicals

All chemicals used were laboratory grade otherwise specified.

Acetic acid	HPLC grade
Acetone	TEM grade
Acetonitrile	HPLC grade
Ammonium acetate	AR grade
β-lactamase type IV from *Enterobacter cloacae*	Sigma
Chloroform	AR grade
Cycloaliphatic Epoxide Resin ERL 4221 (ERL-4221)	TEM grade
DER 736 Epoxy Resin (DER 736 Epoxy)	TEM grade
Dimethyl sulfoxide (DMSO)	AR grade
2-(Dimethylamino) ethanol (DMAE)	TEM grade
Ethanol Absolute	AR grade
95% Ethanol	Lab grade
Ethyl acetate	HPLC grade
Ferric chloride	AR grade
Formic acid	HPLC grade
Gallic acid	HPLC grade
Glacial acetic acid	AR grade
Gelatin	AR grade
Glutaraldehyde	AR grade
HEPES (sodium salt)	AR grade
Hexane	HPLC grade

Hydrochloric acid	AR grade
Iodine	AR grade
Lead acetate	AR grade
Lead citrate	TEM grade
Magnesium ribbon	AR grade
Methanol	AR grade
Methanol	HPLC grade
Mercuric chloride	AR grade
Nonenyl Succinic Anhydride Modified (NSA)	TEM grade
Osmium tetroxide	AR grade
Penicillin G sodium salt (Benzylpenicillin)	Sigma
Potassium ferricyanide	AR grade
Potassium iodide	AR grade
Quercetin	HPLC grade
Rotundine	HPLC grade
Sodium chloride	AR grade
Sodium hydroxide	AR grade
Sodium phosphate	AR grade
Sodium phosphate dibasic	AR grade
Sodium phosphate monobasic	AR grade
Sulfuric acid	AR grade
Uranyl acetate	TEM grade

5. Equipments

5.1 Apparatus

Autoclave	Tomy SX-700
Bacterial vacuum filter	Corning
Biosafety cabinet Class II	Heal force
Column C18 (5 µm, 250 x 4.6 mm)	Bio-sil
Column zorbax sb-C18 RRHD (1.8 µm, 2.1 x 150 mm)	Agilent-technologies
Filter paper No. 1 Ø125 mm	Whatman
Heating mantle	Mtops
High Performance Liquid Chromatography	Water 600
Hot air oven	Shellab
Hot plate	VELP scientific
Incubator	Memmert
Liquid chromatography-tandem mass spectrometry -Agilent Technologies 1290 Infinity -Agilent Technologies 6490 Triple Quad LC/MS	Agilent-technologies
Lyophilizer	Labconco
Micropipettor (2-20 µl)	Witeg
Micropipettor (20-200 µl)	Witeg
Micropipettor (100-1000 µl)	Witeg
Micropipettor (1- 5 ml)	Witeg
Microplate reader	Biorad
Microplate reader (UV visible range)	Biotex

pH meter	Thermo Scientific
Refrigerated centrifuge (Universal 320 R)	Hettich
Refrigerator -80ºC	Thermo Scientific
Refrigerator	Sanyo and Sharp
Rotary evaporator	Buchi
Rotator	FINEPCR
Shaking water bath	GFL
Soxhlet apparatus	Pyrex
Spectrophotometer (Spectronic 21)	Milton Ray
Sterile nylon membrane filter 0.22 µm	Sigma-Aldrich
Sterile 6-well plates	Corning
Sterile 96-well microplates	Corning
Sterile 96-well microplates for UV visible range	Corning
Syringe 5, 10, 30 ml	Nalgene
Syringe filter with sterile cellulose acetate membrane filter 0.22 µm	Labobuy
Syringe filter with sterile cellulose regenerated acetate membrane filter 0.22 µm	Corning
Transmission electron microscope JEM-1230	JEOL
Ultramicrotome	JEM

5.2 Glasswears

Beakers (50, 100, 250, 500, 1000, 2000 ml)

Conical centrifuge tubes (15, 50 ml)

Duran amber-bottles with caps (50, 100, 125, 500 ml)

Duran bottles with caps (50, 100, 125, 500, 1000 ml)

Eppendorf tubes (1.5 ml)

Erlenmeyer flasks (125, 250, 500 ml)

Glass cuvettes

Graduated glass pipettes (1, 5, 10 ml)

Measuring cylinders (10, 50, 100, 250, 500, 1000 ml)

Micropipette tips (2-200 µl)

Micropipette tips (100-1000 µl)

Petri dishes

Test tubes

Volumetric flasks (10, 50, 100, 500, 1000, 2000 ml)

6. Preparation of reagents for phytochemical screening test

6.1 Mayer's reagent

6.1.1 Mercuric chloride solution

Mercuric chloride	1.358	g
Distilled water	60	ml

6.1.2 Potassium iodide solution

Potassium iodide	5	g
Distilled water	10	ml

Thoroughly, mix mercuric chloride solution with potassium iodide solution then make up the volume to 100 ml by distilled water.

