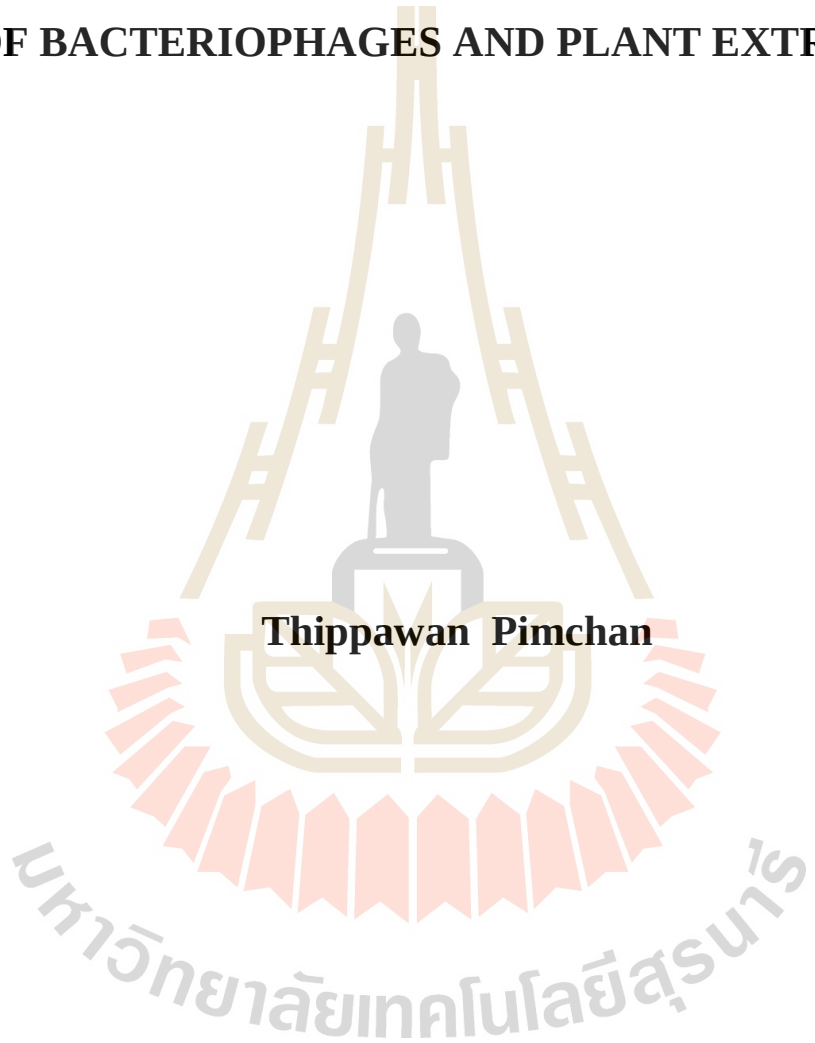
ANTIBACTERIAL EFFECTS OF α-MANGOSTIN AND BETA-LACTAM ANTIBIOTICS AND COMBINATIONS OF BACTERIOPHAGES AND PLANT EXTRACTS

Thippawan Pimchan



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ผลการต้านแบคทีเรียของแอลฟาแมงโกสตินและยาปฏิชีวนะกลุ่มบีตาแลคแทม และการผสมกันของแบคเทอริโอเฟจกับสารสกัดจากพืช

นางสาวทิพวรรณ พิมพ์จันทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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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.

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ทิพวรรณ พิมพ์จันทร์ : ผลการต้านแบคทีเรียของแอลฟาแมงโกสตินและยาปฏิชีวนะกลุ่มบีตาแลคแทม และการผสมกันของแบคเทอริโอเฟจกับสารสกัดจากพืช (ANTIBACTERIAL EFFECTS OF α-MANGOSTIN AND BETA-LACTAM ANTIBIOTICS AND COMBINATIONS OF BACTERIOPHAGES AND PLANT EXTRACTS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ เภสัชกร ดร.เกรียงศักดิ์ เอื้อมเก็บ, 152 หน้า.

การรักษาการติดเชื้อที่เกิดจากเชื้อดื้อยาเป็นปัญหาที่ท้าทาย การศึกษาครั้งนี้จึงได้ทำการศึกษาฤทธิ์การต้านเชื้อแบคทีเรียและการเสริมฤทธิ์ของแอลฟา-แมงโกสตินเมื่อใช้ร่วมกับยาปฏิชีวนะกลุ่มบีตาแลคแทมบางชนิดต่อการต้านเชื้อแบคทีเรียดื้อยา วิทยานิพนธ์นี้ยังได้ทำการศึกษาฤทธิ์ต้านเชื้อแบคทีเรียของสารสกัดจากพืช 3 ชนิดคือ สารสกัดจากกระชาย สารสกัดจากเพกา และสารสกัดจากบอระเพ็ดพุงช้าง ร่วมกับแบคเทอริโอเฟจ เอสยู 16 และ เอสยู 27 ต่อการต้านเชื้อเอสเชอริเชีย โคไล ค่าความเข้มข้นในการยับยั้งต่ำสุด (เอ็มไอซี) ของแอลฟา-แมงโกสตินในการต้านเชื้อเอ็นเทอโรค็อกคัส ฟีเชียม เท่ากับ 2 ไมโครกรัมต่อมิลลิลิตรในขณะที่ค่าเอ็มไอซีมีค่าสูง (>2048 ไมโครกรัมต่อมิลลิลิตร) พบในเชื้อแบคทีเรียแกรมลบที่ถูกนำมาทดสอบทั้งหมด เชื้ออซีเนโตแบคเตอร์ บอมมานิไอที่นำมาทดสอบทั้งหมด และเชื้อซูโดโมแนส แอรูจิโนซาบางสายพันธุ์ดื้อต่อยาเซฟตาซิดิมและ เซฟไตรอะโซน มีค่าเอ็มไอซีเท่ากับ 800 และ >2048 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ นอกจากนี้เชื้อเอ็นเทอโรค็อกคัส ฟีเชียม 2 สายพันธุ์ยังดื้อต่อยาแอมพิซิลลิน มีค่าเอ็มไอซีเท่ากับ 32 และ 128 ไมโครกรัมต่อมิลลิลิตร จากการทดสอบด้วยวิธีเชคเกอร์บอร์ด แสดงให้เห็นการเสริมฤทธิ์กันของแอลฟา-แมงโกสติน กับยาเซฟตาซิดิมและยาเซฟไตรอะโซนต่อการต้านเชื้ออซีเนโตแบคเตอร์ บอมมานิไอทุกสายพันธุ์ โดยมีค่าดัชนีสัดส่วนการยับยั้งเท่ากับ <0.35 และ <0.24 ตามลำดับ กราฟแสดงการตายของเชื้อได้ยืนยันปฏิสัมพันธ์ที่เสริมฤทธิ์กันในการใช้แอลฟา-แมงโกสตินร่วมกับยาเซฟตาซิดิม ในการต้านเชื้ออซีเนโตแบคเตอร์ บอมมานิไอ ดีเอ็มเอสที 45378 ภาพถ่ายด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดและกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านของเซลล์ที่ได้รับสารผสมแอลฟา-แมงโกสตินร่วมกับยาเซฟตาซิดิม แสดงให้เห็นความเสียหายอย่างชัดเจนของผนังเซลล์ เยื่อหุ้มเซลล์ และการเปลี่ยนแปลงของสารภายในเซลล์ พื้นที่ของเซลล์ยังพบว่ามีขนาดเพิ่มขึ้นอย่างมีนัยสำคัญ (พี < 0.01) เมื่อเปรียบเทียบกับกลุ่มอื่น ๆ การใช้แอลฟา-แมงโกสตินร่วมกับยาเซฟตาซิดิม เพิ่มความสามารถในการซึมผ่านของเยื่อหุ้มเซลล์ชั้นในและเยื่อหุ้มเซลล์ชั้นนอกและลดความหนาและความเข้มของแถบโปรตีนที่เกี่ยวข้องกับเยื่อหุ้มชั้นนอกและ

แบคเทอริโอเฟจกับสารสกัดจากพืช พบว่าค่าเอ็มไอซีของสารสกัดจากบอระเพ็ดพุงช้าง สารสกัดจากกระชาย และสารสกัดจากเพกา ต่อเชื้อเอสเชอริเชีย โคไล 16 และ 27 มีค่าเท่ากับ 4 16 และ 16 มิลลิกรัมต่อมิลลิลิตร ขณะที่ค่าเอ็มบีซีเท่ากับ 16 32 และ 32 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ เมื่อนำสารสกัดผสมกับแบคเทอริโอเฟจ ผลจากกราฟแสดงการตายของเชื้อแสดงให้เห็นว่าความแตกต่างของจำนวนเซลล์ที่มีชีวิตในกลุ่มที่ได้รับแบคเทอริโอเฟจหรือสารสกัดจากพืชเพียงอย่างเดียวและเซลล์ที่รับสารผสมแบคเทอริโอเฟจ เอสยู 27 และสารสกัดจากกระชายหรือสารสกัดจากเพกา ต่อการต้านเชื้อเอสเชอริเชีย โคไล 27 โดยการกลับมาเจริญของแบคทีเรียถูกพบหลังผ่านไป 6 ชั่วโมง และสูงขึ้นในระดับที่เท่ากันที่ 24 ชั่วโมง กล่าวโดยสรุปได้ว่า การผสมแอลฟา-แมงโกสตินกับยาเซฟตาซิดิม หรือเซฟไตรอะโซน สามารถแสดงผลการเสริมฤทธิ์กันในการยับยั้งแบคทีเรียที่ดื้อต่อยาเซฟตาซิดิม และยาเซฟไตรอะโซนได้ โดยการทำลายความสามารถในการซึมผ่านของ เยื่อหุ้มเซลล์ชั้นใน เยื่อหุ้มเซลล์ชั้นนอก ลดโปรตีนที่เกี่ยวข้องกับเยื่อหุ้มเซลล์ชั้นนอกและเพปทิโดไกลแคน และยับยั้งการทำงานของเอนไซม์บีตาแลคแทมเมส นอกจากนี้ การผสมกันระหว่างสารสกัดจากพืช กับแบคเทอริโอเฟจไม่แสดงการเสริมฤทธิ์กัน วิธีการเสริมฤทธิ์นี้น่าจะใช้เป็นเครื่องมือใหม่ในการต่อสู้กับแบคทีเรียสายพันธุ์ที่ดื้อยาได้

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THIPPAWAN PIMCHAN : ANTIBACTERIAL EFFECTS OF α-MANGOSTIN AND BETA-LACTAM ANTIBIOTICS AND COMBINATIONS OF BACTERIOPHAGES AND PLANT EXTRACTS. THESIS ADVISOR : ASSOC. PROF. GRIANGSAK EUMKEB, Ph.D. 152 PP.

α-MANGOSTIN / BETA-LACTAM ANTIBIOTIC / BACTERIOPHAGES/ PLANT EXTRACTS /ANTIBACTERIAL EFFECTS

Treatments for antibiotic-resistant infections are of challenging issues. This study, therefore, investigated, antibacterial and synergistic activities of α-mangostin (AMT) and some β-lactam antibiotics combination against antibiotic-resistant bacteria. This thesis also evaluated the combined antibacterial activity of three plant extracts (*Boesenbergia rotunda*: BRE, *Stephania suberosa*: SSE and *Oroxylum indicum*: OIE) with bacteriophages SU16 and SU17 against *Escherichia coli* isolates. The minimum inhibitory concentrations (MICs) of AMT against *Enterococcus faecium* isolates were equally 2 µg/mL, while high MICs (>2048 µg/mL) were observed in all tested Gram-negative bacteria. All tested *Acinetobacter baumannii* and some *Pseudomonas aeruginosa* isolates were resistant to ceftazidime (CAZ) and ceftriaxone (CTX) at the MICs of 800 and 2048 µg/mL, respectively. Two strains of *E. faecium* were also resistant to ampicillin (AMP) at the MICs of 32 and 128 µg/mL. Checkerboard assay demonstrated synergistic activity of AMT with CAZ or CTX against all tested *A. buamannii* at fraction inhibitory concentration indices of <0.35 and <0.24, respectively. The killing curve determination confirmed the synergistic interaction of AMT/CAZ combination against *A. baumannii* DSMT 45378. Scanning

electron microscope and transmission electron microscope imaging of cells treated with AMT/CAZ combination displayed the marked damage to the cell wall, cell membrane and change of intracellular structure. A significant increase in cell area was also observed compared to other groups ($P < 0.01$). AMP/CAZ combination increased the permeability of cytoplasmic (CM) and outer membrane (OM) and reduced the thickness and intensity of outer membrane and peptidoglycan-associated protein (OMPG) bands. AMT inhibited β-lactamase activity in a dose-dependent manner. Regarding the results of bacteriophages/plant extracts, the MICs of SSE, BRE, and OIE against ECOR-16 and ECOR-27 were 4, 16 and 16 mg/mL, while MBCs were 16, 32 and 32 mg/mL, respectively. When the extracts were combined with phages, the results from killing curve assay demonstrated that the marked difference in viabilities of ECOR after phages- or plant extracts-treated alone and cells treated with combined agents was seen only in phage SU 27 plus BRE or OIE against ECOR-27. These bacteria regrew after 6 h of treatment and reached the similar viability of untreated control at 24 h. In conclusion, AMT/CAZ or CTX combination synergistically inhibited CAZ- and CTX-resistant bacteria by damaging to OM and CM permeability, OMPG, and by inhibiting β-lactamase activity. Furthermore, the combinations of extracts/phage were not synergistic. The synergy approach could potentially be a novel tool to combat the resistant strains.

School of Biology

Academic Year 2017

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Thippawan Pimchan

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LIST OF ABBREVIATIONS

AMP	=	Ampicillin
AMT	=	α-Mangostin
ATCC	=	American Type Culture Collection
BRE	=	*Boesenbergia rotunda* (L) Mansf Extract
°C	=	Degree Celsius
CAZ	=	Ceftazidime
CM	=	Cytoplasmic Membrane
CTX	=	Ceftriaxone
CFU	=	Colony Forming Unit
DMSO	=	Dimethyl Sulfoxide
DMST	=	Department of Medical Sciences Thailand
DNA	=	Deoxyribonucleic Acid
ECOR	=	*E. coli* Standard Reference Collection
FIC	=	Fractional Inhibitory Concentration
g	=	Gram
h	=	Hour
HEPES	=	N-2-Hydroxyethyl Piperazine-N'-Ethanesulphonic Acid
HIV	=	Human Immunodeficiency Virus
kDa	=	Kilodalton
LA	=	Lysogeny Agar

LIST OF ABBREVIATIONS (Continued)

LB	=	Lysogeny Broth
LPS	=	Lipopolysaccharide
MBC	=	Minimum Bactericidal Concentration
MIC	=	Minimum Inhibitory Concentration
mL	=	Millilitre
µL	=	Microlitre
MRSA	=	Methicillin-resistant *Staphylococcus aureus*
NaCl	=	Sodium Chloride
nm	=	Nanometre
OD	=	Optical Density
OIE	=	*Oroxylum indicum* Extract
OM	=	Outer Membrane
OMPG	=	Outer Membrane and Peptidoglycan-Associated Protein
PBS	=	Phosphate Buffer Saline
PFU	=	Plaque-Forming Unit
PMX	=	Polymixin B
SEM	=	Scanning Electron Microscopy
SSE	=	*Stephania suberosa* Extract
TEM	=	Transmission Electron Microscopy
Tris-HCl	=	Trizma Hydrochloride

LIST OF ABBREVIATIONS (Continued)

VRE	=	Vancomycin-resistant enterococci
VRSA	=	Vancomycin-resistant *S. aureus*

CHAPTER I

INTRODUCTION

1.1 Introduction

The emergence of antibiotic resistance is increasing rapidly. The World Health Organization (WHO) states that antibiotic-resistant bacteria have become one of the most serious global public health threats of the 21st century (WHO, 2014). Several cases have been reported dead as a result of the infections caused by antibiotic-resistant bacteria (Harbarth et al., 2015). Bacteria can confer resistance to antibiotics through intrinsic mechanisms and by the acquisition of new resistance genes from other bacteria (Alekshun and Levy, 2007). The resistance is mediated by antimicrobial-inactivating enzymes (e.g., β-lactamase), alteration of drug targets, reduction of membrane permeability and an increase of multidrug efflux pumps. More than one resistance mechanisms may be present in the same strain (Rice, 2006). Currently, many bacteria have been documented to resist multiple antibiotics resulting in difficulty in selection of efficient and appropriate antibiotics for the treatment of these recalcitrant bacteria. The spread of antibiotic resistance has globally been reported in numerous pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *enterococci* (VRE), Multidrug-resistant *Acinetobacter baumannii* (MDR), carbapenem-resistant *A. baumannii*, multidrug-resistant *Pseudomonas aeruginosa*, and multidrug- resistant *Escherichia coli*

(Evans et al., 1999; Lister et al., 2009; Moran et al., 2006; Tadesse et al., 2012; Tiwari et al., 2015). Third-generation cephalosporins (ceftazidime, ceftriaxone) and carbapenems are usually the antimicrobial agents of choice for treating serious Gram-negative bacterial infections that are resistant to other β-lactam antibiotics (Falagas and Karveli, 2007). However, currently these bacteria, in particular, *A. baumannii* have developed resistance to this group of antibiotics (Chang et al., 2015; Manchanda et al., 2010). Polymyxin B (PMX) has been used as an alternative antibiotic for the treatment of infections caused by Gram-negative pathogens such as *P. aeruginosa,* and *A. baumannii*. The initial target of polymyxin is lipopolysaccharide (LPS) of the outer membrane. There is a low incidence of *A. baumanii* conferring resistance to PMX. However, to prevent the emergence of the resistance, an increase of the therapeutic dose of this agent or use in combination with other antibiotics has been recommended for the treatment of the infection caused by *A. baumanii* (Kassamali et al., 2015).

Naturally-occurring antimicrobials are among one of the most interesting sources to develop new antimicrobial agents to replace synthetic antimicrobials (Harikrishnan et al., 2003; Immanuel et al., 2004). To develop safer drugs, the research regarding the development of new antimicrobial agents from the herbal plant is objectives of far-reaching importance. Mangosteen (*Garcinia mangostana*), a tropical evergreen fruit, has widely been cultivated in South East Asia. The pericarp of this fruit has been traditionally used for the treatment of chronic diarrhea, infected wounds, skin infections and dysentery (Pedraza-Chaverri et al., 2008). The major bioactive secondary metabolites of mangosteen are xanthone derivatives. These xanthone derivatives have displayed potent pharmacological activities including

antibacterial, antifungal, antioxidant, anti-tumor, anti-inflammatory and anti-allergy properties (Obolskiy et al., 2009; Pedraza-Chaverri et al., 2008). Chomnawang et al. (2007) investigated the activity of Thai medicinal plants on inflammation reduction caused by *Propionibacterium acnes* and found that *G. mangostana* possessed the most significant antioxidant activity and reduced reactive oxygen species production. α-Mangostin, a major active ingredient isolated from mangosteen, has demonstrated antimicrobial activities against Gram-positive bacteria such as MRSA, VRE, *P. acnes*, and *Staphylococcus epidermidis* (Koh et al., 2013; Pothitirat et al., 2010; Sakagami et al., 2005). The bacterial membrane of Gram-positive bacteria is a target of α-mangostin (Koh et al., 2013). However, the antibacterial mechanism on the combination of α-mangostin and cephalosporins was not unknown in Gram-negative bacteria.

Bacteriophages (phages) are considered the viruses that can infect bacteria, resulting in cell lysis (lytic phage) or lysogenization (lysogenic phage) of the infected cell. Phages are very specific to their host cells and not harmful to eukaryotic cells. Phage therapy refers to using lytic phage for the treatment of bacterial infections. Phages were used as therapeutic agents for infection caused by bacteria in the pre-antibiotic era before the first antibiotic was discovered in the 1940s. However, phage therapy has still been used for the treatment of various infections, especially in Eastern Europe countries, such as Georgia and Poland. Due to multidrug-resistant bacteria, phage therapy has caught the interest of researchers worldwide to study the application of phages for the treatment of multidrug-resistant bacteria in the clinical setting. Phages have been used to treat lethally infectious diseases caused by Gram-negative bacteria including *E. coli*, *P. aeruginosa*, *A. baumannii*, *Klebsiella*

pneumoniae, *Vibrio vulnificus* and *Salmonella* spp., and Gram-positive bacteria, such as *Enterococcus faecium* and *S. aureus* (Matsuzaki et al., 2005).

New alternative strategies to treat antibiotic-resistant organisms are highly required. Combination therapy is a common strategy that is often employed to reduce the dissemination of antibiotic-resistant strains. This approach shows better efficiency than a single treatment (Davies and Davies, 2010). Araoka (2012) demonstrated that the combination of β-lactam with aminoglycoside effectively inhibited Gram-negative bacteria including *P. aeruginosa*, *Klebsiella*, *E. coli*, and Enterobacteriaceae. Likewise, Gonzales et al. (2015) reported that β-lactam and meropenem-piperacillin-tazobactam acted synergistically in the combination against MRSA. The major concern of phage therapy is the development of phage resistance. To reduce a chance of the resistance and to improve the effectiveness of the treatment, a combination therapy of antibiotic and phage is used as an alternative approach which is more effective than a single treatment (Davies and Davies, 2010). Research conducted by Comeau et al. (2007) found that the phage-antibiotic combination showed synergistic interaction by inhibiting the growth of *E. coli* at a sub-lethal concentration of β-lactam and quinolone. Moreover, Bedi et al. (2009) also found that the combination of amoxicillin and lytic phage could minimize the formation of *K. pneumoniae* biofilm. Similar to a previous report, both *E. coli* and *P. aeruginosa* biofilms were inhibited by the phage - tobramycin combination (Coulter et al., 2014). However, no study has been investigated on synergistic effects of the combination of phage and plant extract against pathogenic bacteria. Thus, this thesis aims to determine the synergistic interaction of the phage and plant extracts combination against pathogenic bacteria.

1.2. Research objectives

1.2.1 To study the effect of α-mangostin and selected β-lactam antibiotics when used either alone or in combination on β-lactam-resistant bacteria, *A. baumannii*, *E. faecium* and *P. aeruginosa*.

1.2.2 To examine the primary mechanism of actions of α-mangostin when used singly and in combination with selected antibiotics using outer and cytoplasmic membrane permeability test, β-lactamase inhibition assay, gel electrophoresis, scanning electron microscopy (SEM), and transmission electron microscopy (TEM).

1.2.3 To study the potential of bacteriophages and antimicrobial plant extracts (*Stephania suberosa* extract, *Oroxylum indicum* extract, and *Boesenbergia rotunda* extract) against bacterial pathogens.

1.3 Research hypothesis

1.3.1 α-Mangostin could exhibit the potential effect and synergistic activity with selected cephalosporins against cephalosporin-resistant bacteria.

1.3.2 Plant extracts could have a synergistic effect when used in combination with bacteriophages to inhibit bacterial pathogens.

1.4 The significance of the study

The research was expected to provide the following information:

1.4.1 The potential use of α-mangostin either alone or in combination with selected cephalosporins against drug-resistant bacteria *A. baumannii.*

1.4.2 The data suggesting a primary mechanism of action of α-mangostin when used alone or in combination with selected antibiotics.

1.4.3 Insights on the potent combination of bacteriophages and antimicrobial plant bulk extracts to target bacterial pathogens.

CHAPTER II

LITERATURE REVIEW

2.1 Bacterial Structure

Based on the staining characteristics, bacteria are commonly divided into Gram positive and Gram negative bacteria. The bacterial cell comprises of cytoplasmic membrane enclosing the cytoplasm, cell wall (peptidoglycan), outer membrane (only in Gram-negative bacteria). Gram positive bacteria have a thicker peptidoglycan layer than Gram-negative bacteria. To summarize, Gram-positive bacteria consist of three main compartments, the cytoplasm, the cytoplasmic membrane and think cell wall (Figure 2.1a), while Gram-negative bacteria contain two additional compartments including the periplasm and the outer membrane (Figure 2.1b). Some bacteria contain appendages, flagella, and pile, allowing their movements and attachment to cell surfaces (Schumann, 2006). The bacterial cell wall is among of the most important compartment of bacteria. It maintains the shape and controls homeostasis of the cell in the harsh environment. Moreover, the bacterial cell wall is the first barrier that antibiotic agents must overcome. Peptidoglycan is the major component of bacterial cell walls. Gram-positive bacteria have thicker in peptidoglycan layer than Gram-negative bacteria. Differently, in Gram-negative bacteria, their cell wall is composed additional membrane called outer membrane and lipopolysaccharide (Erridge et al., 2002). Disruption or inhibition of bacterial cell wall synthesis results in cell lysis and death. Some antibiotics, for instance, β-lactams

antibiotic and vancomycin, inhibit the growth of bacteria by blocking the synthesis of bacterial cells.

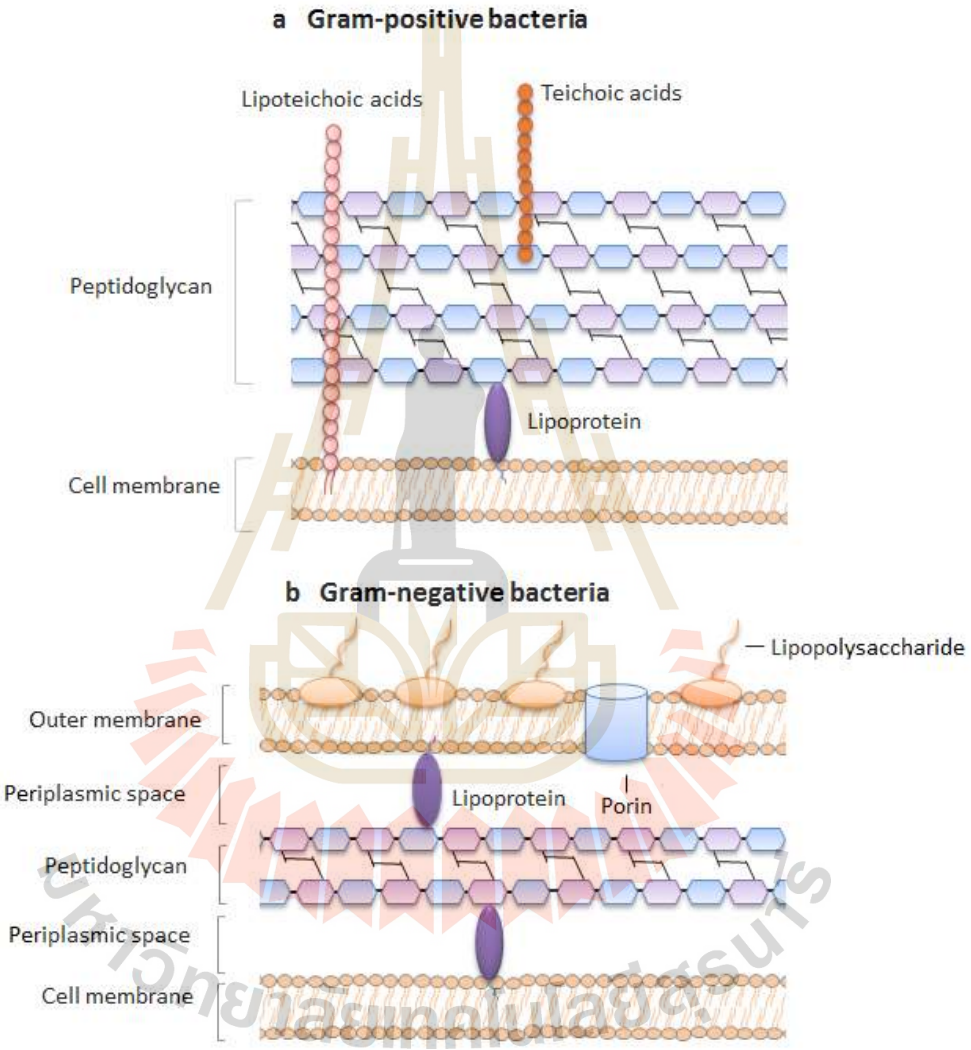


Figure 2.1 Cell wall structure and composition of Gram-negative bacteria (a) and Gram-positive bacteria (b) (Adapted from Brown et al., 2015).

2.1.1 Peptidoglycans

The peptidoglycan, also called murein, is an essential and specific structural component in the cell wall. Its main function is to protect cell integrity by withstanding the turgor. Importantly, the cell wall consisting of peptidoglycan is a unique structure only present in bacteria, so it is the most interesting drug target. The inhibition of cell wall biosynthesis can result in cell lysis. Peptidoglycan also contributes to the maintenance of defined cell shape and serves as a scaffold for anchoring other cell envelope components, including proteins (Dramsi et al., 2008) and teichoic acids (Neuhaus and Baddiley, 2003). Peptidoglycan is a network of glycan chain of β1,4-linked N-acetylglucosamine (Glc*NAc*) and N-acetylmuramic acid (Mur*NAc*) glycan units cross-linked by short peptides (Rogers et al., 1980).

2.1.2 Outer membrane

The outer membrane is a unique compartment found only in the Gram-negative bacteria, which contains clinically significant components called lipopolysaccharide (LPS). This compartment is the outermost layer of bacteria cell forming the periplasmic space, a space between the cytoplasmic membrane and outer membrane, where several degrading enzymes are secreted. The outer membrane is an asymmetric lipid bilayer intercalated with proteins, with its inner leaflet consists of phospholipids that similar to the phosphoglycerides and the outer leaflet comprises of LPS. LPS composes of three components; the proximal hydrophobic lipid A region, the distal hydrophilic O-antigen polysaccharide region that protrudes into the medium, and the core oligosaccharide region that connects the O-antigen polysaccharide region to lipid A. The outer membrane acts as a permeability barrier by controlling the transportation of electrolytes, proteins, nutrients and metabolites

across the hydrophobic portion. Most nutrients pass this barrier via a family of integral outer membrane proteins (OMPs) called porins (Tommassen, 2010). These trimeric proteins form open, water-filled channels in the outer membrane, which allow for the passage of small hydrophilic solutes, such as amino acids and monosaccharides, via passive diffusion (Nikaido, 2003). Other OMPs have more specialized transport functions, such as the secretion of proteins and the excretion of drugs, or function as enzymes or structural components of the outer membrane (Koebnik et al., 2000). Besides integral OMPs, the membrane also contains lipoproteins, which are attached to the membrane via an N-terminal lipid moiety. OMPs usually traverse the membrane and anchor the outer membrane to the underlying peptidoglycan sheet. The outer membrane mass contains approximately 50% of proteins (Koebnik et al., 2000).

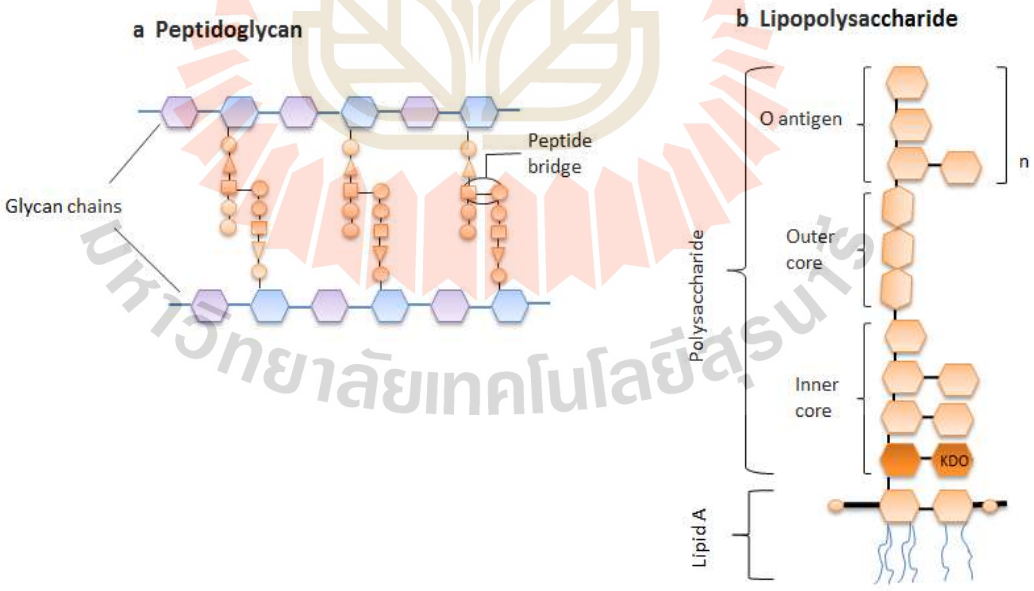


Figure 2.2 Chemical structure of the peptidoglycan and lipopolysaccharide (Adapted from Lebeer et al., 2010).

