ผลของสารสกัดจากกระชาย (*BOESENBERGIA ROTUNDA* (L) MANSF) และยาปฏิชีวนะที่ยับยั้งผนังเซลล์ต้านแบคทีเรียดื้อยาและการพัฒนาวิธีการตรวจฟีโนไทป์เพื่อตรวจหาเอนไซม์บีตาแลคแทเมส

นายโยธิน ตีไธสง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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EFFECTS OF *BOESENBERGIA ROTUNDA* (L) MANSF EXTRACT AND CELL WALL INHIBITOR ANTIBIOTICS AGAINST DRUG-RESISTANT BACTERIA AND THE DEVELOPMENT OF PHENOTYPIC ASSAYS TO DETECT BETA-LACTAMASES

Yothin Teethaisong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biomedical Sciences

Suranaree University of Technology

Academic Year 2016

EFFECTS OF *BOESENBERGIA ROTUNDA* (L) MANSF EXTRACT AND CELL WALL INHIBITOR ANTIBIOTICS AGAINST DRUG-RESISTANT BACTERIA AND THE DEVELOPMENT OF PHENOTYPIC ASSAYS TO DETECT BETA-LACTAMASES

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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โยธิน ตีไธสง : ผลของสารสกัดจากกระชาย (*BOESENBERGIA ROTUNDA* (L) MANSF) และยาปฏิชีวนะที่ยับยั้งผนังเซลล์ต้านแบคทีเรียดื้อยา และการพัฒนาวิธีการตรวจฟีโนไทป์เพื่อตรวจหาเอนไซม์บีตาแลคแทเมส (EFFECTS OF *BOESENBERGIA ROTUNDA* (L) MANSF EXTRACT AND CELL WALL INHIBITOR ANTIBIOTICS AGAINST DRUG-RESISTANT BACTERIA AND THE DEVELOPMENT OF PHENOTYPIC ASSAYS TO DETECT BETA-LACTAMASES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ เภสัชกร ดร.เกรียงศักดิ์ เอื้อมเก็บ, 181 หน้า.

การดื้อยาต้านจุลชีพกำลังเพิ่มขึ้นในระดับที่น่าเป็นห่วง วัตถุประสงค์ของการศึกษาครั้งนี้คือ เพื่อศึกษาผลของสารสกัดกระชายและยาปฏิชีวนะที่ยับยั้งผนังเซลล์ทั้งที่ใช้เดี่ยว ๆ และใช้ผสมกันในการต้านแบคทีเรียที่ดื้อต่อยาปฏิชีวนะ และเพื่อพัฒนาเครื่องมือวินิจฉัยเพื่อตรวจหาและตรวจแยกเอนไซม์บีตาแลคแทเมสชนิดต่าง ๆ ผลการศึกษาพบว่าสารสกัดกระชายมีฤทธิ์ต้านแบคทีเรียอย่างแรงต่อเชื้อ สแตปฟิโลคอคคัส ออเรียสที่ดื้อต่อยาเมทิซิลิน (เอ็มอาร์เอสเอ) ดีเอ็มเอสที 20651 และ ดีเอ็มเอสที 20652 และต้านเชื้อ สแตปฟิโลคอคคัส อีพิเดอร์มิดิส ดีเอ็มเอสที 14932 ที่ค่ายับยั้งต่ำสุด 16 ไมโครกรัมต่อมิลลิลิตร ขณะที่ไม่พบฤทธิ์ต้านแบคทีเรีย (ค่ายับยั้งต่ำสุด > 1024 ไมโครกรัมต่อมิลลิลิตร) ในการต้านเชื้อแบคทีเรียแกรมลบที่ทดสอบทั้งหมด ยาคลอกซาซิลลินมีค่ายับยั้งต่ำสุดที่ 512 ไมโครกรัมต่อมิลลิลิตร ในการต้านเชื้อ เอ็มอาร์เอสเอ 20651 และ 20652 ผลจากการศึกษาด้วยวิธีเชคเกอร์บอร์ดแสดงให้เห็นว่าสารผสมระหว่างสารสกัดกระชายและยาคลอกซาซิลลินมีค่าดัชนีสัดส่วนการยับยั้งต่ำสุดที่ 0.502 ในการต้านเชื้อทั้ง เอ็มอาร์เอสเอ 20651 และ 20652 ทั้งนี้ไม่พบการเสริมฤทธิ์ของสารผสมระหว่างสารสกัดกระชายและยาเซฟตาซิดีมในการต้านเชื้อแบคทีเรียแกรมบวกที่ทดสอบ กราฟแสดงการตายของเชื้อได้แสดงจำนวนเชื้อเอ็มอาร์เอสเอ 20651 ที่มีชีวิตมีจำนวนลดลงเล็กน้อยหลังจากได้รับสารผสมระหว่างสารสกัดกระชายและยาคลอกซาซิลลิน ขณะที่เชื้อนี้เมื่อได้รับการยากลุ่มอื่น ๆ มีจำนวนเซลล์ที่มีชีวิตเพิ่มขึ้นเล็กน้อย จากการศึกษาด้วยกล้องจุลทรรศน์อิเลคตรอนแบบส่องผ่านแสดงให้เห็นอย่างชัดเจนว่าผนังเซลล์ของเชื้อเอ็มอาร์เอสเอ 20651 ที่ได้รับยาคลอกซาซิลลินเดี่ยว ๆ ได้รับความเสียหายและมีการแตกสลาย ขณะที่เชื้อที่ได้รับสารสกัดกระชายเดี่ยว ๆ พบว่าเยื่อหุ้มเซลล์ได้รับความเสียหายและมีการยุบตัว สำหรับเชื้อเอ็มอาร์เอสเอ 20651 ที่ได้รับสารผสมระหว่างสารสกัดกระชายและยาคลอกซาซิลลินได้รับความเสียหายทั้งผนังเซลล์ และเยื่อหุ้มเซลล์ สำหรับขนาดพื้นที่ของเซลล์ในกลุ่มที่ได้รับการทดสอบทุกกลุ่มพบว่ามีการลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มที่ไม่ได้รับการรักษา (พี < 0.05) ผลจากการทดสอบการซึมผ่านของเยื่อหุ้มเซลล์ด้านในพบว่า สารสกัดกระชายเดี่ยว ๆ ยาไน

ซินเดี่ยว ๆ และสารผสมระหว่างสารสกัดกระชายและยาคลอกซาซิลลินมีผลต่อการเพิ่มการซึมผ่านของเยื่อหุ้มเซลล์ด้านในอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มที่ได้รับยาคลอกซาซิลลินเดี่ยว ๆ และกลุ่มที่ไม่ได้รับการทดสอบ (พี < 0.01) สำหรับฤทธิ์ต้านไบโอฟิล์ม พบว่ากลุ่มที่ได้รับสารสกัดกระชายเดี่ยว ๆ ยาคลอกซาซิลลินเดี่ยว ๆ และสารผสมระหว่างสารสกัดกระชายและยาคลอกซาซิลลินมีการลดลงของชีวมวลของไบโอฟิล์มอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มที่ไม่ได้รับการทดสอบ (พี < 0.01) สำหรับการตรวจหาและตรวจแยกชนิดของเอนไซม์บีตาแลคแทเมส การศึกษาครั้งนี้ได้พัฒนาดิสผสมยาปฏิชีวนะและสารยั้บยั้งเอนไซม์บีตาแลคแทเมสที่จำเพาะร่วมการพัฒนาอาหารเลี้ยงเชื้อแบบกึ่งแข็งที่ผสมกับเรสาซูริน (วิธี อาร์ซีเอ) ซึ่งได้ทดสอบเชื้อในวงศ์เอนเทอโรแบคทีริเอซีอีที่ได้รับการระบุชนิดของเอนไซม์ด้วยเทคนิคทางชีวโมเลกุล ทั้งหมด 86 สายพันธ์ เพื่อทดสอบความเที่ยงตรงของวิธีดังกล่าว ผลการศึกษาพบว่า สามารถตรวจหาและแยกเอนไซม์เมทาโล บีตาแลคแทเมส เอนไซม์เครบซีลลา นิวโมนิอี คาร์บาพีเนมเมส และเอนไซม์ ออกซา-48 คาร์บาพีเนมเมส ซึ่งมีความไวและความจำเพาะร้อยละ 100 อีกทั้งวิธีดังกล่าวยังมีความสามารถยอดเยี่ยมในการตรวจหาและแยกเอนไซม์ชนิดอีเอสบีแอล (ความไวร้อยละ 100 และความจำเพาะร้อยละ 98.6) เอนไซม์ชนิดแอมป์ซี (ความไวร้อยละ 100 และความจำเพาะร้อยละ 96.36) และเอนไซม์ชนิดผสม (ความไวร้อยละ 88.89 และความจำเพาะร้อยละ 100) กล่าวโดยสรุป สารผสมระหว่างยาคลอกซาซิลลินและสารสกัดกระชายยับยั้งการเจริญเติบโตของเชื่อ เอ็มอาร์เอส เอ โดยการยับยั้งที่ผนังเซลล์และทำลายเยื่อหุ้มเซลล์ด้านใน วิธีดิสผสมร่วมกับวิธี อาร์ซีเอ เป็นเทคนิคที่ง่าย ราคาไม่แพง และมีความเที่ยงตรงในการตรวจหาและแยกเอนไซม์ชนิดบีตาแลคแทเมสชนิดต่าง ๆ วิธีดังกล่าวสามารถนำไปใช้ห้องปฏิบัติการทางด้านจุลชีววิทยาทุกที่ เพื่อที่จะตรวจหาเอนไซม์ของแบคทีเรียดื้อยาดังกล่าว

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา _____

YOTHIN TEETHAISONG : EFFECTS OF *BOESENBERGIA ROTUNDA* (L) MANSF EXTRACT AND CELL WALL INHIBITOR ANTIBIOTICS AGAINST DRUG-RESISTANT BACTERIA AND THE DEVELOPMENT OF PHENOTYPIC ASSAYS TO DETECT BETA-LACTAMASES. THESIS ADVISOR : ASSOC. PROF. GRIANGSAK EUMKEB, Ph.D. 181 PP.

BOESENBERGIA ROTUNDA / CELL WALL INHIBITOR ANTIBIOTICS /DRUG-RESISTANT BACTERRIA/PHENOTYPIC TEST/BETA-LACTAMASES

Antimicrobial resistance is increasing at an alarming rate. The objectives of this study were to investigate the effect of *B. rotunda* extract (BRE) and cell wall inhibitor antibiotics, either used alone or in combination against antibiotic-resistant bacteria and to develop a diagnostic tool for rapid screening and discrimination of different types of β-lactamases. BRE exhibited a strong antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) DMST 20651 and DMST 20652 and *Staphylococcus epidermidis* DMST 14932 at a MIC value of 16 μg/mL, whereas no inhibitory activity (MIC > 1024 μg/mL) was observed in all Gram-negative bacteria tested. Cloxacillin (CLX) showed the highest MIC value at 512 μg/mL against MRSA 20651 and 20652. The results from the chequerboard assay revealed that the combination of BRE and CLX exhibited the lowest fraction inhibitory concentration (FIC) index at 0.502 against MRSA 20651 and 20652. No synergistic interaction between BRE and ceftazidime was seen in all Gram-negative bacteria tested. Time-kill curve assay displayed a slight reduction in viable counts of MRSA 20651 treated with the combination of BRE and CLX while other treated groups exhibited the steady increase in viable counts. Transmission electron microscope

clearly showed disruption of the cell wall (CW) in MRSA 20651 treated with CLX alone, whilst BRE alone exhibited collapse of the cytoplasmic membrane (CM). Damage of both CW and CM was observed in cells treated with CLX plus BRE. The cell areas of all treated groups were significantly reduced compared with untreated cells ($p < 0.05$). BRE alone, nisin alone and BRE plus CLX significantly induced CM permeability ($p < 0.01$). The biofilm biomass of MRSA 20651 was significantly reduced by BRE alone, CLX alone and BRE plus CLX compared with the control group ($p < 0.01$). To screen and differentiate distinct types of β-lactamases, a combined disc method along with resazurin chromogenic agar (RCA) has been developed. A total of 86-molecularly characterised β-lactamase-producing Enterobacteriaceae were employed to validate this assay. The results showed that the assay successfully detected and discriminated metallo-β-lactamases, *Klebsiella pneumoniae* carbapenemases and OXA-48 carbapenemases with 100% sensitivity and specificity. For the screening and differentiation of Extended-spectrum-β-lactamases (ESBL), AmpC β-lactamases and co-β-lactamases, the RCA assay exhibited excellent performance in detection of ESBL (100% sensitivity and 98.9 % specificity), AmpC (100% sensitivity and 96.36% specificity) and co-production of ESBL and AmpC (88.89% sensitivity and 100% specificity). In conclusion, the combination of CLX and BRE inhibited the growth of MRSA strains by inhibiting CW and damaging CM. A combined disc method along with RCA assay is very simple, inexpensive and reliable in the detection and discrimination of different types of β-lactamases. It could be exploited in any microbiological laboratory for detecting these bacterial enzymes.