6.2 1% HCl

Concentrated HCl	1	ml
Distilled water	100	ml

6.3 Wagner's reagent

Iodine	2	g
Potassium iodide	6	g
Distilled water	100	ml

6.4 10% Lead acetate solution

Lead acetate	10	g
Distilled water	100	ml

6.5 1% Gelatin solution containing 10% NaCl

6.5.1 1% Gelatin solution

Gelatin	1	g
Distilled water	100	ml

6.5.2 1% Gelatin solution containing 10% NaCl

NaCl	10	g
1% Gelatin solution	100	ml

6.6 5% Ferric chloride solution

Ferric chloride	5	g
Distilled water	100	ml

6.7 1% Ferric chloride solution

Ferric chloride	1	g
Distilled water	100	ml

6.8 1% Potassium ferricyanide solution

Potassium ferricyanide	1	g
Distilled water	100	ml

7. Preparation of chemical solution for phytochemical quantitative determination

7.1 Determination of total alkaloid content

7.1.1 50 mM Ammonium formate in water

Ammonium formate	3.153	g
Distilled water	1000	ml

7.1.2 0.1% formic acid and 5 mM ammonium formate in water

Formic acid	1	ml
50 mM Ammonium formate	100	ml
Distilled water	899	ml

7.2 Determination of total flavonoid and polyphenol content

7.2.1 0.1% Formic acid in water

Formic acid	1	ml
Distilled water	999	ml

7.2.2 0.1% formic acid in acetonitrile

Formic acid	1	ml
Acetonitrile	999	ml

8. Preparation of chemical solution for bacterial suspension standard curve, agar disc diffusion, minimum inhibitory concentration by agar dilution technique, checkerboard assay determination and time killing assay

8.1 0.85% NaCl

NaCl	8.5	g
Distilled water	1000	ml

Sterilize in autoclave at 121°C for 15 min.

9. Preparation of chemical solution for cytoplasmic membrane permeability

9.1 0.85% NaCl

NaCl	17	g
Distilled water	2000	ml

Sterilize in autoclave at 121°C for 15 min.

9.2 5 mM sodium HEPES buffer pH 7.0

HEPES (sodium salt)	1.3015	g
Distilled water	800	ml

Add 1 N NaOH dropwise to achieve pH = 7.0, then bring the volume up to 1000 ml of distilled water by using 1000 ml volumetric flask. Filter through syringe filter with sterile cellulose acetate membrane filter 0.22 µm and store at 4°C.

9.3 1000 mM Glucose

Glucose	180	g
Distilled water	1000	ml

Sterilize in autoclave at 121°C for 15 min.

9.4 2.5 mM sodium HEPES buffer pH 7.0 supplemented with 100 mM glucose

5 mM sodium HEPES buffer pH 7.0	500	ml
1000 mM Glucose	100	ml
Distilled water	400	ml

10. Preparation of chemical solution for transmission electronmicroscopy (TEM)

10.1 0.85% NaCl

NaCl	8.5	g
Distilled water	1000	ml

Sterilize in autoclave at 121°C for 15 min.

10.2 0.1 M Sodium phosphate monobasic

$NaH_2PO_4.H_2O$	6.9	g
Distilled water	500	ml

Sterilize in autoclave at 121°C for 15 min.

10.3 0.1 M Sodium phosphate dibasic

$Na_2HPO_4.7H_2O$	13.4125	g
Distilled water	500	ml

Sterilize in autoclave at 121°C for 15 min.

10.4 0.1 M Phosphate buffer pH 7.2

0.1 M Sodium phosphate monobasic	140	ml
0.1 M Sodium phosphate dibasic	360	ml

10.5 2.5% Glutaraldehyde in 0.1 M phosphate buffer

50% Glutaraldehyde	10	ml
0.1 M Phosphate buffer pH 7.2	200	ml

10.6 Resin

ERL-4221	10	g
DER 736 Epoxy	8	g
NSA	26	g
DMAE	0.2-0.3	g

Stir with a magnetic stirrer for 5 min and incubate overnight at -20°C. Stand to allow the temperature to achieve room temperature before use.