2.2 Mechanisms of antimicrobial resistance

The first antibiotic known as penicillin was discovered by Alexander Fleming in the 1940s, which revolutionized the modern era of antibiotics. This discovery is the most important agents for treating infectious diseases in modern medicine. Since then, resistance to penicillin was recognized and identified during the same decade of discovery. The resistance was also reported in other antibiotics within a few years after they were introduced to use in a medical setting. The prevalence of antibiotic resistance in pathogenic bacteria has raised as a major public health concern with high morbidity, mortality, increased health care costs and made the treatment much more challenging (Tiwari et al., 2015). The World Health Organization (WHO) states that antibiotic-resistant bacteria have become one of the most serious global public health threats of the 21st century (WHO, 2014). This problem is a challenging issue to control the dissemination of antibiotic-resistant organisms. Because some antimicrobial agents have lost their original effectiveness, the antibiotic of choice for treating multidrug-resistant bacteria are limited, resulting in the delay in initiation of chemotherapy (Bush, 2010). Treatment of infections is now complicated as a consequence of several bacteria have resistance to multiple antibiotics. Antimicrobial agents are generally classified into 5 groups based on their principal mechanism of action; (1) inhibition of cell wall synthesis (eg, β-lactams and glycopeptide agents), (2) inhibition of protein synthesis (macrolides and tetracyclines), (3) interference with nucleic acid synthesis (fluoroquinolones and rifampin), (4) inhibition of a metabolic pathway (trimethoprim-sulfamethoxazole), and (5) disruption of cell membrane structure (polymyxins and daptomycin) (Tenover, 2006). Several mechanisms of antimicrobial resistance spread rapidly to a variety of bacterial genera. Some species

are innately resistant to more than 1 class of antibiotics. The mechanisms of antibiotic resistance can be grouped into 3 main mechanisms; modification of the receptor or active site, enzymatic degradation, and decreased permeability and efflux pumps (Figure 2.3).

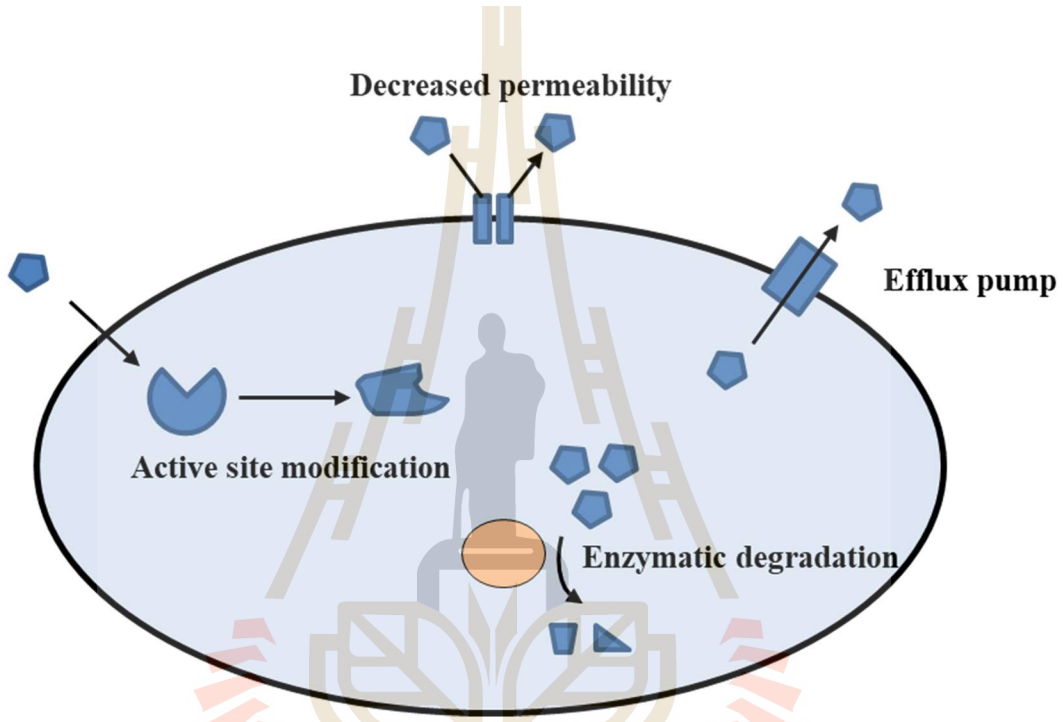


Figure 2.3 Overview of resistance mechanism (Adapted from Sherrard et al., 2014).

2.2.1 Receptor or active site modification

Antibiotics act specifically to their targets or receptors. However, bacteria can alter the target protein of antibiotic by modifying or eliminating the binding site, leading to a reduction in the activity of antibiotic. These alterations can be observed in several bacteria. Conformational change of penicillin-binding proteins (PBPs) in bacteria can decrease the affinity for antibiotic and result in the development of β-lactam resistance eventually. Resistance to methicillin and other β-lactam antibiotics

in MRSA is mediated by acquisition of the *mecA* gene encoded penicillin-binding proteins 2a (PBP2a)(Stapleton and Taylor, 2002). Alterations of DNA gyrase and topoisomerase IV are examples of the target enzymes mutations. Some bacteria can confer resistance to fluoroquinolones by alteration of GyrA or GyrB subunits of DNA gyrase, resulting in a decrease the affinity of the antimicrobial molecule to its targets, leading to drug resistance (Lambert, 2005; Redgrave et al., 2014).

2.2.2 Enzymatic degradation

The bacteria can also develop resistance to the antibiotic by synthesis of degrading enzymes to destroy or modify the antibiotics. There are many enzymes that have been reported to be able to hydrolyze distinct classes of antibiotics, including β-lactams, aminoglycoside, and macrolides (Blair et al., 2015). β-Lactamase-Mediated resistance is the well-known mechanism of resistance to β-lactam antibiotics, which hydrolytically cleave amide bond in β-lactam ring rendering the antibiotic inactive (Bush, 2010). The β-lactamases are encoded by both chromosomal and plasmid genes. In Gram-positive bacteria, the β-lactamase enzymes are secreted into the surrounding environment while these enzymes in Gram-negative are secreted to the periplasmic space (Zeng and Lin, 2013). Although some β-lactamases have been identified recently, they can be classified broadly by two classification schemes based upon amino acid sequence classification (class A-D) and biochemical function (group 1-4). The most clinically important β-lactamase enzymes are AmpC β-lactamases, extended spectrum β-lactamase (ESBL), and carbapenemases that can hydrolyze broad-spectrum cephalosporins (e.g., cefotaxime and ceftazidime) and carbapenem antibiotics (the last resort β-lactam antibiotic for the infection caused by Gram-negative bacteria) (Paterson and Bonomo, 2005).

2.2.3 Decreased permeability and efflux pumps

The outer membrane of Gram-negative bacteria envelope plays a crucial role in controlling molecules crossing the cell by acting as a barrier. Porins, a specific channel for particular molecules, restrict antibiotics and other small organic compound molecules accessing through peptidoglycan, periplasm or the cytoplasm. A mutation in porins can result in the prevention of drug penetration and subsequently decreased cellular antibiotic concentration. As a consequence, the drug cannot kill or inhibit the growth of bacteria (Fernández and Hancock, 2012). Several types of porins have been characterized and found in Gram-negative bacteria such OmpF, OmpC, and PhoE in *E. coli* and OprD in *P. aeruginosa*. Moreover, Gram-negative bacteria can mediate resistance by increasing the expression of efflux pumps to expel the antibiotics out from the cell resulting in reduced antibiotic accumulation in the cells (Alekshun and Levy, 2007). These mechanisms of resistance can be observed in several classes of antibiotics, including the β-lactam drugs, the aminoglycosides, chloramphenicol, and quinolones.

2.3 Important pathogenic bacterial strains

2.3.1 *Acinetobacter baumannii*

Acinetobacter baumannii (family Moraxellaceae), a strictly aerobic Gram-negative coccobacillus, is commonly isolated from the hospital environment and hospitalized patients. *A. baumannii* has become serious infections over the past decade. It causes several infections, including wound infection, ventilator-associated pneumonia, urinary tract infection, meningitis and bacteraemia (Falagas and Karveli, 2007; McConnell et al., 2013) This bacterium can survive under a wide range of

environmental conditions and persist for a prolonged period on surfaces, making it a frequent cause of outbreaks of infection and an endemic, healthcare-associated pathogen (Fournier et al., 2006). Clinical isolates of *A. baumanii* in the ICU have been reported to be 30% resistant to at least three classes of antibiotics, including β-lactams and fluoroquinolones (Lockhart et al., 2007). *Acinetobacter* species possess a wide array of β-lactamases that hydrolyze and confer resistance to penicillins, cephalosporins, and carbapenems. *A. baumannii* inherently produces an AmpC-type cephalosporinase also known as Acinetobacter-derived cephalosporinases (ADCs) These enzymes are highly expressed in this bacterium, which can lead to resistance to the third-generation cephalosporins (Bonomo and Szabo, 2006; Thomson and Bonomo, 2005). OXA-type carbapenemases (mostly OXA-48 and OXA-181) have been identified in carbapenem-resistant *A. baumannii* in many countries, including Scotland, Spain, France, Japan, Singapore, China, Brazil, Cuba and Kuwait (Afzal-Shah et al., 2001; Eliopoulos, 2009; Maragakis and Perl, 2008). Also, *Acinetobacter* strains can produce metallo-β-lactamases (IMP and VIM) that render carbapenem inactive (Thomson and Bonomo, 2005).

2.3.2 *Escherichia coli*

Escherichia coli is a facultative anaerobic, rod shape, a Gram-negative bacterium belonging to the family *Enterobacteriaceae*. *E. coli* is a commensal bacterium that can be found in the gastrointestinal tracts of humans and warm-blooded animals. It is also one of the most important pathogens. Several different *E. coli* strains can cause a variety of intestinal and extraintestinal-associated diseases. There are six well-described categories; enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, and

diffusely adherent *E. coli* (Kaper et al., 2004). The pathogenicity of *E. coli* is facilitated by several virulence factors such as pilli, enterotoxins, Shiga-like toxins, endotoxin (lipopolysaccharide), hemolysin, aerobactin, cytonecrotizing factor, intimin, and biofilm formation (Blanco et al., 1996; Wang et al., 2002). The emerging resistance to fluoroquinolones and the production of extended-spectrum β-lactamases (ESBL) by multidrug-resistant *E. coli* strains are increasing concerns over the last decade due to the limited therapeutic options for infections caused by these strains (Denton, 2007; Garau et al., 1999).

2.3.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa, a rod-shaped gram-negative, is an opportunistic pathogen that commonly found in hospitalized or immune-compromised patients. This bacterium adapts easily to the environment and can also colonize and invade a human host to cause serious infections, such as pneumonia, burn, wound, urinary tract and gastrointestinal infections, otitis media, and keratitis (Coutinho et al., 2008; Silby et al., 2011). This pathogen is one of the most common causes of pneumonia. Infections by *P. aeruginosa* are notoriously difficult to treat due to currently conferred resistance to a broad range of antibiotic, including penicillin G, aminopenicillin, β-lactamase inhibitors, first and second generation cephalosporins, piperacillin, tazobactam, cefepime, ceftazidime, aminoglycosides, quinolones, and carbapenems, as well as colistin and fosfomycin. It has been demonstrated that *P. aeruginosa* strains can produce an AmpC-like inducible chromosomal β-lactamase that can inactivate β-lactams (Hancock and Speert, 2000).

2.3.4 *Enterococcus faecium*

Enterococci are gram-positive cocci, facultatively anaerobic bacteria and belong to family *Enterococcaceae*. *Enterococci* are well recognized as causative pathogens of several infections, including urinary tract, wound, and bloodstream, infective endocarditis and rarely meningitis (Zeana et al., 2001). *E. faecalis and E. faecium* are the most prevalent species isolated from humans, accounting for more than 90% of clinical isolates (de Perio et al., 2006). *E. faecium* are frequently resistant to multiple antibiotics including vancomycin, which is a serious issue that limits the therapeutic options and infection control of these organisms. Enterococci strains can produce β-lactamases that are capable of inactivating penicillin, ampicillin, and relevant antibiotics with transferable plasmids (Vu and Carvalho, 2011). High ampicillin resistance in *E. faecium* is mediated by either mutation or overproduction of PBP5 resulting in the lower affinity for ampicillin. Likewise, resistance to ciprofloxacin and ampicillin of *E. faecium* is mediated by mutations in the gyrA, parC and pbp5 genes (Rathnayake et al., 2012).

2.4 Clinically important antibiotics

β-lactam antibiotics are considered to be the most widely used antibiotics for the treatment of infections caused by a variety of pathogenic bacteria. These antibiotics have high efficacy and low toxicity compared to other synthetic antibiotics. β-lactam antibiotics are classified as bacterial cell wall synthesis inhibitors. Polymyxins inhibit the growth of bacteria via destruction of the cell membrane and lipopolysaccharide. Polymyxins have become increasingly more important as they are the final agents for the treatment of multidrug-resistance bacteria antibiotics that are resistant to almost all available antibiotics.

2.4.1 β-lactam antibiotics

Beta-lactam antibiotics are the oldest and, most commonly used agents for treating bacterial infections. They share a common structure of the β-lactam ring (highlighted in Figure 2.4), and mechanism of action. These agents are active against bacteria by inhibiting the enzymes during cell wall synthesis called Penicillin-binding proteins (PBPs). These particular enzymes are a group of an enzyme found in bacterial membranes and function as peptidoglycan transpeptidase (Georgopapadakou, 1993). There are 4 groups of β-lactam antibiotics; penicillins, cephalosporins, carbapenems, and monobactams (Figure 2.4). β-lactam antibiotics are commonly used agents for the treatment of infection, accounting for 60% of the worldwide antibiotic usage (Livermore and Woodford, 2006).

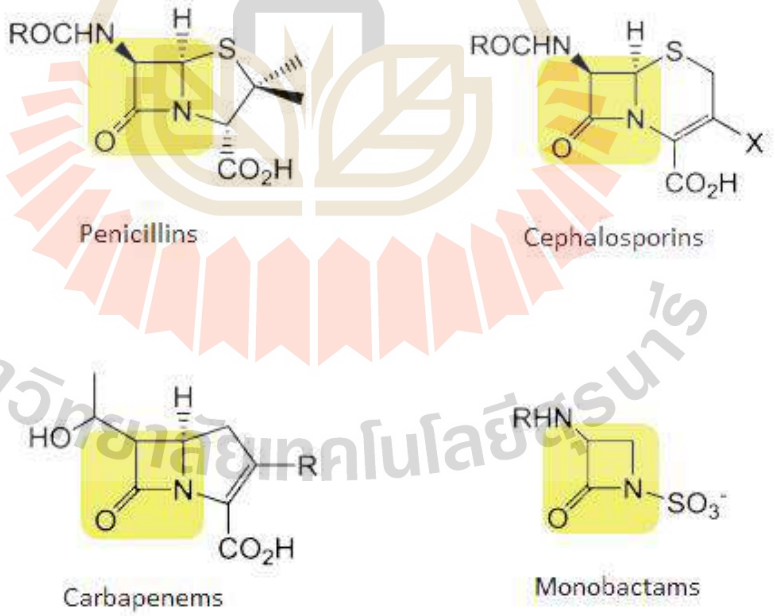


Figure 2.4 General chemical structures of different classes of β-lactam antibiotics (Modified from Konaklieva, 2014).

Penicillin was the first antibiotic discovered by Alexander Fleming in 1928 and the most valuable antibiotics for the treatment of infectious diseases. This group of antibiotics has the basic chemical components of a β-lactam antibiotic, consisting of a β-lactam ring, which is fused to a thiazolidine ring and side chains. The antibacterial mechanisms of all penicillins are the inhibition of the bacterial cell wall synthesis by binding to the PBPs. Inhibition of PBPs results in blocking the cross-links of the peptidoglycan strands, leading to cell lysis and death. Penicillins are classified based on their activity, including natural penicillin (penicillin G, penicillin V), penicillinase-resistant penicillin (methicillin, cloxacillin), extended spectrum penicillin (ampicillin, amoxicillin), broad-spectrum penicillin (carboxypenicillin), and β-lactamase combinations (Augmentin) (Parascandola, 1981).

Cephalosporins are semisynthetic antibiotics derived from soil-inhabiting fungi (*Cephalosporium* spp.) They consist of a β-lactam ring and a dihydrothiazine that differ from the penicillins in that the dihydrothiazine ring of cephalosporins have an additional R2 side chain. These antibiotics have been grouped into four generations based upon their spectrum of activity. The first generation of Cephalosporins is active against Gram-positive cocci but did not have clinically useful activity against enterococci. The second generation cephalosporins are similar to the first generation, but this generation has increased activity against Gram-negative bacilli. The third-generation cephalosporins have potential activity against antibiotic-resistant Gram-positive bacteria and as well show the broad spectrum against a variety of Gram-negative bacteria, including *Haemophilus influenzae*, *Moraxella catarrhalis*, and even *P. aeruginosa* (ceftazidime is a drug of choice). Also, the third generation cephalosporins are an alternative therapy for treating Gram-negative infections that

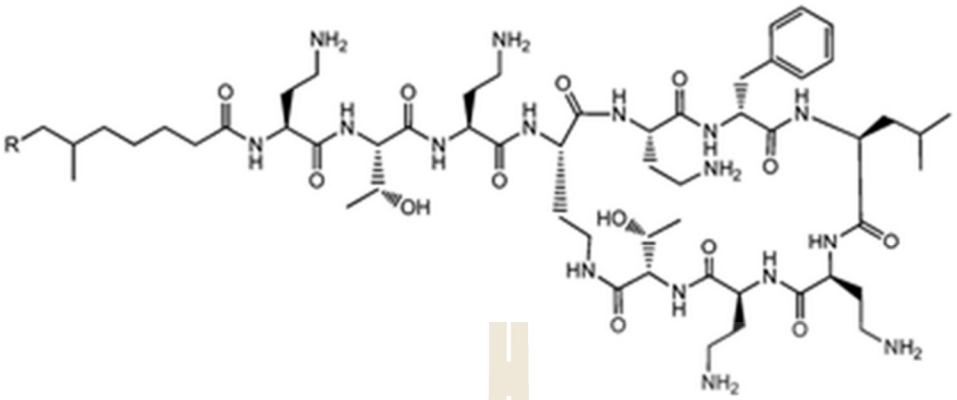
have been resistant to other β-lactams. Due to the fourth generation cephalosporins have a positively charged quaternary ammonium attached to the dihydrothiazine ring, they can penetrate through the outer membrane of gram-negative bacteria better than other groups of cephalosporins, so they have been reserved for the treatment of antibiotic-resistant Gram-negative bacteria (Miller, 2006).

Carbapenems are potent agents against Gram-positive and Gram-negative bacteria. These antibiotics are known to have the highest activity compare to other β-lactam antibiotics. They are often used as the "last line agents." Carbapenems differ from the penicillins at the double bond between C2 and C3 with the substitution of a carbon atom replacing sulfur of the thiazolidine ring. Imipenem, meropenem, ertapenem, and doripenem are the currently available compounds of the carbapenems that are clinically used. Imipenem is distinguished from other beta-lactams by high affinity for PBPs that results in resistance to hydrolysis by β-lactamase (Hashizume et al., 1984).

Monobactams are monocyclic beta-lactam compounds that have been isolated and purified from various soil bacteria. They are active against several aerobic Gram-negative rods, such as *Enterobacters* spp., *Citrobacter* spp., *Klebsiella* spp., *Proteus* spp., and *P. aeruginosa*. However, these antibiotics have no activity against Gram-positive bacteria or anaerobes. Aztreonam is the first commercially monobactam. Aztreonam is a useful alternative for patients with aerobic gram-negative infections, with an allergy to penicillin or cephalosporin.

2.4.2 Other antimicrobial agents targeting bacterial cell wall

Polymyxins are an old class of polypeptide antibiotic discovered in 1947. They can be classified into five different compounds (polymyxin A-E). Polymyxin B (PMX) and polymyxin E (colistin) are commonly used in clinical practice (Garidel and Brandenburg, 2009). PMX and colistin are synthesized from the soil bacterium *Bacillus* spp (Evans et al., 1999). PMX is a lipopeptide comprising a polycationic peptide ring and a tripeptide side chain with a fatty acid tail (Figure 2.5). PMX has rapid bactericidal activity against Gram-negative bacteria by targeting the bacterial cell membrane and disrupting its permeability resulting in leakage of intracellular materials. The mechanism of PMX in destroying bacteria is due to the interaction between cationic molecules of PMX and anionic LPS molecules leading to the derangement of the cell membrane as a consequence of displacement of Ca^{2+} and Mg^{2+} ions on LPS (Zavascki et al., 2007). PMX has been clinically used more than five decades since it has been approved for clinical treatment. The PMX is not initially considered to use as parenteral administration because of the concern of the potential toxicological issue. The main side effects of PMX are nephrotoxicity and neurotoxicity (Falagas and Kasiakou, 2006). However, in recent years the polymyxins have been re-evaluated as antimicrobial agents for the treatment of the infection caused by Gram-negative bacteria, especially bacteria that show resistance to multiple antibiotics. It is also generally prescribed when there is an absence of effective antibiotic availability for treating multidrug-resistant Gram-negative bacteria.



Polymixin B

Figure 2.5 Chemical structure of polymixin B (Hamley, 2015).

2.6 Potential bioactive compound and plant extract

2.6.1 α-Mangostin

Garcinia mangostana Linn., known as mangosteen, is the tropical rainforest fruit plant commonly found in India, Sri Lanka and South East Asian regions, such as Indonesia, Malaysia, Sri Lanka, Philippines, and Thailand. The fruit of the mangosteen is dark purple or reddish and inside contains soft, juicy edible white pulps with slightly acidic, sweet flavor and pleasant smell (Jung et al., 2006). Mangosteen regarded as the queen of fruits because it is uniquely with the best tropical taste. The pericarp of this fruit has been used as traditional medicine for the treatments of abdominal pain, diarrhea, trauma, skin infection, dysentery and wounds (Peres et al., 2000). Various bioactive substances have been identified in mangosteen, such as prenylated and oxygenated xanthones (Pedraza-Chaverri et al., 2008). Xanthones are secondary metabolites commonly found in higher plant families and also in fungi and lichens (Peres et al., 2000). Xanthones are the major bioactive secondary metabolites

of the mangosteen that can be found in the pericarp, whole fruit, bark and leaves (Pedraza-Chaverri et al., 2008). α-Mangostin, a xanthone derivative, is a major bioactive compound isolated from the pericarps of the mangosteen fruit (Schmid, 1855). The compound is a yellowish colouring matter which can be obtained from the other parts of the plant (dried sap and the bark). The chemical structure of xanthone was elucidated by Dragendorff (1930) and Murakami (1932). The molecular formula, type, and position of the substituent groups of α- mangostin were then determined by Yates and Stout (1958). The chemical structure of α-mangostin is illustrated in Figure 2.6. α-Mangostin has long been reported to possess a variety of biological activities, including antibacterial, antifungal, antioxidant, antitumor, anti-inflammatory, antiviral and anti-allergy (Ibrahim et al., 2014). The compound has demonstrated a wide range of antibacterial activities. Sundaram et al. (1983) reported that α-mangostin and its four derivatives have an antibacterial effect against *S. aureus, P. aeruginosa, Salmonella typhimurium, Bacillus subtilis, Klebsiella sp.* and *E. coli*. The study conducted by Iinuma et al. (1996) found that xanthones isolated from the hull of mangosteen fruit inhibit the growth of methicillin-resistant *S. aureus* (MRSA). Furthermore, α-mangostin exhibited the potential anti-tuberculosis activity in the inhibition of *Mycobacterium tuberculosis* (Suksamrarn et al., 2003). According to the study conducted by Sakagami et al. (2005), α-mangostin showed antibacterial activity against vancomycin-resistant *Enterococci* (VRE). Chomnawang et al. (2005) reported that a xanthone derivative had a strong inhibitory effect on *Propionibacterium acnes* and *S. epidermidis*. Similarly, α-Mangostin demonstrated antimicrobial activities against MRSA (Chin and Kinghorn, 2008). In addition, α-mangostin and four of its derivatives also had antifungal activity against *Epidermophyton floccosum,*

Alternaria solani, Mucor sp., *Rhizopus* sp. *and Cunninghamella echinulata* (Sundaram et al., 1983). The antifungal spectrum α-Mangostin-derivatives also covered three phytopathogenic fungi (*Fusarium oxysporum vasinfectum, Alternaria tenuis, and Dreschlera oryzae*) (Gopalakrishnan et al., 1997). In anti-HIV studies, α-mangostin-derivatives have antiviral properties by inhibiting HIV-1 protease (Chen et al., 1996) and the replication cycle of HIV (Vlietinck et al., 1998). Kaomongkolgit et al. reported that α-mangostin showed no toxic effects on human gingival fibroblasts up to 4000 μg/ml (Kaomongkolgit et al., 2009).

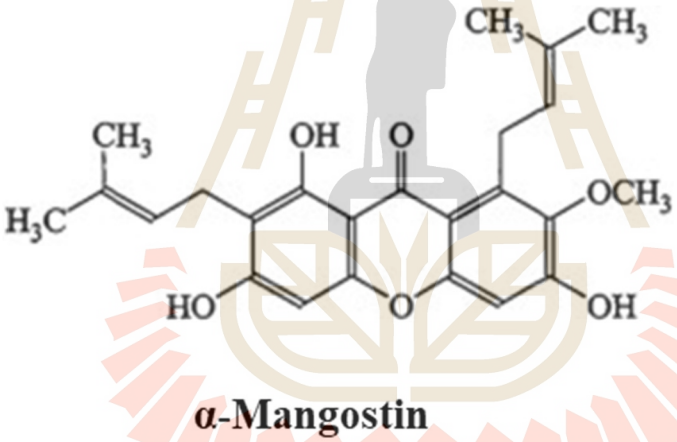


Figure 2.6 Chemical structure of α-mangostin (Mahabusarakam et al., 1987).

2.6.2 *Boesenbergia rotunda* (L.)

Boesenbergia rotunda, locally called in Thai as Kra-chai, is classified in the family of Zingiberaceae and the genus of *Kaempferia*. This herbal plant is widely cultivated in Southeast Asia, India, Sri Lanka and southern China. There are 8 distinct botanical names: *Boesenbergia cochinchinensis* (Gagnep.) Loes. , *Boesenbergia*

pandurata (Roxb.) Schltr., *Curcuma rotunda* L., *Gastrochilus panduratus* (Roxb.) Ridl., *Gastrochilus rotundus* (L.) Alston, *Kaempferia cochinchinensis* Gagnep., *Kaempferia ovate* Roscoe, and *Kaempferia pandurata* Roxb. However, it is now known as *Boesenbergia rotunda* (L.) Mansf (Eng-Chong et al., 2012). The morphologies of the whole plant, flower, and rhizomes of *B. rotunda* are illustrated in Figure 2.7. *B. rotunda* has been used as food ingredients and traditional medicine for treating various human ailments, including rheumatism, muscle ache, fever, gouty arthritis, bowel disorder, abdominal distension, carminative, appetite promotion, and gastric disorders (Chaudhury and Rafei, 2001). Furthermore, many pharmacological activities have been reported in this species including anti-ulcer, anti-inflammation and antioxidant, anti-viral, anti-parasite, and anticancer (Abdelwahab et al., 2011; Isa et al., 2012). Interestingly, this herbal plant exhibits strong wide spectrum antibacterial activity against pathogenic bacteria (Eng-Chong et al., 2012). Pinostrobin and red oil isolated from *B. rotunda* showed potential antibacterial activity against *Helicobacter pylori* with minimum inhibitory concentration (MIC) values of 125 µg/mL and 150 µg/mL, respectively. The minimum bactericidal concentrations (MBCs) for Pinostrobin was 150 µg/mL and for red oil was 175 µg/mL (Bhamarapravati et al., 2006). Furthermore, Panduratin A isolated from *Kaempferia pandurata* Roxb (syn. *B. rotunda*), showed strong anti-staphylococcal activities comparable to commonly used antibiotics against staphylococcal isolates (Rukayadi et al., 2009). Moreover, biofilm-producing enterococcal strains were inhibited by panduratin A at concentrations ranging from ≤ 4 µg/mL to ≤ 16 µg/mL. These MIC lower than those of daptomycin and linezolid, suggesting panduratin A showed stronger antibacterial activity (Rukayadi et al., 2010). Also, Panduratin A displayed

strong anti-periodontal and anti-cariogenic activities against *Porphyromonas gingivalis*, *Prevotella intermedia*, *Prevotella loescheii*, and *S. mutans*. The MIC levels were much lower than those of other antibacterial agents (Park et al., 2005). In addition, a recent study showed that crude ethanolic extract of rhizomes of *B. rotunda* also has a wide antibacterial spectrum against MRSA, methicillin-resistant coagulase-negative Staphylococci (MRCNS), methicillin-susceptible *S. aureus* (MSSA), *Bacillus subtillis*, and *Salmonella typhi* (Sukandar et al., 2014). *B. rotunda* showed potential antifungal activity against *Candida albicans* and *Aspergillus fumigatus* (Cheeptham and Towers, 2002). *C. albicans* treated with *B. rotunda* displayed faster-killing activity than the commercial drug, nystatin (Taweechaisupapong et al., 2010). For antiviral activities, HIV-1 proteases were inhibited by Hydroxypanduratin A and Panduratin A from the methanolic extract of *B. rotunda* at IC_{50} values of 5.6 μM and 18.7 μM. This inhibition was associated with the hydroxylation and prenylation of chalcones (Cheenpracha et al., 2006). The highest anti HIV-1 protease was seen in cells treated with cardamonin compared to pinostrobin, pinocembrin, and alpinetin (Taweechaisupapong et al., 2010).

Figure 2.7 Morphology of *Boesenbergia rotunda*, (a) Whole plant; (b) flower; (c) rhizomes (Eng-Chong et al., 2012).

2.6.3 *Oroxylum indicum*

Oroxylum indicum (L.), a medium-sized deciduous tree, is planted abundantly throughout Southeast Asia and South Asia countries especially in tropical rainforest regions. It belongs to the Bignoniaceae family, genus *Oroxylum,* and species *Oroxylum indicum* (Harminder et al., 2011). The morphologies of the tree, flowers, and pod of *O. indicum* are illustrated in Figure 2.8. Various parts of this herbal plant, including fruit, root and stem bark have been reported to possess a variety of pharmacological activities such as antibacterial activity, antioxidant activity, anti-inflammatory activity, anti-arthritic activity, anti-ulcer activity, anticancer/anti-tumor activity, anti-diarrheal activity and antidiabetic activity (Dinda et al., 2015). Many medicinally important bioactive compounds have been identified in different parts of *O. indicum*. The characterization of major compounds isolated from the root bark of *O. indicum* using RP-HPLC showed the presence of flavonoids oroxylin A, baicalein, and chrysin (Zaveri et al., 2008). The root of *O. indicum* was extracted with dichloromethane also showed flavonoid (baicalein, chrysin, oroxylin-A), naphthalenoids (lapachol) and steroid (β-sitosterol) (Mat Ali et al., 1998). The ethanolic extract of stem bark was reported to contain baicalin, baicalein, and chrysin (Singh and Kakkar, 2013). The extract of stem bark with ethanol showed to contain the highest amount of total phenolic (320.7 mg Gallic acid /g extract) and flavonoid (346.6 mg Quercetin /g extract) (Moirangthem et al., 2013). The major chemical constituents of seeds of the plant also include baicalein, chrysin, baicalein7-O-glucoside, baicalein 7-O-diglucoside and chrysin7-O-diglucoside (Chen et al., 2003). The flavonoid baicalein is an active component in the methanolic extract of *O. indicum*, which represented approximately 4% of freeze-dried fruits (Roy et al.,

2007). The result from identification of flavonoid from leaves of *O. indicum* by high-speed counter-current chromatography system (HPCCC) showed chrysin, baicalein, baicalein-7-O-glucoside, baicalein-7-O-diglucoside, chrysin-7-O-glucuronide, baicalein-7-O-glucuronide, and a chrysin-diglucoside are the major chemical constituents (Yuan et al., 2008). Phytochemical analysis of ethanol extract from the seed of *O. indicum* showed the presence of phenolic and flavonoid contents (10.66 g% gallic acid equivalent and 7.16 g% quercetin equivalent, respectively). Moreover, the thin layer chromatographic profiles of the extracts showed the band that corresponded to a flavonoid baicalein (Sithisarn et al., 2016). The extract from *O. indicum* exhibited broad antibacterial activities against a wide range of bacteria. The ethanolic extract of stem bark showed antibacterial activities against *E. coli*, *Klebsiella* spp., *Proteus* spp., *P. aeruginosa,* and *S. aureus*, with inhibition zones of 20,15,18,18 and 20 mm, respectively. In contrast, the ethanolic extract of the root showed inhibition against *Proteus* spp. and *S. aureus*, with inhibition zones of 20 and 14mm, respectively (Radhika et al., 2011). The ethanolic extract of the leaf demonstrated a moderate antibacterial effect against the nosocomial pathogen *A. baumannii*, with growth inhibition of 67.18% (Phatthalungetal., 2012). The extracts from *O. indicum* fruits and seeds showed antibacterial effects on two clinically isolated bacteria, *Streptococcus suis* and *Staphylococcus intermedius* with an inhibition zone of 15.11 mm and 14.39 mm, respectively (Sithisarn et al., 2016).

Figure 2.8 Morphology of *Oroxylum indicum*, (a) tree; (b) flowers; (c) pod (http://www.plantsofasia.com/index/oroxylum/0-583).