School of Preclinic Student's Signature_____

Academic Year 2016 Advisor's Signature_____

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LIST OF ABBREVIATIONS

ABC	=	ATP-binding cassette
AMP	=	Ampicillin
ATCC	=	American Type Culture Collection
BRE	=	*Boesenbergia rotunda* (L) Mansf extract
CAZ	=	Ceftazidime
CLX	=	Cloxacillin
CFZ	=	Cefazolin
CM	=	Cytoplasmic Membrane
CW	=	Cell wall
CFU	=	Colony Forming Unit
CPD	=	Cefpodoxime
DMST	=	Department of Medical Sciences Thailand
DNA	=	Deoxyribonucleic Acid
EDTA	=	Ethylenediaminetetraacetic Acid
ESBL	=	Extended-spectrum β-lactamase
EPS	=	Extracellular polymeric substance
FIC	=	Fractional Inhibitory Concentration
g	=	Gram

LIST OF ABBREVIATIONS (Continued)

h	=	Hour
HEPES	=	N-2-Hydroxyethyl Piperazine-N'-Ethanesulphonic Acid
HIV	=	Human Immunodeficiency Virus
H_2SO_4	=	Sulfuric acid
HSVI	=	Herpes Virus Type I
MIC	=	Minimum Inhibitory Concentration
mL	=	Millilitre
µL	=	Microlitre
mM	=	Millimolar
MRCNS	=	Methicillin-resistant coagulase-negative *Staphylococci*
MRSA	=	Methicillin-resistant *Staphylococcus aureus*
MSSA	=	Methicillin-susceptible *S. aureus*
MER	=	Meropenem
NaCl	=	Sodium Chloride
NCTC	=	National Collection of Type Cultures
NAT	=	N-Acetyltransferase
NDM-1	=	New Delhi metallo-β-lactamase 1
OD	=	Optical Density
OM	=	Outer Membrane

LIST OF ABBREVIATIONS (Continued)

PBA	=	Phenylboronic acid
RNA	=	Ribonucleic Acid
SCC	=	Staphylococcal chromosomal cassette
TEM	=	Transmission Electron Microscopy
TMC	=	Temocillin
Tris-HCl	=	Trizma Hydrochloride
VRE	=	Vancomycin-resistant *enterococci*
VRSA	=	Vancomycin-resistant *S. aureus*

CHAPTER I

INTRODUCTION

1.1 Introduction

An increasing number of antibiotic-resistant opportunistic pathogens has globally been documented in the recent years resulting in decreasing effective antibiotic availability. Not surprisingly, these problems have created a treatment challenge and pose a serious health risk affecting both hospitalised patients and health care providers (Huttner et al., 2013; National Nosocomial Infections Surveillance System, 2004; Payne, 2008). Infections caused by these bacteria are rapidly widespread by interspecies gene transmission, poor sanitation and hygiene, increasing global trade, international travel and tourism. These refractory bacteria confer resistance by mutations, acquisition of resistant genes and selective pressure facilitated by therapeutic misuse (Aung et al., 2012; Cristino, 1999; Laxminarayan et al., 2013; Rogers et al., 2011; van der Bij and Pitout, 2012). Antibiotic resistance has been reported in such bacteria worldwide, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), carbapenem-resistant *Klebsiella pneumoniae*, fluoroquinolone- and carbapenem-resistant *Pseudomonas aeruginosa*, penicillin- and erythromycin-resistant *Streptococcus pneumoniae*, ampicillin-resistant *Haemophilus influenzae*, multidrug-resistant (MDR) *Acinetobacter baumannii*, extended-spectrum β-lactamase (ESBL)-producing *K. pneumonia*, New Delhi metallo-β-lactamase 1 (NDM-1)-producing

Enterobacteriaceae, MDR *Salmonella enterica* serotypes Choleraesuis and Typhi and carbapenem-resistant *A. baumannii* (de Kraker et al., 2011; Endimiani et al., 2009; Maharat Nakhon Ratchasima Hospital, 2015; Miller and Diep, 2008; National Antimicrobial Resistance Serveillance Centre, 2014; Rahman et al., 2014; Streit et al., 2004).

Currently, MDR Gram-negative bacterial infections, in particular, ESBL- producing Enterobacteriaceae, are among one of the most severe threats to human health (Tacconelli et al., 2014). AmpC β-lactamase producers, ESBL-producers and co-producers of AmpC and ESBL have been reported to be resistant to third-generation cephalosporins, cephamycins or β-lactam/β-lactamase inhibitor combinations (Matsumura et al., 2012). Bacteria co-expressing ESBL and AmpC β-lactamases exhibited higher resistant to fluoroquinolones, aztreonam and β-lactam/lactamase inhibitors than those with either ESBL or AmpC β-lactamases alone (Tsui et al., 2012). Infections caused by AmpC producing organisms are typically associated with resistance to multiple antibiotics, such as penicillins, oxyimino-7-α-methoxycephalosporins and monobactams. These make the selection of an effective antibiotic difficult (Livermore, 1995; Seral et al., 2012). An increasing rate of ESBL in *E. cloacae* have emerged worldwide (Bell et al., 2003; Canton et al., 2002; Crowley and Ratcliffe, 2003; Lahiri et al., 2014; Souna et al., 2014). bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ family genes are the most common ESBL genotypes among *Enterobacter* spp. (Peymani et al., 2014). These ESBL genotypes have characteristically reduced the susceptibility to cefotaxime, ceftazidime, cefepime and/or the monobactam (aztreonam) (Lahlaoui et al., 2012; Livermore and Brown, 2001). Furthermore, AmpC β-lactamase-producing *E. cloacae* is intrinsically resistant to aminopenicillins,

cefazolin and cefoxitin (Zhou et al., 2012). Overproduction of ESBL and AmpC β-lactamases can also be seen among *Citrobacter freundii*, *Serratia marcescens* and *Pseudomonas aeruginosa* (Livermore, 1995).

P. aeruginosa exhibits innately high resistance to a wide spectrum of antibiotics, including anti-pseudomonal penicillins, ceftazidime, carbapenems, aminoglycosides and ciprofloxacin (Dundar and Otkun, 2010). These resistances are mediated by production of AmpC, ESBL and metallo β-lactamase (MBL) enzymes (Bajpai et al., 2014; Manchanda and Singh, 2003; Upadhyay et al., 2010), as well as by restriction of antibiotic uptake through outer membrane and upregulation of efflux pumps (Hancock, 1997; Livermore, 2002). This creates a difficulty for the treatment of infections caused by these bacteria. The carbapenem antibiotics are now the drug of choice for penicillin- or cephalosporin-resistant Gram-negative bacilli (Hurst and Lamb, 2000). However, resistance to carbapenem has been observed in *P. aeruginosa* and *Acinetobacter* spp. (Zafer et al., 2014; Zhao and Hu, 2010). MDR-resistant *A. baumannii* infections have increasingly disseminated in the last decade worldwide. Recently, this pathogen has exhibited resistance to all β-lactams, fluoroquinolones and aminoglycosides by similar mechanisms to those of *P. aeruginosa* and Enterobacteriaceae (Gootz, 2010). Carbapenem-resistant *A. baumannii* is primarily mediated by Ambler's class D OXA-type carbapenemase and class B metallo-β-lactamases (Maragakis and Perl, 2008).

S. aureus is a notoriously causative pathogen of several human diseases, including pneumonia, meningitis, toxic shock syndrome, bacteraemia, wound sepsis, osteomyelitis and endocarditis (Aung et al., 2012; Sandel and McKillip, 2004). It is also the second most frequent bloodstream infection with a fatality rate of

around 15-25% (Laupland et al., 2013). Resistance to penicillin antibiotics has been reported roughly 90-95% worldwide (Casal et al., 2005), 70-80% in the Asian countries were resistant to methicillin (Chambers, 2001) and an epidemic rate in the Middle East (Tokajian, 2014). Methicillin-resistant *S. aureus* (MRSA) has emerged to be one of the most life-threatening pathogens to human health (National Nosocomial Infections Surveillance System, 2004). In Asia, about 67.4% of MRSA infections are hospital-associated infections and 25.5% are community-onset infections (Lai et al., 2014; Song et al., 2011). Resistance to β-lactam in this bacterium has rapidly emerged by the acquisition of an additional penicillin-binding protein 2a (PBP2a) encoded by the *mecA* gene (Pinho et al., 2001; Zapun et al., 2008) and production of β-lactamase mediated by *blaZ* gene (Zhang et al., 2001).

Antibiotic resistance of bacteria in biofilms is well established. A biofilm is a structured community of bacteria embedded in a self-produced extracellular polymeric substance (EPS). In general, EPS composes of polysaccharides, proteins, nucleic acids, lipids and humorous-like substances (Flemming et al., 2007). This matrix makes it difficult to eradicate and causes chronicity of infection (Lewis, 2001). Biofilms can form on many implanted medical devices, such as central venous catheters, heart valves, urinary catheters orthopaedic devices and even contact lenses (Mah and O'Toole, 2001; Stewart and Costerton, 2001). The bacteria growing in a biofilm can become as much as 10-1000 times more resistant to antimicrobial agents compared with normally seen in planktonic cells (Mah and O'Toole, 2001). Failed penetration of the agents, delayed diffusion of antibiotic, and slow-growing or non-growing state are the contributing factors for resistance to antibiotics (Costerton et al., 1999; Donlan and Costerton, 2002).

Accumulation of β-lactamase with an increased upregulation of efflux pumps allows bacteria to survive in the biofilm (Hoiby et al., 2010).

Antimicrobial resistance crisis has increased pharmaceutical industry interest in research and development of new antimicrobial agents (Page and Bush, 2014). Plant-based antimicrobials are always one of the most interesting sources for novel therapeutics because they produce a tremendous variety of secondary metabolites to protect themselves from environmental pathogens (Cowan, 1999; Savoia, 2012). However, plant's small molecules have weaker antimicrobial potency compared with that of bacteria and fungi derived antibiotics. Thus, it should be considered for use in either phytochemical/ phytochemical or phytochemical/antibiotic combination for the treatment of drug-resistant bacteria (Hemaiswarya et al., 2008). Phytochemical and antibiotic combinations have been recommended and studied for combating multidrug-resistant bacteria. This is a promising approach, capable of attacking multiple synergistic drug targets, interacting with drug resistance mechanisms of bacteria, and neutralising and eliminating unwanted adverse effects (Eumkeb and Chukrathok, 2013; Wagner, 2011; Worthington and Melander, 2013).

With regard to these issues, they present an urgent necessity for research and development of novel antibacterial compounds and new strategies to overcome antibiotic-resistant bacteria. To achieve these aims, this thesis, therefore, investigate the antibacterial and antibiofilm activities of *Boesenbergia rotunda* (L) Mansf extract (BRE) and its synergism with cell wall inhibitor antibiotics against drug-resistant bacteria. *B. rotunda* (syn. *Kaempferia pandurata* Roxb or *Boesenbergia pandurata* Roxb), locally known in Thai as "Krachai or Krachai-Dang" and finger

root in English, has been widely cultivated and used as a food ingredient and in folk medicine in several Asian countries (Eng-Chong et al., 2012). It has traditionally been used for the treatments of rheumatism, muscle ache, fever, gouty arthritis, bowel disorder, abdominal distension, carminative, appetite promotion and gastric disorders (Chaudhury and Rafei, 2001), as well as anti-ulcer (Abdelwahab et al., 2011), anti-inflammation, cytotoxic and antioxidant (Isa et al., 2012), anti-dengue-2 virus (Kiat et al., 2006) and anticancer (Kirana et al., 2007). Furthermore, there is a wealth of evidence that panduratin A from *B. pandurata* has strong antimicrobial activity against *Staphylococcus* strains, enterococci, multi-species oral biofilm, *Streptococcus mutans*, *Porphyromonas gingivalis* and *Helicobacter pylori* (Bhamarapravati et al., 2006; Hwang et al., 2004a; Hwang et al., 2004b; Park et al., 2005; Rukayadi et al., 2010; Rukayadi et al., 2009). However, antibacterial and antibiofilm activities of *B. rotunda* extract (BRE) against β-lactam-resistant bacteria have not yet been examined. Also, no work has been carried out on the combination of BRE and cell wall inhibitor antibiotics against those tolerant bacteria. Hence, the present study evaluated the antibacterial and antibiofilm actions of BRE either used singly or in combination with cell wall inhibitor antibiotics against drug-resistant bacteria. The elementary mechanisms of actions, such as cytoplasmic membrane (CM) permeabilisation, transmission electron microscopy, and antibiofilm activity, were also elucidated in this thesis. Development of a diagnostic method for rapid screening and discrimination of extended-spectrum β-lactamase (ESBL), AmpC β-lactamase and co-production of ESBL and AmpC, and *Klebsiella pnuemoniae* carbapenemase (KPC), metallo- β-lactamase (MBL) and OXA-48 carbapenemase is also the aim of the present thesis.

1.2 Research objectives

The objectives of this thesis were:

1.2.1 To investigate antibacterial properties of the *Boesenbergia rotunda* extract (BRE) and cell wall inhibitor antibiotics against drug-resistant bacteria, including *Enterobacter cloacae*, *Escherichia coli*, *Acinetobacter baumannii*, Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis*.

1.2.2 To study the synergistic activity of BRE and cell wall inhibitor antibiotics against antibiotic-resistant bacteria.

1.2.3 To evaluate the elementary mechanism of action of BRE when used singly and in combination with cell wall inhibitor antibiotics by examining bacterial morphology with TEM, cytoplasmic membrane permeability and antibiofim activity.

1.2.4 To develop a combined disc method utilising resazurin chromogenic agar plate for early screening and discrimination of KPC, MBL, OXA-48 carbapenemases, ESBL, AmpC and co-existence of ESBL and AmpC-β-lactamases.

1.3 Research hypothesis

BRE could exhibit potent antibacterial activity, antibiofilm and synergistic interaction with cell wall inhibitor antibiotics against drug-resistant bacteria and their biofilms. The phenotypic tests developed by the present study could show an excellent performance in detection and differentiation of extended-spectrum β-lactamase (ESBL), AmpC β-lactamase and co-production of ESBL and AmpC, and *Klebsiella*

pnuemoniae carbapenemase (KPC), metallo- β-lactamases (MBL) and OXA-48 carbapenemases among Enterobacteriaceae.

1.4 Scope and limitations of the study

The rhizomes of *B. rotunda* or kra-chai were harvested from a local field in Pakthongchai district, Nakhon Ratchasima province, Thailand during August 2014. The clinical isolates of antibiotic-resistant bacteria were obtained from the Department of Medical Science. The reference bacterial strains have been achieved from the American Type Culture Collection (ATCC), USA. The β-lactam antibiotics, other cell wall synthesis inhibitor antibiotics and resazurin were obtained from Sigma-Aldrich, Germany. For bacterial strains used to validate the diagnostic test developed in the present study, 86 β-lactamases-producing Enterobacteriaceae were used. These organisms were locally collected in England during 2012-2015. The characteristic and strain codes are detailed in Appendix B. *E. coli* NCTC 13352 (bla_{TEM-10}) and *E. coli* NCTC 13353 ($bla_{CTX-M-15}$) obtained from the National Collection of Type Cultures (NCTC), the UK was used as ESBL positive controls and *E. cloacae* ATCC BAA-1143 from the American Type Culture Collection, USA was used as a positive control for AmpC β-lactmases.

The antibiotic showing the best synergy effect with BRE against selected resistant bacteria was chosen for further investigation of the mechanism of action.

1.5 Significance of the study

No work has previously been conducted on the effect and mode of action of *B. rotunda* extract (BRE) plus cell wall inhibitor antibiotics against drug-

resistant bacteria, such as *E. cloacae*, *E. coli*, *A. baumannii*, MRSA, and *S. epidermidis*. So, the purpose of this research was to examine the antibacterial and the synergistic effect of naturally occurring phytochemicals from *B. rotunda* and cell wall inhibitor antibiotics, such as ampicillin (AMP), cloxacillin (CLX), cefazolin (CFZ) and ceftazidime (CAZ) when used singly and in combination against these drug-resistant bacteria. Apart from this, the identification of a novel phytochemical extract that showed a synergistic effect with cell wall inhibitors that had lost their original effectiveness, which would enable their use to treat diseases was a research objective of far-reaching importance. A resazurin chromogenic agar (RCA) plate along with a combined disc method developed in the present study. This assay was very simple, inexpensive and reliable in the detection and differentiation carbapenemase-producing Enterobacteriaceae (KPC, MBL and OXA-48), as well as ESBL, AmpC and co-production of ESBL and AmpC β-lactamases. It allowed observation of results within 7 h and could be applicable in any microbiological laboratory. The rapid diagnostic method that could detect and differentiate different types of β-lactamase would improve the effectiveness of antibiotic administration and would also help to control the dissemination of the infection caused by these refractory bacteria.