11. Preparation of chemical solution for β-lactamase enzyme assay for β-lactamase enzyme inhibitor

11.1 10 mM Ammonium acetate, pH 4.5 (acetic acid)

Ammonium acetate	1.5416	g
Distilled water	1800	ml

Add concentrated acetic acid dropwise to achieve pH = 4.5, then bring the volume up to 2000 ml of distilled water by using 2000 ml volumetric flask.

11.2 10 mM Ammonium acetate (pH 4.5 acetic acid) : acetonitrile (75:25)

10 mM Ammonium acetate, pH 4.5 (acetic acid)	1500	g
Acetonitrile	500	ml

11.3 0.1 M Phosphate buffer pH 7.0

0.1 M Sodium phosphate monobasic	195	ml
0.1 M Sodium phosphate dibasic	305	ml

11.4 50 mM Phosphate buffer pH 7.0

0.1 M Phosphate buffer pH 7.0	250	ml
Distilled water	250	ml

11.5 Methanol : acetic acid (100:1)

Methanol	500	ml
Acetic acid	5	ml

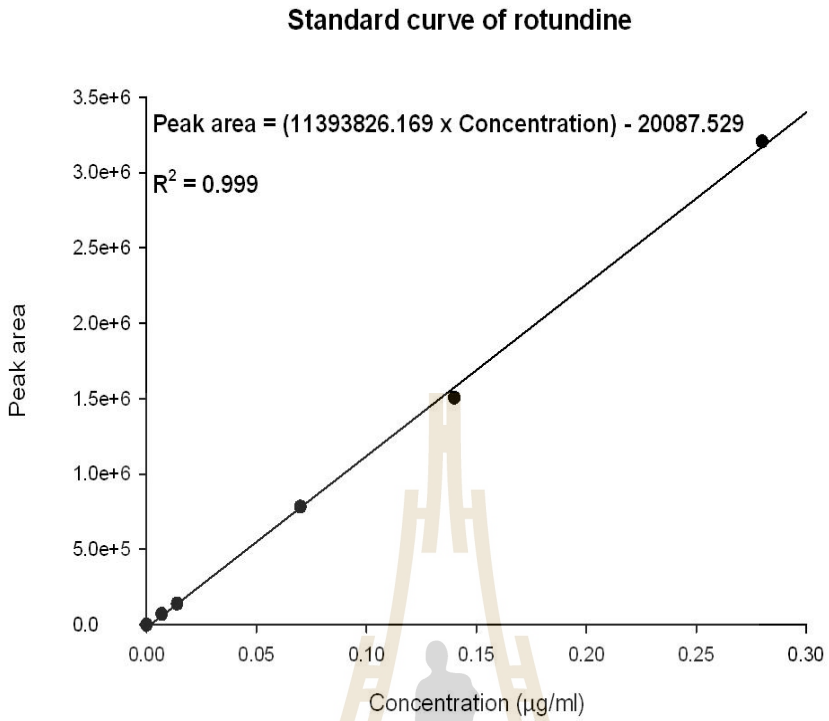

Figure A Standard curve of rotundine.