2.6.4 *Stephania suberosa*

Stephania suberosa Forman is traditional widely cultivated in Africa, India, Southeast Asia and Australia. It is classified in family Menisperamaceae and genus *Stephania.* Characteristics of *S. suberosa* are showed in Figure 2.9. Plants in this genus have been used extensively for the treatments of several human diseases such as asthma, tuberculosis, dysentery, dyspepsia, diarrhea, urinary complaints, intestinal diseases hyperglycemia, cancer, fever, sleep disturbances, and inflammation (Chopra, 1958; Gaur, 1999; Kirtikar and Basu, 2004; Makarasen et al., 2011). The tubers of *S. suberosa* and *S. venosa* have been used traditionally in Thailand as medicinal plants (Patra et al., 1987). Alkaloids have been found to be the major chemical constituents present in this genus (Yang et al., 2010). Moreover, many compounds have been isolated and identified including tannins, glycosides, steroids, anthocyanin, (+)-Cepharanthine, (+)-2-norcepharanthine, (+)-cepharanthine 2'-β-N-oxide, (+)-stephasubine, (+)-norstephasubine, stephasubimine, etc. (Semwal et al., 2010). Accordingly, this could be a potential source of biologically active compounds which might be used for the development of new drugs (Blanchfield et al., 2003). In the previous work, *S. suberosa* extract showed antibacterial and synergistic activities against ampicillin-resistant *Staphylococcus aureus* by reversing bacterial resistance to its original susceptibility via following three primary modes of actions: (1) inhibits peptidoglycan synthesis, resulting in morphological damage, (2) inhibits β-lactamases activity, and (3) increases CM permeability (Teethaisong et al., 2014). Xylopinine and cepharanthine alkaloid compounds isolated from *Stephania rotunda* were reported against *Plasmodium falciparum* W2 in vitro. Cepharanthine showed the best activity against W2 with inhibitory concentration at 50% (IC50) at 0.61 μM. Furthermore,

cepharanthine was tested with *Plasmodium berghei*. The parasite was inhibited at a concentration of 10 mg/ kg, against 47% of parasitaemia from the intraperitoneal injection and 50% of parasitaemia from oral administration and had antioxidant activity. Interestingly, these compounds were also found in *S. suberosa* (Chea et al., 2007; Semwal et al., 2010).

Figure 2.9 Morphology of *Stephania suberosa*, (a) leaves; (b) flowers; (c) tuberous root (http://www.bihrmann.com/caudiciforms/subs/stesub sub.asp).

2.7 Bacteriophages (Phages)

Bacteriophages are viruses that specifically infect their host bacteria. They can be found abundantly in nature and also can be used to control the bacterial population in the natural system (Carlton, 1999; Imbeault et al. , 2006) . They were first discovered and identified by Twort in 1915 and D' Herelle in 1917. Bacteriophages are thought to be one of the most ubiquitous organisms on the earth. The amounts of phages were estimated to range from 10^4 to 10^8 virions/mL in fresh water and about 10^9 virions/mL in soils, with an estimated total number of 10^{32} bacteriophages on the planet (Ashelford et al. , 2003; Hanlon, 2007) . Phages were first exploited for the treatment of diseases caused by bacteria during pre-antibiotic era. In the 1920s and most of the 1930s, the only available was serum therapy for selected pathogens such as pneumococci and diphtheria. Since the first antibiotic was discovered by Alexander Fleming and was clinically used, the attention on phage therapy was decreasing (Wittebole et al., 2014). However, phage therapy has regained its attention following an increasing emergence of antibiotic-resistant bacteria (Bragg et al., 2014).

2.7.1 Phage morphology and classification

Phages were taxonomically classified by Bradly in 1967 based on their nucleic acid content (dsDNA, ssDNA, dsRNA, and ssRNA) and structural morphology. They were divided into groups A-F based on morphological classification: group A (*Myoviridae*) phages attribute to long contractile tails, group B (*Siphoviridae*) phages possess long non-contractile tails, group C (*Podoviridae*) phages have short non-contractile tails, both group D, and E phages are tailless with characterizable different sized capsomeres, and group F phages are filamentous phages that contain long protein filaments filled by ssDNA. These morphological classifications can be further

sub-divided based on their head size (Figure 2.11). For instance, The C morphotype phages can be sub-divided into C1, C2, and C3. The number is assigned corresponding to increased head length (Bradley, 1967).

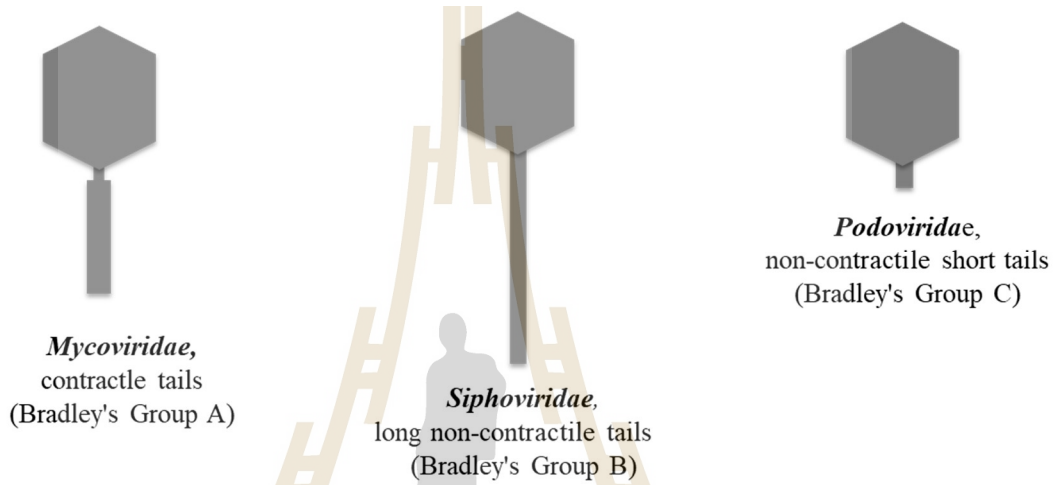


Figure 2.10 Morphological classification of phages (Adapted from Hull et al., 1990).

A phage particle is composed of three main components; the head, tail and tail fibers. The head of a phage Viron is a protein shell in which stores phage genome (mostly dsDNA). The tail and tail fibers of phages are the component that helps adhesion to the host and important in the delivery of the packed genome in the head (Rohwer, 2003). In addition to phage metrological classification by Bradley, the International Committee on Taxonomy of Viruses (ICTV) classifies phages into 1 major order, 13 families and 31 genera based upon nucleic acid content, morphology characteristics, and genome data. Phages mostly belong to the *Caudovirales* order (approximately 96%), composing of 3 major families (25% *Myoviridae*, 61% *Siphoviridae* and 14% *Podoviridae*). These phages comprise of the tail. The remaining 4% of isolates are with no tail and varying structure (Ackermann, 2003).

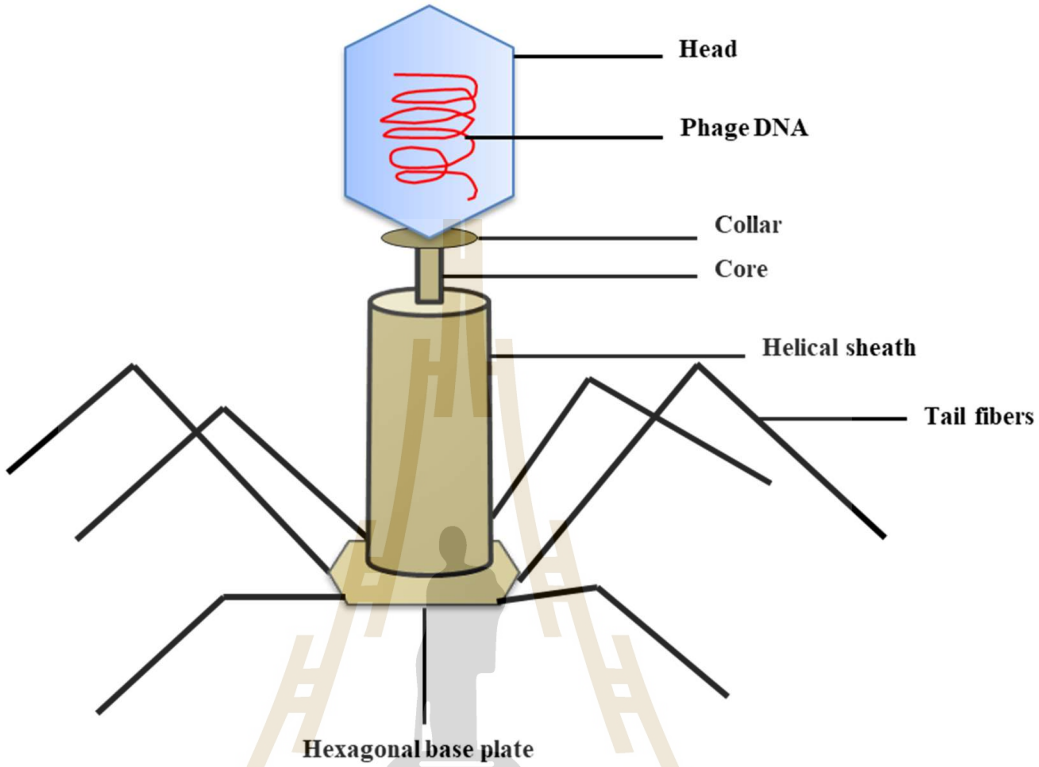


Figure 2.11 Structure of Myoviridae phage T4 (Adapted from Chernomordik, 1989).

2.7.2 Phage life cycle

Phages can be roughly divided into 2 groups based upon their replication cycle; lytic phages and lysogenic phages. Lytic phages are more significant in treatment purpose than lysogenic phages. The lytic cycle composes a series of events that occur between attachments of phage particle to a bacterial cell and subsequently host cells are lysed to release daughter phage particles. It consists of 5 steps in the cycle; (1) adsorption of phage to host cell, (2) injection of phage nucleic acid, (3)

intracellular development and replication, (iv) assembly of phage particles and v) finally release of progeny phage particles by lysing the host cell (Keary et al., 2013). The lysogenic cycle comprises of the replication of phage nucleic acid together with the host genes for several generations without major metabolic consequences for the cell. This is a dormant mode of infection, and it occurs at a very low frequency. The phage genes in this state may occasionally revert to lytic cycle, leading to the release of phages particles and lysis of the host cell. In addition, some phages can develop both lytically and lysogenic cycles. They are so-called temperate phages (Clokie et al., 2011).

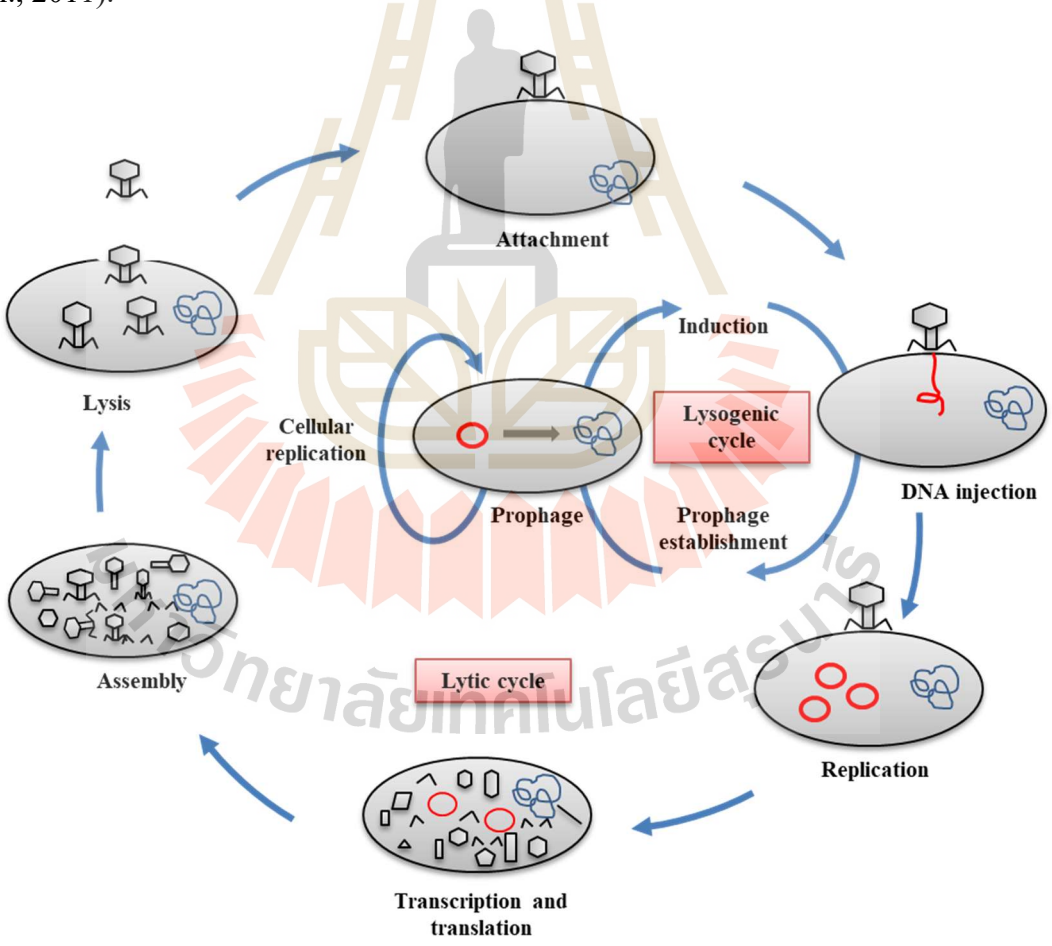


Figure 2.12 Phage life cycle (Adapted from Salmond and Fineran, 2015).

2.7.3 Phages therapy

Phage therapy is the application of treatment of bacterial infections using lytic bacteriophage to attack pathogenic bacteria specifically. Bacteriophages are known to be active against bacteria but cannot infect eukaryotic cells. Bacteriophages have specific properties which support them to be used as therapeutic agents (Table 2.1). Bacteriophage specifically is more specific in targeting their host than antibiotics, therefore preventing harm to normal flora and resulting in less of secondary infection. Bacteriophages replicate at the local infection, which increases concentration at the site of infection (Mathur et al., 2003). Phage therapy was widely used in the 1920s and 1930s for the treatment of bacterial infection. Since the first antibiotic was discovered in the 1940s, the phage therapy, and relevant research was considerably detracted from many researchers. Over the last decade, bacteriophage therapy has regained the attention from researchers and clinician as an alternative solution to treat the infections caused by bacteria as a consequence of the significant emergence of antibiotic-resistant bacteria. Also, the effective antibiotic to handle these resistant bacteria have been decreasing consciously at an alarming rate. Previous studies demonstrated that phage therapy can effectively treat pathogenic bacterial infection, including *K. pneumonia*, *P. acnes, S. aureus, P. aeruginosa, E. coli* (Abuladze et al., 2008; Azizian et al., 2015; Chhibber et al., 2008; O'flaherty et al., 2005; Sheng et al., 2006). Also, phages showed to be effective in an animal model for treating the infections caused by antibiotic-resistant bacteria, including MRSA, VRE, and multidrug-resistant *P. aeruginosa* and *E. coli* (Biswas et al., 2002; Matsuzaki et al., 2003; Wang et al., 2006a; Wang et al., 2006b). Most reports on phage therapy over the past ninety years have been studied by researchers from Georgia and Poland. The

safety and efficacy of clinical trials for the treatment of bacterial infection by phages were studied in 153 patients infected with a broad range of bacteria. The data was collected between January 2008 and December 2010. The results showed that 18% of the patients were reported for complete pathogen elimination and recovery. A positive clinical response was observed in 40% of patients, while less than 4% of patients did not complete the treatment as a result of adverse effect (Miedzybrodzki et al., 2012). Moreover, phage has also been examined in combination with conventional antibiotics for improving antimicrobial activity. Increasing of phage production by the sub-lethal concentration of antibiotics is reported to be due to cell filamentation in *E. coli* (Comeau et al., 2007). A combination of sub-inhibitory concentrations of ceftriaxone and *P. aeruginosa* phages had a synergistic effect, a result of cell morphological changes and reduced bacterial growth (Knezevic et al., 2013). The presence of a sub-lethal concentration of cefotaxime stimulated plaque size and phage concentration in *E. coli* (Ryan et al., 2012).

Table 2.1 Comparison of the therapeutic use of phages and antibiotics (Adapted from Sulakvelidze et al., 2001).

	Bacteriophages	**Antibiotics**
Targeting Ability	Phage usually affects only the targeted bacterial species. Therefore, dysbiosis and chances of developing secondary infections are avoided.	Antibiotics target both pathogenic microorganisms and normal microflora. This affects the microbial balance in the patient, which may lead to serious secondary infections.
Site Specificity	Replicate at the site of infection and are thus available where they are most needed.	They are metabolized and eliminated from the body and do not necessarily concentrate at the site of infection.
Side Effects	No serious side effects have been described.	Multiple side effects, including intestinal disorders, allergies, and secondary infections have been reported.
Resistance	Phage-resistant bacteria remain susceptible to other phages having a similar target range.	Resistance to antibiotics is not limited to targeted bacteria.
Treatment Development	Selecting new phages is a relatively rapid process that can frequently be accomplished in days or weeks.	Developing a new antibiotic is a time-consuming process and may take several years.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Compound and plant specimens

α-Mangostin was obtained from Indofine Chemical Company, New Jersey, USA. Fresh samples of *Stephania suberosa* (root), *Oroxylum indicum* (fruit), and *Boesenbergia rotunda* (root) were obtained locally from Pak Thong Chai district, Nakhon Ratchasima province, Thailand.

3.1.2 Bacterial isolates and bacteriophage

The clinical isolates of *A. baumannii*, and *E. faecium* and *P. aeruginosa* were obtained from the Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand. The reference strains, *E. coli* ATCC 25922, and *Enterococcus faecalis* ATCC 29212 were obtained from the American Type Culture Collection (ATCC), USA. *E. coli* strains ECOR-16 and ECOR-27 were obtained from the ECOR standard reference collection of *E. coli* (Ochman and Selander, 1984). Bacteriophage SU16 and SU27 were obtained from Stockholm University.

3.1.3 Antibiotics, chemicals, and equipment

All tested antibiotics were purchased from Sigma Aldrich, UK. All chemicals and equipment used are detailed in Appendix C. for a combination of bacteriophages

and antimicrobial plant extracts, all chemicals and reagents were obtained from either Sigma Aldrich (Stockholm, Sweden) or VWR (Stockholm, Sweden).

3.2 Methods

3.2.1 Monitoring of antibacterial activity and mechanism of action of α-mangostin

3.2.1.1 Determination of viable bacterial cell count

The bacterial suspensions standard curve method was used to reveal viable count. The experiment followed the method of Eumkeb et al. (2012) with slight modifications. Briefly, twenty microlitres of bacteria in 30% glycerol stock was transferred to 50 mL sterile Cation-adjusted Mueller-Hinton Broth (CAMHB). The cultures were incubated overnight at 37 °C with shaking at 120 RPM. The bacterial cells were harvested by centrifuging at 3,000 ×g for 10 minutes (min) and then washed twice by suspending in 0.9% NaCl and centrifuging at 3,000 ×g for 5 min. The cells were resuspended in 0.9% NaCl and measured for optical density at wavelength 500 nm. The ideal absorbance read should fall in the range of approximately 0.05-0.25. The dilutions of each absorbance reading that were 10-fold diluted in 900 µL of 0.9% NaCl was spread onto agar plates for viable cell counting. The number of colony forming units (CFU/mL) was calculated and then plotted as a bacterial suspension standard curve.

3.2.1.2 Minimum inhibitory concentration (MIC) determination

The MIC was determined using a broth microdilution assay with resazurin reduction assay according to the modified method described by the Clinical Laboratory Standards Institute; Taneja and Tyagi (CLSI 2013; Taneja and Tyagi, 2007). Stock solutions of antibiotics at a concentration of 20,480 µg/mL were

prepared following the manufacturer's instruction. The stock solution of α-mangostin was dissolved in 5% dimethyl sulfoxide (DMSO). Serial two-fold dilutions of α-mangostin and selected antibiotics (ampicillin, ceftriaxone, and ceftazidime) solutions were prepared in the culture medium in the 96-well polystyrene microtitre plate. Wells without drug were served as a control for bacterial growth. Twenty microliters of 0.02% (w/v) resazurin were added to all wells. The microtitre plates were inoculated with 20 µL per well of bacteria cell suspension obtained during the logarithmic phase of growth at a final concentration of 5×10^6 CFU/mL. After incubation at 37 °C for 18-24 h, the MIC was defined as the lowest concentration of test agents that inhibited bacterial growth, which showed no colorimetric change from blue (resazurin) to pink (resorufin). The assay was carried out in three replicates and three independent experiments.

3.2.1.3 Checkerboard determination

The synergistic activity between α-mangostin and selected antibiotic against drug-resistant bacteria was assayed using checkerboard determination following the method of Bonapace et al. (Bonapace et al., 2002). Checkerboard synergy testing was performed in triplicate with 96-well polystyrene microtiter plate using an 8-by-8 well configuration. Dilutions of each antimicrobial agent were carried out using an automated dilution with concentrations ranging from 0.008×MIC to 1×MIC. The final inoculum was prepared similarly to the MIC determination, approximately 5×10^5 CFU/mL. Microtitre plates were incubated at 37 °C for 18 h. The synergy activity was determined by the lowest concentration of two agents in combination with calculation of the fractional inhibitory concentration index (FICI).

The FICI was calculated to determine drug interaction, and interpreted as follows (Marques et al., 1997).

$$\text{FIC index} = \frac{\text{Conc.of A in MIC of A+B}}{\text{MIC of A alone}} + \frac{\text{Conc.of B in MIC of A+B}}{\text{MIC of B alone}}$$

FICI ≤ 0.5 denoting synergism

FICI > 0.5 – <1.0 denoting partial synergism

FICI = 1.0 denoting additions

FICI > 1.0 - ≤ 4.0 denoting indifferent

FICI > 4.0 denoting antagonism

3.2.1.4 Time-kill assay

The effectiveness of the α-mangostin and selected antibiotic combinations against drug-resistant bacterial strain was determined by time-kill curve assay. This experiment is performed to confirm antibacterial and synergistic activities by the number of remaining viable bacteria at varying time points. The assay followed the previous method with some modifications (Richards et al., 1993). Changes in bacterial count during incubation period were determined in four test tubes containing the following: (1) only bacteria (5×10^5CFU/mL); (2) bacteria and a sub-inhibitory concentration of antibiotics; (3) bacteria and sub-inhibitory concentration of α-mangostin and (4) bacteria, inhibitory concentration of antibiotics and α-mangostin combination. The bacterial counts were determined at 0, 0.5, 1, 2, 3, 4, 5, 6, and 24 h of incubation by spreading appropriate dilutions on an agar plate. The plates were

incubated at 37 °C overnight, and bacterial colonies were counted. Time-kill curve was plotted and interpreted as follows: The interactions were considered synergistic if there was a reduction in ≥ 2log10 CFU/mL in comparison to the initial inoculum. Bactericidal activity was defined as a decrease of ≥ 3 log10 CFU/mL in comparison to the initial inoculum (Belley et al., 2008). The sensitivity limit of detection was less than 10^2 CFU/mL.

3.2.1.5 Scanning electron microscopic study of cell morphology

Scanning electron microscope (SEM) experiment was performed to observe the alteration in cell morphology change. Suspensions of overnight broth cultures were prepared. The cells were then treated with the half-MIC value of α-mangostin, and selected antibiotic alone and the 3/4 MIC of these combinations that gave synergistic FIC, for 4 h with shaking 120 RPM at 37 °C. Untreated controls were also prepared as a control. Then, the samples were harvested by centrifugation at 6,000 ×g for 15 min at 4 °C, washed twice with phosphate buffered saline (PBS). The precipitated cells were fixed with 2.5% (w/v) glutaraldehyde (Electron Microscope Sciences; EMS) in 0.1M phosphate buffer (pH 7.2) for 12 h. The samples were rinsed twice with 0.1 M phosphate buffer and fixed again with 1% osmium tetroxide (EMS) in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4 °C. The samples were dehydrated in 30%, 20%, 40%, 60%, 80%, and 100%, graded acetone dilutions for 15 min and then air dried. The cells were transferred into glass slides and mounted on the stubs using double-sided carbon tape and the coated thin layer of gold. The samples were observed with a JSM-6010LV electron microscope (JEOL, Japan). Secondary electron images were taken at low electron energies between 2 keV and 5.0 keV.

3.2.1.6 Transmission electron microscopic study of cell ultrastructure damage

Ultrastructure damage of bacterial cell was examined using TEM following the method of Eumkeb and Chukrathok (2013). Bacteria for TEM study were grown and treated the same as SEM preparation. The treated cells were centrifuged, washed twice with PBS and fixed in with 2.5% (w/v) glutaraldehyde in 0.1M phosphate buffer (pH 7.2). The fixed bacterial cells were fixed again with 1% osmium tetroxide (EMS) in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4 °C. Subsequently, the samples were then dehydrated in graded acetone series. Infiltration and embedding were performed with various ratios of propylene oxide and Spurr's resin and polymerization in 100% Spurr's resin at 70 °C for 8 h. Ultrathin sections were prepared on copper grids and stained with 2% (w/v) uranyl acetate and 0.25% (w/v) lead citrate for 15 min. The prepared samples were viewed with a Tecnai G2 electron microscope (FEI, USA) at 120 kV. Furthermore, for each group of cells, the morphological parameters including cell area were measured to confirm the effects of either used α-mangostin singly and in combination on cell size.

3.2.1.7 Cytoplasmic membrane (CM) permeability

The CM permeabilisation was performed by measurement the release of 260 nm absorbing material concentrations using UV-VIS spectrophotometer following the method of Zhou et al. (2008) and Devi et al. (2010). The cultures were adjusted to a final cell suspension of 5×10^5 CFU/mL in PBS and incubated with 1/2 MIC of selected antibiotic, 1/2 MIC of α-mangostin, and selected antibiotic plus α-mangostin (3/4 FIC). A cell without an antibacterial agent was used as the negative control and with (1/2 MIC). Nisin was used as a positive control. Samples were

removed at 0, 0.5, 1, 2, 3, and 4 h and then centrifuged at 13,400 ×*g* for 15 min. The supernatants were filtered through a 0.2 μm pore size filter, and the UV-absorbing materials concentration at each time point was determined by UV–VIS spectrophotometer at a wavelength of 260 nm. The assay was done in triplicate. Mean ratios for each treatment and time were calculated and compared to the means of the corresponding control samples.

3.2.1.8 Outer membrane (OM) permeability

β-lactamase is an enzyme generally located in the periplasmic space of most Gram-negative bacteria (Ciofu et al., 2000; Pradel et al., 2009). Nitrocefin (NCF), a chromogenic cephalosporin, is commonly used as a substrate for this enzyme. Hence, the OM permeability was determined by measuring β-lactamase activity using NCF as substrate (Eriksson et al., 2002; Junkes et al., 2008). The bacteria were grown in CAMHB for 18-20 h and adjusted to 10^6 CFU/mL. The adjusted cells 15 μL (5×10^6 CFU/mL) were added to 135 μL of 10 mM Tris buffer containing 2.5 mM NCF and 1/2 MIC of selected antibiotic, 1/2 MIC of α-mangostin, and selected antibiotic plus α-mangostin at half-FIC concentrations. A control sample was similarly prepared without antibiotic treatment. Polymyxin B at half-MIC concentrations was used as positive control. The rate of permeability was evaluated by measuring absorbance at 500 nm on Biotex microplate reader.

3.2.1.9 Enzyme assay

The ability of α-mangostin to inhibit the activity of β-lactamase type IV from *Enterobacter cloacae* was assayed following the method previously described by Eumkeb et al. (2010) with slight modifications. Benzylpenicillin, a substrate for β-lactamase, was adjusted to a concentration that allows 50-60% hydrolysis within 5

min. The α-mangostin was preincubated with the enzyme in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C for 5 min before adding the substrate. The time-course assay was performed at 0, 5, 10, 15 and 20 min using methanol/acetic acid (100:1) as a stopping agent. Ten microlitres of each sample were injected to HPLC to analyse the remaining benzylpenicillin. 10 mM ammonium acetate (pH 4.5 acetic acid): acetonitrile (75:25) was used as the mobile phase. The HPLC conditions were carried out at flow rate 1 mL/min, UV detector at 200 nm, Ascentis C18 column, and 35 °C for column temperature. The quantity of remaining benzylpenicillin was calculated by comparing the area under the chromatographic curve.

3.2.1.10 Gel electrophoresis

To examine the effect of α-mangostin and selected antibiotic on the outer membrane and peptidoglycan-associated protein (OMPG) profiles of gram-negative bacteria, the protein profile was examined by gel electrophoresis following the method of Eumkeb (1999) and Fernández-Cuenca et al. (2003) with few modifications. The cell culture was incubated at 37 °C overnight. Four mL of 18 h culture was added to 400 mL CAMHB and incubated for 4 h at 37 °C. Then the logarithmically growing culture was divided into four portions of 100 ml and treated with 1/2 MIC of selected antibiotic, 1/2 MIC of α-mangostin alone, and plus selected antibiotic at 3/4 FIC concentration for 4 h at 37 °C. The cultures without antibacterial agents were used as a control. The samples were harvested by centrifugation at 6000 ×*g* for 15 min at 4 °C then the supernatant fractions were removed. Then the sediment fractions were washed twice with HEPES buffer (10mM, pH 6.8). The pellets were then resuspended in 10 mL HEPES buffer (10mM, pH 7.4) and disintegrated by sonication (400 W, 4×2 min, at 4 °C). Cell debris was removed by centrifugation at 10,000 ×*g* for 10 min at 4

°C. The supernatant (10 mL) was collected, and 1 mL of 20 % (w/v) sodium-N-lauroylsarcosine (Sarkosyl) were added and incubated for 30 min at room temperature. The mixture was ultracentrifuged at 100,000 ×*g* for 60 min at 4 °C (Backman 70.1 Ti, 40,000 rpm). The pelleted proteins were suspended in 5 mL of 2 % (w/v) N-lauroylsarcosine solution and then incubated for 30 min at room temperature and recovered by ultracentrifugation at 100,000 ×*g* for 60 min at 4 °C. The sediment fractions containing outer membrane proteins were collected and stored at -80 °C. The OMPG profiles of the samples were analysed by Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE).

3.2.2 Combination of bacteriophages and antimicrobial plant extracts

3.2.2.1 Plant extracts

Fresh samples of *Stephania suberosa* (root), *Oroxylum indicum* (fruit), and *Boesenbergia rotunda* (root) were washed thoroughly with tap water. Samples were cut into small pieces and dried in a hot air oven at 40 °C for 2 days. The dried pieces were pulverized, using a mechanical grinder to a coarse powder. Powdered of the extracts (500 g) were extracted by the soxhlation technique with 2 L of ethanol for 8 h. The extracts were filtered by passing through Whatman number 1 filter paper, and the solvents were evaporated under reduced pressure in a Buchi rotary evaporator at 50 °C. The remaining of the extract was lyophilized to obtain dried extracts.

3.2.2.2 Bacteriophage and bacterial strains

ECOR-16 and ECOR-27 were stored in glycerol at -80 ºC and maintained on lysogeny agar (LA) plates at 4 °C. ECOR-16 and ECOR-27 were routinely cultured in lysogeny broth (LB) at 37 °C overnight with shaking at 120 rpm. Prior to use, suspensions were centrifuged at 3864 ×*g* for 15 minutes, the supernatant

discarded and the pellet suspended in 10 mL PBS and further diluted in PBS to contain 1-5 × 10^8 CFU/mL.

Previously characterized phages SU16 and SU27 (Khan Mirzaei and Nilsson, 2015) stocks were stored at -80 °C. Phages were cultured in 10mL LB using the appropriate bacterial strain (100 µL) as a routine host at 37 °C overnight with shaking. Following incubation, suspensions were centrifuged at 3864 ×g for 15 minutes at 4 °C and passed through 0.45 and 0.2µm syringe filters. Phage suspensions were enumerated using the agar overlay method (Adams, 1959) and stored at 4 °C until required. Prior to use, phages were diluted in PBS to contain 1-3 × 10^8 PFU/mL.

3.2.2.3 MIC/MBC determination and activity of preservatives against phage

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts were determined against ECOR-16, and ECOR-27 was determined using a modified broth method (Clinical and Laboratory Standards Institute, 2014). In brief, standardised bacterial suspensions were diluted 1:100 in double strength LB (dsLB) and the bacterial content determined by the drop count method (Miles et al., 1938). To triplicate wells of the first row of a 96 well microtitre plate, 200 µL of plant extract was added. A 100 µL aliquot was removed and serially diluted 1:2 in PBS over the remainder of the plate. To each well, 100 µL of the diluted bacterial suspension was added and the plate incubated at 37 °C overnight. The MIC value was determined to be the lowest concentration where no visible growth could be observed. The MBC was determined by spreading 100 µL of suspension from wells showing no visible growth over the surface of pre-prepared LA

plates and then incubated at 37 °C overnight. The MBC value was determined to be the lowest concentration where no visible growth could be observed.