CHAPTER II

LITERATURE REVIEW

2.1 Overview of *Boesenbergia rotunda* (L) Mansf

Plant-derived antimicrobial mining has become more popular, since the increased dissemination of antibiotic-resistant microbes throughout the world. This is due to plants containing many phytochemicals such as tannins, terpenoids, alkaloids, flavonoids, and polyphenols, which have been reported to have antimicrobial characteristics (Cowan, 1999). To develop novel ethnomedicine, this thesis focuses on *B. rotunda* because it possesses various pharmacological activities, particularly antibacterial activity. *B. rotunda* belongs to the family of Zingiberaceae that is commonly 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 nevertheless 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 2.1.

2.1.1 Phytochemical constituents

Many bioactive compounds have been identified in *B. rotunda,* including Panduratin A, Pinostrobin, Pinocembrin Cardamonin, Boesenbergin A, Boesenbergin

B, Alpinetin, essential oils, (2S)-6-Geranylpinostrobin, Geranyl-2,4-dihydroxy-6-phenethylbenzoate,2′,4′-Dihydroxy-3′-(1′-geranyl)-6′-methoxychalcone, 1′R,2′S,6′R)-2-Hydroxyisopanduratin A, Tectochrysin, (±)-6-Methoxypanduratin A, (2R)-8-Geranylpinostrobin, (2S)-7,8-Dihydro-5-hydroxy-2-methyl-2-(4″-methyl-3″ pentenyl)-8-phenyl-2H, 6H-benzo[1,2-b:5,4-b′]dipyran-6-one, 5,6-Dehydrokawain, Flavokawain C, Nicolaioidesin B, Isopanduratin A1, Isopanduratin A2 and Polyphenols (Eng-Chong et al., 2012; Jantan et al., 2001; Jing et al., 2010; Tan, 2005; Tewtrakul et al., 2003; Tuntiwachwuttikul et al., 1984; Win et al., 2007).

2.1.2 Ethnopharmacological properties of *B. rotunda*

B. rotunda has been used as traditional ethnomedicine for treating several human ailments including rheumatism, muscle ache, fever, gouty arthritis, bowel disorder, abdominal distension, carminative, appetite promotion and gastric disturbances (Chaudhury and Rafei, 2001), as well as anti-ulcer, anti-inflammation, antioxidant, anti-viral, anti-parasite and anticancer (Abdelwahab et al., 2011; Isa et al., 2012; Kiat et al., 2006; Kirana et al., 2007; Sawangjaroen et al., 2005). Interestingly, this herbal plant exhibits strong antibacterial activity with a wide spectrum against pathogenic bacteria (Eng-Chong et al., 2012).

(a)

(b)

(c)

Figure 2.1 Morphology of *B. rotunda*. Whole plant (a), flower (b) and rhizomes (c) (Eng-Chong et al., 2012).

2.1.3 Antimicrobial spectrum of *B. rotunda*

Pinostrobin and a red oil isolated from the rhizomes of *B. rotunda* exhibited 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) of pinostrobin and red oils were 150 and 175 µg/mL, respectively, against this strain (Bhamarapravati et al., 2006). Also, flavonoids from the ethanolic extract of *B. rotunda* exhibited significantly reduced *H. pylori* infection in *Mongolian gerbils* (Mahady et al., 2006). Research on panduratin A, a natural chalcone compound isolated from *Kaempferia pandurata* Roxb (syn. *B. rotunda*), showed stronger *in vitro* anti-staphylococcal activities (MIC 1 µg/mL) compared with more commonly used antimicrobials against clinical staphylococcal isolates (Rukayadi et al., 2009). Moreover, biofilm-producing Enterococcal bacteria were inhibited and eradicated by panduratin A at concentrations of ≤ 4 µg/mL and ≤ 16 µg/mL, respectively, which was stronger than daptomycin and linezolid (Rukayadi et al., 2010). In addition, panduratin A exhibited preventive and inhibitory effects against multi-species oral biofilms, including *Streptococcus mutans*, *Streptococcus sanguis* and *Actinomyces viscosus*. Biofilm mass was reduced > 50% after exposure to panduratin A at 10 µg/mL for 15 min (Yanti et al., 2009). Likewise, panduratin A displayed stronger 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). Crude ethanol extract of rhizomes of *K. pandurata* also showed 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). In addition, isopanduratin A from *K. pandurata* also possessed anti-cariogenic *S. mutans* activity with MIC 4 mg/L, which was much lower than that of sanguinarine, green tea extract, carvacrol, thymol, isoeugenol and eucalyptol (Hwang et al., 2004a). These studies provide evidence that *B. rotunda*, especially panduratin A, could be a good candidate for development of new antibacterial agents.

B. rotunda shows not only a wide spectrum antibacterial activity, but it also exhibits a potential antifungal role. The ethanolic extract of *B. rotunda* revealed inhibitory activity against *Candida albicans* and *Aspergillus fumigatus* (Cheeptham and Towers, 2002). In a time-kill curve assay, *C. albicans* treated with *B. rotunda* displayed faster killing activity than the commercial drug, nystatin. The cell treated with oil extract showed fungistatic activity at concentrations of 1 and 1.5x MIC, while fungicidal activity was observed at concentrations of 2 and 2.5x MIC (Taweechaisupapong et al., 2010). Regarding antiviral activities, hydroxypanduratin A and panduratin A from the methanolic extract of *B. rotunda* rhizome showed high inhibitory action on HIV-1 protease, with IC_{50} values of 5.6 µM and 18.7 µM, respectively. This HIV-1 protease inhibition is associated with the hydroxylation and prenylation of chalcones (Cheenpracha et al., 2006). The highest anti HIV-1 protease activity was seen in HIV-1 treated with cardamonin compared with pinostrobin, pinocembrin, and alpinetin (Tewtrakul et al., 2003). Furthermore, cyclohexenyl chalcone derivatives, 4-hydroxypanduratin A, and panduratin A isolated from fingerroot (*B. rotunda*) exhibited good activities against dengue-2 virus NS3 protease (Kiat et al., 2006). These results, therefore, lead us to believe that hydroxypanduratin

A and panduratin A have a potential effect to inhibit HIV-1 protease and dengue-2 virus NS3 protease.

2.1.4 Bacterial cell architecture and composition

Bacteria are most commonly divided into Gram-positive and Gram-negative bacteria, depending on their staining characteristics. Both types of bacteria have a cytoplasmic membrane that acts as a permeability barrier controlling the transportation of substances into and out of the cell. It also serves as anchoring sites for many proteins and enzymes, including transpeptidase and carboxypeptidase enzymes, as well as serving as cytochrome activity, carrier-mediated transport, and generation of proton motive force (PMF).

The periplasm is the space between the inner and outer membrane of a Gram negative bacterium, and the peptidoglycan lies within it. The periplasm contains enzymes that hydrolyse large molecules, antibiotics, and binding proteins for facilitating transport. The cell wall is a web-like structure that is sometimes called the murein sacculus, composed of peptidoglycan (Figure 2.2). The cell wall allows the cell to maintain shape and osmotic stability. Only Gram-negative bacteria have an outer membrane. Porins and porin-like proteins in the outer membrane allow the membrane to act as a molecular sieve, restricting the accession of some molecules to the cell wall and periplasm. The most clinically significant components of the outer membrane are a phospholipid-like molecule called lipopolysaccharide (LPS) (Madigan et al., 2009; Murray et al., 2009).

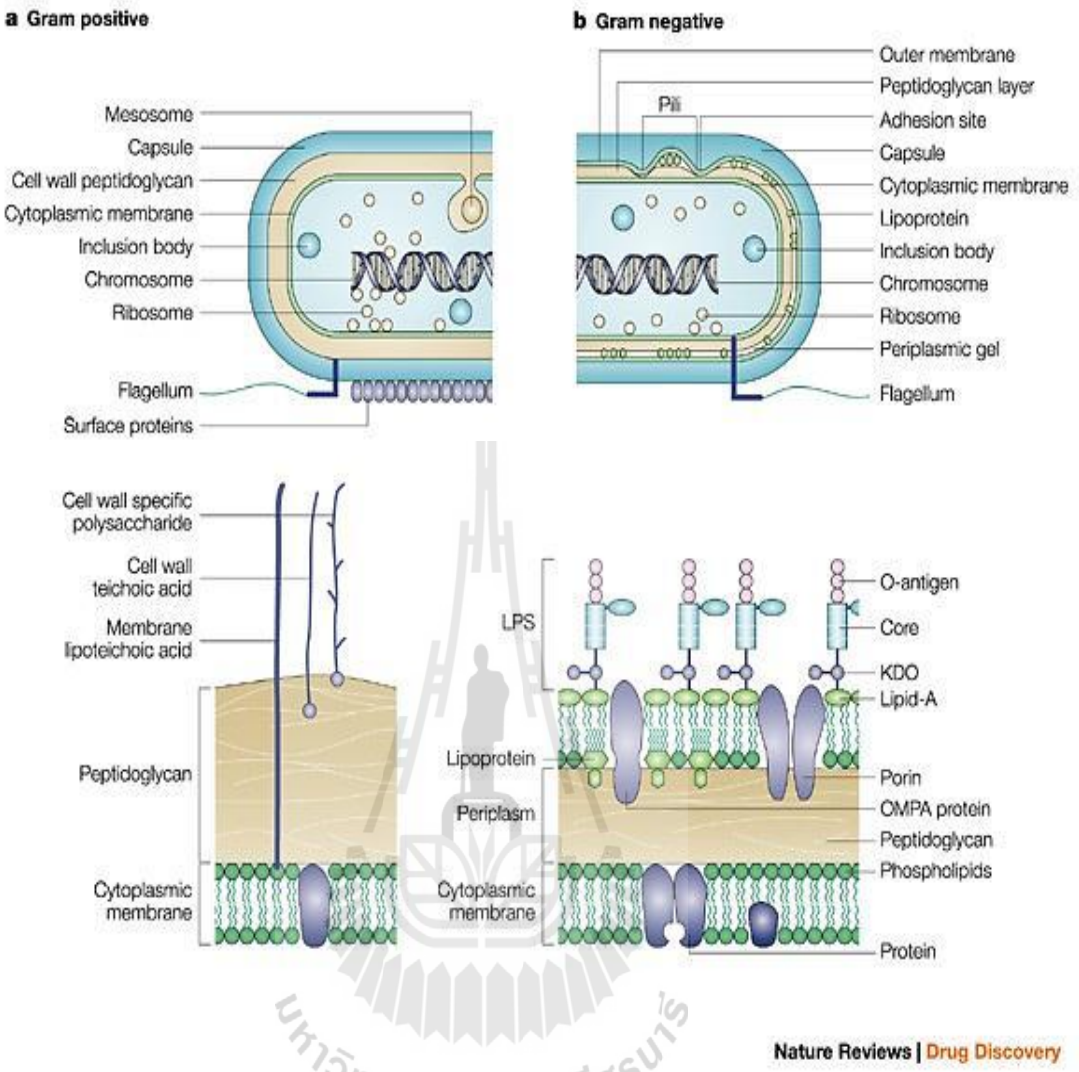


Figure 2.2 Structure and composition of Gram positive bacteria (a) and Gram-negative bacteria (b) (Lolis and Bucala, 2003).

2.1.5 Bacterial pathogens

2.1.5.1 *Staphylococcus aureus*

Staphylococcus aureus, a Gram-positive coccus, belongs to the family Staphylococcaceae. It colonises as the normal microbiota in human mucous, nasopharynx and skin. This bacterium has been recognised to be one of the greatest threats to human health in both community onset and healthcare-acquired infections.

It attributes to a variety of human illnesses, including pneumonia, meningitis, toxic shock syndrome, bacteraemia, wound sepsis, osteomyelitis, and endocarditis (Sandel and McKillip, 2004). Methicillin-resistant *S. aureus* (MRSA) was recorded in the year of the drug's launch. The resistance was conferred by alteration of additional PBP2a encoded by *mecA*, which is a gene located on a mobile element, named staphylococcal chromosomal cassette (SCC) and production of β-lactamase mediated by *blaZ* (Zhang et al., 2001). Normally, susceptible *S. aureus* employs three penicillin-binding proteins, PBPs 1, 2, and 3, to catalyse cross-linking of peptidoglycan. Whereas, PBP2a has low affinity for β-lactams and subsequently is resistant to β-lactams (Livermore, 2000; Pinho et al., 2001). Infections caused by MRSA have higher morbidity and mortality in both community and hospital setting compared with methicillin-sensitive *S. aureus* (Jimenez et al., 2013). In addition to β-lactam resistance, the resistance to vancomycin was also reported in 1996 from a Japanese patient with post-operative wound infection that was refractory to long-term vancomycin therapy. Later, vancomycin-resistant *S. aureus* (VRSA) strains have been increasing reported in several countries, including the USA, France, Korea, South Africa, and Brazil. The emerging problem has confirmed that resistance to vancomycin in *S. aureus* is a global concern (Hiramatsu, 2001).

2.1.5.2 *Staphylococcus epidermidis*

S. epidermidis also belongs to the family Staphylococcaceae. It is primarily a normal inhabitant of the healthy human skin and mucosal microbiota. This bacterium causes numerous nosocomial infections, such as bloodstream infections, cardiovascular infections, and infections of the eye, ear, nose, and throat. *S. epidermidis* is one of the most important causative pathogens in drug abusers and

immuno-compromised patients (patients under immunosuppressive therapy, AIDS patients, and premature newborns). The entry route for invading into the human is usually via an intravascular catheter (Lim and Webb, 2005).

Appoximately 80% of *S. epidermidis* isolates from device-associated infections were resistant to methicillin. Nosocomial *S. epidermidis* isolates are characterised by their pronounced resistance against many practically used antibiotics including methicillin. This resistance is also mediated by the *mecA* gene that is located on large DNA elements that are termed staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* carries a set of recombinases and a wide variety of mobile DNA elements such as transposons, insertion sequences or integrated plasmids (Kozitskaya et al., 2004). Currently, five major SCC*mec* types have been identified, ranging in size from 21 kb to 67 kb, and a recent study of SCC*mec* distribution has provided evidence that *S. epidermidis* can harbour all types of SCC*mec* (Wisplinghoff et al., 2003). Interestingly, SCC*mec* has been shown to be transferable among staphylococcal species. Genome sequencing of the methicillin-resistant *S. epidermidis* RP62A recently revealed the presence of an SCC*mec* type II cassette (Hanssen et al., 2004). Antibiotic resistance and the ability of many nosocomial *S. epidermidis* isolates to form biofilms on inert surfaces made these infections difficult to treat (Ziebuhr et al., 2006).

2.1.5.3 *Acinetobacter baumannii*

Acinetobacter baumannii, belonging to family Moraxellaceae, is a Gram negative coccobacillus and is a strictly aerobic bacterium. *A. baumannii* has become medically significant over the past decade. It causes several infections, including pneumonia (both hospital and community-acquired infections), bacteraemia,

endocarditis, skin and soft tissue infections, urinary tract infections and meningitis (Falagas and Karveli, 2007; McConnell et al., 2013). This bacterium can survive in hospital environments and on medical devices for prolonged periods. Among clinical isolates of *A. baumanii* from ICU patients, approximately 30% were reported to be resistant to at least three classes of antibiotics, including β-lactams and fluoroquinolones (Lockhart et al., 2007). OXA-type β-lactamases 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; Maragakis and Perl, 2008). Furthermore, *Acinetobacter* strains can produce metallo-β-lactamases (IMP and VIM) that render carbapenem antibiotics inactive (Thomson and Bonomo, 2005).