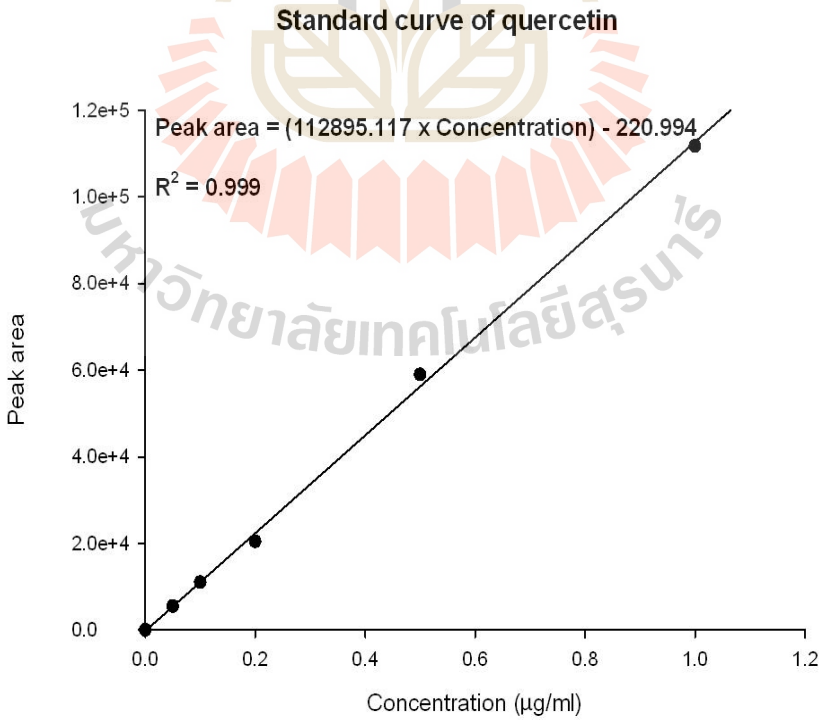

Figure B Standard curve of quercetin.

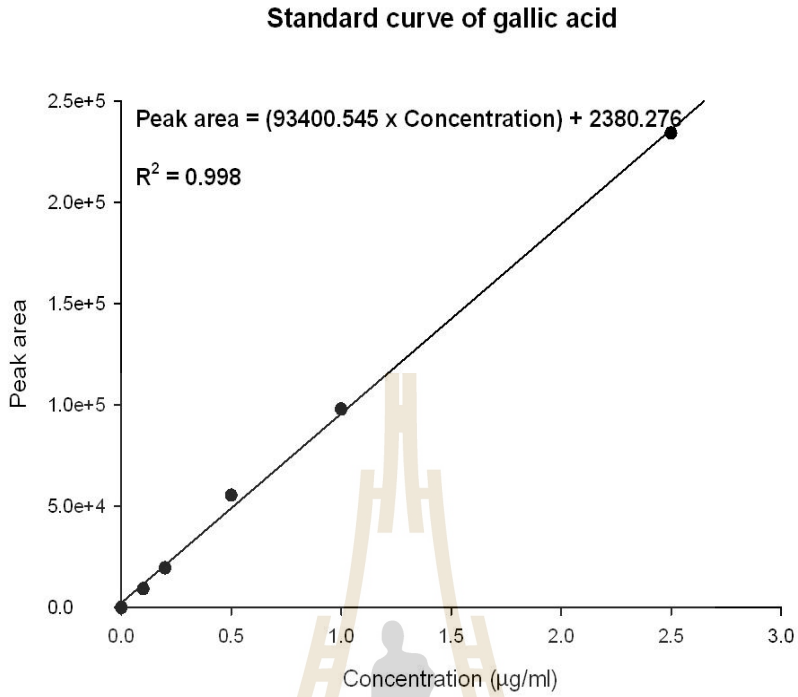

Figure C Standard curve of gallic acid.

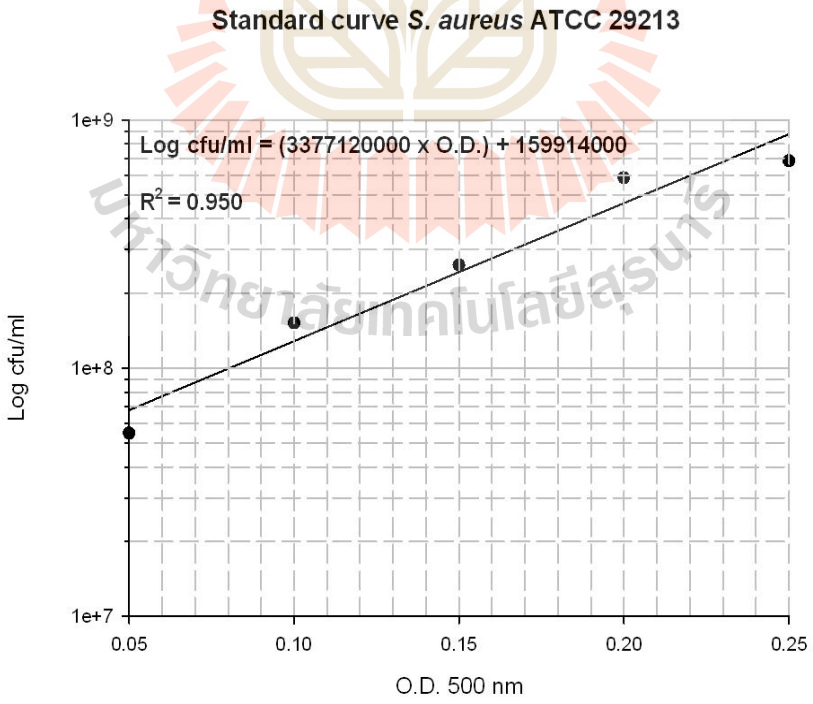

Figure D Bacterial suspension standard curve of *S. aureus* ATCC 29213.