The activity of plant extracts against SU16 and SU27 was assessed using a modified suspension test assay (Pinto et al., 2010). In brief, 1mL of standardised phage suspension was added to 9 mL plant extract at different concentrations (100, 10, and 1% of MIC) and incubated for 24 h at room temperature (approx. 37 °C). Extracts which maintained significant antiviral activity at 1% MIC were further diluted as necessary. After 24 h incubation, 100 µL aliquots were removed and added to 9.9 mL PBS. Suspensions were then serially diluted in PBS and the phage content determined using the agar overlay method (Adams, 1959).

3.2.2.4 Combined antibacterial activity of phage/extract combinations

Twenty-five mL of dsLB was added to 5 mL of extracts in PBS (or PBS with 5% (v/v) DMSO where appropriate), 5 mL of standardized phage suspension and 5 mL standardised bacterial suspension. The total volume was then increased to a final volume of 50 mL with PBS and flasks were then incubated at 37 °C with shaking at 150 rpm. At defined time points (t= 2, 4, 6, 8, and 24 h) 1 mL aliquots were removed from each flask and 1mL of fresh LB was added to each flask to maintain volume. For phage containing samples, a 500 µL aliquot was added to 50 µL chloroform, vortexes and phage content determined using the agar overlay method (Adams, 1959). One hundred µL of the original sample was then serially diluted 1:10 in PBS and bacterial content determined by spreading 100 µL of each dilution over the surface of duplicate LA plates. For non-phage containing samples, bacterial content was determined using the drop count method (Miles et al., 1938). Plates were

incubated overnight at 37 °C and the CFU/mL or PFU/mL content determined. Controls containing PBS (untreated) or phage only (no extracts) were also performed.

3.3 Statistical analysis

All experiments were performed in triplicate unless stated in the text. Data were expressed as a mean ± standard deviation (SD). Significant differences in the viable bacterial count among each of treated groups at the same time were analysed by one-way ANOVA followed by Tukey's HSD posthoc test. The $P < 0.05$ was considered as the statistically significant difference. Also, the significant differences between each tested group and the control were determined using the independent T-test at $P < 0.05$.

CHAPTER IV

RESULTS

4.1 Antibacterial activity and mechanism of action of α-mangostin

4.1.1 Minimum inhibitory concentration (MIC) determination

The antibacterial activity of ampicillin, ceftazidime, and ceftriaxone were evaluated against the clinical isolates *A. baumannii* DMST 45378, *A. baumannii* DMST 44689, *A. baumannii* DMST 43105, *E. faecium* DMST 12829, *E. faecium* DMST 12852, *E. faecium* DMST 12970, *E. faecalis* ATCC 29212 (a reference strain for enterococci), *P. aeruginosa* DMST 37169, *P. aeruginosa* DMST 37186, *P. aeruginosa* DMST 37166 and *E. coli* ATCC 25922 (a reference strain for Gram-negative bacteria). The MIC values of these particular antibiotics are illustrated in Table 4.1. MICs values of α-mangostin were 2 µg/mL against *E. faecium* strains and were >2048 µg/mL against all tested Gram-negative bacteria. Furthermore, the MIC values of ceftazidime against *A. baumannii* DMST 45378, *A. baumannii* DMST 44689, *A. baumannii* DMST 43105, *P. aeruginosa* DMST 37169, *P. aeruginosa* DMST 37186, *P. aeruginosa* DMST 37166 and *E. coli* ATCC 25922 were found to be 800, 800, 800, 800,100, 1, and ≤ 1 µg/mL, respectively. These clinical isolates had MIC values for ceftriaxone of 2048, 2048, 2048, 400, 2048, 2, and ≤ 1 µg/mL, respectively. In addition, the MIC values of ampicillin against *E. faecium* DMST 12829, *E. faecium* DMST 12852, *E. faecium* DMST 12970, *E. faecalis* ATCC 29212 were 32, 128, 2, and 2 µg/mL, respectively.

Table 4.2 shows the interpretation criteria according to the CLSI susceptibility breakpoints for commonly-used antibiotics against different types of pathogens (Clinical and Laboratory Standard Institute, 2014). The results indicated that all tested *A. baumannii* isolates as well as *P. aeruginosa* DMST 37169, and *P. aeruginosa* DMST 37186 exhibited high resistance to ceftazidime and ceftriaxone. Likewise, resistance to ampicillin was observed in *E. faecium* DMST 12829 and *E. faecium* DMST 12852. The following bacterial strains were found to be susceptible to antibiotics: *P. aeruginosa* DMST 37166, *E. faecium* DMST 12970, a reference strain *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212.

Table 4.1 Minimum inhibitory concentration (MIC) of selected antibiotics and α-mangostin against important pathogenic Gram-positive bacteria and Gram-negative bacteria. The data were obtained from three replications.

Bacterial Strains	Minimum inhibitory concentration (µg/mL)			
	AMT	CAZ	CTX	AMP
A. baumannii DMST 45378	>2048NA	800^{R}	2048^{R}	-
A. baumannii DMST 44689	>2048NA	800^{R}	2048^{R}	-
A. baumannii DMST 43105	>2048NA	800^{R}	2048^{R}	-
P. aeruginosa DMST 37169	>2048NA	800^{R}	400^{R}	-
P. aeruginosa DMST 37186	>2048NA	100^{R}	2048NA	-
P. aeruginosa DMST 37166	>2048NA	1^{S}	2NA	-
E. faecium DMST 12829	2NA	-	-	32^{R}
E. faecium DMST 12852	2NA	-	-	128^{R}
E. faecium DMST 12970	2NA	-	-	2^{S}
E. coli ATCC 25922*	>1024NA	≤1^{S}	≤1^{S}	-
E. faecalis ATCC 29212**	1NA	-	-	4^{S}

S = susceptible; R = resistant; NA = not applicable; AMP = ampicillin; AMT = α-mangostin; CAZ = ceftazidime; CTX = ceftriaxone; * A reference strain for Gram-negative bacteria; ** A reference strain for Gram-positive bacteria.

Table 4.2 CLSI susceptibility breakpoint interpretive criteria for commonly-used antibiotics against different types of pathogens (Clinical Laboratory Standard Institute, 2016).

Antimicrobial Agent	MIC Interpretive Criteria		
	S	I	R
Acinetobacter spp.			
Ceftazidime	≤8	16	≥32
Cefotaxime	≤8	16–32	≥64
Pseudomonas spp.			
Ceftazidime	≤8	16	≥32
Cefotaxime	NA	NA	NA
Enterobacteriaceae			
Ceftazidime	≤4	8	≥16
Cefotaxime	NA	NA	NA
Enterococcus spp.			
Ampicillin	≤8	NA	≥16

[S] = susceptible; [I] = intermediate; [R] = resistant; [NA] = not applicable

4.1.2 Checkerboard determinations

Due to an increasing emergence of antimicrobial-resistant microbes, using drug combination therapy is about a promising approach for the treatment of infections caused by these resistant microorganisms. The results of drug interaction determined by checkerboard assay for the interaction of α-mangostin in combination with antibiotics against drug-resistant bacteria are demonstrated in Table 4.3. The lowest fraction inhibitory concentration (FIC) index was seen in the combination of α-mangostin and ceftazidime against *A. baumannii* DMST 45378, *A. baumannii* DMST 44689, and *A. baumannii* DMST 43105 at an FIC index of <0.35, which indicated synergistic interaction. In addition, α-mangostin and ceftriaxone combination was also found to be synergistic interaction against *A. baumannii* strains at FIC index of <0.24. In *P. aeruginosa* DMST 37186, there was synergistic activity in the combinations of α-mangostin plus ceftazidime or ceftriaxone. The FIC indices of these combinations were 0.53 and 0.52, respectively.

In addition, the partial synergistic interaction was observed with the combination of α-mangostin and ceftazidime against *P. aeruginosa* DMST 37169 at an FIC index of 0.75. Furthermore, when α-mangostin was combined with ampicillin, it was able to inhibit the growth of *E. faecium* DMST 12829 and DMST 12852 at an FIC index of 0.75. This FIC index was classified in partial synergistic interaction.The additive interactions were observed with the combination of α-mangostin and ceftazidime against *P. aeruginosa* DMST 37169 (FIC index = 1.00).

Table 4.3 FICs for checkerboard assay of α-mangostin used alone and in combination with selected antibiotics.

Bacterial strains	Combination of agents	MIC (μg/mL)	FIC index	Type of interaction
A. baumannii DMST 45378	AMT	200	<0.35	Synergism
	CAZ	200		
	AMT	250	<0.24	Synergism
	CTX	250		
A. baumannii DMST 44689	AMT	200	<0.35	Synergism
	CAZ	200		
	AMT	250	<0.24	Synergism
	CTX	250		
A.baumannii DMST 43105	AMT	200	<0.35	Synergism
	CAZ	200		
	AMT	250	<0.24	Synergism
	CTX	250		
P. aeruginosa DMST 37169	AMT	1024	0.75	Partial synergism
	CAZ	200		
	AMT	1024	1.00	Addition
	CTX	200		
P. aeruginosa DMST 37186	AMT	1024	≥0.53	Partial synergism
	CAZ	3.125		
	AMT	1024	≥0.52	Partial synergism
	CTX	50		
E. faecium DMST 12829	AMT	1	0.75	Partial synergism
	AMP	8		
E. faecium DMST 12852	AMT	0.5	0.75	Partial synergism
	AMP	64		

4.1.3 Killing curve determinations

A. baumanii DMST 45378 and *E. faecium* DMST 12829 were selected for further investigation with the killing curve assay. This experiment was performed to confirm the antibacterial activity and the interaction of α-mangostin plus ceftazidime, or plus ampicillin. Figure 4.1 presents the viable counts of *A. baumanii* DMST 45378 after exposure to α-mangostin, either singly or in combination with ceftazidime. The control cells grew rapidly during 3 h of incubation and then the growth rate slightly increased. In cells treated with ceftazidime at sub-MIC (400 µg/mL), the growth was inhibited until 4 h of incubation when growth increased again growing until 24 h of incubation. The similar growth pattern was seen in cells treated with α-mangostin (1,024 µg/mL). In cells treated with α-mangostin (200 µg/mL) plus ceftazidime (200 µg/mL), the viable counts of *A. baumanii* DMST 45378 were markedly reduced to approximately 10^2 CFU/mL at 6 h of incubation. The inhibition was continued for 24 h of incubation. At 24 h of treatment, in the combined group, a reduction in the viable count for ≥2-log 10 CFU/mL when compared with α-mangostin and ceftazidime alone was observed. This finding confirmed that the combination of α-mangostin and ceftazidime synergistically inhibited the growth of *A. baumanii* DMST 45378. In addition, this combination possessed bactericidal activity as a decrease in the viable count for ≥3-log10 CFU/mL was observed compared with starting inoculum.

Figure 4.2 describes the viabilities of *E. faecium* DMST 12829 after exposure to α-mangostin alone (1 µg/mL), ampicillin alone (16 µg/mL) and the combination of α-mangostin (1 µg/mL) plus ampicillin (8 µg/mL). *E. faecium* DMST 12829 grew slightly in the absence of any antibacterial agent until 5 h of incubation. Then, no substantial change in viability was seen through 24 h of incubation. The growth of

cultures treated with either ampicillin alone or combination with α-mangostin slightly decreased and stayed steady until 24 of incubation. When the combined group was compared with α-mangostin and ampicillin alone, reduction in viable count <2-log10 CFU/mL, indicating that this combination showed no synergistic interaction in inhibiting the growth of *E. faecium* DMST 12829. Furthermore, cell viabilities of *E. faecium* DMST 12829 after exposure to the combination of α-mangostin and ampicillin were slightly reduced to <3-log10 CFU/mL. This result suggested that this combination possessed bacteriostatic activity.

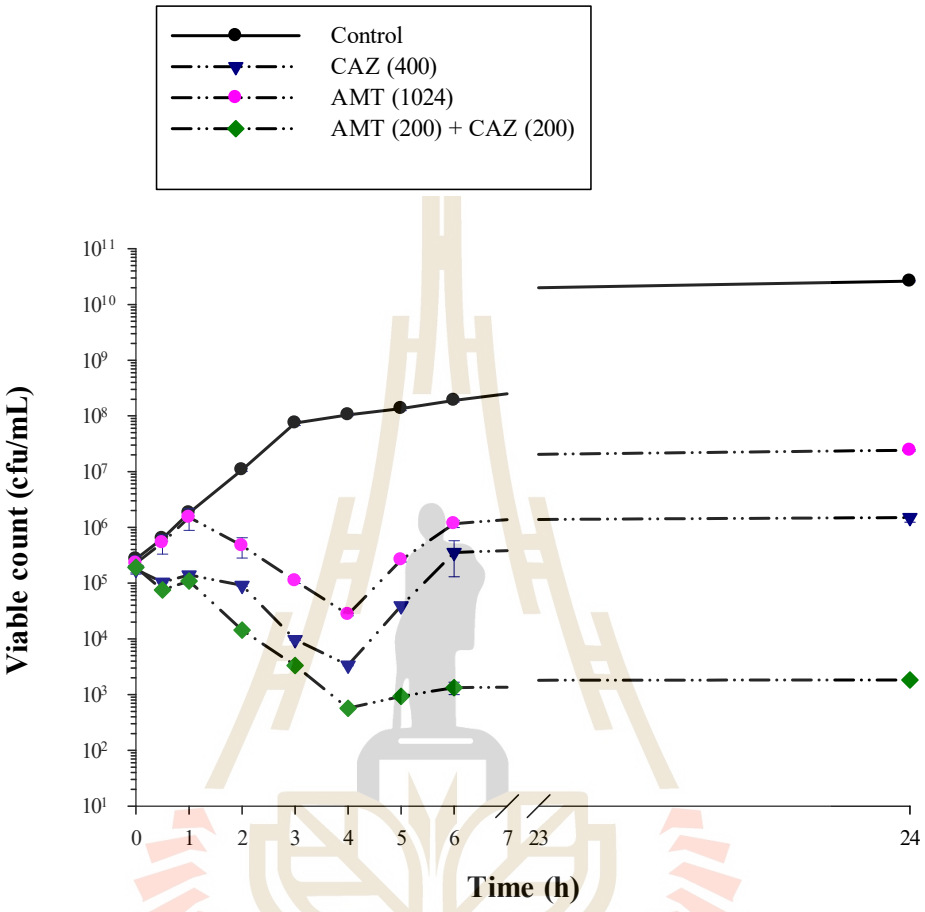


Figure 4.1 Time killing-curve of *A. baumannii* DMST 45378 after exposure to α-mangostin (AMT), ceftazidime (CAZ) either alone or in combination. Con = control (drug free); CAZ (400) = ceftazidime at 400 µg/mL; AMT (1024) = α-mangostin at 1,024 µg/mL; AMT (200) + CAZ (200) = α-mangostin at 200 µg/mL plus ceftazidime at 200 µg/mL. The values plotted are the means of 3 observations, and the vertical bars indicate the standard errors of the means.

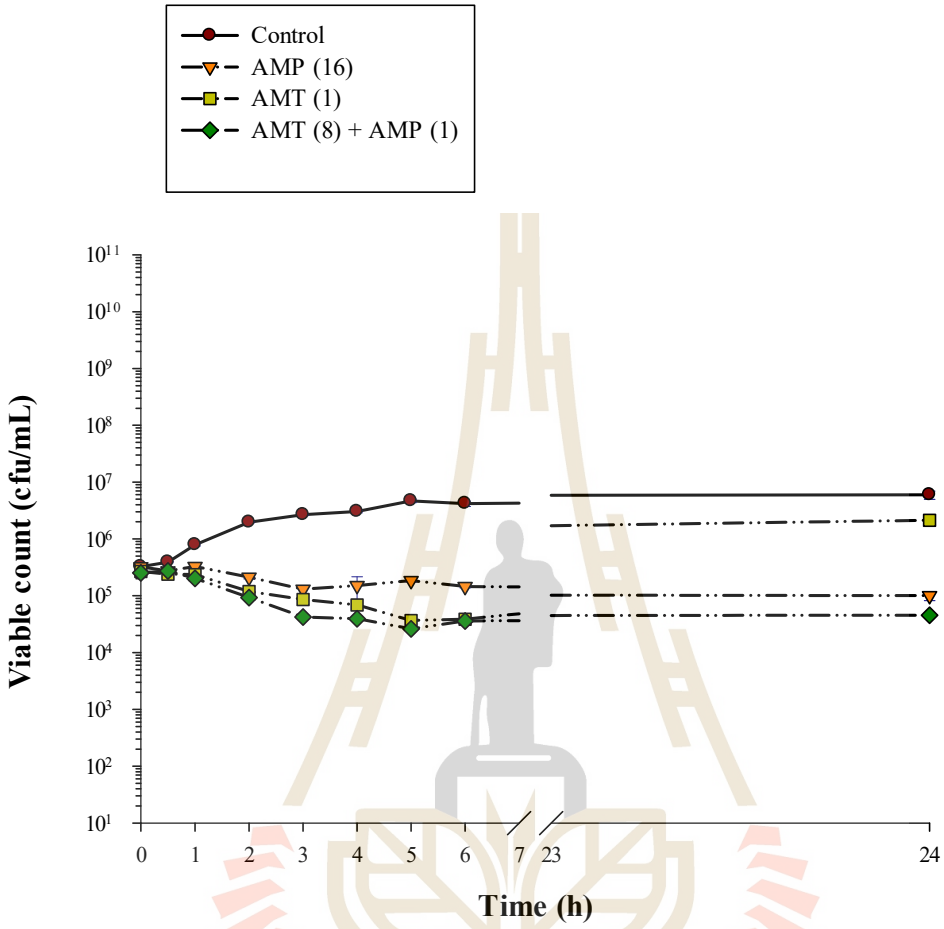


Figure 4.2 Time killing-curve of *E. faecium* DMST 12829 after exposure to α-mangostin (AMT), ampicillin (AMP) either alone or in combination. Con = control (drug free); AMP (16) = ampicillin at 16 µg/mL; AMT (1) = α-mangostin at 1 µg/mL; AMT (1) + AMP (8) = α-mangostin at 1 µg/mL plus ampicillin at 8 µg/mL. The values plotted are the means of 3 observations, and the vertical bars indicate the standard errors of the means.

4.1.4 Morphological change of bacterial cells as evaluated by SEM

SEM study was performed to observe the change in the cell morphology. In the Figure 4.3A and 4.3B, the untreated cells displayed a normal rod-shaped with intact envelope, whereas cells treated with ceftazidime were found to be longer compared with untreated cells. No bacterial septum was observed in these treated cells (Figure 4.3C and 4.3D). In α-mangostin-treated cells, cell surface was dimpled and protruded. The irregular cell surface was also observed in this treated group (Figure 4.4A and 4.4B). After treatment of *A. baumannii* with α-mangostin plus ceftazidime (Figure 4.4C and 4.4D), the cell envelope was markedly damaged and dimpled. Rough and irregular cell surface were also seen in cells treated with the combination of α-mangostin plus ceftazidime.

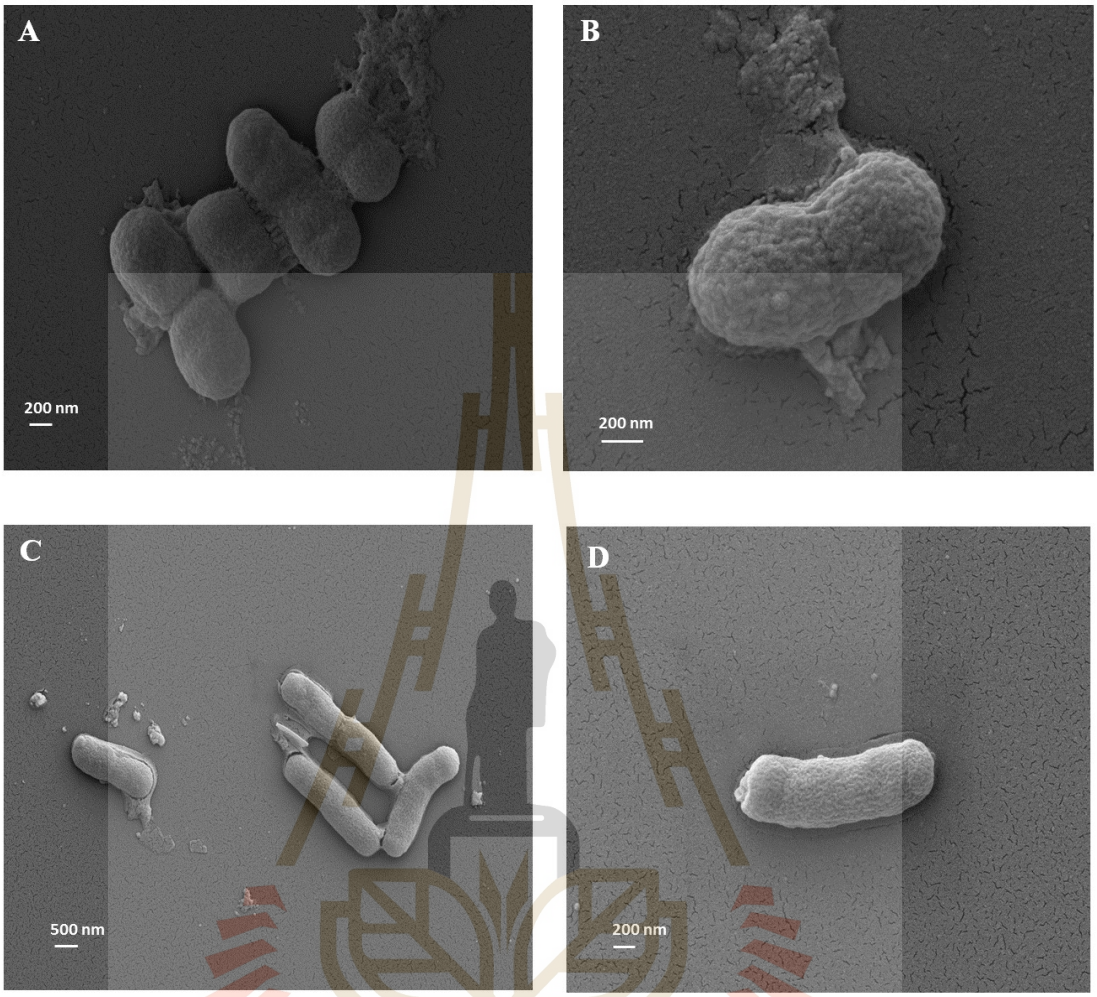


Figure 4.3 SEM micrographs of *A. baumannii* DMST 45378 grown in the Mueller-Hinton broth in the absence of the antimicrobial agent (A, B) and the presence of ceftazidime at a concentration of 400 µg/mL (C, D).

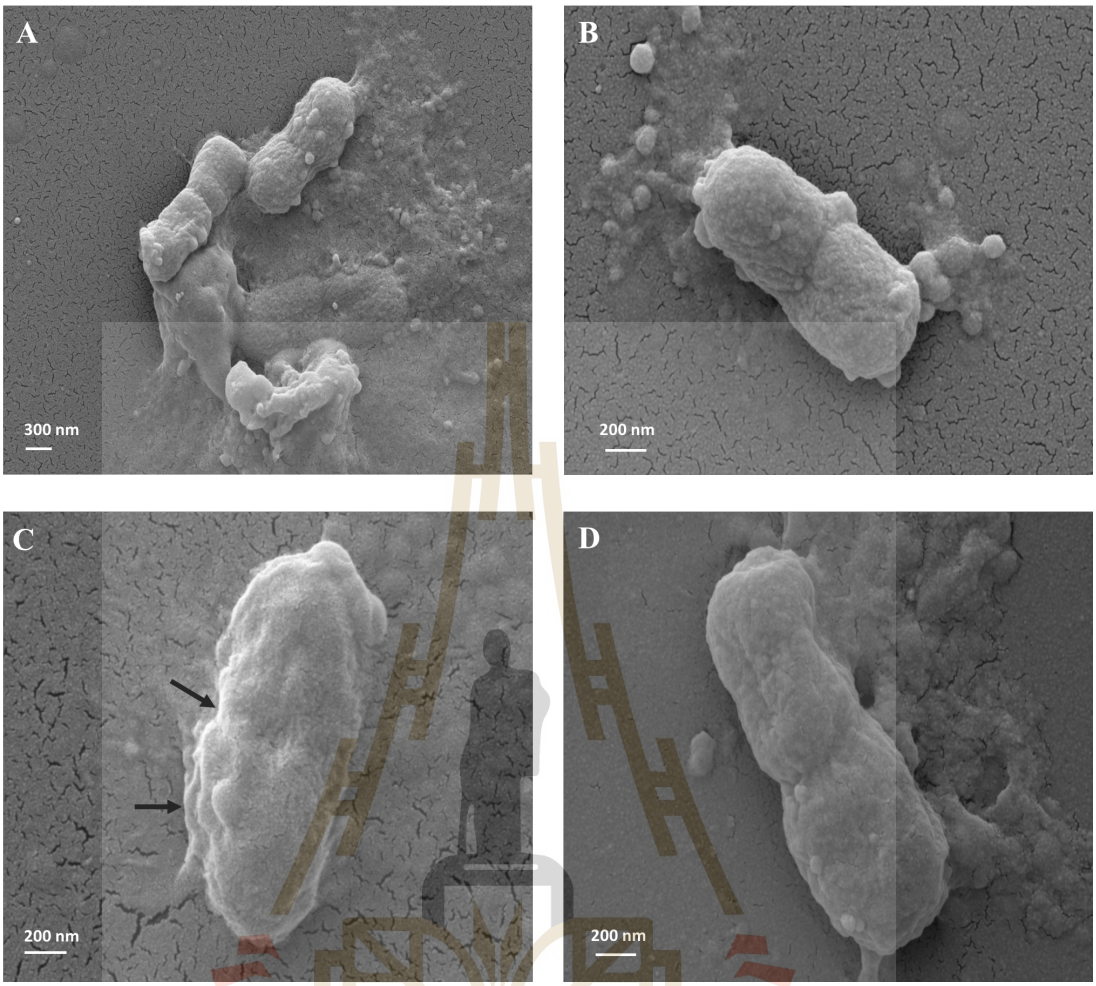


Figure 4.4 SEM micrographs of *A. baumannii* DMST 45378 grown in the presence of α-mangostin at a concentration of 1,024 µg/mL (A, B) and grown in the presence of α-mangostin at 150 µg/mL plus ceftazidime at 150 µg/mL (C, D). The black arrows indicate the sites of damage.

4.1.5 Morphological change of bacterial cells as evaluated by TEM

TEM imaging was conducted to observe the structural damage of bacterial cells treated with α-mangostin or ceftazidime alone and in combination. Figure 4.5 shows the electron micrographs of log phase of *A. baumannii* DMST 45378 cells grown in the absence of any antimicrobial agent. The untreated cells showed a normal cell shape, intact cell envelope and normal intracellular structure. In the cells treated with ceftazidime at a sub-MIC concentration (Figure 4.6), disruption and change in the intracellular structure were seen in these treated cells. When cells were exposed to α-mangostin, the cells exhibited damage to the cell wall and cell membrane. (Figure 4.7). Leakage of their intracellular contents as a result of the disintegration of cell envelope was also demonstrated in those cells treated with α-mangostin. Interestingly, the bacterial cells treated with the combination of ceftazidime and α-mangostin remarkably elucidated destruction of the cell wall and cell membrane. These treated cells obviously revealed leakage of cytoplasmic materials as shown in Figure 4.8. Cell debris and residual substances were also observed in the combined treated group. Furthermore, the effect of ceftazidime and α-mangostin, either used alone or in combination on cell size was also investigated in the present thesis as indicated by cell area. The average cell area of the treated cells with ceftazidime/α-mangostin combination was significantly larger than untreated controls and cells treated with either agent alone ($P < 0.01$). The significant difference (higher) of cell area was also observed in cells treated with α-mangostin alone when compared to untreated control and ceftazidime alone (Figure 4.9).

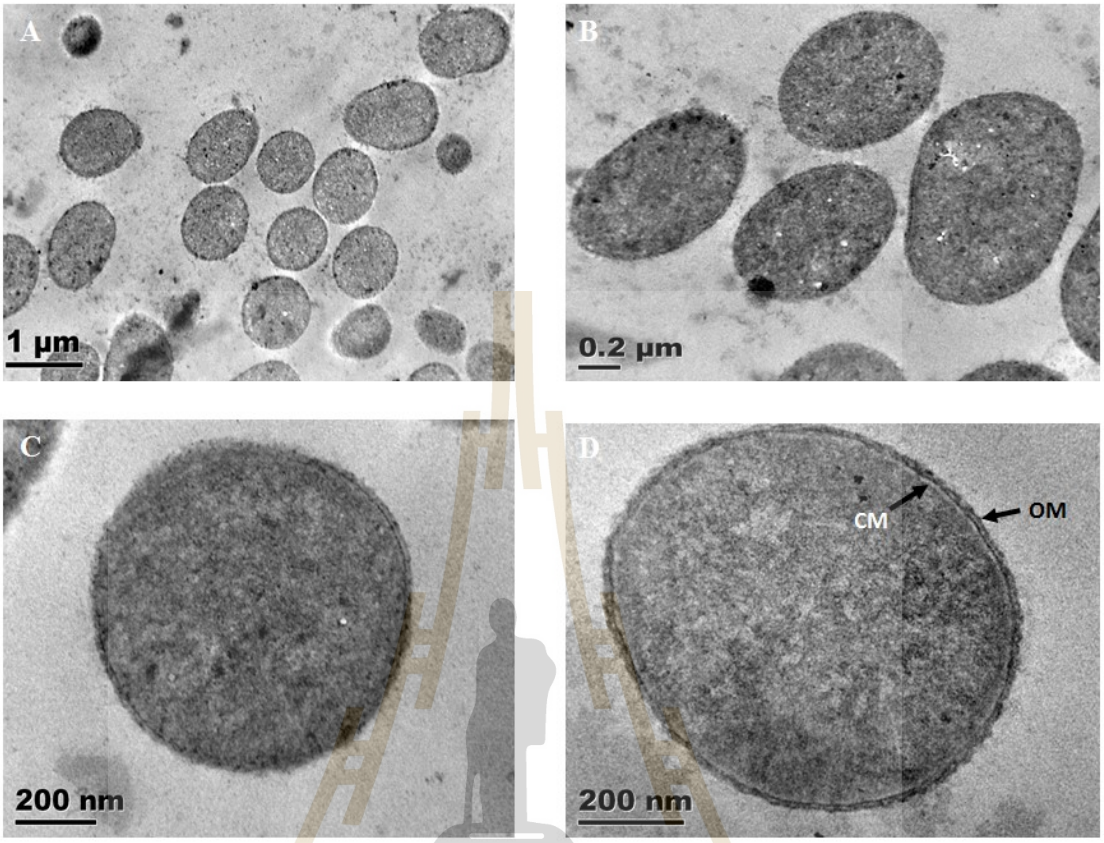


Figure 4.5 TEM micrographs of *A. baumannii* DMST 45378 grown in the Mueller-Hinton broth in the absence of the antimicrobial agent. A) bar = 1 µm and magnification 9,000x; B) bar = 0.2 µm and magnification 12,000x; C) bar = 200 nm and magnification 35,000x; D) bar = 100 nm and magnification 38,000x; CM = cytoplasmic membrane; OM = outer membrane.

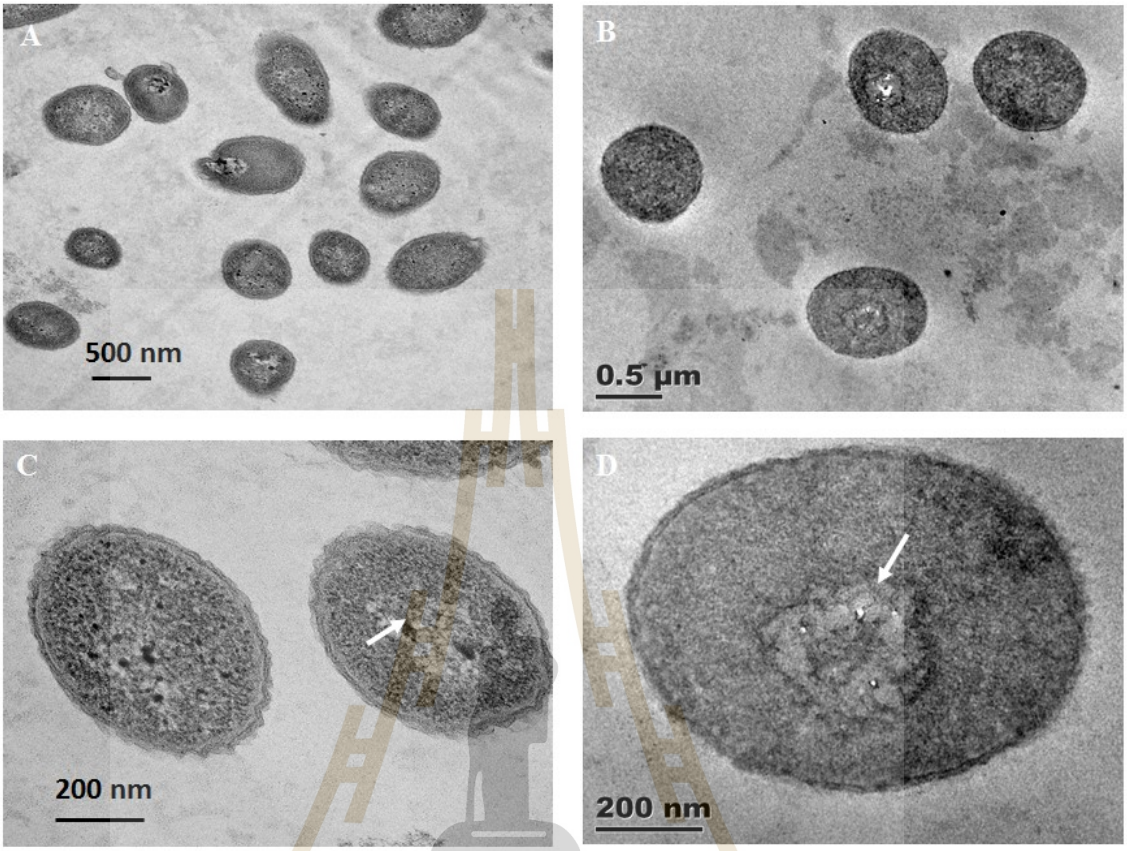


Figure 4.6 TEM micrographs of *A. baumannii* DMST 45378 grown in the presence of ceftazidime at 400 µg/mL. A) bar = 500 nm and magnification 9,000x; B) bar = 0.5 µm and magnification 19,000x; C) bar = 200 nm and magnification 34,000x; D) bar = 200 nm and magnification 38,000x. The white arrows indicate the change of cellular contents.