2.1.5.4 *Escherichia coli*

Escherichia coli is a facultative anaerobic and Gram negative rod-shaped bacterium. This bacterium belongs to the family Enterobacteriaceae. It is one of the best and most extensively studied free-living organisms. It is a remarkably diverse species because some *E. coli* strains live as harmless commensals in human and animal intestines, whereas other distinct genotypes are the enteropathogenic, enterohemorrhagic, enteroinvasive, enterotoxigenic and enteroaggregative human intestinal pathogens and exhibit significant morbidity and mortality. Extraintestinal *E. coli* is another varied group of life-threatening pathogens of this manifestly versatile species (Hooton and Stamm, 1997). 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 treating the infections caused by these strains (Denton, 2007; Garau et al., 1999).

2.1.5.5 *Enterobacter cloacae*

Enterobacter cloacae is a Gram-negative rod-shaped and facultative anaerobic bacterium that is classified in the family Enterobacteriaceae. This bacterium is an important nosocomial pathogen causing a variety of infections, including urinary tract, lower respiratory system, skin and soft tissue, biliary tract, wounds, intravenous catheters and the central nervous system (Sanders and Sanders, 1997). The presence of ESBL genotypes has characteristically reduced susceptibility to oxyimino-aminothiazolyl cephalosporins, such as cefotaxime, ceftazidime, cefepime and/or the monobactam (aztreonam) (Lahlaoui et al., 2012; Livermore and Brown, 2001). Furthermore, *E. cloacae* can intrinsically be resistant to aminopenicillins, cefazolin, and cefoxitin due to the production of AmpC β-lactamases (Kluytmans et al., 2013). Likewise, the prevalence of infection by plasmid-mediated AmpC β-lactamases (pAmpC)-producing *E. cloacae*, which can hydrolyse penicillins, oxyimino-, 7-α-methoxycephalosporins, and monobactams, varies depending upon the type of enzyme and geographical location. Infection caused by these enzymes is typically associated with resistance to multiple antibiotics resulting in difficulty when selecting an effective and appropriate antibiotic (Livermore, 1995; Seral et al., 2012). Also, antibiotics currently available for the treatment of multi-drugs resistant *E. cloacae* infections are frequently associated with undesired side effects. Imipenem/cilastatin, often reserved for more serious hospital-acquired infections, is thought to be associated with a higher risk of seizures than other penicillins and carbapenems (Hoffman et al., 2009).

2.1.6 Antimicrobial chemotherapy

Antimicrobial agents can be categorised in some ways by their sites of the mechanism of action, their chemical structure, or the particular types of organisms upon which they are effective (Harvey et al., 2012). However, they are commonly classified based on their mechanisms of action, including cell wall synthesis inhibitors, protein synthesis inhibitors, metabolism and nucleic acid inhibitors, and cell membrane inhibitors, as depicted in Figure 2.2.

2.1.6.1 Cell wall synthesis inhibitors

Inhibitors of bacterial cell wall synthesis selectively inhibit or interfere with some steps in the synthesis of the bacterial cell wall that are composed of a polymer called peptidoglycan. They can inhibit cell wall formation without harming the host cell because the cell wall is not found in mammalian cells. Inhibitors of bacterial cell wall synthesis are more active against rapidly dividing bacteria than dormant or non-dividing bacteria. Drugs grouped in this class, are broadly divided into β-lactams and other antibiotics (non- β-lactams) (Figure 2.3).

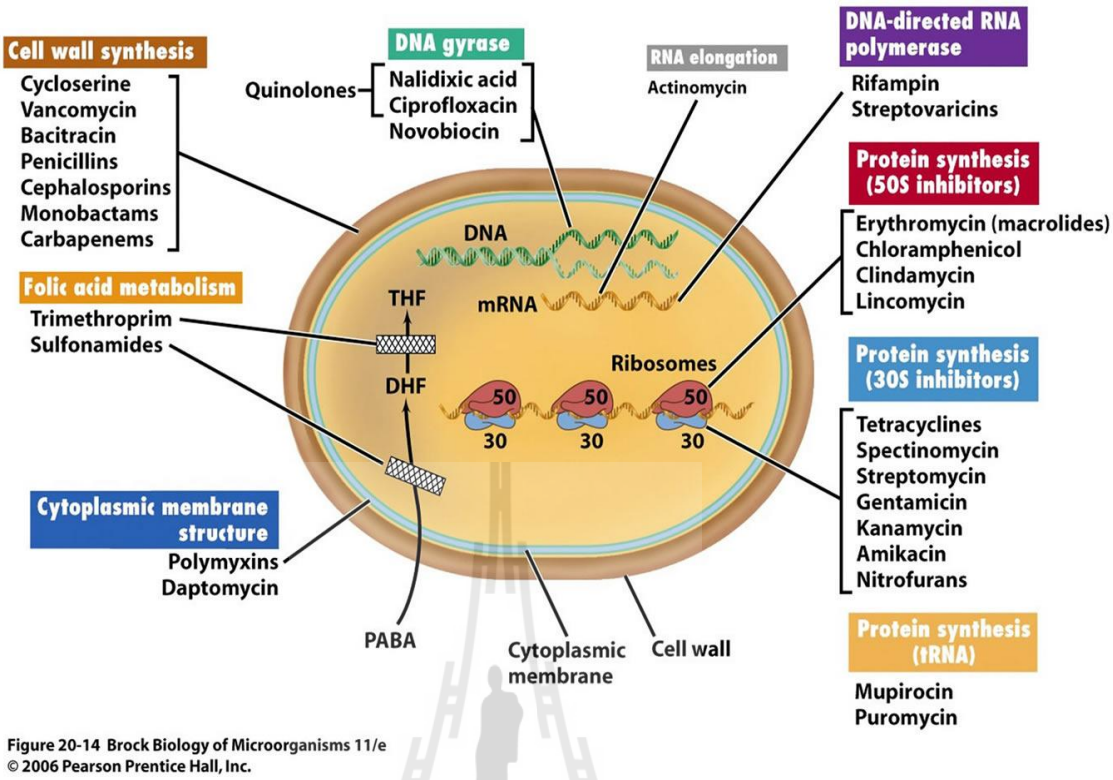


Figure 2.3 Classification of some antimicrobial drugs by their sites of action. THF = Tetrahydrofolate; DHF = Dihydrofolate; PABA= *p*-aminobenzoic acid. (Naghmouchi et al., 2012).

2.1.6.2 β-lactam antibiotics

β-Lactams make up the most well-known antibiotics and are designated by a β-lactam ring that is responsible for their activities. They bind covalently to penicillin binding proteins, (PBPs), a group of enzymes that localise in the cytoplasmic membrane and extend into the periplasm. The PBPs play a crucial role in an assembly, maintenance and regulation of peptidoglycan. β-lactams weaken peptidoglycan by inhibition of some PBPs, resulting in preventing elongation or cross-linking of peptidoglycan (Brenner and Stevens, 2010; Harvey et al., 2012)

There are 4 groups of β-lactam antibiotics; penicillins, cephalosporins (subdivided into 1st generation, 2nd generation, 3rd generation and 4th generation), carbapenems, and monobactams (Figure 2.4). β-lactam antibiotics are widely prescribed in clinical practice because of their high bactericidal activity against a broad selection of bacteria (De Sarro et al., 1995).

Penicillins were the first antibiotics discovered by Alexander Fleming and the most effective antibiotics consisting of a β-lactam ring fused to a thiazolidine ring, with an R group. Drugs in this family are differentiated from others by R substitution (Figure 2.5A). Antibacterial and pharmacological characteristics of a particular type of penicillin are dependent upon the side chain. Penicillins can be grouped based upon their antimicrobial activity, including narrow-spectrum penicillins (penicillin G and penicillin V), penicillinase-resistant penicillins (dicloxacillin and nafcillin), extended-spectrum penicillins (amoxicillin, ampicillin, and antipseudomonal piperacillin and ticarcillin) (Brenner and Stevens, 2010; Harvey et al., 2012).

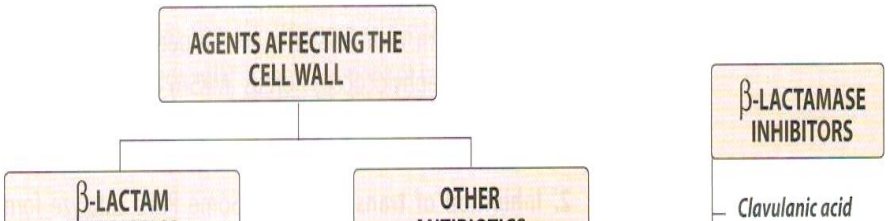


Figure 2.4 Schematic representation of the summary of antimicrobial agents that inhibit bacterial cell wall synthesis. β-lactamase inhibitors are also presented (Harvey et al., 2009).

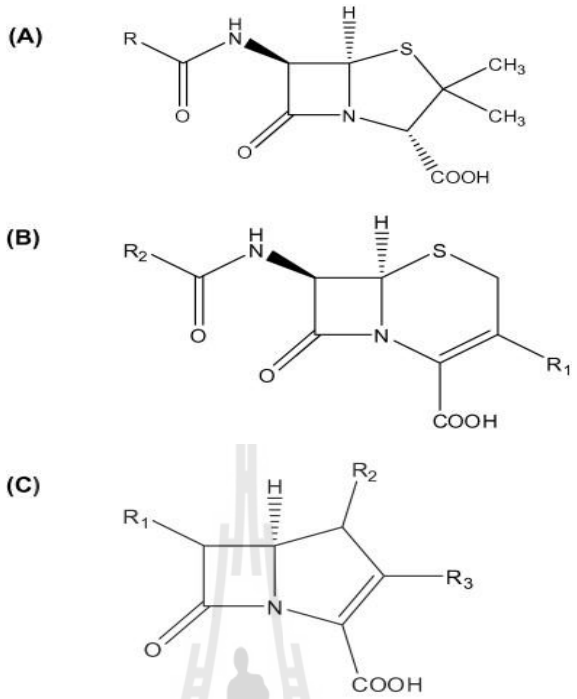


Figure 2.5 General chemical structures of penicillins (A), cephalosporins (B), and carbapenems (C) (Lara et al., 2012).

Cephalosporins, semisynthetic drugs derived from *Cephalosporium* spp., are one of the largest and the most frequently used groups of antibiotics. They have a β-lactam ring and a dihydrothiazine, but unlike penicillin, the cephalosporins have at least two R groups fused to the molecule (Figure 2.5B). The drugs in this class have greater resistance to bacterial β-lactamase with a broader range of antimicrobial spectrum than penicillins. Cephalosporins are subdivided into the 1st generation to 4th generation. The first generation of Cephalosporins shows good activity against most Streptococci. They also cover enteric bacilli, including *E. coli* and *K. pneumonia*. The second-generation cephalosporins are similar to the 1st generation, but this generation cover *Hemophilus influenzae*. The third-generation cephalosporins are active against a wider range of Gram-negative bacteria, which extend to Enterobacteriaceae, *H.*

influenzae, *Moraxella catarrhalis*, and even *P. aeruginosa* (ceftazidime is a drug of choice). Finally, cefepime, a drug in a fourth generation of the cephalosporin, is active against several Gram-negative bacilli with multiple PBPs target and it commonly serves as an alternative drug for cephalosporin resistance (Brenner and Stevens, 2010; Harvey et al., 2012).

Carbapenems are different in structure from penicillin in that the sulphur atom in the thiazolidine ring is substituted with a carbon atom (Figure 2.5C). These agents are active against a wide range of Gram-positive and Gram-negative bacteria, including many aerobic and anaerobic Gram-negative bacilli. Imipenem, meropenem, ertapenem, and doripenem are the only drugs currently commercially available that are classified in this group. Imipenem is highly resistant to hydrolysis by most β-lactamases with an exception for metallo-β-lactamases. So, this drug plays a role in empirical therapy. Meropenem has similar antibacterial characteristics to those of imipenem (Brenner and Stevens, 2010; Harvey et al., 2012).

Monobactams are monocyclic β-lactams (the β-lactam ring is not attached to another ring) that can inhibit the growth of several aerobic Gram-negative bacilli, such as *Enterobacter*, *Citrobacter*, *Klebsiella*, *Proteus* species, and *P. aeruginosa*. Aztreonam is only the drug commercially available. This agent cannot inhibit Gram-positive or anaerobic bacteria (Brenner and Stevens, 2010; Harvey et al., 2012).

2.1.6.3 Other bacterial cell wall synthesis inhibitors

Vancomycin is a tricyclic glycopeptide antibiotic that inhibits cell wall synthesis by binding tightly to the D-alanyl-D-alanine portion of the peptidoglycan precursor. This precludes cross-linking of peptidoglycan by preventing bonding of the penultimate D-alanine to the pentaglycine peptide resulting in weakening the cell wall. This drug has become more increasingly important since multiple drug resistant *S. aureus* and enterococci, as well as methicillin-resistant *S. epidermidis*, have rapidly emerged. It is commonly used as the drug of choice for the treatment of skin and soft tissue infections, penicillin-resistant streptococcal and enterococcal infections-causing endocarditis and necrotizing fasciitis. However, replacement of D-alanine with D-lactate, and decreased drug affinity can confer vancomycin resistance in some strains of staphylococci and enterococci (Brenner and Stevens, 2010; Harvey et al., 2012).

Polymyxins, cyclic polypeptide antibiotics, have gained attention since MDR Gram-negative bacilli have rapidly emerged. Polymyxins have a strong positive charge and a hydrophobic acyl chain that interact electrostatically to the lipid portion of lipopolysaccharide (LPS) resulting in disruption of the membrane, increased membrane permeability, leakage of intracellular components, and subsequently, cell death (Yahav et al., 2012). There are polymyxin A-E, of which only polymyxin B and Polymyxin E (also called colistin) are currently available for sale. Colistin has extensively been used as a salvage therapy that is active against Enterobacteriaceae (including carbapenemase-producing bacteria), *Haemophilus influenzae*, *Legionella pneumophila*, MDR *P. aeruginosa* and *Acinetobacter* spp., *Stenotrophomonas maltophilia* and most pandrug-resistant bacteria (El Solh and Alhajhusain, 2009; Giamarellou, 2010).

2.1.7 General mechanisms of antimicrobial resistance

Bacteria acquire resistance to antimicrobial agents by either passive processes that result from intrinsic mechanisms or active processes that include new mobile genetic elements. The major mechanisms of antimicrobial resistance included the production of degrading enzymes, modification of drug targets, elimination of the drugs, increased efflux pumps and decreased drug uptake (Wright and Sutherland, 2007). These mechanisms can be grouped into three primary mechanisms; those that result in inactivated drugs by enzymatic strategies (alterers), decreased intracellular accumulations (expellers), and altered target sites (blockers) (Figure 2.6).