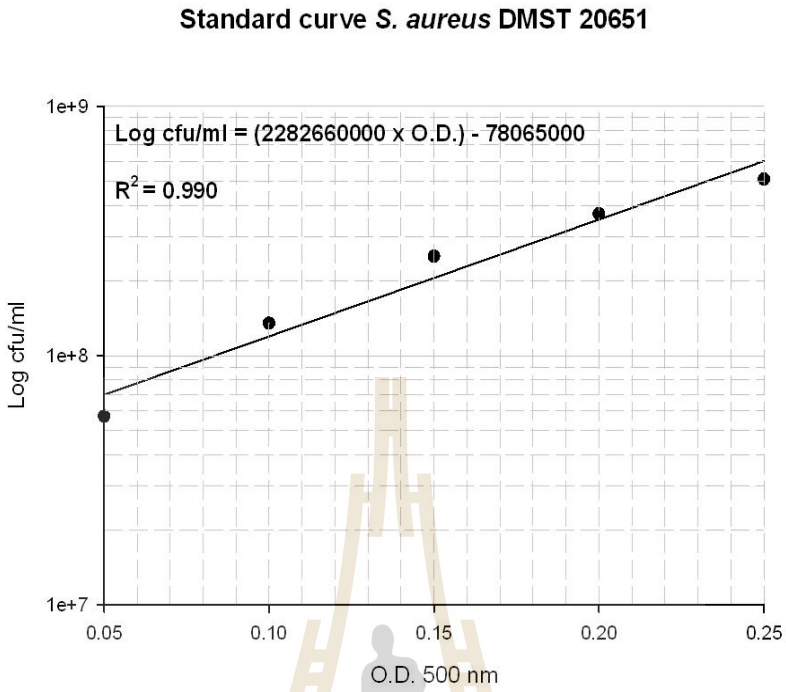

Figure E Bacterial suspension standard curve of MRSA.

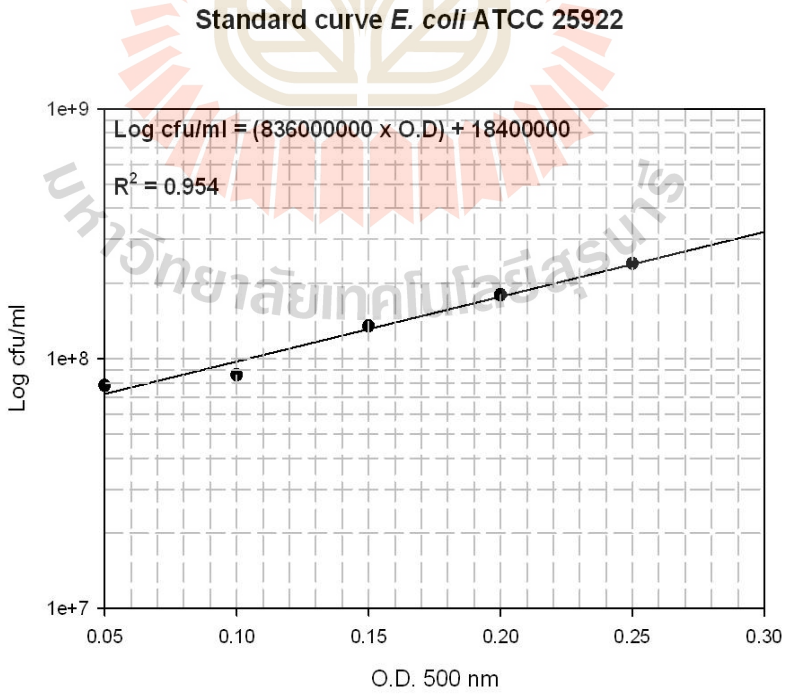

Figure F Bacterial suspension standard curve of *E. coli* ATCC 25922.