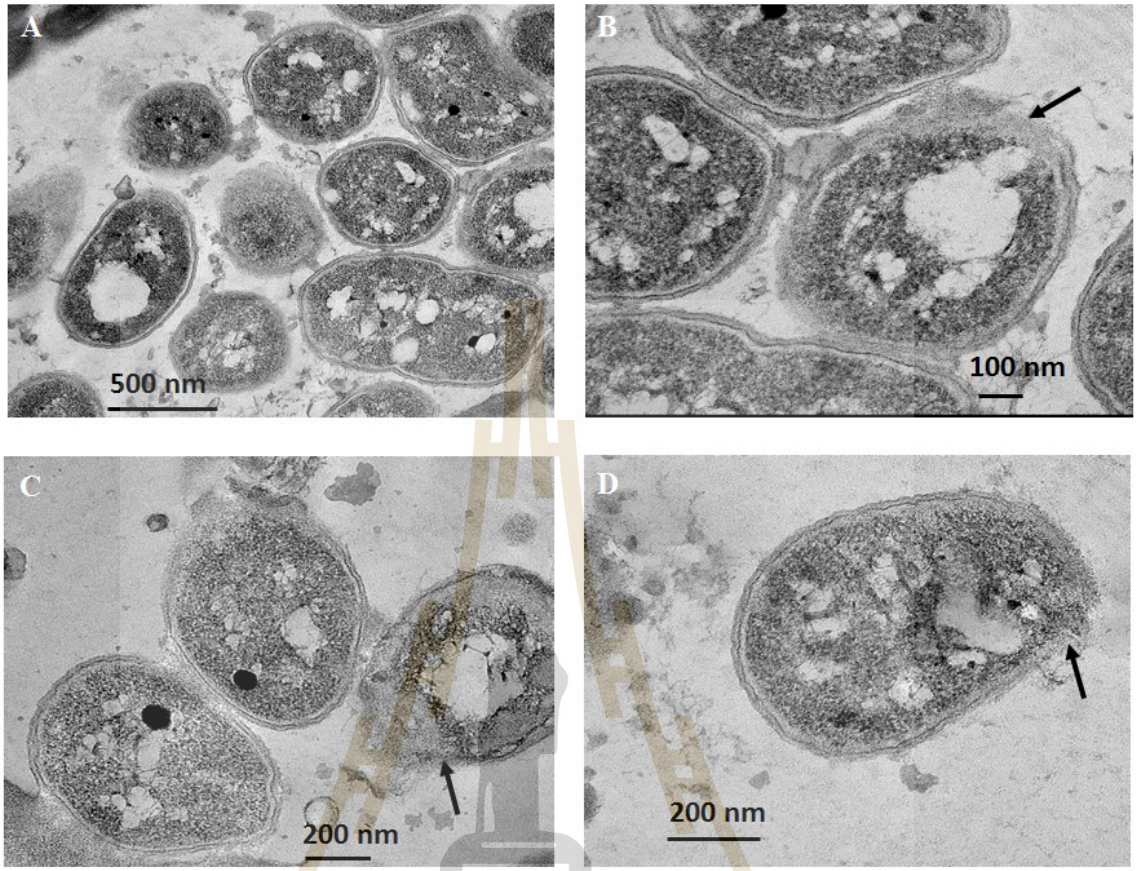


Figure 4.7 TEM micrographs of *A. baumannii* DMST 45378 grown in the presence α-mangostin at 1,024 µg/mL. A) bar = 500 nm and magnification 17,000x; B) bar = 500 nm µm and magnification 38,000x; C) bar = 200 nm and magnification 26,000x; D) bar = 200 nm and magnification 36,000x. The black arrows indicate the sites of damage.

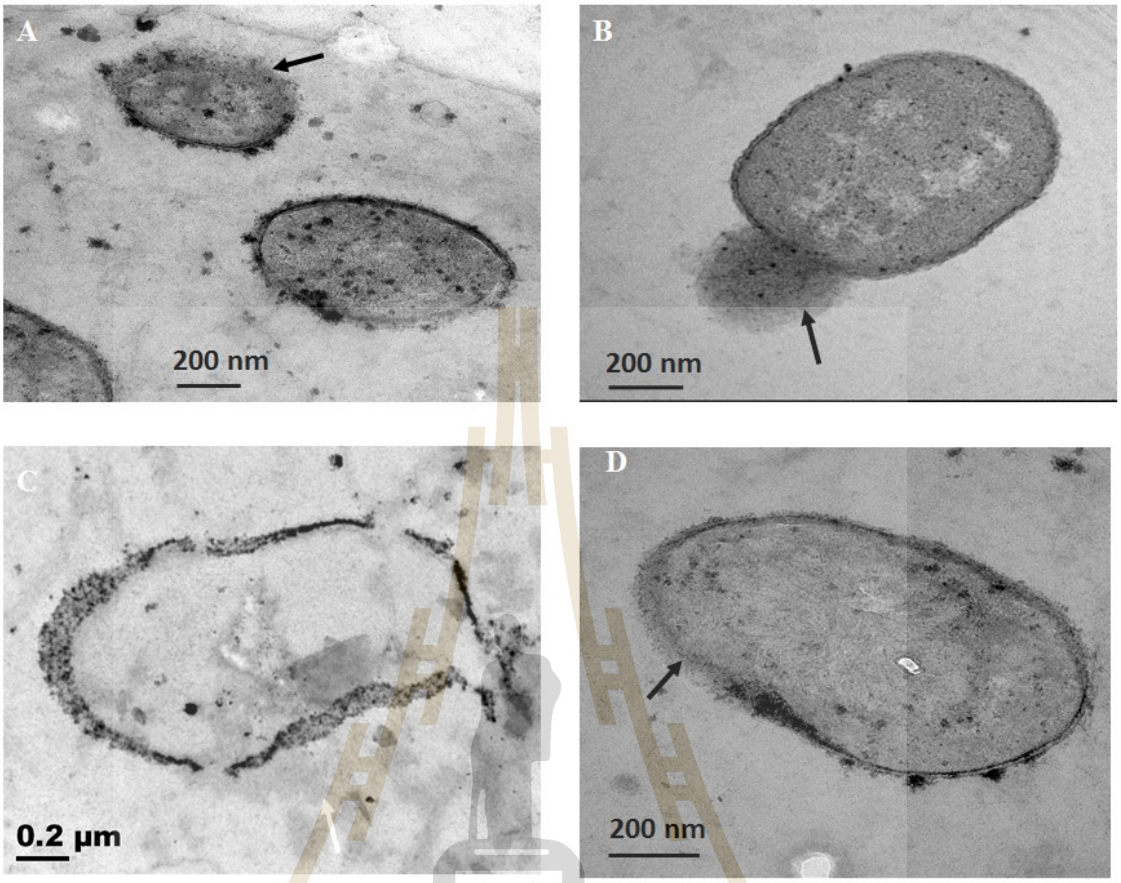


Figure 4.8 TEM micrographs of *A. baumannii* DMST 45378 grown in the presence α-mangostin at 150 µg/mL+ ceftazidime at 150 µg/mL. A) bar = 200 nm and magnification 26,000x; B) bar = 200 nm µm and magnification 19,500x; C) bar = 0.2 µm and magnification 20,000x; D) bar = 200 nm and magnification 26,000x. The black arrows indicate the sites of damage. The white arrows indicate the cell debris.

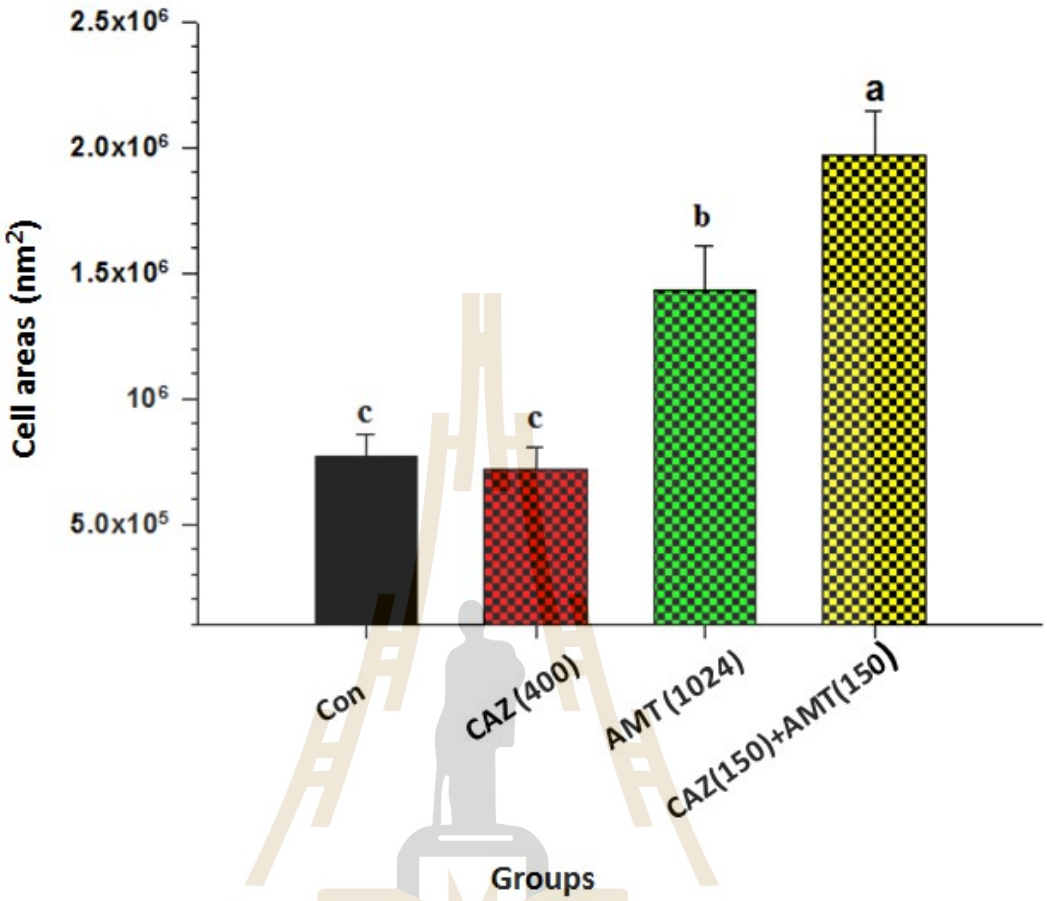


Figure 4.9 The cell area of *A. baumannii* DMST 45378 after exposure to α-mangostin, ceftazidime either alone or in combination. Con = control (drug free); CAZ (400) = ceftazidime at 400 µg/mL; AMT (1024) = α-mangostin at 1,024 µg/mL; AMT (150) + CAZ (150) = α-mangostin at 150 µg/mL plus ceftazidime at 150 µg/mL. Data are the mean of three replicated ± standard deviation (SD). The different superscript alphabet represents a statistically significant difference using one-way ANOVA with Tukey's HSD ($P < 0.01$).

4.1.6 Effects of the treatments on cytoplasmic membrane (CM) permeabilization

The effect of 400 µg/mL ceftazidime, 1,024 µg/mL α-mangostin and the combination of 150 µg/mL ceftazidime plus 150 µg/mL α-mangostin on the alteration of CM membrane permeability of *A. baumannii* DMST 45378 was determined by measuring the release of OD_{260}-absorbing materials. Nisin at a concentration of 128 µg/mL was used as a positive control. The results of this study are presented in Figure 4.10. After *A. baumannii* cells were treated with ceftazidime alone, no marked change in the amount of OD_{260}-absorbing materials compared with untreated control throughout 4 h of treatment. In an α-mangostin-treated group, a slight increase in optical density was observed. This treated group exhibited significantly higher in optical density than untreated and ceftazidime-treated groups, but lower than the nisin-treated group and combined group ($P < 0.01$). Interestingly, the combination of α-mangostin plus ceftazidime displayed the highest potent activity in inducing CM permeability of *A. baumannii* compared with nisin, α-mangostin, ceftazidime alone and untreated control.

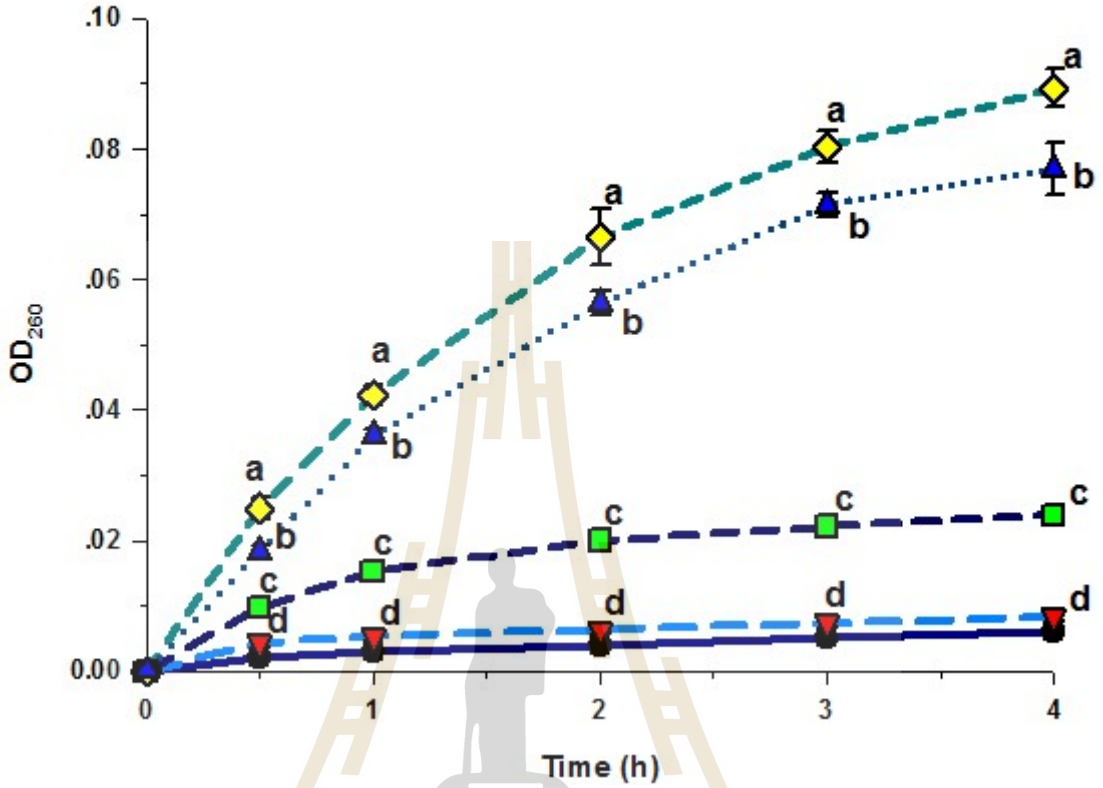


Figure 4.10 The presence of 260 nm absorbing the material in the supernatants of *A. baumannii* DMST 45378 after exposure to α-mangostin, ceftazidime either alone or in combination. Untreated control; 400 µg/mL CAZ; 1024 µg/mL AMT; 150 µg/mL CAZ and 150 µg/mL AMT; 128 µg/mL nisin (positive control). Data are the mean of three replicated ± standard deviation (SD). Means with the different superscript at the same time point are significantly different (Tukey's HSD, $P < 0.01$).

4.1.7 Effects of the treatments on outer membrane (OM) permeabilization

Nitrocefin, a substrate for β-lactamases (enzymes generally localized in the periplasmic space of Gram-negative bacteria), was used to investigate the effect of 400 μg/mL ceftazidime, 1,024 μg/mL α-mangostin and the combination of 150 μg/mL ceftazidime plus 150 μg/mL α-mangostin on the alteration of the OM membrane permeability of *A. baumannii* DMST 45378. Polymixin B (10 μg/mL) was used as a positive control. The results of this investigation are illustrated in Figure 4.11. Untreated control and the ceftazidime-treated group showed no substantial change in OM permeabilization. Whereas, α-mangostin-treated group significantly altered OM permeabilization, but showed a significantly lower effect than the combined-treated group and polymyxin B treated group ($P < 0.01$). The combination of α-mangostin and ceftazidime also significantly increased OM permeabilization compared with individual components and the untreated control ($P < 0.01$). Furthermore, polymixin B demonstrated the highest activity in permeabilizing OM of *A. baumannii* DMST 45378 compared to other groups.

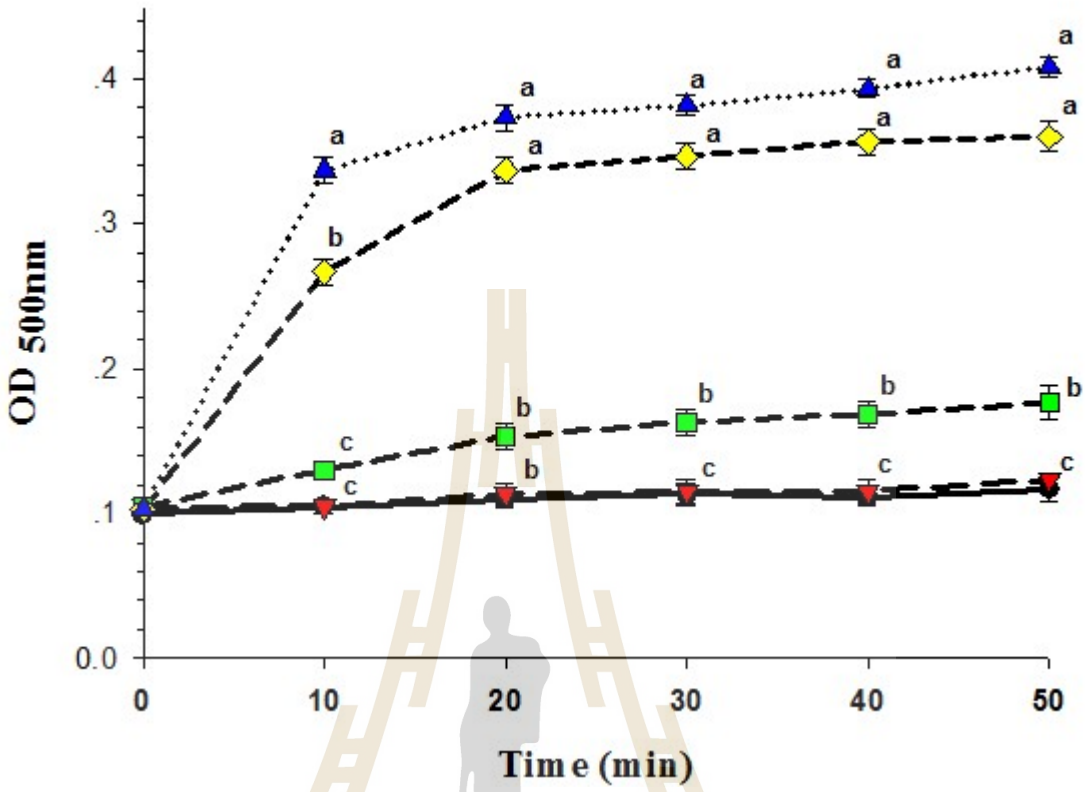


Figure 4.11 Outer membrane permeability of ceftazidime-resistant *A. baumannii* DMST 45378 after exposure to α-mangostin, ceftazidime either alone or in combination. ● untreated control; ▼ 400 µg/mL ceftazidime; ■ 1024 µg/mL α-mangostin; ◆ 150 µg/mL ceftazidime and 150 µg/mL α-mangostin; ▲ 10 µg/mL polymixin B (positive control). Data are the mean of three replicates ± standard deviation (SD). The mean sharing the same superscript are not significantly different (Tukey's HSD test, $P < 0.01$).

4.1.8 Outer membrane and peptidoglycan-associated protein (OMPG)

The effect of the α-mangostin alone or in combination with ceftazidime on the OMPG of ceftazidime-resistant *A. baumannii* DMST 45378 is shown in Figure 4.12. The combination of α-mangostin and ceftazidime reduced the size and intensity of OMPG bands at 22, 25 and 30 kDa compared to controls. The effect of the α-mangostin alone or in combination with ceftazidime on the adjusted relative density of ceftazidime-resistant *A. baumannii* DMST 45378 -OMPG is shown in Figure 4.13. The combination of α-mangostin and ceftazidime exhibited significantly lower adjusted relative density than other groups in all OMPG bands ($P < 0.01$), whereas the adjusted relative density OMPG of ceftazidime band displayed significantly higher than others ($P < 0.01$). These findings suggest that the combination displayed outer membrane and peptidoglycan damage resulting in the loss of OMPG.

4.1.9 Inhibitory activity of α-mangostin on β-lactamase

The enzyme assay was used to determine the inhibited activity of β-lactamase by α-mangostin. The remaining of benzylpenicillin from HPLC indicated the inhibitory activity of α-mangostin to inhibit β-lactamase. The capability of α-mangostin to inhibit the activity of β-lactamase enzyme type IV isolated from *E. cloacae* is shown in Figure 4.14. The higher benzylpenicillin remainder means, the lesser β-lactamase activity. The results demonstrated that α-mangostin displayed β-lactamase inhibitory activity in a concentration dependent manner.

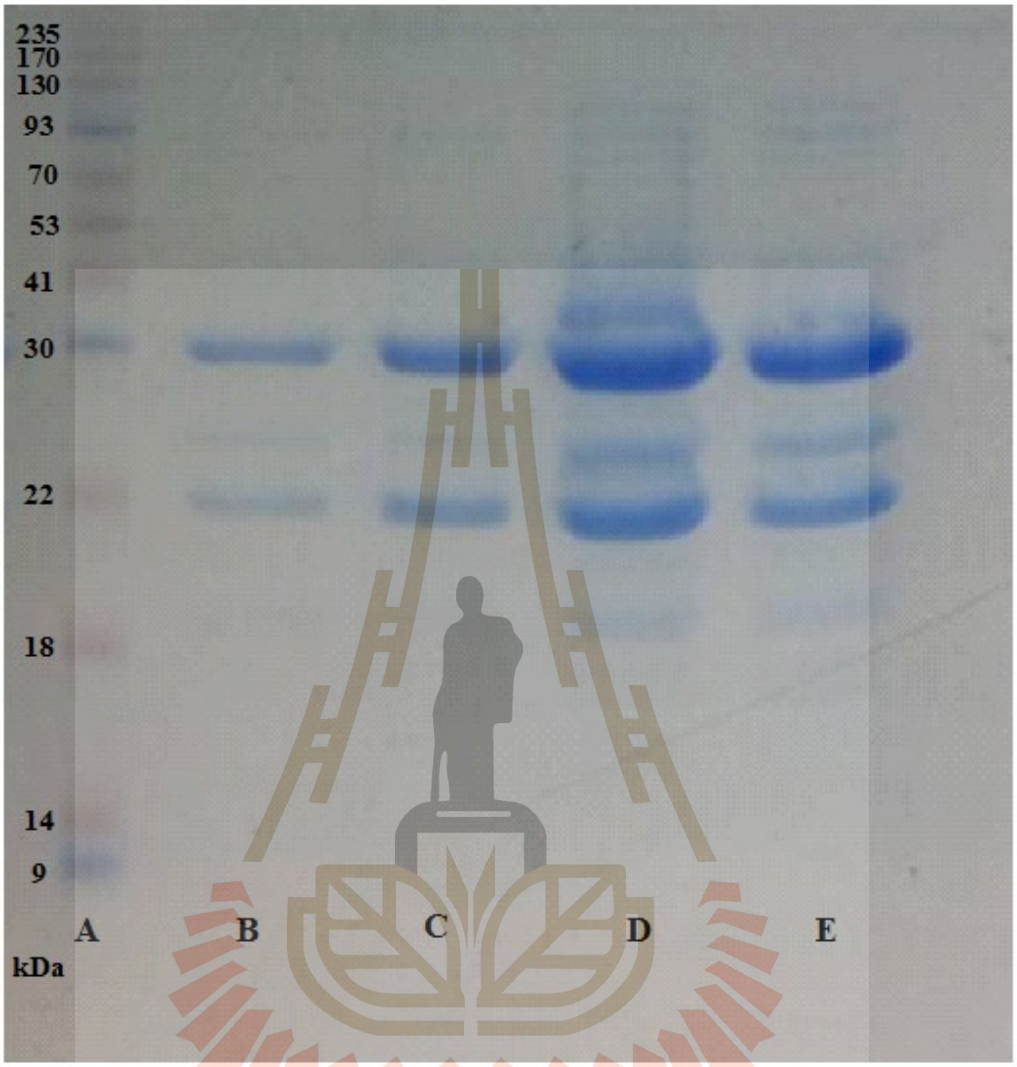


Figure 4.12 SDS-PAGE showing the OMPG of *A. baumannii* DMST 45378 after exposure to α-mangostin, ceftazidime either alone or in combination. (A): MW marker (kDa); (B): 150 µg/mL ceftazidime and 150 µg/mL α-mangostin; (C): 1024 µg/mL α-mangostin; (D): 400 µg/mL ceftazidime; (E): untreated control.

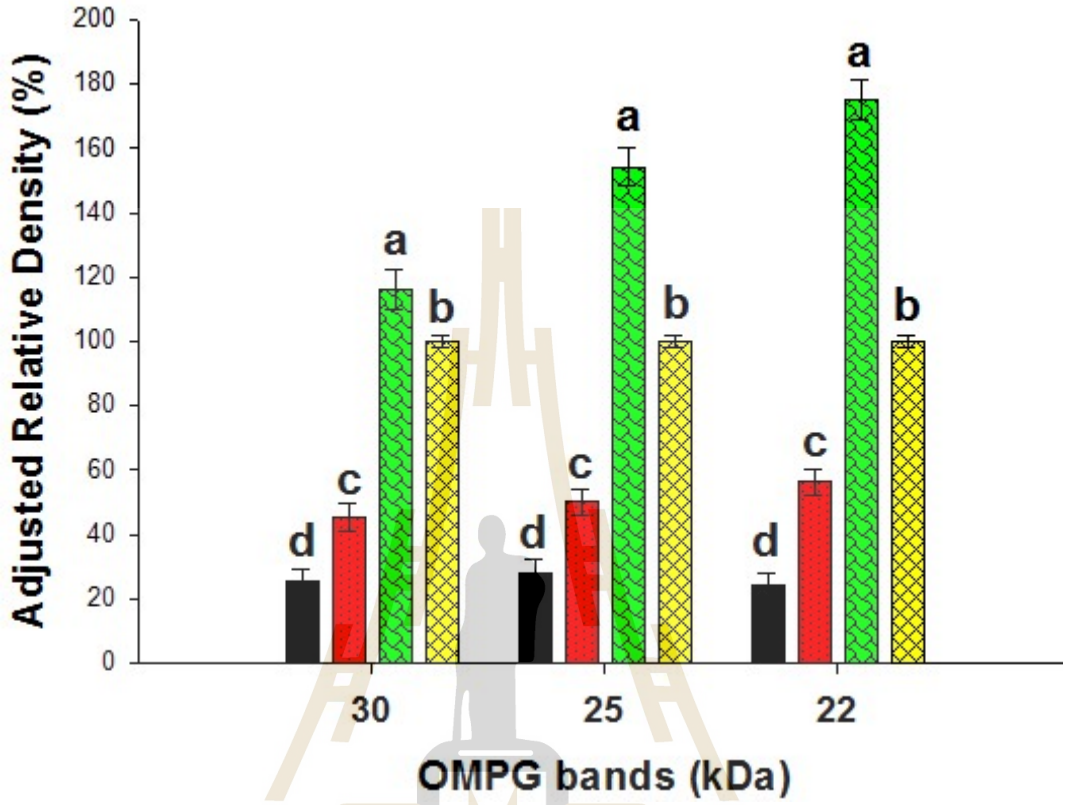


Figure 4.13 The adjusted relative density (% of control) obtained from quantitative densitometry of SDS-PAGE of each *A. baumannii* DMST 45378 OMPG band. 150 µg/mL ceftazidime and 150 µg/mL α-mangostin; 1024 µg/mL α-mangostin; 400 µg/mL ceftazidime; untreated control. Data are the mean of three replicates ± standard deviation (SD). Means with the same superscript are not significantly different from each other (Tukey's HSD test, $P < 0.01$).

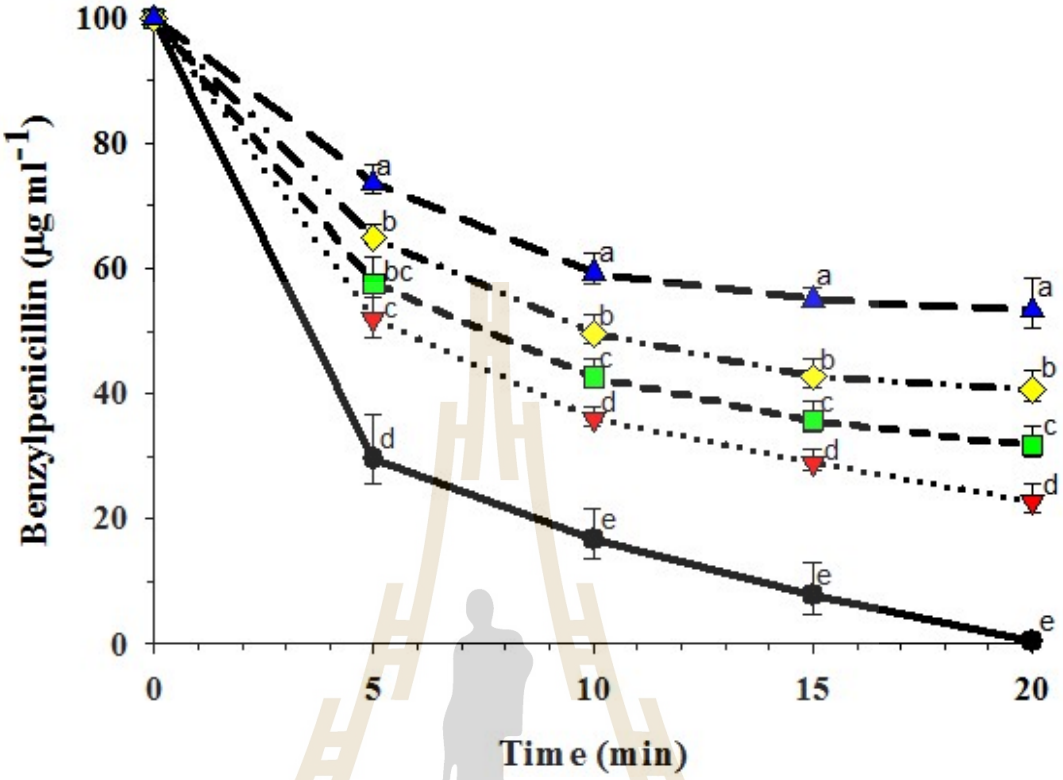


Figure 4.14 The inhibitory activity of α-mangostin against β-lactamase hydrolysis of benzylpenicillin. ● untreated control; ▼ 25 µg/mL α-mangostin; ■ 50 µg/mL α-mangostin; ◆ 100 µg/mL α-mangostin; ▲ 150 µg/mL α-mangostin. Data are the mean of three replicates ± standard deviation (SD). Means with the different superscript at the same time point are significantly different (Tukey's HSD, $P < 0.01$).

4.2 *In vitro* activity of the combination of bacteriophages and antimicrobial plant extracts

4.2.1 MIC/MBC of extracts

The antibacterial activity of plant extracts against *E. coli* strains ECOR-16 and ECOR-27 is presented in Table 4.3. The MIC values of SSE, BRE and OIE against ECOR-16 and ECOR-27 were 4 mg/mL, 16 mg/mL and 16 mg/mL, while MBC of these particular extracts were 16 mg/mL, 32 mg/mL and 32 mg/mL, respectively. When the bulk extracts were tested for antiviral properties (Figure 4.15), SSE exhibited toxicity against SU16 and SU27 at 100% MIC concentration. The non-significant reduction of phage concentration was observed at 10% MIC concentration. Following 24 h of exposure to the OIE and BRE, the result was showed no toxic effect on bacteriophage SU27 at 100% MIC concentration as indicated by no significant reduction in plaque forming unit. Whereas, the MIC concentration of these extracted obviously inhibited the growth of bacteriophage SU16. Non-toxicity concentrations of OIE and BRE toward bacteriophage SU16 was found at 0.1% MIC and 0.01% MIC, respectively.