Bacteria can inactivate antimicrobial drugs by altering or modifying their structure via hydrolysis, group transfer or reduction. β-lactamase, a good example of hydrolytic degradation, can hydrolytically destroy the β-lactam ring of penicillins and cephalosporins. This hydrolytic cleavage is achieved through two main molecular mechanisms: 1) action of an active site serine nucleophile or 2) through activation of water via a Zn^{2+}. Group transfer includes acylation, phosphorylation, thiolation, glycosylation, nucleotidylation, and ribosylation. They partly contribute to aminoglycoside resistance. Moreover, lyases can inactivate antimicrobial drugs via oxidation or non-oxidative carbon cleavage (Edson and Kwon, 2014; Wright, 2005).

Decreased intracellular antibiotic accumulation is mainly due to increase active efflux pumps. Five families of drug effluxes have been identified in prokaryotic systems, including the ATP-binding cassette (ABC) family, major facilitator superfamily (MFS), resistance-nodulation-division (RND) family, multi-antimicrobial extrusion (MATE) family, and small multidrug resistance (SMR)

family. ATP-binding cassette (ABC) requires ATP to be active while other transporters require a proton motive force (Edson and Kwon, 2014).

Additionally, bacteria achieve resistance by alteration or modification in binding sites of antibiotics, which is a common mechanism of resistance. This is due to the reduction in affinity of the target macromolecules for antimicrobial agents. This type of drug resistance arises as a result of antibiotic selection pressure and bacterial mutation. For instance, resistance to rifamycins and quinolone are mediated by mutations in RNA polymerase and DNA gyrase. In addition, *mecA* encoded transpeptidase PBP2a is a major cause of *S. aureus* resistance to methicillin and other β-lactams (Brenner and Stevens, 2010; Edson and Kwon, 2014; Lambert, 2005).

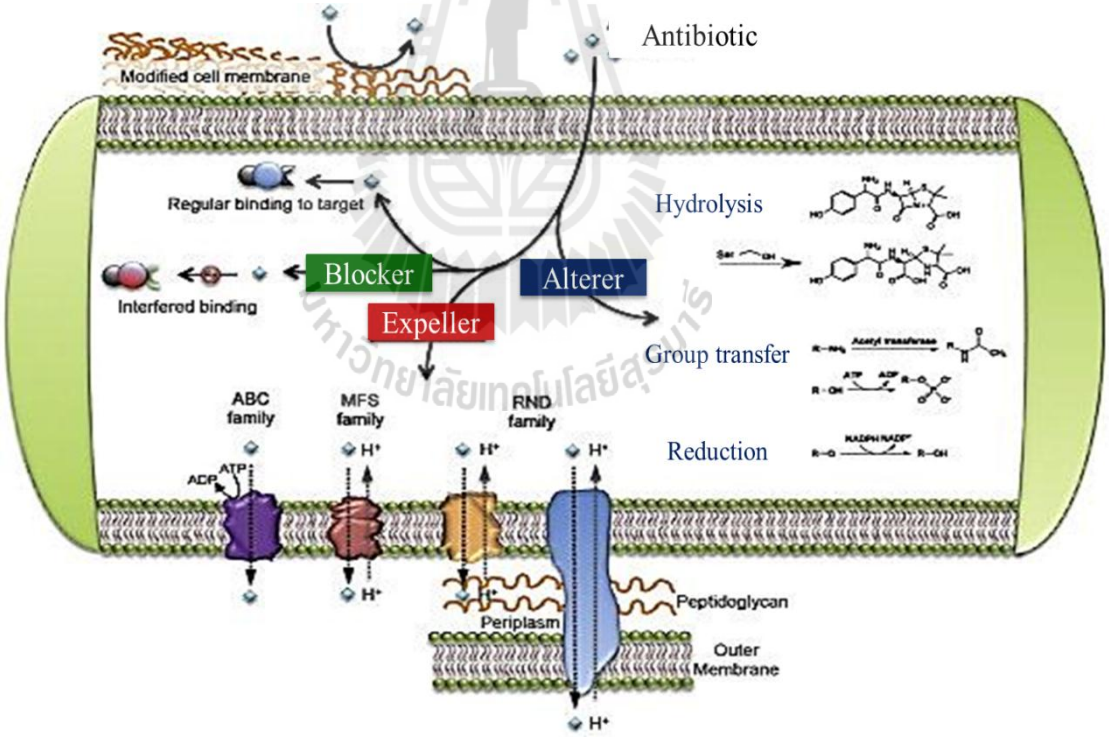


Figure 2.6 Three primary mechanisms of antimicrobial resistance, including 1) inactivation of antimicrobial agents by hydrolysis, group transfer, reduction (alterers), 2) decrease in intracellular drug accumulation by membrane efflux pumps (expellers), and 3) alteration of binding sites (blockers) (Edson and Kwon, 2014).

2.1.8 Beta-lactamase activity and classification

Production of β-lactamase plays a predominant role in resistance to penicillin and other β-lactam antibiotics by cleaving the amide bond in the β-lactam ring resulting in loss bactericidal effectiveness (Gupta, 2007). β-Lactamases are encoded by both chromosomal and plasmid genes. β-Lactamases in Gram-positive bacteria are secreted and released as exoenzymes into the surrounding environment. In Gram-negative bacteria, these enzymes occupy the periplasmic space, where they destroy the β-lactams before they bind PBPs (Brenner and Stevens, 2010). Some Gram-negative rod bacteria can produce powerful β-lactamases called extended-spectrum β-lactamases (ESBL), especially in Enterobacteriaceae bacteria as well as in *P. aeruginosa* and *A. baumannii.* A common working definition of ESBLs is "β-lactamase capable of conferring bacterial resistance to the penicillins, 1^{st}-, 2^{nd}-, and 3^{rd}–generation cephalosporins and aztreonam (monobactam) (but cannot hydrolyse cephamycin and carbapenem) with a hydrolysis rate of at least 10% of benzylpenicillin and inhibited by clavulanate (Bradford, 2001; Naas et al., 2008)).

Although numerous β-lactamases have been identified, these enzymes are most generally classified according to two schemes: basis of functional or biochemical classification by Bush et al. (1995) (Groups 1-4), and molecular classification based upon amino acid sequences (Class A through D) by Ambler et al. (1991) as shown in Table 2.1.

Table 2.1 Ambler molecular classification of β-lactamases. (Modified from (Ambler et al., 1991; Brenner and Stevens, 2010; Gupta, 2007; Sidjabat et al., 2013)

Bush classification	Ambler classification	Characteristics	Examples
Group 1 Cephalosporinases	Class C (AmpC – serine β-lactamase)	-Resistant to β-lactams, except carbapenems. -Not inhibited by clavulanate.	CMY, DHA
Group 2 Penicillinases	Class A (Serine β-lactamase)	-Penicillinase, cephalosporinase are inhibited by clavulanic acid	-TEM, SHV, CTX-M
		-Carbapenemases are inhibited by clavulanic acid	-KPC
	Class D (Serine β-lactamase)	Oxacillinases that are poorly inhibited by clavulanate	OXA-1 OXA-2
	Class D with carbapenemases activity	Carbapenemases that are less inhibited by clavulanate	OXA-48, OXA-181
Group 3 Metallo-β-lactamases	Class B (zinc or metallo-β-lactamase)	Carbapenemases that are resistant to clavulanate	IMP, VIM, NDM
Group 4 Penicillinases	-	Penicillinases that are not inhibited by clavulanate	-

Extended-spectrum β-lactamases (ESBL) are enzymes that have a high and epidemiological importance as a major resistance mechanism of Enterobacteriaceae bacteria and other Gram-negative bacilli to a variety of β-lactams, including cephalosporins and monobactams. The majority of clinically isolated ESBL-producing bacteria are bla_{TEM}, bla_{SHV} and bla_{CTX-M} genotypes (Peymani et al., 2014). The SHV-ESBLs were first discovered in *Klebsiella ozaenae* and found to hydrolyse cefotaxime and ceftazidime efficiently. These enzymes are constitutive the majority of *K. pneumoniae* strains. In addition, SHV-β-lactamases have now been detected in Enterobacteriaceae, *P. aeruginosa*, and *Acinetobacter* spp. (Paterson and

Bonomo, 2005). TEM-β-lactamase was first identified from a patient named "Temoneira", in Athens, Greece so-called TEM-ESBL. These enzymes are more capable of hydrolysing ampicillin at greater rates than oxacillin or cephalothin. TEM-type ESBLs have been reported in genera of Enterobacteriaceae, such as *Enterobacter aerogenes*, *Morganella morganii*, *Proteus mirabilis*, *Proteus rettgeri*, and *Salmonella* spp. However, these enzymes can be inhibited by clavulanate (Bradford, 2001; Paterson and Bonomo, 2005). CTX-M-type-ESBLs (cefotaximase-Munich) are a new family of plasmid-mediated ESBLs that exhibit high hydrolytic activity against, in particular, cefotaxime. Some CTX-M-type ESBLs may hydrolyse ceftazidime resulting in this cephalosporin becoming inactive (Bradford, 2001; Paterson and Bonomo, 2005; Rossolini et al., 2008). Although CTX-M-type ESBLs were discovered after TEM- and SHV-ESBLs, these enzymes have become the predominant ESBLs replacing TEM- and SHV-ESBLs in many European countries and East Asia (Livermore et al., 2007). Characterisation of TEM, SHV, CTX-M-ESBLs in clinically isolated *E. cloacae* in the west of Algeria showed that most strains of *E. cloacae* produced CTX-M type ESBLs (CTX-M-15 and CTX-M-3) while only 5 produced SHV-type ESBLs (SHV-12) (Souna et al., 2014). ESBL-producing Enterobacteriaceae isolated from wastewater treatment plants were studied by PCR and sequencing analysis. The results showed that the predominant ESBL genotypes were bla_{CTX-M} (67.4%) followed by bla_{TEM} (47%), bla_{SHV} (17.4%) and bla_{OXA} (8.3%) (Ojer-Usoz et al., 2014). Identifications of ESBL genes in *P. aeruginosa* from burn patients revealed that the bla_{CTX-M} (2.43%), bla_{VEB-1}(100%), bla_{PER-1}(68.3%), bla_{GES-1} (24.4%), bla_{OXA-1} (70.7%), bla_{OXA-4} (17.1%) and bla_{OXA-10} (92.7%) genes were detected (Shacheraghi et al., 2010).

AmpC β-lactamases are cephalosporinases that have been classified in Amble molecular class C or group 1 of the Bush-Jacoby-Medeiros classification system. These enzymes exhibit less inhibition by clavulanic acid. Plasmid-encoded AmpC β-lactamases-producing bacteria can be detected in numerous pathogens, including *K. pneumoniae*, *E. coli*, *Salmonella* spp., *Proteus mirabilis* and *Citrobacter freundii* (Bauernfeind et al., 1998). AmpC β-lactamases were the first bacterial enzymes found to inactivate penicillin, first identified in *Escherichia coli* in 1940 prior to penicillins being medically introduced (Abraham and Chain, 1940; Jacoby, 2009). Overexpression of chromosomally-encoded AmpC β-lactamases in many Enterobacteriaceae and few other Gram-negative bacilli can mediate resistance to a wide range of β-lactams, including cephalothin, cefazolin, cefoxitin, most penicillins, cefotaxime, ceftazidime and β-lactamase inhibitor/β-lactam combinations. AmpC β-lactamase encoded by both chromosomal and plasmid genes, which can hydrolyse broad-spectrum cephalosporins. Carbapenems are usually used as a drug of choice for treating infections due to AmpC-producing bacteria, but resistance to carbapenems can occur by increasing drug efflux (Jacoby, 2009). CMY-type β-lactamases, especially CMY-2, are highly prevalent pAmpC in *E. coli*, and have been commonly identified in many regions of the world (Doi et al., 2010; Liebana et al., 2013). These enzymes are known to be inhibited by boronic acid (BA), and its derivatives (Beesley et al., 1983) along with cloxacillin (CX) (Jacoby, 2009). BA and its derivatives are known to form reversible covalent bonds with serine protease inhibiting their activities, Kiener and Waley (1978) first demonstrated that BA derivatives inhibit a β-lactamase produced by *Bacillus cereus.*

Carbapenemases have been recognised since imipenem was approved for clinical use in the 1980s (Walsh, 2010). The first carbapenemase (NmcA) was identified in the clinical isolate of *Enterobacter cloacae* in 1993, and since then numerous carbapenemase-encoded genes in Enterobacteriaceae have been identified (Naas and Nordmann, 1994). Three major classes of clinically-important carbapenemases have molecularly been classified; Ambler class A (mostly KPC), class B metallo-β-lactamase (MBL: IMP, VIM, and NDM) and class D enzymes with carbapenemase activity (mostly OXA-48 and OXA-181) (Patel and Bonomo, 2013). Currently, the USA, Israel, Greece, and Italy are endemic for KPC, while OXA-48-producing *K. pneumoniae* and *E. coli* have extensively been identified in North Africa and Turkey. The Indian subcontinent is an important reservoir of NDM-producing-Enterobacteriaceae, especially in *K. pneumoniae* and *E. coli*, as well as KPC and OXA-48 like-producing isolates (mostly OXA-181) (Nordmann and Poirel, 2014).

2.1.9 Detection of β-lactamase-associated resistance

2.1.9.1 Detection of carbapenemases (KPC, MBL, and OXA-48)

Detection of carbapenemase production among Enterobacteriaceae and other Gram negative bacteria is more challenging, as a result of multiple resistance mechanisms in the same strain (Nordmann et al., 2012a). Earlier carbapenemase screening methods relied upon the antimicrobial susceptibility profile determined by the disc diffusion test and minimum inhibitory concentration (MIC) value in the broth dilution method or by an automated system (Miriagou et al., 2010). The cloverleaf test or the Modified Hodge Test (MHT) has been recommended by the Clinical and Laboratory Standards Institute (CLSI) in 2009 as a confirmatory test for carbapenemase production in isolates demonstrating reduced susceptibility to

carbapenem antibiotics. There are several shortcomings of these tests as they have poor specificity and sensitivity and relatively slow turnaround times (Cavalcanti et al., 2012).

Combined disc-inhibitor synergy tests have been used widely to discriminate different classes of carbapenemases. Boronic acids, particularly phenylboronic acid (PBA), have been used to inhibit class A KPC activity, while metal chelating agents such as EDTA and dipicolinic acid (DPA) have been used to inhibit MBL activity (Giske et al., 2011; Nordmann et al., 2012a; Tsakris et al., 2011). The temocillin (TMC) resistance profile (MIC≥ 128 µg ml^{-1} or zone diameter of 30 µg disc ≤ 10 mm) has been suggested as a phenotypic marker of OXA-48-producing Gram negative bacteria where there is a decrease in carbapenem susceptibility and the absence of synergistic effect of carbapenem plus KPC or MBL inhibitors (Hartl et al., 2013; van Dijk et al., 2014; Woodford et al., 2014). A time to result of a disc diffusion method usually takes at least 18 h or overnight. The colorimetric plate containing resazurin showed excellent performance and reproducibility for disc diffusion susceptibility testing in *E. coli* isolates (Sener et al., 2011). Hence, the present study has developed the resazurin chromogenic agar (RCA) plate along with combined disc-inhibitor synergy test for early screening and differentiation of KPC, MBL, and OXA-48 carbapenemases.