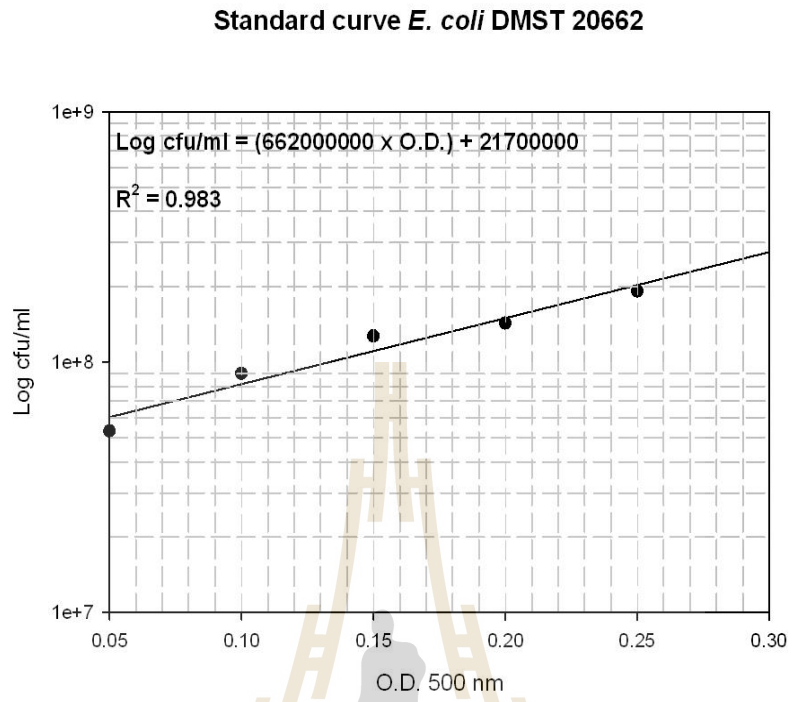

Figure G Bacterial suspension standard curve of CREC.

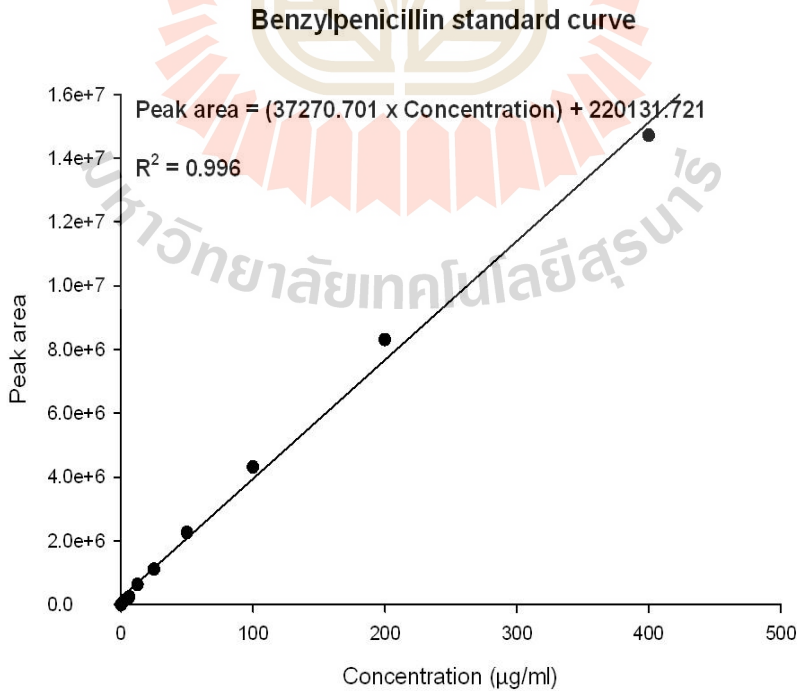

Figure H Standard curve of Benzylpenicillin.

CURRICULUM VITAE

Name Miss. Prairadda Cheypratub

Date of Birth May 19, 1972

Place of Birth Bangkok, Thailand

Education

1992-1996 Microbiology, B.Sc. (Hons), Faculty of Science, Srinakharinwirot University, Thailand

1997-1998 M.Sc. in Bioprocess Technology, Asian Institute of Technology (AIT), Thailand

Proceedings and presentations

1. Cheypratub, P., Leeanansaksiri, W., and Eumkeb, G. (2014) (May 6-8, 2014). Antibacterial activity of *Cyperus rotundus* extract against methicillin-resistant *Staphylococcus aureus*. Paper presented at the 5th international conference on natural products for health and beauty. (NATPRO 5), Phuket, Thailand. 235-242.