Table 4.4 MIC and MBC of plant extracts (mg/mL) used in the current investigation of bacterial host strains.

Compound/Extract	Bacterial Strain			
	ECOR-16		ECOR-27	
	MIC	MBC	MIC	MBC
BRE	16 ± 0	32 ± 0	16 ± 0	32 ± 0
OIE	13.33 ± 4.62	42.67 ± 18.48	13.33 ± 4.62	42.67 ± 18.48
SSE	4 ± 0	16 ± 0	4 ± 0	16 ± 0

BRE = *Boesenbergia rotunda* extract; OIE = *Oroxylum indicum* extract; SSE = *Stephania suberosa* extract. Data are the mean of three replicates ±SD.

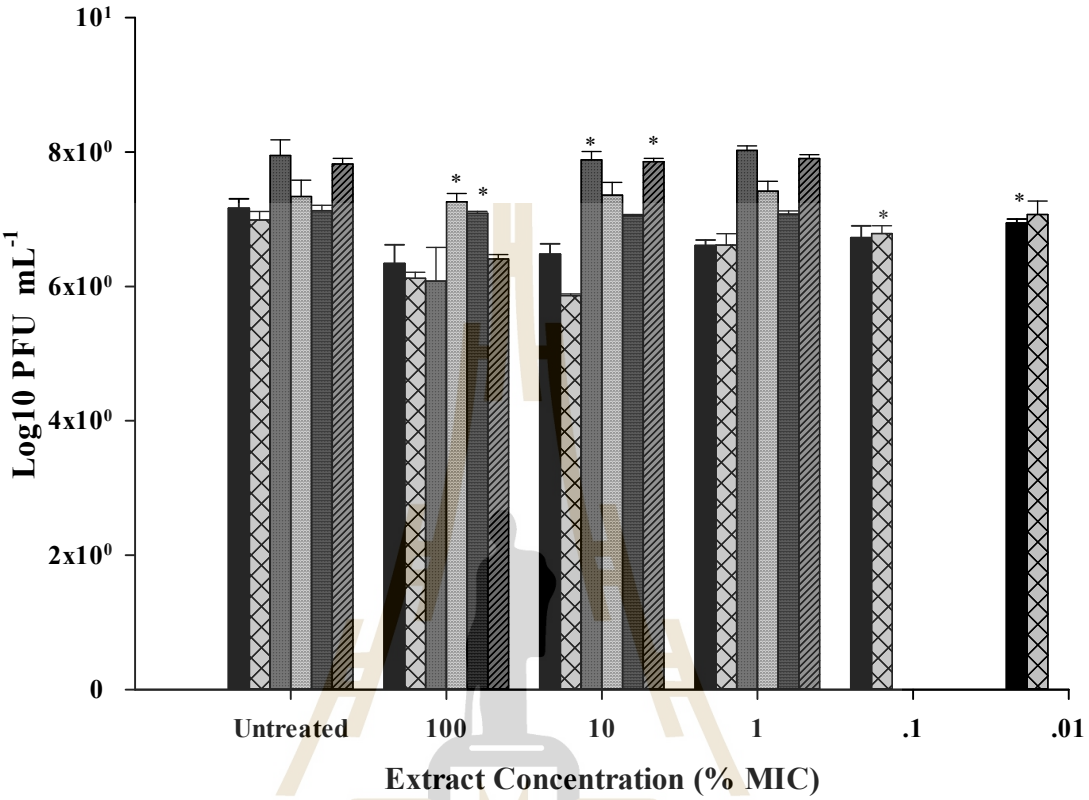


Figure 4.15 Antiviral activity of plant extracts against bacteriophages SU16 and SU27 after 24 h exposure. SU16 + BRE; SU16 + OIE; SU16 + SSE; SU27 + BRE; SU27 + OIE; SU27 + SSE. Data are the mean of 3 replicates ± standard deviation (SD). The MIC of extract concentrations were 16 mg/mL for BRE/OIE and 4mg/mL for SSE against both bacterial host strains. * indicates non-significant difference to the untreated control ($P > 0.05$).

4.2.2 Combined activity of bacteriophages and bulk plant extracts

Figure 4.16 Illustrates the kinetic inhibition of after exposure to bacteriophage (SU16 or SU27), either separately or in combination with non-toxicity concentration of BRE against corresponding ECOR-16 and ECOR-27. The result showed a substantial reduction in the viable bacterial count of cells treated with phage alone and in combination with the plant extracts during 2 h of incubation compared with untreated control and BRE alone. Then, regrowth of these treated cells was seen, reaching the level comparable with that of the untreated control at 24 of exposure. The combined group showed a more inhibitory effect than phage SU27 alone. However, there was no markedly different in the viability of ECOR-16 after treatment with phage SU16 alone and the combined phage SU16 plus BRE (Figure 4.16A).

Likewise, phage SU16 and SU27 alone and in combination with OIE dramatically inhibited the growth of ECOR-16 (Figure 4.17A) and ECOR-27 (Figure 4.17B). After 2 h of treatment, these bacteria regrew reaching similar viabilities of cells treated with OIE alone and untreated control cells. Interestingly, the combination of phage SU27 plus OIE noticeably exhibited higher antibacterial activity than phage-treated separately in inhibition of ECOR-27. Furthermore, phage SU16 and SU27 alone and in combination with SSE were also found to substantially reduce the bacterial content during 2 h. While no reduction in viable counts of ECOR-16 (Figure 4.18A) and ECOR-27 (Figure 4.18B) was observed in SSE treated alone and untreated control.

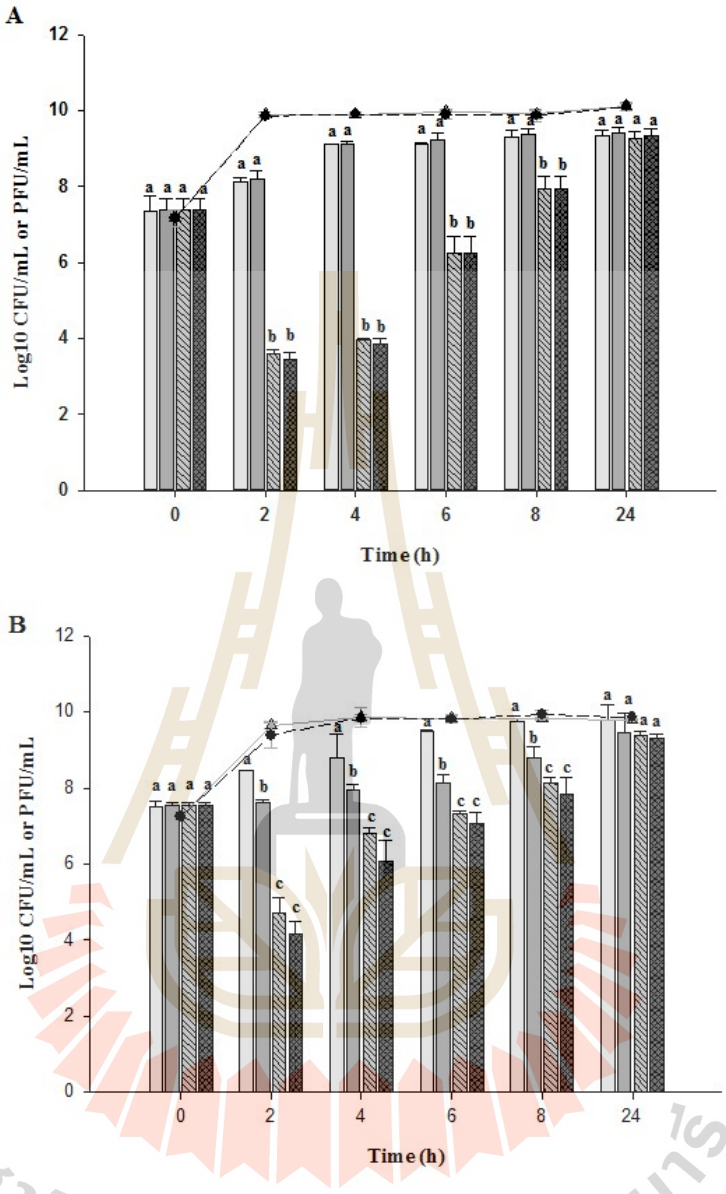


Figure 4.16 Activity of bacteriophages SU16 and SU27 in combination with BRE. (A) SU16; (B) SU27 Untreated control; Extract only; Phage only; Extract and phage. Data are the mean of three replicates ± standard deviation (SD). Means sharing the same superscript at the same time are not significantly different from each other (Tukey's HSD test, $P < 0.05$). Lines indicate phage counts. Phage only; Extract and phage.

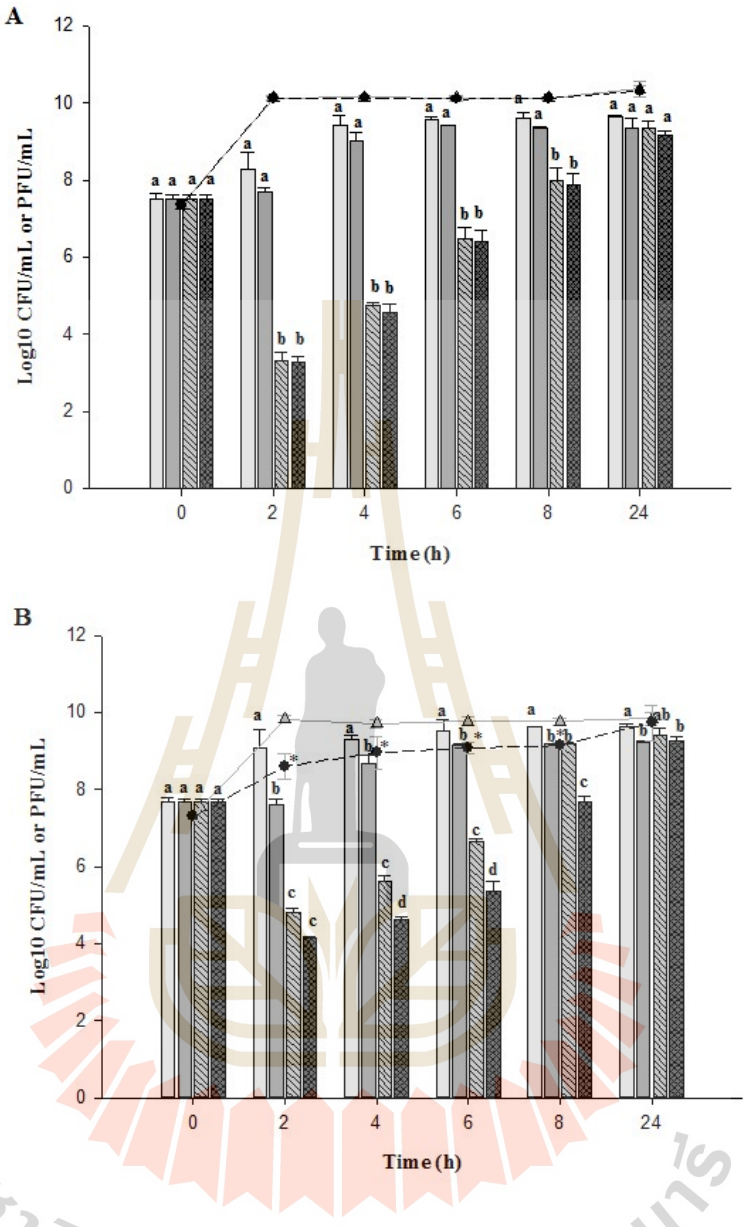


Figure 4.17 Activity of bacteriophages SU16 and SU27 in combination with OIE. (A) SU16; (B) SU27 Untreated control; Extract only; Phage only; Extract and phage. Data are the mean of three replicates ± standard deviation (SD). Means sharing the same superscript at the same time are not significantly different from each other (Tukey's HSD test, $P < 0.05$). Lines indicate phage counts. Phage only; Extract and phage. * indicates significant difference to the phage at the same time ($P > 0.05$).

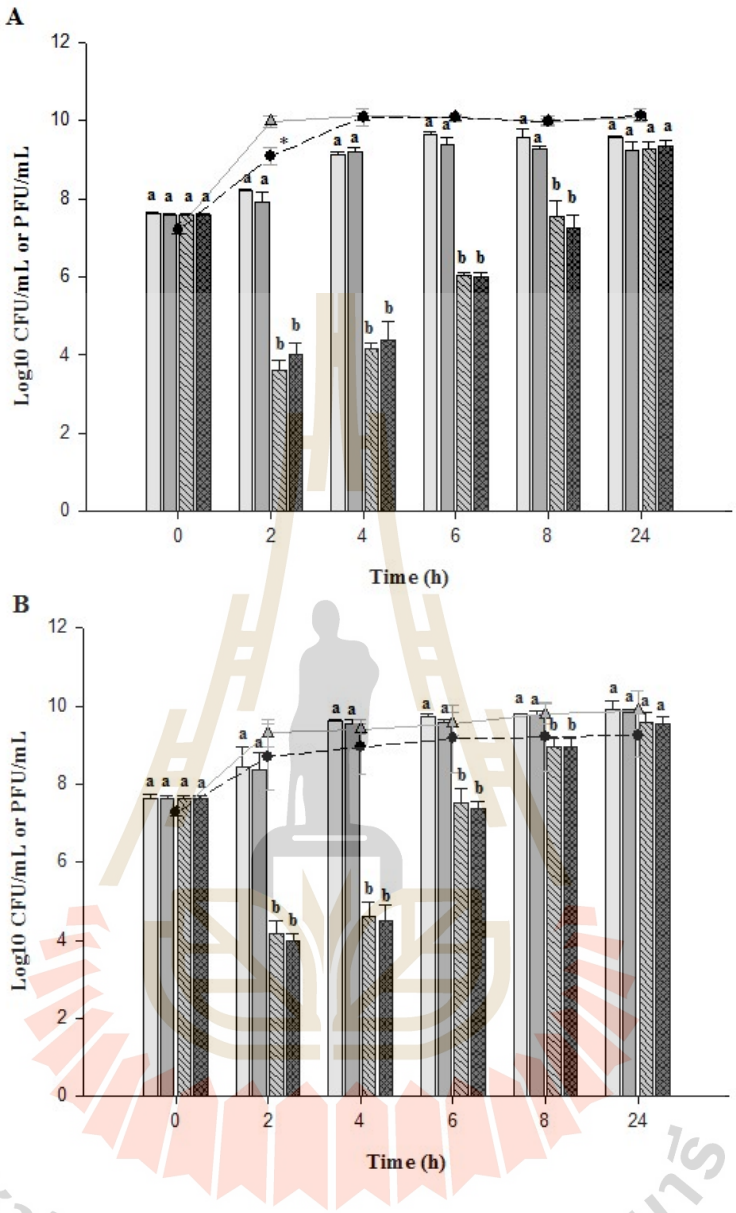


Figure 4.18 Activity of bacteriophages SU16 and SU27 in combination with SSE. (A) SU16; (B) SU27 Untreated control; Extract only; Phage only; Extract and phage. Data are the mean of three replicates ± standard deviation (SD). Means sharing the same superscript at the same time are not significantly different from each other (Tukey's HSD test, $P < 0.05$). Lines indicate phage counts. Phage only; Extract and phage. * indicates significant difference to the phage at the same time ($P > 0.05$).

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Discussion

The emergence of resistance to conventional antibiotics in microorganisms is becoming a critical global healthcare problem in all parts of the world. The treatment of bacterial infections, in particular, antibiotic-resistant bacteria has become a challenging issue. This motivates researchers to develop new antibacterial agents and new strategies to combat bacterial infections. Many herbal plant extracts have been extensively used for the treatment of the infections caused by pathogenic bacteria for centuries (Savoia, 2012; Srivastava et al., 2014). However, some of them have relatively low antibacterial activities. To potentiate their antibacterial action, the combination of phytochemical/phytochemical or phytochemical/antibiotic is recommended (Eumkeb and Chukrathok, 2013; Hemaiswarya et al., 2008; Wagner, 2011). Recently, several researchers have proposed the combination of bacteriophage/antibiotic for an alternative treatment of bacterial infections, especially for treating recalcitrant bacteria. There is a paucity of evidence regarding bacteriophage/phytochemical combination against bacterial infections (Coulter et al., 2014; Jo et al., 2016; Knezevic et al., 2013; Torres-Barceló et al., 2016). The presented thesis, therefore, investigated the antibacterial activity and synergistic effect of α-mangostin and antibiotic combinations against antibiotic-resistant bacteria. The combined antibacterial activity of three herbal plant extracts (BRE, SSE, and OIE)

with bacteriophage SU16 and SU27 against *E. coli* isolates were also evaluated in the current thesis.

5.1.1 Antibacterial and synergistic activity of α-mangostin and antibiotic combination

The antimicrobial properties of α-mangostin have been studied extensively. α-Mangostin can inhibit the growth of various pathogenic bacteria including MRSA, and vancomycin-resistant Enterococci (VRE) (Chomnawang et al., 2009; Dharmaratne et al., 2013; Sakagami et al., 2005). It has been reported that α-mangostin rapidly disrupts the integrity of the cytoplasmic membrane of Gram-positive bacteria including MRSA (Koh et al., 2013). Moreover, α-mangostin can attenuate the development of *Streptococcus mutans* biofilms (Nguyen et al., 2014). However, there is a scarce study regarding the mechanism of action of α-mangostin either used alone or in combination with practically-used antibiotics against Gram-negative bacteria. Hence, the antimicrobial activity of α-mangostin against a wide range of bacterial pathogens alone and in combination with cell wall inhibitor antibiotics was investigated in the present thesis. This study also examined some elementary mechanism of action using scanning electron microscope (SEM), transmission electron microscopy (TEM), cytoplasmic membrane (CM) permeability, outer membrane (OM) permeability, outer membrane peptidoglycan-associated protein (OMPG) and β-lactamase activity.

The MIC results indicated that α-mangostin exhibited antibacterial activity against the all tested *E. feacium* strains at low MIC values. In the Gram-negative isolates, α-mangostin inhibited their growth at the high-MIC value of > 2048 μg/mL. *E. feacium* DMST 12829 and *E. feacium* DMST 12852 were resistant to ampicillin,

according to CLSI guidelines with MICs exceeding ≥ 32μg/ mL (Clinical and Laboratory Standards Institute, 2014). Clinical isolates of *A. baumannii* and *P. aeruginosa* were highly resistant to ceftazidime and ceftriaxone, but *P. aeruginosa* DMST 37166 was still susceptible to these antibiotics. Ampicillin, ceftazidime, and ceftriaxone are β-lactam antibiotics that inhibit cell wall synthesis through binding and inhibition of penicillin-binding proteins (PBP_s) (Hayes and Orr, 1983; Liao and Hancock, 1997). Resistance to these antibiotics are predominantly involved three broad mechanisms: (1) antimicrobial-inactivating enzymes (in particular β-lactamases, e.g., extended-spectrum-β-lactamase, AmpC β-lactamase, and carbapenemases), (2) reduced access to bacterial targets (decreased porin and outer membrane permeability) and (3) altered and modified drug target and cellular function (Garnacho-Montero and Amaya-Villar, 2010; Gordon and Wareham, 2010; Rice, 2006). The antibacterial activity of a bioactive compound isolated from the pericarp of *Garcinia mangostana* has been previously reported to be against various clinical isolates of bacteria, especially those of Gram-positive bacteria including MRSA, *Staphylococcus epidermidis* and *Propionibacterium acnes* (Chomnawang et al., 2005; Iinuma et al., 1996). Moreover, α-mangostin has cytoplasmic membrane disruption property, increasing their permeability and leading to release of intracellular components resulting in cell lysis and death (Koh et al., 2013).

Due to the paucity of study on the interaction between α-mangostin and conventional antibiotics against antibiotic-resistant bacteria, this study aimed to investigate its interaction. A marked reduction in MIC value in both α-mangostin and ceftazidime or ceftriaxone was observed when they were used in combination against *A. baumannii* isolates. The combination of α-mangostin plus ceftazidime and

ceftriaxone synergistically inhibited the growth of all tested *A. baumannii* strains at FIC index of < 0.37 and < 0.24, respectively. The presented thesis is the first study reporting the synergistic interaction between α-mangostin and ceftazidime or ceftriaxone against recalcitrant *A. baumanni* isolates. For *E. faecium* strains, the partial synergistic interaction between α-mangostin and ampicillin was observed against these bacteria. A previous study showed that α-mangostin isolated from the pericarp of *Garcinia mangostana* extract in combination with gentamicin (GM) and vancomycin hydrochloride (VCM) exhibited a synergistic effect against VRE and MRSA, respectively (Sakagami et al., 2005). Recently, it has been reported that α-mangostin isolated from *G. mangostana* inhibited the growth of oxacillin-resistant *Staphylococcus saprophyticus*. It also exhibited synergistic interaction with oxacillin against this particular refractory isolate (Phitaktim et al., 2016).

The result of time-kill kinetic assay confirmed that the combination of α-mangostin and ceftazidime synergistically inhibited the growth of *A. baumannii* DMST 45378. The finding from time-kill assay also suggested that α-mangostin in combination with ceftazidime showed bactericidal activity by reducing cell count of *A. baumannii* DMST 45378 higher than 3-log10 CFU/mL. It is suggested that the activity of this combination is increased because they have a different mode of action and act at the distinct target site. Furthermore, time-kill assay exhibited that no synergistic activity was observed in the combination of α-mangostin plus ampicillin against *E. faecium* DMST 12829. There are no interpretative criteria for partial synergism using time-kill assay. However, the result of checkerboard assay demonstrated that this combination showed partial synergistic interaction.

To understand the mechanism of action, scanning electron microscopy (SEM) has demonstrated that *A. baumannii* treated with ceftazidime had long cell shape and absence of bacterial septum. This is consistent with earlier findings suggesting that the presence of subinhibitory concentrations of β-lactam antibiotics causes elongation, filamentation, and absence of septation (Zhanel et al., 1992; Knezevic et al., 2013). In cells treated with α-mangostin, irregular cell wall structure was observed. These results may be explained that α-mangostin targets the bacterial membrane by penetrating into lipid bilayer resulting in membrane breakdown (Koh et al., 2013). *A. baumannii* DMST 45378 treated with α-mangostin plus ceftazidime showed damage to the cell surface envelope, such as membrane hole, deep craters, and cell debris. In according to the TEM results, the results showed that ceftazidime plus α-mangostin combination could damage and disrupt the cellular membrane and peptidoglycan layer. When compared to untreated controls, the overall of these treated cells increased significantly. These findings suggest that the mechanism of action of the combination α-mangostin plus ceftazidime is due to the destruction of cell membrane together with damage to the cell wall. These observations seem to correspond with previous finding reporting that the combination of isolated α-mangostin and oxacillin exhibited peptidoglycan and cytoplasmic membrane damage, irregular cell shapes of oxacillin-resistant *Staphylococcus saprophyticus* and average cell areas became significantly larger than those of the controls (Phitaktim et al., 2016).

CM and OM permeability studies exhibited a significant enhancement in the absorbance values of OD_{260}-absorbing material, and β-lactamase inhibitory activity was observed in *A. baumannii* treated with α -mangostin plus ceftazidime. This finding suggests that this combination interacts with CM and OM of *A. baumannii*

resulting in leakage of intracellular constituents. α-Mangostin contains isoprenyl group which can penetrate into the phospholipid lipid bilayer. This penetration of the cell membrane coupled with the action of β-lactam on the transpeptidation of the cell membrane leading to an enhanced antibacterial effect of the combination. This result is in substantial correspondence with a previous investigation that luteolin and amoxicillin altered both the outer and inner membrane of amoxicillin-resistant *E. coli* (Eumkeb et al., 2012). Moreover, decreases in the intensity of membrane proteins is another hallmark of the membrane damage and loss of membrane permeability. The intensity of some protein bands decreased or disappeared after treated with α-Mangostin plus ceftazidime. The expression of bacterial OMPG may be modified by combination treatment. This might be explained by the induction of stress proteins in the bacterial cells or by changed cell functions manifesting as a different OMPG expression. The protein profile revealed OM and peptidoglycan damage resulting in the loss of OMPG. This finding is in corresponding with TEM observations made.

β-lactamase inhibition assay revealed that α -mangostin significantly prevented β-lactamase to hydrolyze benzylpenicillin levels in a concentration-dependent manner compared to the untreated control. These findings are consistent with those of Phitaktim et al. who reported that α -mangostin isolated from *G. mangostana* inhibited β-lactamase activity in a dose-dependent manner (Phitaktim et al., 2016). This result may enhance the rapid killing by a membrane-targeting antimicrobial, and that β-lactamase inhibition could be a secondary effect of the membrane disruption (Koh et al., 2013).

The synergistic paradigm of combining the conventional antibiotic with phytochemical compounds has been shown to be an effective avenue to treat

infectious diseases caused by drug-resistant bacteria (Eumkeb and Chukrathok, 2013; Wagner and Ulrich-Merzenich, 2009). Simulations of the molecular dynamics of this synergy revealed that the isoprenyl groups of α-mangostin, occupying the chromone structure, play a major role in penetrating the lipid bilayer of the MRSA membrane, which results in increased permeability and breakdown of the membrane and (Koh et al., 2013). While not conclusive, these findings could support an assumption that the isoprenyl groups and the chromone structure of α-mangostin play a significant role in inhibiting *A. baumannii* strains through direct interactions with the bacterial membrane (Koh et al., 2013). It has been previously reported that AMT provided a sufficient margin of safety for therapeutic use (Kaomongkolgit et al., 2009; Phitaktim et al., 2016) and would suggest that the AMT could potentially be a useful adjuvant to ceftazidime or ceftriaxone for the treatment of the infection caused by ceftazidime- and ceftriaxone-resistant *A. baumannii*.

5.1.2 Combined antibacterial activity of plant extracts (BRE, SSE, and OIE) and bacteriophages

Since phages were discovered in 1915 and 1917, they have increasingly gained attention to be developed as a novel agent for the treatment of bacterial infections in human (O'Flaherty et al., 2009; Sulakvelidze et al., 2001). Phages can destroy a variety of pathogenic bacteria, including *P. aeruginosa* and *S. aureus* (Kutter et al., 2010). Their abilities in prevention and disruption of bacterial biofilm have also been reported (Harper et al., 2014; Meng et al., 2011). Due to increasing emergence of antibiotic-resistant bacteria, a combination therapy of two or more practically-used antibiotics is of promising solutions to conquer the infections caused by refractory bacteria (Knezevic et al., 2013). Both phages and herbal plant extracts

are amongst those being studied either as monotherapies (Bonifácio et al., 2014; Semler et al., 2014; Takemura-Uchiyama et al., 2014) or as adjuvants to conventional antibiotics (Cushnie et al., 2014; Kamal and Dennis, 2015). No such studies have been investigated in bacteriophage/phytochemical combination. Thus, the present thesis evaluated the combined effects of bacteriophage SU16 and SU27 in combination with antibacterial herbal plant extracts (BRE, SSE, and OIE) against corresponding *E. coli* strains.

The present study found that that MICs of BRE, OIE, and SSE were 4, 16 and 16 mg/mL, while MBCs were 16, 32, and 32 mg/mL, respectively. This study found that SSE showed higher antibacterial activity against ECOR than to BRE and OIE, probably as a result of their different chemical compounds. The antibacterial properties of the extracts from these plants have been reported to be against several bacteria. The extracts from *O. indicum* fruits exhibited antibacterial activities against clinically isolated bacteria *Streptococcus suis* and *Staphylococcus intermedius* in the disc diffusion assay (Sithisarn et al., 2016). Pinostrobin and red oil isolated from the rhizomes of *B. rotunda* showed inhibition effects on *Helicobacter pylori* (Bhamarapravati et al., 2006). The compound 1 and 3 isolated from the tubers of *Stephania succifera* showed antimicrobial activities against *Staphylococcus aureus* with diameters of the inhibition zones of 14.0 mm and 18.0 mm, respectively (Yang et al., 2010).

When the extracts were tested for antiviral properties, there was a significant difference in the susceptibility of the two phages SU16 and SU27, suggesting that the high concentration of extract may interfere the adsorption step of the phage on their host cell. Several plant species have been reported to have antiviral activity.

The time-kill assay was used to assess the effect of combinations of the extracts and ECOR phages. The result showed that significant differences were not observed when phages SU16 and SU27 were combined with plant extract, although combination OIE and SU27 exhibited a greater reduction the bacterial cell count of EOR27 than SU27 when used alone. Moreover, Changes in the number of phage plaque of plant extract plus phage were compared to phage only and revealed that the numbers of phage plaque in the combined groups were slightly decreased in the initiate of incubation time. The efficacy of bacteriophages in combination with antibiotics has been examined. Comeau et al. (2007) observed that increased phage production by the sub-lethal concentration of antibiotics was due to cell filamentation in *E. coli*. A combination of sub-inhibitory concentrations of ceftriaxone and *P. aeruginosa* phages had a synergistic effect resulting in cell morphological changes and reduced bacterial growth (Knezevic et al., 2013). This result demonstrated that the decrease of bacterial cells was not induced by the plant extracts. It may be driven by phage alone. This experiment was performed with cure extract, the presence of non-purify components might hinder the ability of phage by suppressing bacteriophage development. However, this result cannot be compared to the data of other reports regarding a combination of plant extract and bacteriophage, because this is the first report the effect of a combination of plant extract and phage.

Noticeably, the marked difference in viable count between cells treated with bacteriophage or herbal plant extract alone and cells treated with the combined agent was observed only in BRE plus phage SU16 combination against ECOR-16 and OIE plus phage SU27 against ECOR-27. These plant extracts were investigated at MIC concentration, while others were used at concentrations much lower than MIC

concentration. These findings suggest that the very low concentration of the extract may result in no synergistic activity. It is accepted that the synergistic activity happens when the two compounds target different mechanisms. Whereas, the additive interaction occurs when different compounds target the same mechanism of action (Basri and Sandra, 2016). The present thesis also speculates that the combination of phage plus herbal plant extract cannot achieve synergistic effect may be due to phage and herbal plant extracts inhibit the growth of tested *E. coli* strains with the same mechanism of action.

5.2 Conclusions

To summarize, α-mangostin treated separately has low antibacterial activity against ceftazidime-and ceftriaxone-resistant *A. baumannii*. Its antibacterial action is synergistically enhanced when it was used in combination with ceftazidime or ceftriaxone. The mechanism of action of this inhibition is by following three possible modes of action: 1) increased OM and CM permeability; 2) direct damage to the OMPG and disruption of peptidoglycan structure and 3) the inhibition of β-lactamase activity. Antibiotic-resistant *A. baumannii* isolates have been classified as a critical priority according to the urgency of the need for new antibiotics. Therefore, the combination of α-mangostin and ceftazidime or plus ceftriaxone is a promising candidate to be developed as a new adjunct agent for the treatment of the infection caused by ceftazidime-and ceftriaxone-resistant *A. baumannii*. Future studies should be investigated and confirmed in an animal test or humans. Also, the synergistic effect on blood and tissue should be evaluated.

The combination of phage and antibacterial plant extract is of an interesting approach to combat antibiotic-resistant bacteria. The current thesis is the first study to elucidate the plant extracts (BRE, OIE, and SSE)/phage combination. The results suggest that the combination cannot achieve the potential effect on inhibiting the growth of ECOR when compared to using phages alone. It may be due to the use of low concentrations of plant extracts as the marked difference in phage and plant extract treated alone compared to the combination is observed in only the MIC concentration of the plant extract is employed. Furthermore, this work is the first investigation reporting the interaction of the antimicrobial potential of plant extracts (BRE, OIE, and SSE) in combination with phages. The current proof of concept study has highlighted the variation which can be introduced by including such additional components, especially when they are poorly characterized bulk compounds derived from natural products.