2.1.9.2 Detection of ESBL, AmpC and ESBL plus AmpC

A rapid diagnostic test can play a major role in guiding clinicians to appropriate antibiotic administration and minimising treatment failure. β-Lactamase-producing isolates are often resistant to multiple antibiotics, making the decision of the appropriate antibiotic difficult (Tangden and Giske, 2015). Although

simple phenotypic tests for ESBL, AmpC, and MBL, have been proposed by several researchers, and indeed conducted in clinical laboratory practice (Jeong et al., 2009; Yagi et al., 2005), there is no standard guideline for phenotypic detection of AmpC that has been recommended by the CLSI (Clinical Laboratory Standards Institute, 2012). Broth microdilution methods have become more popular in clinical diagnostic laboratories since semi-automated antimicrobial susceptibility test systems have become commercially available. This test generally involves overnight incubation in the determination of antibiotic susceptibility profiling (Yagi et al., 2005). The present study uses cefpodoxime-combined disc method for rapid screening of ESBLs, AmpCs, and co-production of ESBL and AmpC β-lactamases in Enterobacteriaceae.

2.1.10 Biofilm formation and antibiotic resistance

A biofilm is known to be problematic for several human ailments. Bacteria that live in a biofilm present high resistance to antibiotics leading to treatment failure. This is due to biofilms being highly resistant to immune killing and clearance, and to treatment with antimicrobial agents (Bjarnsholt, 2013). Bacterial biofilm can form on either environmental abiotic surfaces or biotic surfaces, and this formation is carried out through 5 stages (Figure 2.7). The first stage is cells attach reversibly to surfaces using a variety of extracellular organelles and proteins, including flagella, pili, fimbriae, curli fibers, and outer membrane proteins. The second stage is irreversible attachment, in which cells secrete an extracellular polymeric substance (EPS) to facilitate adhesion between cells and surfaces. Next is maturation-I. At this stage, bacteria replicate and grow into microcolonies with increased production of EPS. Quorum sensing plays a central role in biofilm formation by modulating cellular functions, including pathogenesis, nutrient uptake,

conjugation, motility, and virulence factors. After this comes maturation-II, in which the biofilm grows into a three-dimensional structure reaching its maximum thickness. The cells in this stage are experiencing no oxygen or reduced oxygen conditions. The matured biofilm can prevent mechanical stress and detach from the surface of the substrate. Finally, in the dispersion stage, bacteria within the community can swim away from cell clusters. This process probably allows bacteria to access better nutrients and environment for propagation and self-renewal of the clusters (Renner and Weibel, 2011; Sauer et al., 2002).

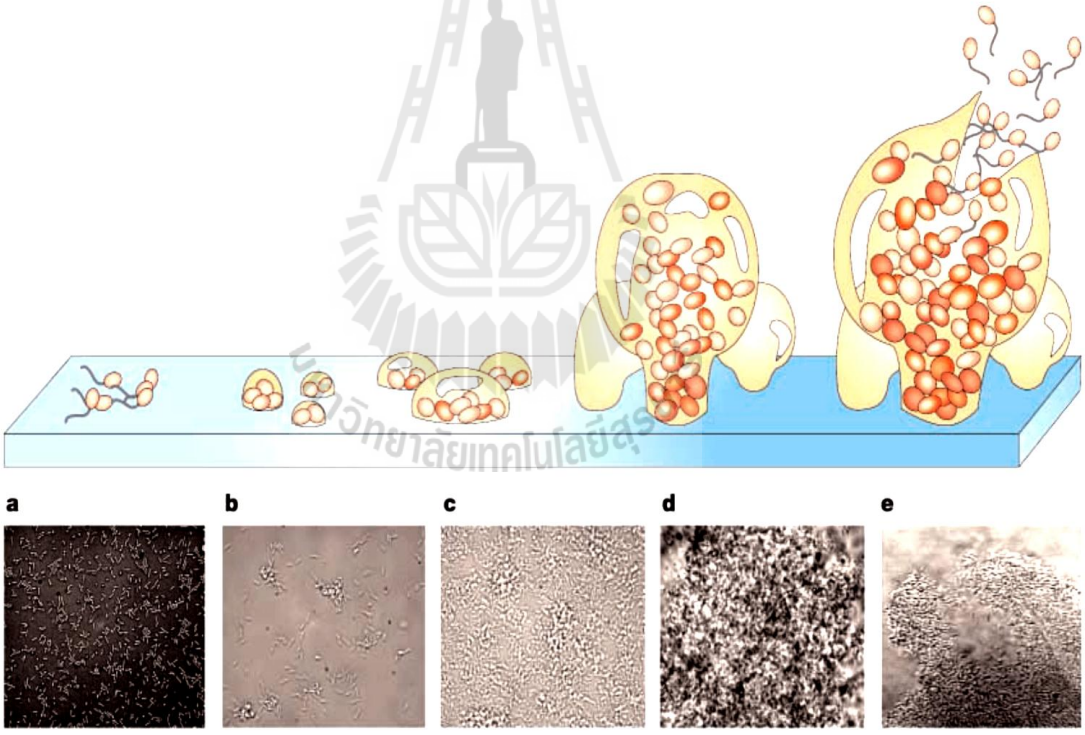


Figure 2.7 Schematic representation of bacterial biofilm development by 5 stages. a = Reversible attachment; b = Irreversible attachment; c = Maturation-I; d = Maturation-II; e = Dispersion stage. (Davies, 2003)

Biofilm formation is mainly governed by cyclic-dimeric-guanosine monophosphate (c-di-GMP), which is a ubiquitous second messenger widely used by

bacteria to control the switch between the motile and sessile form of bacteria, as well as virulence factors. Intracellular levels of this messenger are directly proportional to biofilm formation and EPS production (Karatan and Watnick, 2009). The role of c-di-GMP in governing biofilm formation was first reported in *Vibrio parahaemolyticus* by Boles and McCarter (2002). A small molecule called c-di-GMP is a central regulator of biofilm formation and exopolysaccharide production in *Pseudomonas* and *Burkholderia* species (Borlee et al., 2010; Fazli et al., 2014). Interestingly, an increase in c-di-GMP levels contributes to antimicrobial resistance in *P. aeruginosa* (Gupta et al., 2014).

Treatment of bacteria in the biofilm with antibiotic often fails to kill. This is due to the restriction of drug penetration into its deeper layer, slow growth rate, the presence of persister cells, accumulation of degrading enzyme, and multidrug efflux pumps (Jolivet-Gougeon and Bonnaure-Mallet, 2014; Stewart, 2002). Bacteria embedded in a biofilm exhibit higher antimicrobial resistance compared to cells in planktonic form. For instance, the biofilm-formed cultures of *Actinomyces pyogenes*, *Corynebacterium renale*, *Corynebacterium pseudotuberculosis*, *S. aureus*, *Staphylococcus hyicus* and *Streptococcus agalactiae* in biofilm were resistant to ampicillin, ceftiofur, cloxacillin, oxytetracycline, penicillin G, streptomycin, tetracycline, enrofloxacin, and erythromycin. Whereas, all of these antibiotics were effective against those strains in planktonic cells (Davies, 2003). Likewise, treatment of biofilm-encased *S. aureus* with chlorine requires concentration 600 times higher than that effective on the free-living form of the same species (Luppens et al., 2002). Ito and colleagues (2009) reported that ampicillin treatment did not kill *E. coli* living in mature biofilms, but ofloxacin and kanamycin were effective against biofilm cells.

Currently available antibiotics are most effective against planktonic bacteria, but they are typically ineffective to eradicate bacterial biofilm and persistent infections. So, this indicates a necessity to research and develop novel agents targeting biofilms (Lynch and Abbanat, 2010). Plant-derived antibiofilm agents are of interest. The inhibitory activity of citrus flavonoids, including naringenin, kaempferol, quercetin and apigenin, effectively suppressed cell-cell signalling and biofilm formation of *Vibrio harveyi* and *E. coli* (Vikram et al., 2010). Similarly, flavonoids isoloated from *Moringa oleifera* seed extract had inhibitory effects on biofilms of *S. aureus*, *P. aeruginosa* and *C. albicans* (Osei Sekyere et al., 2015). Riihinen et al. (2014) also reported that flavonol glycosides and procyanidins purified from lingonberry (*Vaccinium vitis-idaea* L.) showed anti-biofilm activity against *Streptococcus mutans*. Salvipisone extracted from hairy roots of *Salvia sclarea* L. also showed anti-biofilm activity against *S. aureus* and *S. epidermidis* (Kuzma et al., 2007). In addition, phytochemical/antibiotic or antibiotic/antibiotic combinations are a promising approach to eradicate bacterial biofilms. Coelho and Pereira (2013) also found the anti-biofilm activity of three essential oils, including cinnamon (*Cinnamomum zeylanicum*), tea tree (*Melaleuca alternifolia*) and palmarosa (*Cymbopogon martini*), either used singly or in combination with ciprofloxacin. They found that *P. aeruginosa* biofilms were inhibited by each of these essential oils. Lower concentrations were observed to inhibit biofilm formation when they were used in combination. N-acetylcysteine, EDTA, ethanol and talactoferrin (TLF) also revealed synergy with some antibiotics against *S. epidermidis* and *C. albicans* biofilms, which are common catheters-indwelling pathogens. These combinations

were proposed to be new regimens for treatment of infected catheters (Venkatesh et al., 2009).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant specimen

B. rotunda or Kra-chai was obtained locally from Pakthongchai district, Nakhon Ratchasima province, Thailand during August 2014. The plant specimen was identified and authenticated by the Forest Herbarium, Bangkok, Thailand. A voucher specimen (BKF No. 192160) was deposited in The Forest Herbarium of Thailand.

3.1.2 Bacterial isolates

The following clinical isolates were employed in the present study; clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) DMST 20651 and DMST 20652, *Staphylococcus epidermidis* DMST 14932, *Enterobacter cloacae* DMST 19719 and *Escherichia coli* DMST 20662 (CREC). These strains were obtained from the Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand. The control strains included *S. aureus* ATCC 29213, *E. coli* ATCC 25922 were obtained the American Type Culture Collection (ATCC), USA.

For detection and differentiation of distinct types of β-lactamases, including ESBL, AmpC, co-production of ESBL and AmpC, KPC, MBL and OXA-48, a total of 86-β-lactamase-producing Enterobacteriaceae clinical isolates were used in this study to validate the performance of rezasurin chromogenic agar plate assay

along with combined disc methods for rapid screening and discrimination of different types of β-lactamases (ESBL, AmpC, co-production of ESBL) and carbapenemases (KPC, MBL, and OXA-48). The bacterial strains employed were *E. coli* (n=25), *K. pneumonia*e (n=29), *E. aerogenes* (n=12), *E. cloacae* (n=13), *Morganella morganii* (n=1), *Citrobacter freundii* (n=4), and *Klebsiella ozaenae* (n=1). These organisms were UK clinical isolates collected from 2012-2015. Their characteristics and strain codes are detailed in Appendix B. *E. coli* NCTC 13352 (bla_{TEM-10}) and *E. coli* NCTC 13353 ($bla_{CTX-M-15}$) obtained from the National Collection of Type Cultures (NCTC), the UK, were used as ESBL positive controls and *E. cloacae* ATCC BAA-1143 from the American Type Culture Collection, USA, was used as a positive control for AmpC β-lactamase.

3.1.3 Antibiotics, chemicals, and equipment

All antibiotics employed in the present study, including ceftazidime, cloxacillin, vancomycin, nisin, ampicillin, cefazolin, meropenem and phenylboronic acid were obtained from Sigma, Bristol-Myers. All chemicals and equipment used are detailed in Appendix C.

3.2 Methods

3.2.1 Extraction of *B. rotunda*

The rhizomes of *B. rotunda* were washed thoroughly with tap water, dried in hot air oven (40 °C) until completely dry and were pulverised using a mechanical grinder. One hundred grammes of the finely ground of dried *B. rontunda* were macerated with 500 ml of 99.9% ethanol for a week at room temperature. The

extracts were filtered, concentrated using a rotatory evaporator and lyophilised (Rukayadi et al., 2008; Salama et al., 2012).

3.2.2 Preliminary qualitative phytochemical screening test

Preliminary qualitative phytochemical screening analysis was executed in accordance with previously described literature with slight modifications (Al-Daihan et al., 2013; Savithramma et al., 2011; Xavier and Johnson, 2013; Yadav and Agarwala, 2011). The extract was tested for its constituent: alkaloids, tannins, flavonoids, saponins, glycosides, steroids and coumarins.

3.2.2.1 Test for alkaloids

Two millilitres of 100 mg/mL ethanol crude extract were added to 2 ml of 1% HCl and was then mixed gently. Two hundred millilitres of Mayer's or Wagner's reagents were added to the extraction mixture. The presence of brown precipitate is evidence of alkaloids.

3.2.2.2 Test for tannins

3.2.2.2.1 Ferric chloride

Two millilitres of 100 mg/mL exact were mixed with 2 ml of 2% $FeCl_3$. The development of blue-green (cathechic tannin) or blue-black (gallic tannin) indicated the presence of tannin

3.2.2.2.2 Gelatine test

Two millilitres of 1% gelatin solution containing 10% NaCl were added to 1 ml of 100 mg/mL extract. The development of white precipitate indicated the presence of tannin.

3.2.2.3 Test for flavonoids

3.2.2.3.1 Alkaline reagent

Two millilitres of the extract (100 mg/mL) were mixed with 2 ml of 2% NaOH. The formation of an intense yellow and turning to colourless after adding few drops of dilute acid (1% HCL), indicating the existence of flavonoids. Quercetin was used as a positive control.

3.2.2.3.2 Lead acetate test

Two millilitres of the extract (100 mg/mL) were mixed with 2 ml of 10% lead acetate solution. The formation of yellow precipitate indicated the presence of flavonoids.

3.2.2.4 Test for saponins

Four millilitres of the extract (100 mg/mL) were shaken vigorously for 15 minutes. The production of persistent foam for 10 min was considered as the presence of saponins.

3.2.2.5 Tests for Glycosides

3.2.2.5.1 Liebermann's test

Two millilitres of the extract at the concentration of 100 mg/mL were mixed with 2 mL of chloroform and 2 mL of acetic acid. The mixture was then cooled in ice and carefully added a few drops of concentrated H_2SO_4. A change in colour from violet to blue or green indicated the presence of glycosides.

3.2.2.5.2 Salkowski's test

Two millilitres of the extract were added to 2 mL of chloroform. Then, 2 mL of concentrated H_2SO_4 was added carefully to the mixture and shaken gently. A reddish brown colour indicated the presence of glycoside.

3.2.2.6 Test for steroids

Two millilitres of the extract were mixed with 2 mL of chloroform and concentrated H_2SO_4. Production of a red colour in the lower chloroform layer was considered as the existence of steroids.

3.2.3 Preliminary quantitative flavonoids screening analysis

Total flavonoid content was quantified following the previous reports (Chatatikun and Chiabchalard, 2013; Miliauskas et al., 2004). The aluminium chloride colorimetric assay was employed. The BRE extract and standard quercetin were prepared by dissolving in 80% ethanol prior to adding 50 µL of extract (100 mg/mL) or quercetin at varying concentrations to 10 µL of 10% aluminium chloride accompanied by 150 µL of 95% ethanol. An aliquot of 10 µL of 1 M sodium acetate was then added to the mixture in 96 well-plate. The mixture was incubated at room temperature for 40 min in the absence of light. The absorbance in this assay was measured at a wavelength of 415 nm using a microplate reader. A solution of 95% ethanol was used as a blank solution. The total flavonoid content of the extract was quantified by comparing with standard quercetin. The data were expressed in mg quercetin equivalents (QE) per 100 mg extract. The standard curve of quercetin was carried out at concentrations of 1, 2, 3, 4 and 5 mg/mL.

3.2.4 Antibacterial activity and mechanism of action of *B. rotunda* extract

3.2.4.1 Bacterial suspension standard curve

This assay was carried out to select bacterial suspensions with a known viable count. Twenty microlitres of bacteria in 30% glycerol stock that have been stored in -80 °C were transferred to 50 mL of Mueller Hinton broth and incubated at 37 °C for 18 h with shaking at 120 oscillations/min. The cells were

pelleted by centrifugation at 3,000 x g for 10 min and then washed twice by resuspending in 0.85% NaCl and centrifuging at 3,000 x g for 5 min. The cells were adjusted with 0.85% of sterile NaCl to obtain optical densities (OD) at 0.05, 0.1, 0.15, 0.2, 0.25 at a wavelength of 600 nm. One hundred microlitres of dilutions with each optical density were 10-fold diluted in 900 μL of normal saline to enumerate 3-50 colonies. Ten microlitres from each dilution were dropped onto agar medium. Following 20 h of incubation at 37 °C, the growing colonies were counted and then plotted as a bacterial suspension standard curve. The experiment was performed in six replications. The number of colony forming units (CFU) per millilitre was calculated as follows; mean counted colonies x dilution factor x 100 (Eumkeb, 1999; Munsch-Alatossava et al., 2007; Richards et al., 1993).

3.2.4.2 Minimum inhibitory concentration (MIC) determinations

Microdilution with the resazurin reduction assay was conducted to determine MIC values for *B. rotunda* extract (BRE) and antibiotics. Resazurin was first used to evaluate the antibacterial activity in 1995 by Zabransky and colleagues (Zabransky et al., 1995). It has been extensively used as an indicator of cell growth, cell viability and toxicity (Palomino et al., 2002), in particular antibacterial screening of phytochemicals (Mann and Markham, 1998; Salvat et al., 2001; Sarker et al., 2007), as well as for screening of antitubercular activity against *Mycobacterium* spp. (Taneja and Tyagi, 2007). The *in vitro* antimicrobial susceptibility testing was performed using a 96-well microtitre plate with resazurin dye according to the modified method previously described by the Clinical Laboratory Standards Institute (2014); Elavarasan et al. (2013); Taneja and Tyagi (2007).

Antibiotics were prepared following the manufacturer's instruction to obtain 10,240 µg/mL as a stock solution. BRE was dissolved in 5% DMSO to obtain a stock solution at a concentration of 10,240 µg/mL. Resazurin was prepared by dissolving in sterile distilled water to obtain 0.02% (w/v) and was then sterilised by filtration through a 0.2 µm-pore syringe filter. Antibiotics and BRE were twofold serially diluted to obtain stock solutions at varying concentrations. Twenty microlitres of each concentration were added to the well containing 120 µL of culture medium and 20 µL of 0.02% resazurin was added to all wells. Prior to adding 20 µL of test bacterium, an 18 h-culture was adjusted according to bacterial suspension standard curve to obtain approximately 1 x 10^8 CFU/mL and then diluted with 0.85% NaCl to give 5 x 10^6 CFU/mL so that the final concentration of inoculum was 5 x 10^5 CFU/mL. The total volume in each well was 200 µL, and the final concentration of BRE and antimicrobial agents ranged from 0.5-1024 µg/mL. Wells without antibacterial agents and bacteria were used as controls. Wells containing 10% and 5% DMSO were also used as controls. The microplate was then incubated at 37 °C for 20 h. The lowest concentration showing no colorimetric change from blue (resazurin) to pink (resorufin) was considered as the MIC value.

3.2.4.3 Chequerboard determinations

The interaction between BRE and the antibiotic combination was evaluated by a chequerboard assay following Bonapace and colleagues (Bonapace et al., 2002). The bacterial culture and antibacterial agents were prepared and performed similarly to the MIC determination. Differently, the BRE and antibiotic were combined and incubated at 37 °C for 20 h. The lowest concentration of antibacterial agents in combination showing no visible growth was selected to

calculate the fraction inhibitory concentration (FIC). The FIC index (FICI) was calculated to determine drug interaction and interpreted as shown below (Marques et al., 1997)

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

FICI $\leq$ 0.5 denoting synergism

FICI > 0.5- <1.0 denoting partial synergism

FICI = 1 denoting addition

FICI > 1- $\leq$ 4.0 denoting indifference

FICI > 4.0 denoting antagonism

3.2.4.4 Time-kill curve determination

Killing curve determination was carried out to confirm antibacterial and synergistic activities of BRE when used alone and in combination with and antibiotic by selecting the lowest FIC index. The viabilities of drug-resistant bacteria after exposure to BRE or antibiotics alone and in combination at nine distinct exposure times (0, 0.5, 1, 2, 4, 6, 8 and 24 h) were counted. The assay was performed according to a previous report with some modifications (Aung et al., 2012). Inocula (5 x 10^5 CFU/mL) were challenged with BRE, either singly or in combination with antibiotics. Aliquots (0.1 mL) of each exposed time were diluted in 0.9 mL normal salines to enumerate 3-50 colonies. Ten microlitres from each dilution were dropped on plates containing particular agar medium. Following incubation at 37 °C for 20 h, the growing colonies were counted and then plotted as a time-kill curve. The

experiment was performed in six replications. Decreases of ≥ 2 log10 in CFU/mL between the combination and these obtained by the agents alone at 24 h were termed synergistic interactions. Additive or indifferent interactions were defined as < 2 log10 CFU/mL reduction. Increases of ≥ 2 log10 in CFU/mL at 24 h were denoted as antagonism. In addition, the bactericidal and bacteriostatic activity of antibacterial agents could be seen in this experiment by which ≥ 3 log10 increase or < 3 log10 reduction in CFU, respectively at 24 h compared with the starting inoculum (Lee and Burgess, 2013).

3.2.4.5 Transmission electronmicroscopy (TEM) method

TEM was conducted to examine ultrastructure damages of bacteria treated with BRE either alone or in combination with antibiotic. The specimens were prepared in accordance with a previous report (Richards et al., 1995) with slight modifications. An 18 h culture of bacteria was adjusted spectrophotometrically to reach a final concentration of approximately 5 x 10^6 CFU/mL. The culture was allowed to grow in the absence of antibacterial agent (control), BRE alone, antibiotics alone and BRE plus antibiotic at sub-MIC, for 4 h with shaking 110 oscillations/min in a water bath at 37 °C. Then, the culture was harvested by centrifugation at 6000 x g for 15 min at 4 °C and the pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 12 h. The samples were then carefully washed twice with 0.1 M phosphate buffer. The post-fixation was carried out with 1% osmium tetroxide (EMS) in 0.1 M phosphate buffer (pH 7.2) for 2 h at room temperature. After washing in the buffer, the samples were gently dehydrated with graded acetone solutions (20%, 40%, 60%, 80% and 100%, respectively) for 15 min each. Afterwards, infiltration and embedding were performed

using Spurs resin (EMS), the block resin was sectioned using an ultramicrotome with a diamond knife and mounted on copper grids. Ultimately, the ultrathin sections were counterstained with 2% (w/v) uranyl acetate for 15 min and then 0.25% (w/v) lead citrate for 15 min. Following staining, the specimens were visualised and images captured with a Tecnai G2 electron microscope (FEI, USA), operating at 100 kV. In addition, the cell area from micrographs was calculated by measuring cell width multiplied by cell length (nm^2) to confirm the effects of BRE either used singly or in combination with antibiotic on cell size.

3.2.4.6 Cytoplasmic membrane permeability

The cytoplasmic membrane permeabilisation experiment was executed as previously described by Carson et al. (2002); Shen et al. (2012) with some modifications. BRE alone or in combination with antibiotic-induced cytoplasmic membrane damage was measured as the release of OD_{260}-absorbing material using a UV-VIS spectrophotometer. The bacteria were prepared and cultured according to CLSI. Two millilitres of inocula were added to 98.0 mL of specific media and shaken at 110 r.p.m. at 37 °C for 4 h. Bacterial cultures were harvested and adjusted in normal saline to give 5 x 10^6 CFU/mL. Five millilitres of adjusted cultures were added to 45 mL of 2.5 mM sodium HEPES buffer (pH 7.2) supplemented with 100 mM glucose plus of BRE or antibiotic alone at half-MIC concentrations or the combination of antibiotic and BRE at FIC concentrations. The flask containing no antibacterial was used as a negative control and with nisin (for Gram-positive bacteria) or polymixin B (for Gram-negative bacteria) used as a positive control. The bacterial suspensions were incubated at 37 °C in a shaking water bath. CM permeability was determined after a contact time of 0, 0.5, 1.0, 2.0, 3.0 and 4.0 h. by

taking 3.0 mL of each sample from each contact time and then filtered through a sterile nitrate cellulose membrane (0.2 µm). An OD_{260} value of the supernatant was indicated by UV-absorbing materials released by cells. All the measurements were carried out in three replicate experiments in a Varian Cary 1E UV/ VIS spectrophotometer.

3.2.5 Antibiofilm activity of *B. rotunda* Extract

3.2.5.1 Quantification of biofilm formation inhibition

The inhibitory effects of BRE either alone or in combination on biofilm development were evaluated as previously described by Gopal et al. (2014) with some modifications. An 18-h culture of test bacteria was adjusted in saline to give 5 x 10^6 CFU/mL. Twenty microlitres of adjusted bacterial suspension were added to 190 µL of broth medium supplemented with 0.2% glucose plus BRE, or antibiotic alone, or in combination. The concentration at half-MIC or FIC of BRE or antibiotic was employed. Following incubation at 37 °C for 48 h, the culture medium was discarded, and free-floating planktonic cells were washed with distilled water. The adhered biofilm in the well bottoms was stained with 0.4% (w/v) crystal violet solution for 30 min. The excess stain was washed off thoroughly with distilled water. After the wells are air-dried, 100% ethanol was added to each well to solubilise stained biofilm before measuring and absorbance using a microplate reader at 595 nm. The optical density values represented adhering bacteria and forming a biofilm.

3.2.5.2 Microscopic analysis of bacterial biofilm inhibition

The light microscope analysis of bacterial biofilm was examined following the method of Nithya and Pandian (2010) and Packiavathy et al. (2014) with few modifications. Two hundred microlitres of bacteria adjusted from

18-h culture to 5 x 10^6 CFU/mL were added to 1800 µl of fresh medium (supplemented with 0.2% glucose) containing BRE either alone or in combination with antibiotics in 24-well polystyrene plate. Wells with and without test bacteria were used as controls. The plate was incubated at 37 °C for 48 h. Afterwards, the planktonic cells were removed by rinsing with PBS buffer, and the adhered cells were stained with 0.4% crystal violet solution. The excess crystal violet was washed off by repeatedly submerging the plate into reservoirs of distilled water, which was then shaken. The plate was then placed in an incubator at 37 °C until dry. The stained biofilm was viewed under a light microscope.

3.2.6 Development of diagnostic methods for detection of β-lactamases

The development of quick and accurate methods for the detection and characterisation of different types of β-lactamase remains an urgent necessity. Early detection is crucial in guiding appropriate antimicrobial therapy. Current methods of detection include disc diffusion assays, broth microdilution methods and multiplex PCR (Gupta et al., 2012; Jacoby, 2009; Jeong et al., 2009). Moreover, some phenotypic assays require a freeze-thawed (five to seven times), and concentrated pellet rather than the whole cell culture (Lee et al., 2005; Nasim et al., 2004). All the above detection methods for the production of β-lactamase are either time-consuming (approximately 24 to 48 h after initial isolation of the pathogen) or expensive (Nordmann et al., 2012b).

Phenotypic tests for β-lactamase production are carried out in most clinical diagnostic laboratories because they are easy to implement and are cost effective. This detection is important regarding epidemiological purposes and to restrict the dissemination of β-lactamase-mediated resistance mechanisms (Poulou et

al., 2014). Phenotypic tests alone cannot distinguish between the specific *bla* genes of β-lactamases (TEM, SHV, CTX for ESBLs, CMY, MOX for AmpCs, and IMP, NDM, VIM for MBLs) (Pitout and Laupland, 2008; Pitout et al., 2010), therefore, several reference laboratories use molecular methods for identification of those β-lactamase-encoding genes. Molecular approaches are restricted largely to reference laboratories for epidemiological studies because of the complexity and diversity of distinct point mutations of β-lactamases (Sundsfjord et al., 2004)

3.2.6.1 Genotypic test using multiplex PCR and sequencing

Molecular typing of a total of 86 β-lactamases-producing Enterobacteriaceae were was carried out by multiplex PCR and sequencing. The primer sets used were β-lactamases specific, including bla_{ESBL} family genes, bla_{ampC} family genes and carbapenemase genes (bla_{NDM}, bla_{KPC}, bla_{IMP}, bla_{VIM} and bla_{OXA-48}. The primer sequences and primer concentration used in this study are shown in Table 3.1, 3.2 and 3.3, respectively.

To prepare template DNA, 1 mL of 18 h-culture was taken to a microcentrifuge tube and was then pelleted by centrifugation at 3,000 x g. Cells were resuspended in 1 ml of distilled water prior to lysing by boiling at 95 °C for 10 min. Cellular debris was removed by centrifugation at 17,000 x g for 5 min, and 2 mL of supernatant was used a template DNA for amplification (Dallenne et al., 2010; Pérez-Pérez and Hanson, 2002).

A multiplex PCR was carried out with a final volume of 50 μL using a one-step PCR master mix (Thermo Scientific, UK). The experiment was performed according to a previous report by Pagès and Amaral (2009) with slight modification. Each reaction contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM each

deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 1.2 U of *Taq* DNA polymerase, primers and 2 μL of template DNA. The tube containing DNase-free water in place of template DNA was used as a negative control. Amplification of *ESBL* family genes and carbapenemase genes were performed as follows: an initial denaturation at 95 °C for 5 min; 30 cycles of a DNA denaturation at 95 °C for 30 s, an annealing at 60 °C for 30 s, an extension at 72 °C for 1 min. The final extension was performed after the last cycle at 72 °C for 7 min. For amplification of bla_{ampC} family genes, the programme consisted of an initial denaturation at 95 °C for 5 min; 30 cycles of a DNA denaturation at 95 °C for 40 s, an annealing at 64 °C for 40 s, an extension at 72 °C for 1 min and a final extension at 72 °C for 7 min.