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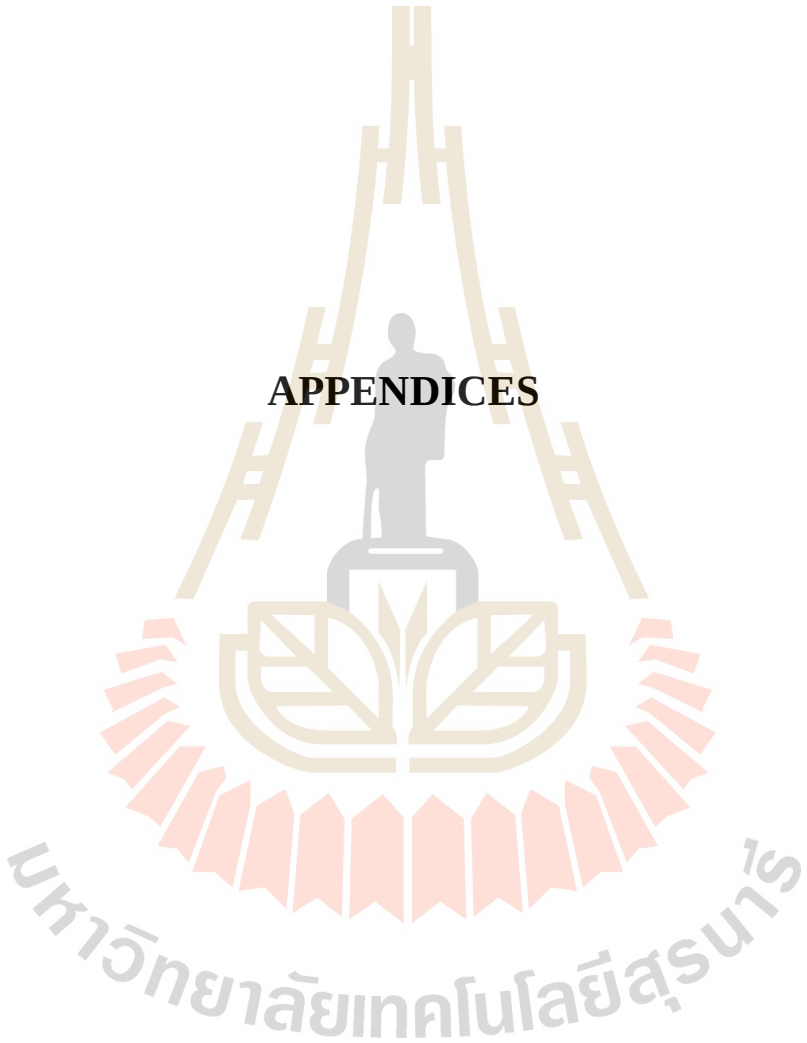
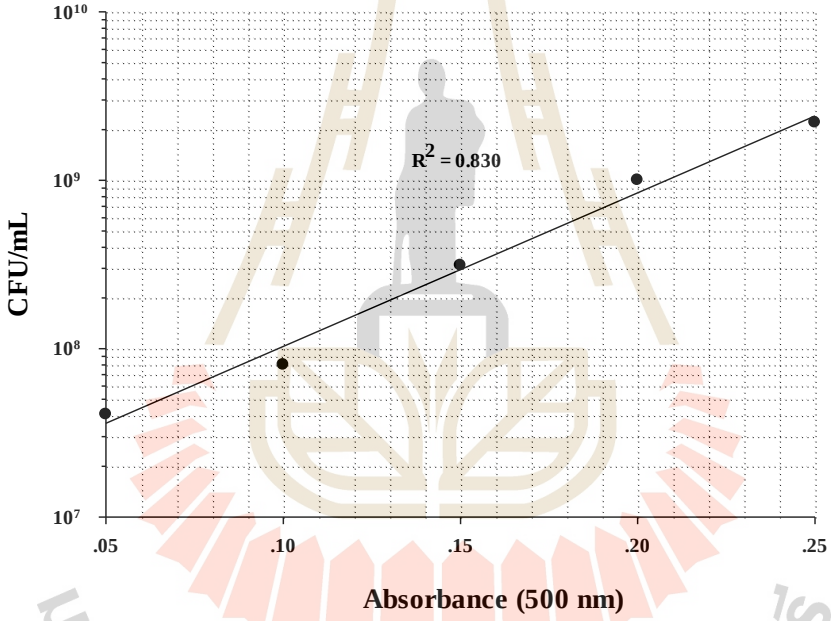
APPENDICES



APPENDIX A

BACTERIAL SUSPENSION STANDARD CURVE

Bacterial suspension standard curve of *A. baumannii* DMST 45378



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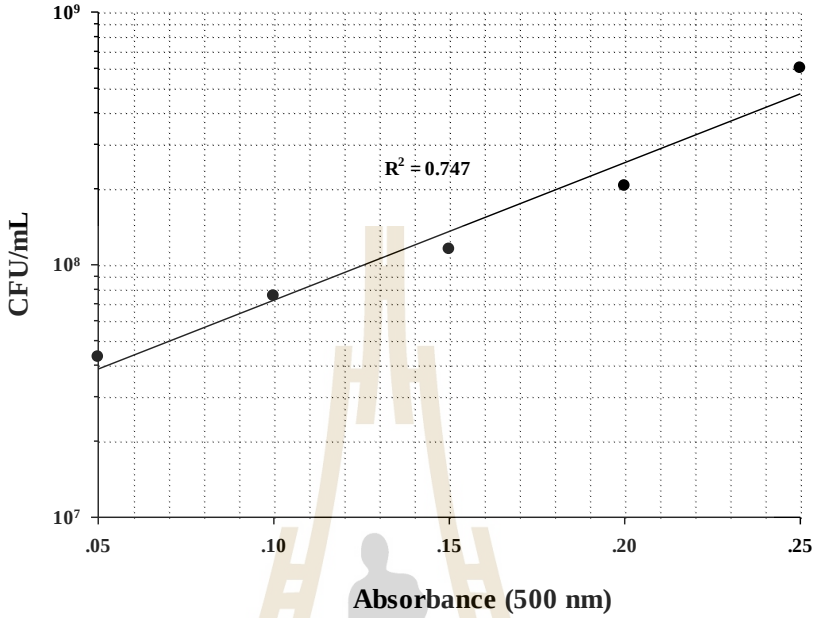

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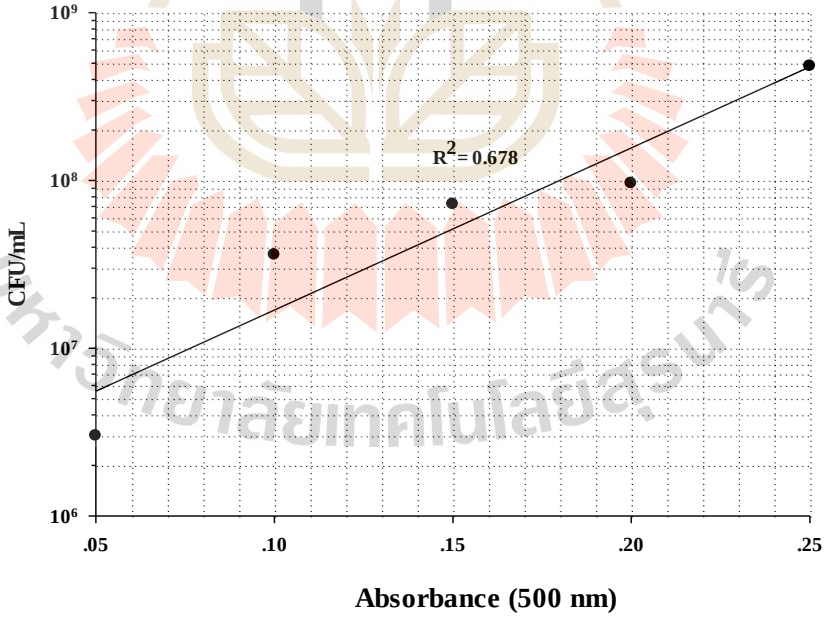
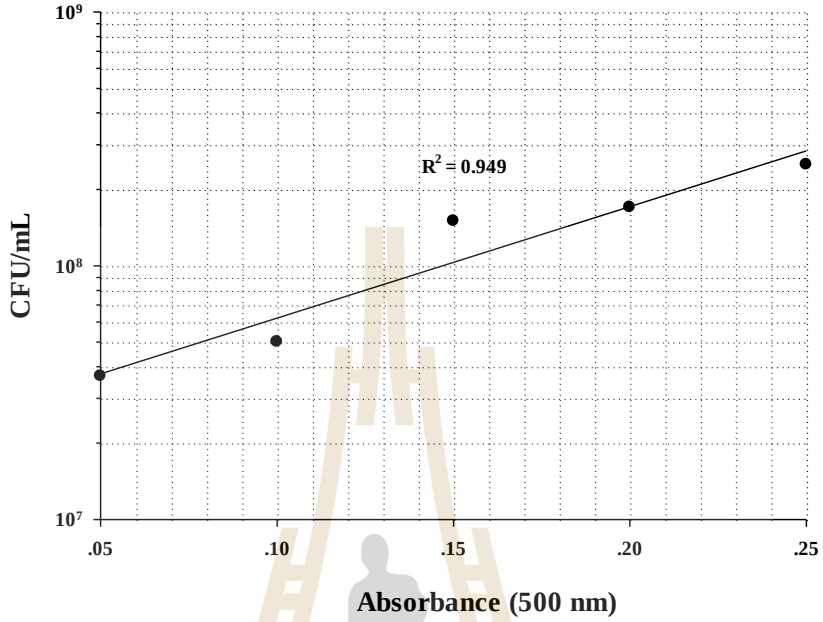
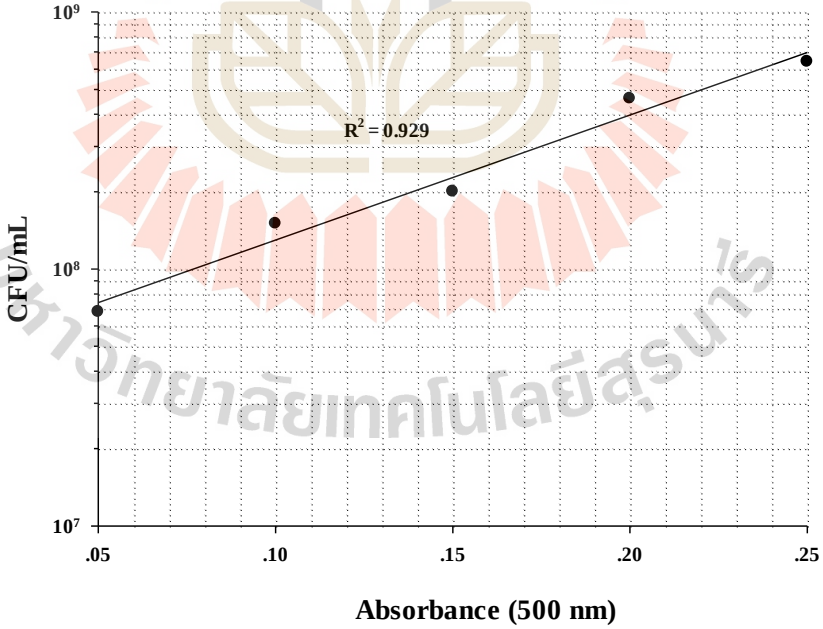


Bacterial suspension standard curve of *E. coli* ATCC 25922



Bacterial suspension standard curve of *E. faecalis* ATCC 29212



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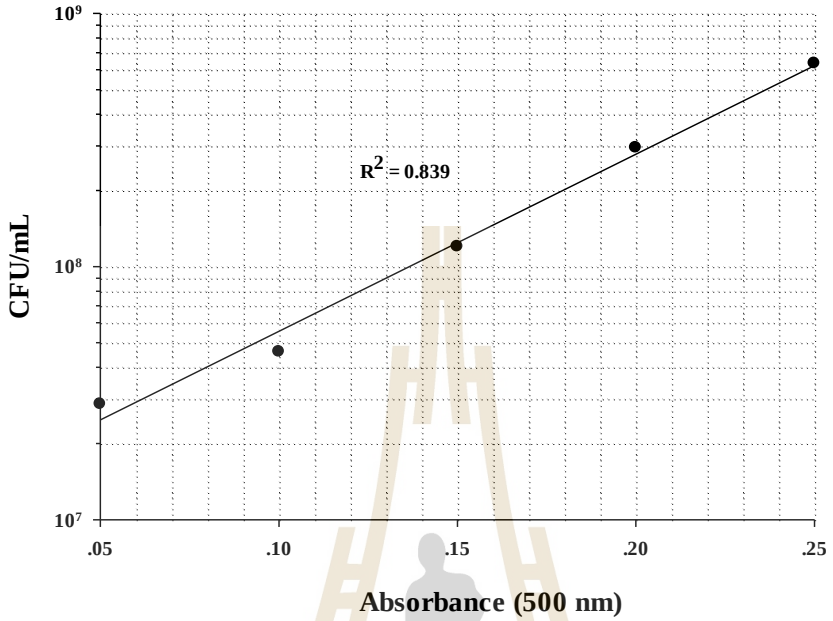
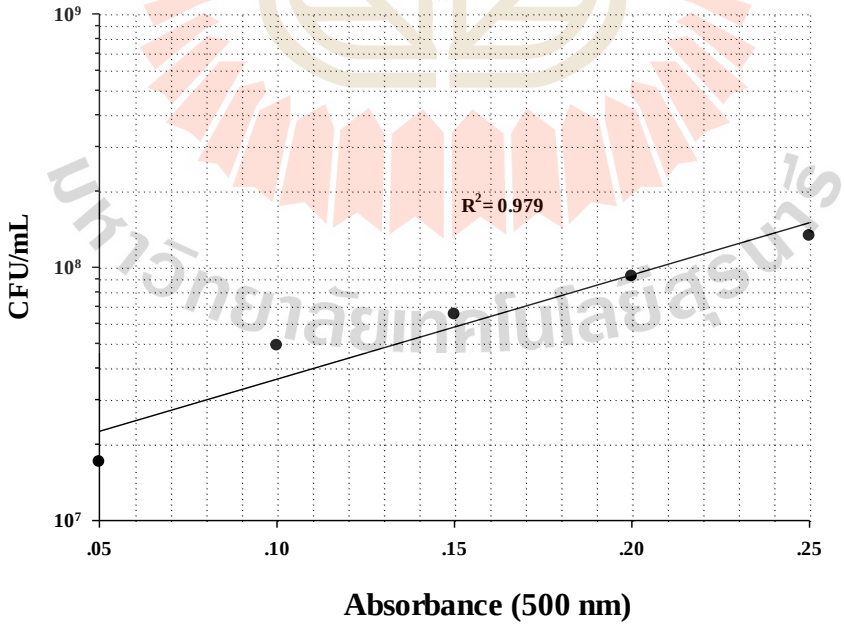
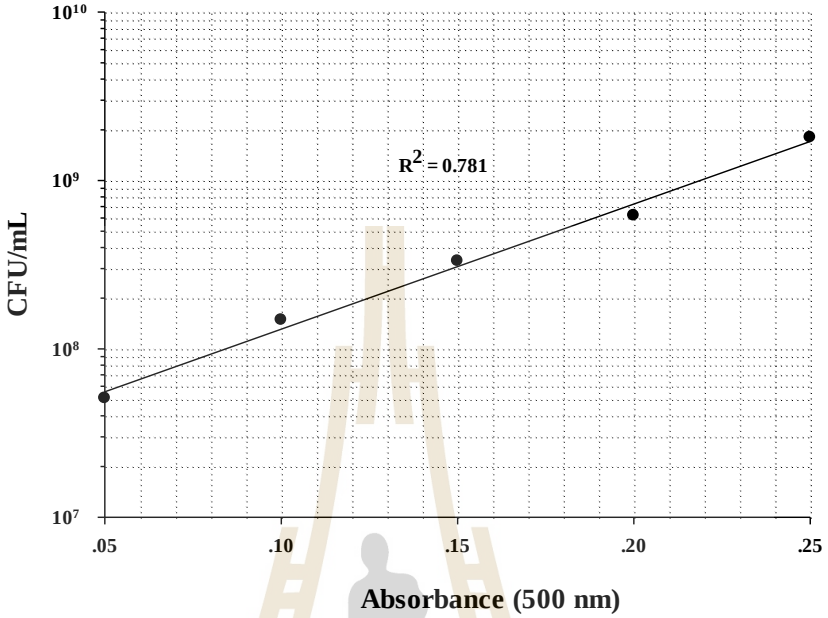


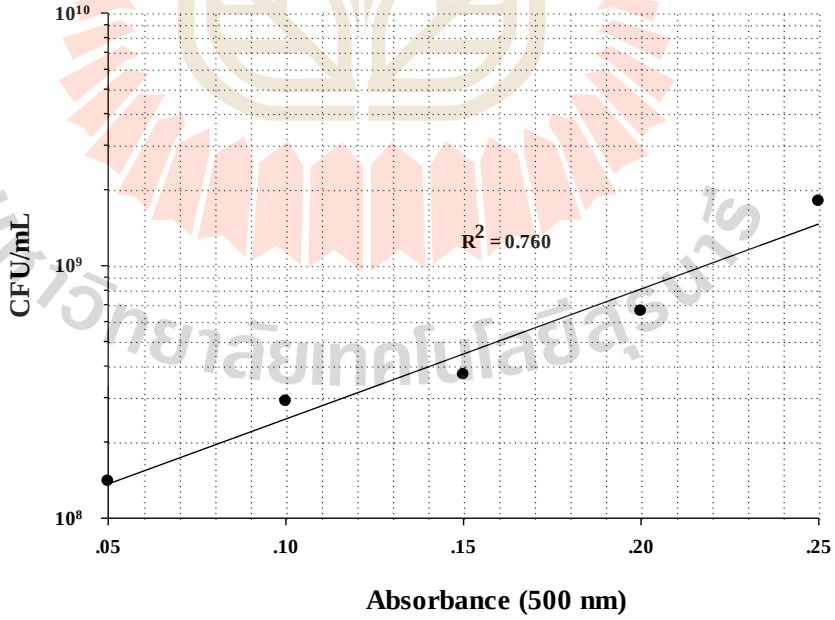
Becterial suspension standard curve *E. faecium* DMST 12829



Bacterial suspension standard curve of *E. faecium* DMST 12970



Bacterial suspension standard curve of *P. aeruginosa* DMST 37166



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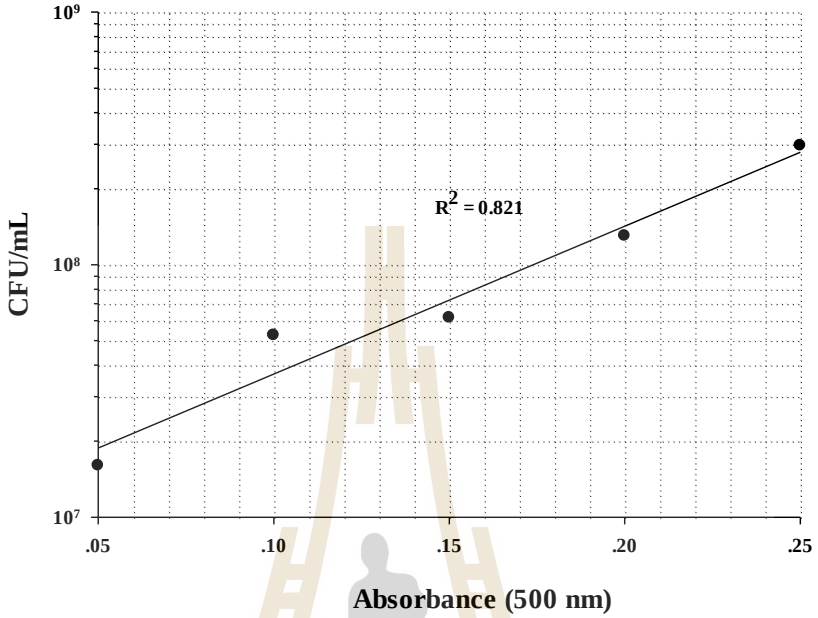
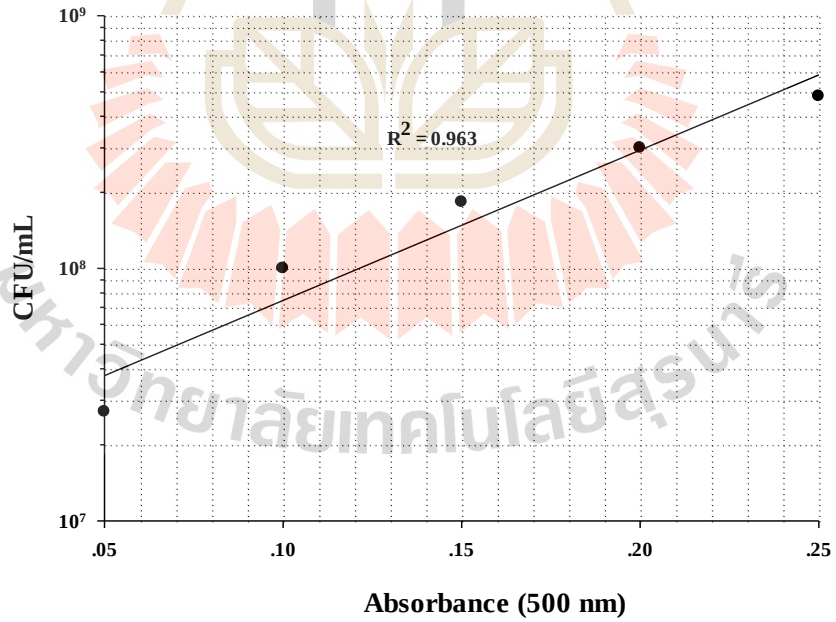


Bacterial suspension standard curve of *P. aeruginosa* DMST 37186



APPENDIX B

CULTURE MEDIA, CHEMICALS AND EQUIPMENT

1. Culture media

Nutrient agar, Mueller-Hinton broth and agar were obtained from Oxoid.

Approximate formulae per liter of each medium was as follows:

1.1 Nutrient agar

HiMedia nutrient agar was used for preparation of stock cultures on agar slopes and the basic agar culture of bacterial cells for colony counting.

The formula was:

	g/litre
Peptic digest 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

1.2 Mueller-Hinton broth (MHB)

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

The formula was:

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

pH 7.4 ± 0.2 at 37 °C

Mueller-Hinton had been cation-adjusted that had the corrected concentrations of the divalent cations of Ca2+20 mg/l and Mg2+10 mg/L (MBH).

All culture media were dissolved by water.

1.3 Mueller-Hinton agar (MHA)

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

The formula was:

	g/litre
Agar	17.0
Beef heart infusion	2.0
Casein acid hydrolysate	17.5
Soluble starch	1.5

pH 7.3±0.2 at 25 °C

2. Chemicals

All chemicals used were laboratory grade otherwise specified.

Tris-HCl	Lab grade
Q-Sepharose	Lab grade
Sephadex G-50	Lab grade
Ethanol Absolute	Lab grade
Ethyl acetate	Lab grade
Sodium chloride	AR grade
Sodium phosphate	Lab grade
Sodium hydroxide	Lab grade
95% Ethanol	Lab grade
Amonium acetate	AR grade
Acetronitrile	Lab grade
Albumin	AR grade
Lecithin	Lab grade
Tween 80	Lab grade
Paraformaldehyde	Lab grade
Glutaraldehyde	Lab grade
Osmiun tetroxide	Lab grade
Methanol	Lab grade
Araldite	Lab grade
Agarose	Lab grade
Uranyl acetate	Lab grade

Lead acetate	Lab grade
HEPES buffer	Lab grade
Phosphate	Lab grade
PMSF	Lab grade
Ceftazidime	AR grade
Ceftriaxone	AR grade
Ampicillin	AR grade

3. Equipment

3.1 Apparatus

Mixer (Model 5000)	Buchi
Column chromatography	Merck
Filter paper	Whatman
Spectronic 21	Milton Roy
Labofuge	400R Heraeus
Autoclave	Yamato
Laminar air flow	Woerden
Hot air oven	Shellab
Hot plate	VELP scientifica
Refrigerated Incubator	VELP scientifica
Ultramicrotome	JEM
Micropipettors (2-20 µL)	Witeg
Micropipettors (2-200µL)	Witeg

Micropipettors (100-1000 µL)	Witeg
Centrifuge tubes	Pyrex
Spectraphysics	Agilent
Micro titer plate (96 wells)	Bio-Rad
xMark™ Microplate Absorbance Spectrophotometer	Bio-Rad

3.2 Glassware

Beakers (50, 100, 250, 500, 1000 mL)

Pipettes (1, 5, 10 µL)

Measuring cylinder (10, 20 µL)

Petri dishes

Test tubes

APPENDIX C

PUBLICATIONS

Pimchan, T., Maensiri, D., and Eumkeb, G. (2017). Synergy and mechanism of action of α-mangostin and ceftazidime against ceftazidime-resistant *Acinetobacter baumannii*. **Letters in Applied Microbiology**. 65: 285-291.

Pimchan T, Cooper, C. J., Eumkeb, G., and Nilsson A. S. (2017). In vitro activity of a combination of bacteriophages and antimicrobial plant extracts. **Letters in Applied Microbiology**. 66: 182-187.

Teethaisong, Y., **Pimchan, T.,** Srisawat, R., Hobbs, G., and Eumkeb, G. (2018). *Boesenbergia rotunda* (L.) Mansf. extract potentiate the antibacterial activity of some β-lactams against β-lactam-resistant Staphylococci. **Journal of Global Antimicrobial Resistance**. 12: 207-213.

Siriwong, S., **Pimchan, T**., Naknarong, W., and Eumkeb, G. (2015). Mode of Action and Synergy of Ceftazidime and Baicalein against *Streptococcus pyogenes*. **Tropical Journal of Pharmaceutical Research**. 14 (4): 641-648**.**

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ORIGINAL ARTICLE

Synergy and mechanism of action of α-mangostin and ceftazidime against ceftazidime-resistant *Acinetobacter baumannii*

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Significance and Impact of the Study: *Acinetobacter baumannii* strains cause serious infections, patient mortality, and have been reported to rise of multidrug resistance. This article represents the first report of using α-mangostin plus ceftazidime against these resistant strains and its mechanism of action. α-mangostin has no cytotoxic effects. Therefore, α-mangostin has strong potential for development as a useful, novel adjunct phytopharmaceutical to ceftazidime synergistically for the treatment of these strains. The synergy approach could potentially be a novel tool to combat the resistant strains.

Keywords
antibacterial activity, β-lactam antibiotics, checkerboard assays, combination therapy, ESBL phenotypes, synergistic activity.

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Abstract

To address the resistance of *Acinetobacter baumannii* to β-lactam antibiotics, combination therapy between different antibiotic classes is increasingly used. The antibacterial activity of α-mangostin (AMT) alone or in combination with ceftazidime (CTZ) was investigated against ceftazidime-resistant *A. baumannii* DMST 45378 (CRAB). Initial screening showed that *A. baumannii* strains possessed AmpC β-lactamase (AmpC), extended-spectrum beta-lactamase (ESBL) and metallo-β-lactamases (MBL). Minimum inhibitory concentrations (MICs) of all test agents were >800 μg ml^{-1} against CRAB. The combination of AMT/CTZ exhibited a fractional inhibitory concentration index (FICI) of <0·35 suggestive of synergy. Time-kill curves showed that the AMT/CTZ combination was significantly more efficient ($P < 0·01$) at reducing CRAB than the individual components. Structural analysis revealed that AMT/CTZ-treated cells exhibited increased cell volume, increased cytoplasmic and outer membrane permeability and a decrease in outer membrane peptidoglycan-associated protein (OMPG) bands. In addition, it was shown that Type IV β-lactamase was inhibited by AMT. The data suggest that AMT in combination with CTZ is synergistic and efficient against CRAB. The data also indicate that the AMT/CTZ combination may target multiple structures on the bacterial cell surface. This represents the first report of this effect on CRAB and could potentially be expanded into *in vivo* studies.

Introduction

Acinetobacter baumannii is a Gram-negative opportunistic pathogen that can cause serious infections and is often associated with increased patient mortality (Chen *et al.* 2005; Eliopoulos *et al.* 2008). *A. baumannii* has become resistant to carbapenems and third-generation cephalosporins, which are the best available antibiotics for treating multidrug-resistant bacteria and have been classified as a critical priority according to the urgency of the need for new antibiotics (WHO, 2017). Colistin-resistant *A. baumannii* has also emerged due to the increasing use of colistin methanesulfonate to treat carbapenem-resistant *A. baumannii* infections (Qureshi *et al.* 2015). The combination of colistin with ceftazidime against local carbapenem-resistant *A. baumannii* showed only 7%

synergistic activity *in vitro* (Le Minh *et al.* 2015). *A. baumannii* antimicrobial resistance falls into three broad categories: (i) antimicrobial-inactivating enzymes (in particular β-lactamases), (ii) reduced access to bacterial targets (decreased outer membrane permeability) and (iii) mutations that change targets or cellular function (Rice 2006). The increase in the type and overall level of *A. baumannii* antimicrobial resistance highlights the need for the development of novel treatment strategies. While combination therapy using multiple antibiotic classes may be effective at reducing mortality (Lopez-Cortes *et al.* 2014), the combination of antibiotics and naturally derived products remains relatively understudied.

α-Mangostin, a plant-derived compound, has demonstrated active antimicrobial activities against Gram-positive bacteria including *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) by disrupting the cytoplasmic membrane (Iinuma *et al.* 1996; Sakagami *et al.* 2005; Koh *et al.* 2013). Although several studies have focused on the synergy between α-mangostin and antibiotics (Iinuma *et al.* 1996; Sakagami *et al.* 2005; Phitaktim *et al.* 2016), there is currently little to no information on the potential synergy when targeting cephalosporin-resistant *A. baumannii* strains. This study sought to evaluate the *in vitro* antimicrobial effect of α-mangostin alone and combined with ceftazidime against CRAB strains and to investigate any potential synergistic mechanism.

Results and discussion

β-lactamase phenotypic assays showed that all *A. baumannii* strains tested possessed AmpC, extended-spectrum beta-lactamase (ESBL) and metallo-β-lactamases (MBL). These findings suggest that coexistence of AmpC-ESBL-MBL combinations within these strains can cause cephalosporin resistance.

The minimum inhibitory concentration (MIC) for all conditions tested was >800 μg ml^{-1} and is shown in Table 1. These results show that all *A. baumannii* strains tested are resistant to ceftazidime (CTZ) and ceftriaxone (CTX). These results correspond with those of Rynga *et al.* who demonstrated that *A. baumannii* isolates from hospitals in Delhi were resistant to ceftazidime and cefotaxime and also tested positive for ESBL, AmpC and MBL (Rynga *et al.* 2015). The fractional inhibitory concentration (FIC) indices for AMT plus CTZ or CTX against all tested *A. baumannii* strains were <0·35 or <0·24, respectively, and suggest that the AMT/antibiotic combination exhibited synergistic activity against these strains (Odds 2003).

The effects of AMT, CTZ alone and together on the viable counts of CRAB are presented in Fig. 1. The combination of 200 μg ml^{-1} AMT and 200 μg ml^{-1} CTZ was shown to be the most efficient significantly reducing the bacterial concentration within 4 hours when compared with the untreated control ($P < 0·01$) and also the individual components ($P < 0·01$) by the conclusion of the experiment. These results confirmed the results of the checkerboard assay, which indicated that the synergistic activity of the combination produced a decrease of ≥2 $\log_{10}$ CFU per ml compared with the CTZ treatment alone (Eliopoulos and Moellering 1996).

TEM imaging of CRAB cells that were untreated (Fig. 2I) or treated with CTZ (Fig. 2II) showed no structural changes to the cell membrane. While CRAB cells showed an increase in a peptidoglycan and cytoplasmic membrane damage when treated with AMT alone (Fig. 2III), the most marked changes were demonstrated by those cells treated with the AMT/CTZ combination (Fig. 2IV). When compared with untreated controls, the overall area of these cells increased significantly ($P < 0·01$) (Fig. S1), suggesting that α-mangostin in combination with CTZ can damage or disrupt the cellular membrane and peptidoglycan layer. These findings seem to correspond with previous findings showing that the combination of isolated α-mangostin and oxacillin exhibited peptidoglycan and cytoplasmic membrane damage, irregular cell shapes of oxacillin-resistant *Staphylococcus*

Table 1 Minimum inhibitory concentrations (MICs) and fractional inhibitory concentration index (FICI) of α-mangostin (AMT) alone and in combination with ceftazidime (CTZ) or ceftriaxone (CTX)

	MIC (μg ml^{-1})			FIC (μg ml^{-1})		FIC index	
Strains	AMT	CTZ	CTX	AMT + CTZ	AMT + CTX	AMT + CTZ	AMT + CTX
A. baumannii DMST 45378	>2048	800^{R}	>2048^{R}	200 + 200	250 + 250	<0·35^{SI}	<0·24^{SI}
A. baumannii DMST 44689	>2048	800^{R}	>2048^{R}	200 + 200	250 + 250	<0·35^{SI}	<0·24^{SI}
A. baumannii DMST 43105	>2048	800^{R}	>2048^{R}	200 + 200	250 + 250	<0·35^{SI}	<0·24^{SI}
E. coli ATCC 25922	>1024	≤1^{S}	≤1^{S}	*N/D*	*N/D*	*N/D*	*N/D*

^{S}Susceptible; ^{R}Resistant; ^{SI}Synergistic interaction; *N/D*, not determined, FIC, fractional inhibitory concentration.
The minimum inhibitory concentrations (MICs) are presented as the median values measured from three independent experiments.

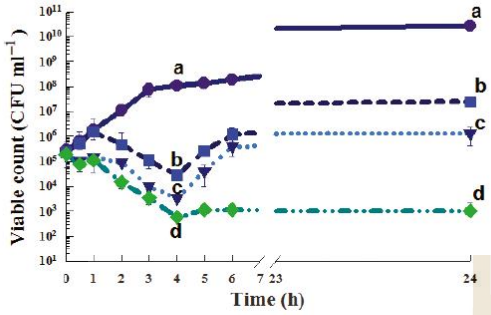

Figure 1 The bactericidal effects of α-mangostin alone or in combination with ceftazidime, against ceftazidime-resistant *Acinetobacter baumannii* DMST 45378. (●) untreated control; (▼) 400 μg ml^{-1} CTZ; (■) 1024 μg ml^{-1} α-mangostin (AMT) and (◆) 200 μg ml^{-1} CTZ and 200 μg ml^{-1} AMT. Data are the mean of four replicates ±SD. Means with the different superscript at the same time point are significantly different (Tukey's HSD, $P < 0{\cdot}01$). [Colour figure can be viewed at wileyonlinelibrary.com]

saprophyticus and average cell areas significantly larger than those of the controls (Phitaktim *et al.* 2016).

CRAB cells treated with AMT alone and in combination with CTZ and also nisin released significantly more material that absorbed at 260 nm compared with CTZ or the untreated control (Fig. 3; $P < 0{\cdot}01$). This suggests that the AMT/CTZ combination increases the permeability of the cellular membrane to DNA, RNA, and cellular metabolites and leads to the deactivation of β-lactamase activity (Shen *et al.* 2012). These data support previous findings, which showed that α-mangostin rapidly disrupted the integrity of the cytoplasmic membrane of MRSA cells, leading to the loss of intracellular components in a concentration-dependent manner (Koh *et al.* 2013).

The effect of the AMT alone or in combination with CTZ on the OMPG of CRAB is shown in Fig. S2. The combination of AMT and CTZ reduced the size and intensity of OMPG bands at 22, 25 and 30 kDa compared with controls. The effect of the AMT alone or in combination with CTZ on the adjusted relative density of CRAB-OMPG is shown in Fig. S3. The combination of AMT and CTZ exhibited significantly lower adjusted relative density than other groups in all OMPG bands ($P < 0{\cdot}01$), whereas the adjusted relative density OMPG of CTZ band displayed significantly higher than others ($P < 0{\cdot}01$). These findings suggest that the combination displayed outer membrane and peptidoglycan damage resulting in the loss of OMPG and would support TEM observations made in the current investigation.

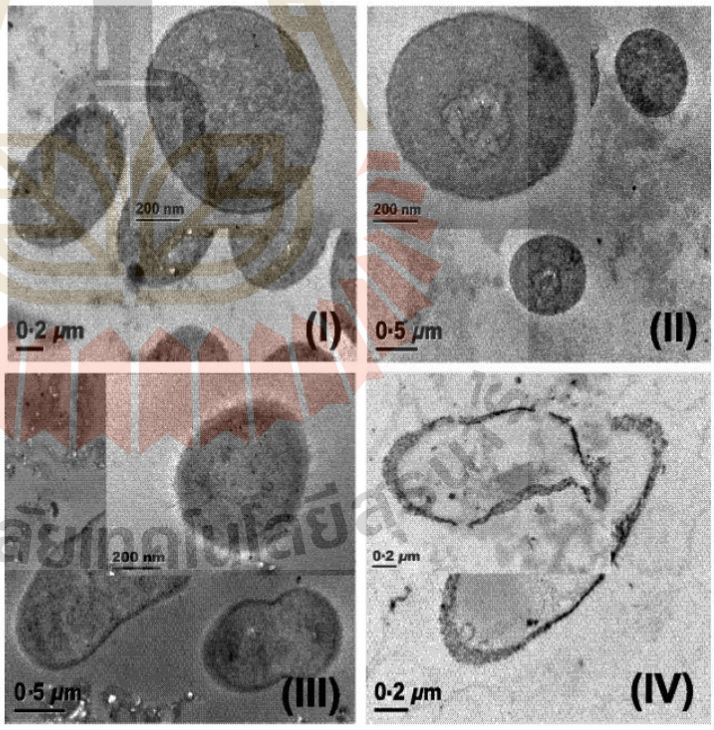

Figure 2 Impact of α-mangostin (AMT) alone and in combination with CTZ on the surface membrane of *Acinetobacter baumannii* DMST 45378. (I), untreated control; (II), 400 μg ml^{-1} CTZ; (III), 1024 μg ml^{-1} AMT; (IV), 150 μg ml^{-1} CTZ and 150 μg ml^{-1} AMT. Magnification: (I), 10 000×, bar = 0·2 μm; (II), 8000×, bar = 0·5 μm; (III), 10 000×, bar = 0·2 μm; (IV), 16 000×, bar = 0·23 μm; Inset magnification; (I), (II) 15 000×; (III), (IV) 16 000×; bars: (I), (II), (III), (IV) 0·2 μm).

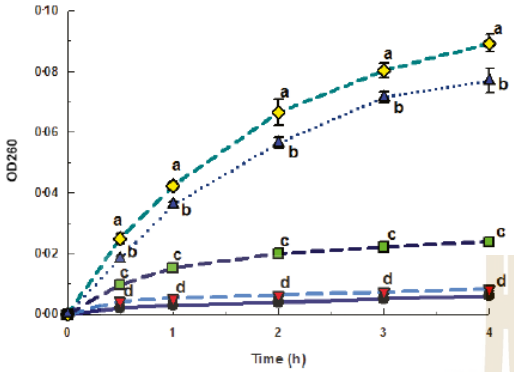

Figure 3 Release of intracellular material from *Acinetobacter baumannii* DMST 45378 treated with α-mangostin (AMT) alone or in combination with CTZ. (●) untreated control; (▼) 400 μg ml^{-1} CTZ; (■) 1024 μg ml^{-1} AMT and (◆) 150 μg ml^{-1} CTZ and 150 μg ml^{-1} AMT; (▲) 128 μg ml^{-1} nisin (positive control). Data are the mean of three replicated ± SD. Means with the different superscript at the same time point are significantly different (Tukey's HSD, $P < 0{\cdot}01$). [Colour figure can be viewed at wileyonlinelibrary.com]

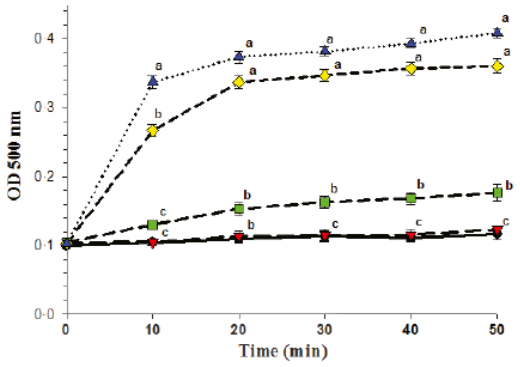

Figure 4 Outer membrane permeability of ceftazidime-resistant *Acinetobacter baumannii* DMST 45378. (●) untreated control; (▼) 400 μg ml^{-1} ceftazidime; (■) 1024 μg ml^{-1} α-mangostin; (◆) 150 μg ml^{-1} ceftazidime and 150 μg ml^{-1} α-mangostin and (▲) 10 μg ml^{-1} polymyxin B (positive control). Data are the mean of three replicates ± SD. The mean sharing the same superscript is not significantly different (Tukey's HSD test, $P < 0{\cdot}01$). [Colour figure can be viewed at wileyonlinelibrary.com]

Polymyxin B was significantly better than all other test conditions over the duration of the experiment (Fig. 4; $P < 0{\cdot}01$). However, the AMT/CTZ combination also significantly increased OM permeabilization of CRAB compared with individual components and the untreated control (Fig. 4; $P < 0{\cdot}01$).

α-mangostin was able to significantly reduce the breakdown of benzylpenicillin levels in a concentration-dependent manner compared with the untreated control (Fig. S4; $P < 0{\cdot}01$). These results are in substantial agreement with those of Phitaktim *et al.* that the isolated α-mangostin inhibited β-lactamase in a dose-dependent manner (Phitaktim *et al.* 2016). This result may enhance the rapid killing by a membrane-targeting antimicrobial, and that β-lactamase inhibition could be a secondary effect of the membrane disruption (Koh *et al.* 2013).

The synergistic paradigm of combining the conventional antibiotic with phytochemical compounds has been shown to be an effective avenue to treat infectious diseases caused by drug-resistant bacteria in several studies (Wagner and Ulrich-Merzenich 2009; Eumkeb and Chukrathok 2013). Simulations of the molecular dynamics of this synergy revealed that the isoprenyl groups of α-mangostin, occupying the chromone structure (Fig. S5), play a major role in penetrating the lipid bilayer of the MRSA membrane, which results in increased permeability and breakdown of the membrane (Koh *et al.* 2013). While not conclusive, these findings could support an assumption that the isoprenyl groups and the chromone structure of α-mangostin play a significant role in inhibiting CRAB strains through direct interactions with the bacterial membrane (Koh *et al.* 2013). It has been previously reported that AMT provided a sufficient margin of safety for therapeutic use (Kaomongkolgit *et al.* 2009; Phitaktim *et al.* 2016) and would suggest that the AMT could potentially be a useful adjuvant to CTZ for the treatment of CRAB.

In conclusion, our findings indicate that AMT alone has limited activity against CRAB, but increased antibacterial activity when combined with CTZ. Further investigation into the potential mechanisms of this synergy suggests three possible modes of action: (i) increased OM and CM permeability; (ii) direct damage to the OMPG and peptidoglycan structure disruption and (iii) the inhibition of β-lactamase activity.

Materials and methods

Bacterial strains

Acinetobacter baumannii DMST strains, three strains, were obtained from the Department of Medical Science, Ministry of Public Health, Thailand. *Escherichia coli* ATCC 25922, one strain, was obtained from the American Type Culture Collection (ATCC).

Bacterial suspension standard curve

Bacterial suspensions were routinely cultured in Cation-adjusted Mueller–Hinton broth and viable counts determined on Mueller-Hinton agar (Oxoid, Basingstoke, UK). The bacterial suspension standard curve method as

described by Liu *et al.* was used to standardize the bacterial count of suspensions prior to each test (Liu *et al.* 2000).

Screening test of AmpC, ESBL and MBL

The presence of AmpC, ESBL and MBL in *A. baumannii* DMST strains was phenotypically determined as previously described using a resazurin microtitre plate assay with inhibitor-based methods (Teethaisong *et al.* 2016).

MICs and checkerboard determinations

The MIC of ceftazidime (Sigma-Aldrich, Dorset, UK), ceftriaxone (Sigma-Aldrich) and AMT (Indofine Chemical Company, Hillsborough Township, NJ) were determined using Clinical and Laboratory Standard Institute broth microdilution and resazurin dye methods (Taneja and Tyagi 2007; Clinical and Laboratory Standards Institute, 2013; Elavarasan *et al.* 2013). The checkerboard method as described by Phitaktim *et al.* was used to assess the activity of AMT in combination with CTZ or CTX (Phitaktim *et al.* 2016). The FIC index was calculated using the following formula:

FIC of AMT
= MIC of AMT in combination/MIC of single AMT

Therefore, the FIC index = FIC of AMT + FIC of CTZ

A FIC index of ≤0·5 is defined as synergistic; 0·5–4·0 is defined as 'no interaction' between the agents and a value >4·0 indicates antagonism (Eliopoulos and Moellering 1996; Odds 2003).

Time-killing assays

Time-killing assays were performed as previously described (Richards *et al.* 1995; Mun *et al.* 2013) to determine the overall antibacterial activity of AMT when used alone and in combination with CTZ. The synergy of an antimicrobial combination was defined by at least 2 $\log_{10}$ decreases in CFU per ml compared with its more active constituent (Eliopoulos and Moellering 1996). The experiment was performed in three observations, each observation was performed in triplicate, and data are shown as mean ± SD.

TEM

The ultrastructural morphology of CRAB cells after treatment with AMT alone or in combination with CTZ was determined as previously described by Eumkeb and Chukrathok (2013).

The total cell area (nm^2) was determined for both compounds when exposed to 50% MIC of individual compounds and 75% of the FICs for combinations. The experiment was performed on 10 different fields of view, at least 10 counts per field, and the cell areas are displayed as the mean ± SD.

CM permeability

The effect of AMT alone and in combination with CTZ on the permeability of the CM was performed as previously described with some modifications (Zhou *et al.* 2008; Shen *et al.* 2012).

Effect on OMPG

The effect of AMT alone and in combination with CTZ on the OMPG of *A. baumannii* was determined as previously described (Eumkeb 1999). Furthermore, Image J 1.8.0_112 (Windows version of NIH Image, https://imagej.nih.gov/ij/download.html) was used to quantify and compared the OMPG band images on an SDS–PAGE gel (Gassmann *et al.* 2009; Miller 2010). The experiment was performed in three replicates.

OM permeability

α-mangostin-induced permeabilization of the OM of CRAB was determined as previously described (Eriksson *et al.* 2002). In a word, membrane permeabilization was assayed in 96-well microtitre plates. The OM permeabilization assay was carried out with wells filled with 400 $\mu g\ ml^{-1}$ CTZ, 1024 $\mu g\ ml^{-1}$ AMT, 150 $\mu g\ ml^{-1}$ CTZ plus 150 $\mu g\ ml^{-1}$ AMT and 10 $\mu g\ ml^{-1}$ PMX and 50 μl of the NCF stock solution.

Inhibition of β lactamase by AMT

The ability of AMT to inhibit the activity of β-lactamase type IV isolated from *Enterobacter cloacae* was determined as previously described by Richards *et al.* (1995).

Statistical analysis

All experiments were performed in triplicate unless otherwise stated in the text and the data expressed as the mean ± SD. Data were analysed by one-way ANOVA, followed by Tukey's HSD *post hoc* test at the 99% confidence interval ($P < 0·01$) (Eumkeb and Chukrathok 2013).

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Conflict of Interest

The authors have declared that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effects of CTZ and α-mangostin (AMT) on the average cross-sectional area of ceftazidime-resistant *Acinetobacter baumannii* DMST 45378 cells.

Figure S2. SDS–PAGE showing the OMPG of ceftazidime-resistant *Acinetobacter baumannii* DMST 45378.

Figure S3. The adjusted relative density (% of control) obtained from quantitative densitometry of SDS–PAGE of each ceftazidime-resistant *Acinetobacter baumannii* OMPG band.

Figure S4. Inhibitory activity of α-mangostin against *β*-lactamase hydrolysis of benzylpenicillin.

Figure S5. The structure of α-mangostin, chromone and isoprenyl.

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ORIGINAL ARTICLE

In vitro activity of a combination of bacteriophages and antimicrobial plant extracts

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Significance and Impact of Study: This preliminary study provides insights into the potential combination of bacteriophages and antimicrobial plant bulk extracts to target bacterial pathogens. It is to our knowledge the first time in which virulent bacteriophages have been combined with antimicrobial plant extracts.

Keywords
antimicrobial compounds, bacteriophages, combination therapy, natural products, phage therapy.

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Abstract

The continuing threat of antimicrobial resistance presents a considerable challenge to researchers to develop novel strategies ensuring that bacterial infections remain treatable. Many plant extracts have been shown to have antibacterial properties and could potentially be combined with other antibacterial agents to create more effective formulations. In this study, the antibacterial activity of three plant extracts and virulent bacteriophages have been assessed as individual components and in combination. When assessed with a modified suspension test, these plant extracts also exhibit antiviral activity at bacterial inhibitory concentrations. Hence, to investigate any potential additive effects between the extracts and virulent phages, the extracts were tested at subantiviral concentrations. Phages alone and in combination with plant extracts significantly reduced ($P < 0{\cdot}05$) the bacterial concentration compared to untreated and extract treated controls up to 6 h (2–3$\log_{10}$), but this reduction did not extend to 24 h. In most cases, the phage and extract combinations did not significantly reduce bacterial content compared to phages alone. Additionally, there was little impact on the ability of the phages to reproduce within their bacterial hosts. To our knowledge, this study represents the first of its kind, in which antimicrobial plant extracts have been combined with virulent phages and has highlighted the necessity for plant extracts to be functionally characterized prior to the design of combinatorial therapies.

Introduction

The potential for a future in which bacterial infections become untreatable is undeniable, with a number of bacterial infections failing to respond to drugs of last resort or antibiotic combination therapy (Fifer *et al.* 2016; Bi *et al.* 2017). While this is due not only to an increase in the level and type of resistance that exists (Zhu *et al.* 2013; Oz *et al.* 2014), the overuse and misuse of antibiotics in both human and veterinary applications cannot be understated (Berendonk *et al.* 2015; Meek *et al.* 2015; Fleming-Dutra *et al.* 2016) and has shifted focus towards new strategies for combatting multi-resistant infections, including reducing antibiotic consumption (Sabuncu *et al.* 2009) and the development of new therapies (Brown and Wright 2016). Both bacteriophages and plant extracts are amongst those being investigated either as individual therapies (Bonifácio *et al.* 2014; Semler *et al.* 2014; Takemura-Uchiyama *et al.* 2014) or as adjuvants to conventional antibiotics (Coulter *et al.* 2014; Cushnie *et al.* 2014; Kamal and Dennis 2015).

A number of strategies are being pursued to reduce antibiotic resistance levels, including the development of new types of therapy and reduction of antibiotic consumption. Although combinatorial therapy between antibiotics and phages has been shown to be successful *in vitro*, and furthermore against resistant phenotypes (Comeau *et al.* 2007; Torres-Barceló *et al.* 2016), it is perhaps counteractive to employ at the same time as trying to reduce overall consumption of antibiotics, particularly in veterinary applications where antibiotic resistance is a major issue (Allen 2014; Van Boeckel *et al.* 2015). As such, combinations of naturally antimicrobial plant extracts with virulent phages could potentially address such issues.

In the current study, the antimicrobial potential of subinhibitory concentrations of three antimicrobial plant extracts in combination with virulent phages has been investigated.

The plant extracts were derived from *Stephania suberosa* roots (SSE, Menispermaceae), *Oroxylum indicum* fruits (OIE, Bignoniaceae) and *Boesenbergia rotunda* rhizomes (BRE, Zingiberaceae), all used in traditional Asian Medicine. The virulent bacteriophages SU16 (Myoviridae) and SU27 (Siphoviridae) were isolated from wastewater in 2010 and have been previously shown to be able to infect 16 and 4 strains of *Eshcherichia coli* at high or moderate efficiency of plating, respectively (Khan Mirzaei and Nilsson 2015).

Results and discussion

MIC/MBC determination and anti-phage activity of plant extracts

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination showed that both host bacterial strains (ECOR16 and ECOR27 from the *E. coli* standard reference collection; ECOR; Ochman and Selander 1984) were inhibited by the bulk plant extracts at the same inhibitory concentrations (Table 1). When the bulk extracts were tested for antiviral properties, all three extracts produced a statistically significant reduction (approx. 0·5–2 $\log_{10}$ PFU per ml; $P < 0{\cdot}05$; Fig. 1) of phage SU16 at MIC concentrations. There was a significant difference in the susceptibility of the two phages, SU16 and SU27, when tested against at multiple concentrations of BRE and OIE (100-1% MIC; $P < 0{\cdot}05$; Fig. 1), while SSE showed no significant difference between the two phages at concentrations of 10% MIC or lower ($P > 0{\cdot}05$; Fig. 1). Due to the differences in anti-phage activity exhibited by the different compounds, the concentration of the plant extracts was optimized on a phage+compound basis for time kill analyses.

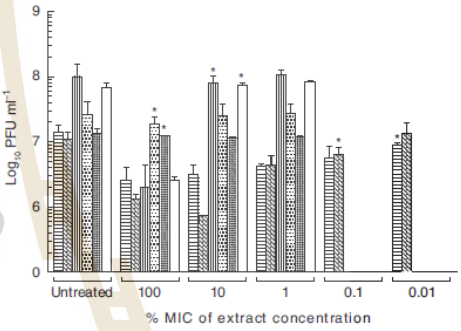

Figure 1 Antiviral activity of plant extracts against bacteriophages SU16 and SU27 after 24 h exposure. (▤) SU16 + *Boesenbergia rotunda* rhizomes (BRE); (▩) SU16 + *Oroxylum indicum* fruits (OIE); (▮) SU16 + *Stephania suberosa* roots (SSE); (□) SU27 + BRE; (▦) SU27 + OIE; (□) SU27 + SSE. Data are the mean of three replicates ±SD. The minimum inhibitory concentration (MIC) of extract concentrations were 16 mg ml^{-1} for BRE and OIE and 4 mg ml^{-1} for SSE against both bacterial host strains. *Indicates selected concentration that shows nonsignificant difference to the untreated control ($P > 0{\cdot}05$).

Table 1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extracts (mg ml^{-1}) used in the current investigation to bacterial host strains

	Bacterial strain			
	ECOR16		ECOR27	
Compound/Extract	MIC	MBC	MIC	MBC
SSE	4 ± 0	16 ± 0	4 ± 0	16 ± 0
OIE	13·33 ± 4·62	42·67 ± 18·48	13·33 ± 4·62	42·67 ± 18·48
BRE	16 ± 0	32 ± 0	16 ± 0	32 ± 0

Data are the mean of three replicates ± SD. *Stephania suberosa* root (SSE), *Oroxylum indicum* fruit (OIE), and *Boesenbergia rotunda* rhizome (BRE) extracts.

Combined activity of bacteriophages and bulk plant extracts

√Two bacteriophages (one *Myoviridae*, SU16 and one *Siphoviridae*, SU27) which infect *E. coli* were investigated for their antibacterial activity when combined with bulk plant extracts with known antimicrobial properties. The phages were only tested against the strains from which they were originally isolated, but have been previously shown to be active against several *E. coli* strains (Khan Mirzaei and Nilsson 2015). Overall, the combination of bulk plant extracts and bacteriophages exhibited no significant difference in antibacterial activity at 24 h when compared to the untreated control or the individual components ($P > 0{\cdot}05$; Figs 2 and 3). However, both phage only and phage with extract significantly reduced bacterial content in all cases for up to 6 h compared to the untreated and extract only controls ($P < 0{\cdot}05$; Figs 2 and 3), which suggests that the reduction was primarily driven by the phage component. No phage SU16 + extract-treated samples showed significant differences compared to the phage only samples ($P > 0{\cdot}05$; Fig. 2). In the case of phage SU27 and extract treated samples, a significant additive effect could be seen when BRE (up to 4 h) and OIE (up to 8 h) extracts were added (Fig. 3a,b). Interestingly, a noticeable shift in the bacterial growth could be observed in the BRE and OIE extracts only compared to control which may be responsible for this additive effect. In addition, a significant reduction ($P < 0{\cdot}05$) in phage titre for the OIE-treated sample was observed at 2 to 8 h (Fig. 3b).

The concentrations of extracts tested in the current investigation were chosen to be subinhibitory to bacterial growth and also of limited toxicity to phages. While this appears to be the case with phage SU16 and ECOR16 (Fig. 2), with modest and often statistically insignificant differences ($P > 0{\cdot}05$) in phage and BRE or OIE extract titre compared to nonextract containing controls (Fig. 2a,b), there are differences in susceptibilities when used in concentration with SU27, most notably for OIE and SSE phage titres.

The combination of phages with adjuvant components is a currently understudied topic, with great focus being placed on the combination of phages and conventional antibiotics (Chaudhry *et al.* 2017; Oechslin *et al.* 2017; Scanlan *et al.* 2017) and purified phage cocktails (Gundogdu *et al.* 2016; Regeimbal *et al.* 2016; Yen *et al.* 2017). However, in order to further develop phages as antimicrobial agents, it is necessary to investigate the impact of more complex formulations which include additional active and excipient components (Abedon 2017). The current proof of concept study has highlighted the variation which can be introduced by including such additional components, particularly when they are poorly characterized bulk compounds derived from natural products.

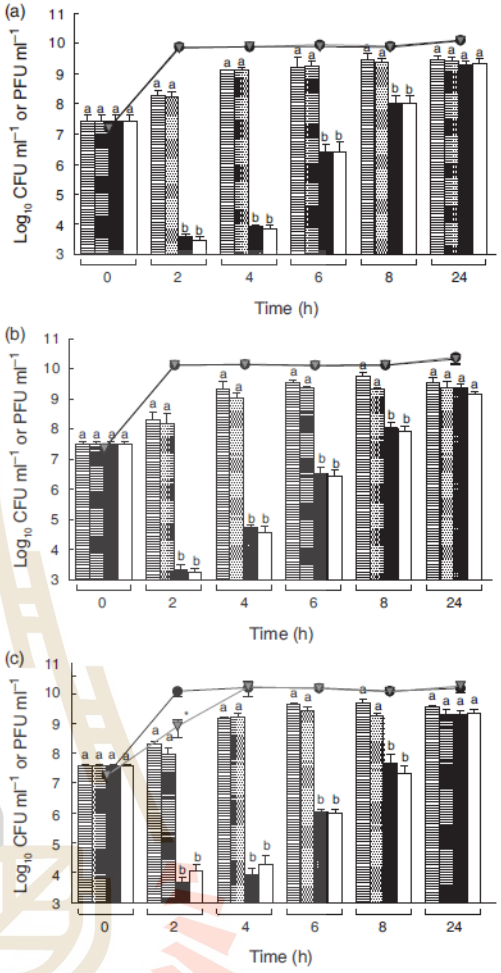

Figure 2 Activity of phage SU16 in combination with different plant extracts against ECOR16. (a) *Boesenbergia rotunda* rhizomes (BRE) (1·6 μg ml^{-1}); (b) *Oroxylum indicum* fruits (OIE) (16 μg ml^{-1}); (c) *Stephania suberosa* roots (SSE) (400 μg ml^{-1}). (▤) Untreated control; (▨) Extract Only; (▩) Phage Only; (□) Extract and phage. The mean ± SD for three replicates are illustrated. Means sharing the same superscript at the same time are not significantly different from each other (Tukey's HSD test, $P < 0{\cdot}05$). Lines indicate phage counts. (–●–) phage only; (–▽–) Extract and phage. *Indicates significant difference to the phage at the same time ($P < 0{\cdot}05$).

Materials and methods

All chemicals and reagents were obtained from either Sigma Aldrich (Stockholm, Sweden) or VWR (Stockholm, Sweden) and of general laboratory grade unless otherwise stated in the text.

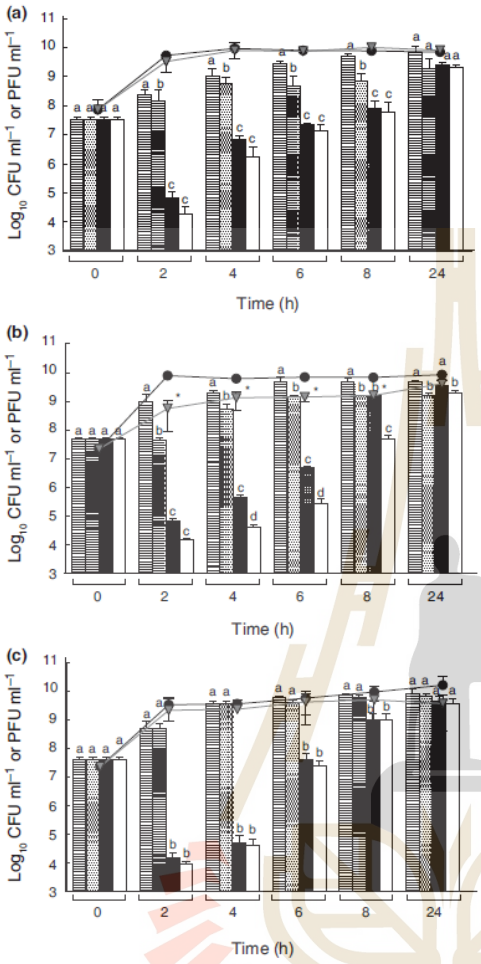

Figure 3 Activity of phage SU27 in combination with different plant extracts against ECOR27. (a) *Boesenbergia rotunda* rhizomes (BRE) (16 mg ml^{-1}); (b) *Oroxylum indicum* fruits (OIE) (16 mg ml^{-1}); (c) *Stephania suberosa* roots (SSE) (400 μg ml^{-1}). (▤) Untreated control; (▽) Extract Only; (□) Phage Only; (▥) Extract and phage. The mean ± SD for three replicates are illustrated. Means sharing the same superscript at the same time are not significantly different from each other (Tukey's HSD test, $P < 0{\cdot}05$). Lines indicate phage counts. (—●—) phage only; (—▽—) Extract and phage. *Indicates significant difference to the phage at the same time ($P < 0{\cdot}05$).

Plant extracts

Fresh samples of SSE, OIE and BRE were obtained locally from Nakhon Ratchasima province, Thailand. Samples were cut into small pieces, dried for 2 days at 40°C and ground into a coarse powder with a mechanical grinder. Crude antimicrobial mixtures were extracted from 500 g of powdered samples with soxhlation extraction in 2 l of ethanol for 8 h. Extracts were passed through Whatman number 1 filter paper to remove large debris and the solvent removed by rotatory evaporation under reduced pressure at 50°C. The remaining sample was lyophilized to obtain dried extracts. Extracts were stored at −20°C until required and suspended in PBS (for SSE) or PBS with 5% DMSO (for BRE and OIE).

Bacteriophage and bacterial strains

Escherichia coli strains ECOR16 and ECOR27 were obtained from the *E. coli* standard reference collection (ECOR; Ochman and Selander 1984). These strains are the hosts on which phages SU16 and SU27 were originally isolated. Working stocks were produced from −80°C glycerol stocks and maintained on lysogeny agar (LA) plates stored at 4°C. ECOR16 and ECOR27 were routinely cultured in lysogeny broth (LB) at 37°C overnight with shaking at 150 rev min^{-1}. Prior to use, suspensions were centrifuged at 3864*g* for 15 min, the supernatant discarded and the pellet suspended in 10 ml phosphate buffered saline (PBS) and further diluted in PBS to contain 1–5 × 10^8 CFU per ml.

Previously characterized phages SU16 and SU27 (Khan Mirzaei and Nilsson 2015) were obtained from Stockholm University stocks stored at −80°C in glycerol. Phages were cultured in 10 ml lysogeny broth (LB) using the appropriate bacterial strain (100 μl inoculum of overnight culture) as the regular host at 37°C overnight with shaking. Following incubation, suspensions were centrifuged at 3864*g* for 15 min at 4°C and passed through 0·45 and 0·2 μm syringe filters. Phage suspensions were enumerated, using the agar overlay method (Adams 1959) and stored at 4°C until required. Prior to use, phages were diluted in PBS to contain 1–3 × 10^8 PFU per ml.

MIC/MBC determination and activity of extracts against phage

The minimum inhibitory concentration and MBC of plant extracts were determined against ECOR16 and ECOR27 using a modified broth method (Clinical and Laboratory Standards Institute 2014). In brief, standardized bacterial suspensions were diluted 1 : 100 in double strength LB (dsLB) and the bacterial content determined by the drop count method (Miles *et al.* 1938). To triplicate wells of the first row of a 96-well microtitre plate, 200 μl of plant extract was added. A 100 μl aliquot was removed and serially diluted 1 : 2 in PBS over the

remainder of the plate. To each well, 100 μl of the diluted bacterial suspension was added and the plate incubated at 37°C overnight. The MIC value was determined to be the lowest concentration where no visible growth could be observed. MBC was determined by spreading 100 μl of suspension from wells showing no visible growth over the surface of pre-prepared LA plates which were then incubated at 37°C overnight. The MBC value was determined to be the lowest concentration where no visible growth could be observed.

The activity of plant extracts against phages SU16 and SU27 was assessed using a modified suspension test assay (Pinto *et al.* 2010). In brief, 1 ml of standardized phage suspension was added to 9 ml plant extract at different concentrations (100, 10, and 1% of MIC) and incubated for 24 h at 37°C). Extracts which maintained significant antiviral activity at 1% MIC were further diluted as necessary. After 24 h incubation, 100 μl aliquots were removed and added to 9·9 ml PBS. Suspensions were then serially diluted in PBS and the phage content determined, using the agar overlay method (Adams 1959).

Combined antibacterial activity of phage+extract combinations

Twenty-five milliliter of dsLB was added to 5 ml of extracts in PBS (or PBS with 5% (v/v) DMSO where appropriate), 5 ml of standardised phage suspension and 5 ml standardised bacterial suspension. The total volume was then increased with PBS to a final volume of 50 ml and flasks incubated at 37°C with shaking at 150 rpm min^{-1}. At defined time points (t = 2, 4, 6, 8 and 24 h) 1 ml aliquots were removed from each flask, and 1 ml of fresh LB was added to each flask to maintain volume. For phage containing samples, a 500 μl aliquot was added to 50 μl chloroform, vortexed and phage content determined, using the agar overlay method (Adams 1959). One hundred μl of the original sample was then serially diluted 1 : 10 in PBS and bacterial content determined by spreading 100 μl of each dilution over the surface of duplicate LA plates. For nonphage containing samples, the bacterial content was determined using the drop count method (Miles *et al.* 1938). Plates were incubated overnight at 37°C and the CFU per ml or PFU per ml content determined. Controls containing PBS/PBS+DMSO (untreated) or phage only (no extracts) were also performed.

Statistical analysis

All experiments were performed in triplicate unless stated in the text. Data were expressed as the mean ± standard deviation. Significant differences in the bacterial viable count among each of treated groups at the same time were analysed by one-way ANOVA followed by Tukey's HSD *post hoc* test. The $P < 0·05$ was considered as the statistically significant difference. Also, the significant differences between each tested group and the control were determined, using independent T-tests at $P < 0·05$.

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Conflict of Interest

No conflict of interest to declare.

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