Table 3.1 Primer sequences for detection of bla_{ampC} family genes (Pérez-Pérez and Hanson, 2002)

Target Genes	Sequences (5′→3′)	Primer concentration (μM)	Product size
bla_{MOXM} (F)	GCTGCTCAAGGAGCACAGGAT	0.5	520
bla_{MOXM} (R)	CACATTGACATAGGTGTGGTGC	0.5	
bla_{CITM} (F)	TGGCCAGAACTGACAGGCAAA	0.5	462
bla_{CITM} (R)	TTTCTCCTGAACGTGGCTGGC	0.5	
bla_{DHAM} (F)	AACTTTCACAGGTGTGCTGGGT	0.5	405
bla_{DHAM} (R)	CCGTACGCATACTGGCTTTGC	0.5	
bla_{ACCM}(F)	AACAGCCTCAGCAGCCGGTTA	0.5	346
bla_{ACCM} (R)	TTCGCCGCAATCATCCCTAGC	0.5	
bla_{EBCM}(F)	TCGGTAAAGCCGATGTTGCGG	0.5	302
bla_{EBCM}(R)	CTTCCACTGCGGCTGCCAGTT	0.5	
bla_{FOXM} (F)	AACATGGGGTATCAGGGAGATG	0.5	190
bla_{FOX-M} (R)	CAAAGCGCGTAACCGGATTGG	0.5	

Table 3.2 Primer sequences for detection of *bla*$_{ESBL}$ family genes (Dallenne et al., 2010)

Target Genes	Sequences (5′→3′)	Primer concentration (μM)	Product size
Multiplex I			
bla$_{\text{TEM}}$ (F)	CATTTCCGTGTCGCCCTTATTC	0.5	800
bla$_{\text{TEM}}$ (R)	CGTTCATCCATAGTTGCCTGAC	0.5	
bla$_{\text{SHV}}$ (F)	AGCCGCTTGAGCAAATTAAAC	0.5	713
bla$_{\text{SHV}}$ (R)	ATCCCGCAGATAAATCACCAC	0.5	
Multiplex II			
bla$_{\text{CTX-M group 1}}$ (F)	TTAGGAARTGTGCCGCTGYA[a]	0.5	688
bla$_{\text{CTX-M group 1}}$ (R)	CGATATCGTTGGTGGTRCCAT[a]	0.5	
bla$_{\text{CTX-M group 2}}$ (F)	CGTTAACGGCACGATGAC	0.5	404
bla$_{\text{CTX-M group 2}}$ (R)	CGATATCGTTGGTGGTRCCAT[a]	0.5	
bla$_{\text{CTX-M group 9}}$(F)	TCAAGCCTGCCGATCTGGT	0.5	561
bla$_{\text{CTX-M group 9}}$ (R)	TGATTCTCGCCGCTGAAG	0.5	
bla$_{\text{CTX-M group 8/15}}$ (F)	AACRCRCAGACGCTCTAC[a]	0.5	326
bla$_{\text{CTX-M group 8/15}}$ (R)	TCGAGCCGGAASGTGTYAT[a]	0.5	

[a] = Y=T or C; R=A or G; S=G or C; D=A or G or T.

Table 3.3 Primer sequences for detection of carbapenemase family genes (Dallenne et al., 2010; Kaase et al., 2012)

Target Genes	Sequences (5′→3′)	Primer concentration (μM)	Product size
bla$_{\text{NDM-type}}$ (F)	CCTCAACTGGATCAAGCAGG	0.4	341
bla$_{\text{NDM-type}}$ (R)	CCTTGCTGTCCTTGATCAGG	0.4	
bla$_{\text{KPC-type}}$ (F)	CATTCAAGGGCTTTCTTGCTGC	0.4	538
bla$_{\text{KPC-type}}$ (R)	ACGACGGCATAGTCATTTGC	0.4	
bla$_{\text{VIM-type}}$ (F)	GATGGTGTTTGGTCGCATA	0.5	390
bla$_{\text{VIM-type}}$ (R)	CGAATGCGCAGCACCAG	0.5	
bla$_{\text{OXA-48-like}}$ (F)	GCTTGATCGCCCTCGATT	0.6	281
bla$_{\text{OXA-48-like}}$ (R)	GATTTGCTCCGTGGCCGAAA	0.6	
bla$_{\text{IMP-type}}$ (F)	TTGACACTCCATTTACDG	0.6	139
bla$_{\text{IMP-type}}$ (R)	GATYGAGAATTAAGCCACYCT	0.6	

Amplicons were analysed by agarose gel electrophoresis after running at 100 V for 1-2 h by gel electrophoresis (Pérez-Pérez and Hanson, 2002). Five microlitres of PCR products were loaded on a 2% agarose gel (Sigma-Aldrich, UK) containing ethidium bromide (Sigma-Aldrich, UK) at a concentration of 10 μg/mL. Markers employed were GelPilot® (QIAGEN, Germany) 100 bp for bla_{ampC} family genes and GelPilot® 100 bp plus (QIAGEN, UK) for bla_{ESBL} and carbapenemase family genes. The gels were visualised by UV transilluminator. Prior to confirmation of molecular types by sequencing (Source Bioscience, UK), the PCR products were purified by using the QIAquick® PCR Purification Kit (QIAGEN, UK). The purified PCR product was sent for sequencing with its forward primer. Each sequence obtained was compared with already known β-lactamases genes by the NCBI nucleotide blast.

3.2.6.2 Phenotypic test using combined disc method and resazurin chromogenic agar plate assay for carbapenemases

To prepare the RCA plate, 25 mg of resazurin sodium salt (Sigma-Aldrich) was dissolved in 10 ml of sterile water and sterilised by filtration through a 0.2 μm syringe filter. The sterile resazurin solution was added to 990 ml sterile Mueller-Hinton (MH) agar (Oxoid, UK) when the temperature of the cooling medium reached approximately 45-50 °C (to a final concentration of 25 μg/mL). The resazurin-containing MH agar was gently mixed prior to pouring 25 mL of the solution or approximately 4 mm depth into 90 mm circular petri dishes. Uninoculated RCA plates were stored in the refrigerator (4 °C) for up to a week and kept away from the light (Sener et al., 2011).

For the preparation of antibiotic-containing discs, Meropenem (MER) discs (10 µg) were prepared by supplementing blank discs (6.5 mm, MAST Diagnostic Group, UK) with 10 µl of 1 mg/mL MER (Sigma-Aldrich). Dried MER discs were then impregnated with 10 µl of PBA (Sigma-Aldrich) at a concentration of 40 mg/mL and EDTA at 75 mg/mL to obtain the final amount of 400 µg/disc and 750 µg/disc, respectively. PBA was dissolved in dimethylsulphoxide (Sigma-Aldrich) and sterile water as previously recommended (Coudron, 2005). EDTA and MER were dissolved in sterile water. Discs were air-dried in the cabinet for one hour before use.

The disc susceptibility testing was performed in accordance with the CLSI guideline (Clinical Laboratory Standards Institute, 2012). A sterile swab was dipped in a 0.5 McFarland standard suspension of test bacteria and spread thoroughly on the entire RCA plate surface. Five discs, including MER, MER+PBA, MER+EDTA, MER+PBA+EDTA and 30 µg TMC (MAST Diagnostic Group, UK), were firmly placed at equidistant points on the surface of the RCA plate. Following incubation at 37 °C for seven hours, the diameters of the blue zones of inhibition were measured. A change in the colour of medium from blue (resazurin) to pink (resorufin) was visually observed in live bacteria. No colorimetric change was indicative of bacterial growth inhibition. The results were interpreted according to previously described assays with additional modifications (Table 3.4) (Miriagou et al., 2013; van Dijk et al., 2014). The synergy between MER and EDTA and PBA was considered as positive results for MBL and KPC, respectively. The absence of synergy between MER and EDTA or PBA with TMC zone diameter (≤ 10 mm) was denoted as a positive result for OXA-48-producing isolates (Table 3.4). Sensitivity and specificity values were calculated by comparing results from RCA assay to molecular

characterisation results. SPSS statistical analysis software (SPSS Inc, USA) was used to analyse the data and create a box-and-whisker plot.

Table 3.4. Interpretive criteria for combined disc synergy test and Performance of combined disc test along with RCA assay for the early detection of carbapenemase-producing Enterobacteriaceae.

Test	Definition of the test	MBL	KPC	OXA-48
EDTA synergy test	MER+EDTA vs MER ≥ 5 mm and MER+EDTA+PBA vs MER+PBA ≥ 5 mm	+	-	-
PBA synergy test	MER+PBA vs MER ≥ 5 mm and MER+EDTA+PBA vs MER+EDTA ≥ 5 mm	-	+	-
TMC zone ≤ 10 mm	Considered when the absence of EDTA, PBA, and EDTA+PBA synergy tests	-	-	+

MER = meropenem disc (10 µg), PBA = phenylboronic acid disc (400 µg) TMC = temocillin disc (30 µg). EDTA = Ethylenediaminetetraacetic acid (750 µg).

3.2.6.3 Phenotypic test using a combined disc method and resazurin chromogenic agar plate assay for ESBL and AmpC β-lactamases

The procedure for screening of ESBL, AmpC β-lactamases and co-production of ESBL and AmpC β-lactamases is summarised in Figure 3.1. For the preparation of the antibiotic- or β-lactamase inhibitor-containing discs, 10 µg of CPD discs (MAST Group, UK) were supplemented with 10 µl of 1 mg/mL CA (Sigma-Aldrich,UK), 10 µl of CX (Sigma-Aldrich,UK) at a concentration of 50 mg/mL, or impregnated with both CA and CX. Meropenem (MER) discs (10 µg) were prepared by adding 10 µl of MER (Sigma-Aldrich, UK) at a concentration of 1 mg/mL to blank

discs (6.5 mm diameter, MAST Group, UK). Before performing disc diffusion susceptibility testing, the discs were air-dried in the biosafety cabinet for 1 h. The disc susceptibility test was similarly performed as mentioned above.

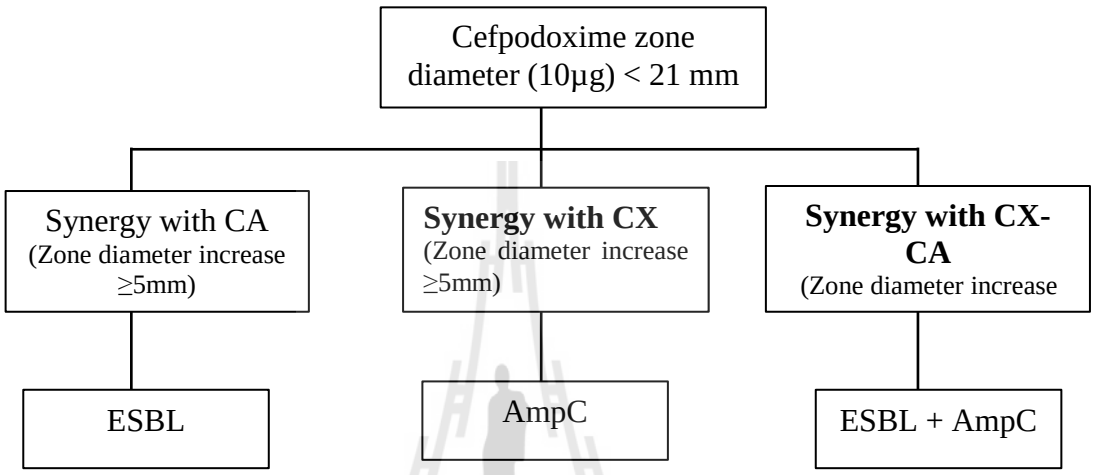


Figure 3.1 The procedure for screening of ESBL, AmpC, and co-production of ESBL and AmpC in Enterobacteriaceae**.** CA = clavulanic acid (10 µg); CX = cloxacillin (500 µg).

The interpretation criteria in screening and differentiation of ESBL, AmpC, and co-β-lactamases were based upon a previous report as presented in Table 3.5 (Nourrisson et al., 2015). An increase in zone diameter (≥ 5 mm) of CPD supplemented with β-lactamase inhibitor compared with CPD alone was considered as synergistic activity. To interpret the results, CA synergy was considered as a positive result for ESBL, while CX synergy and CA plus CX synergy were noted as positive results for AmpC and co-production of ESBL and AmpC, respectively. A zone diameter of MER < 25 mm was used as a cutoff point to screen for the presence of carbapenemases. Sensitivity and specificity of the RCA assay along with combined

disc method were calculated by comparing the results with molecular types from PCR and sequencing results. A box-and-whisker plot was analysed using SPSS statistical analysis program version 18 (SPSS Inc, Chicago, IL, USA) to elucidate the distribution of zone diameters of the discs against different β-lactamase producers.

Table 3.5 Interpretation criteria of a combined disc synergy method along with RCA assay for rapid screening of ESBL, AmpC, and co-producers of ESBL and AmpC among Enterobacteriaceae

Synergy test	**Definition of the test**	**ESBL**	**AmpC**	**ESBL+ AmpC**
CA synergy test	CPD+CA vs CPD ≥ 5 mm and CPD+CA+CX vs CPD+CX ≥ 5 mm	+	-	-
CX synergy test	CPD+CX vs CPD ≥ 5 mm and CPD+CA+CX vs CPD+CA ≥ 5mm	-	+	-
CA+CX synergy test	Both CPD+CA+CX vs CPD+CX ≥ 5 mm and CPD+CA+CX vs CPD+CA ≥ 5 mm	-	-	+

CPD = cefpodoxime (10 µg); CA = clavulanic acid (10 µg); CX = cloxacillin (500 µg)

3.2.7 Statistic analysis

The experiments were carried out in three replications with an exception for suspension standard curve, time-kill curve (6 replications) quantitative biofilm formation assay (5 replications); data were expressed as mean ± standard error of the mean (SEM). Significant differences of cell area in each treated group from TEM, CM permeability and inhibition of biofilm formation were analysed by one-way ANOVA. A *p*-value < 0.05 and < 0.01 with Tukey's HSD *post-hoc* test was considered as statistically significant difference.

CHAPTER IV

RESULTS

4.1 Antibacterial activity of *B. rotunda* Extract (BRE)

4.1.1 Preliminary phytochemical analysis

The lyophilised ethanol extracts of *B. rotunda* were weighed to calculate the percentage yield of the extract. The percentage of yield obtained was 10.3% (w/w). The preliminary qualitative phytochemical screening tests showed that there were alkaloids, flavonoids, tannins, glycoside and steroids in BRE, while saponin and coumarins were found to be absent in this extract (Table 4.1). The total flavonoids content was 4.73 ± 1.02 mg quercetin equivalent per 100 mg extract.

Table 4.1 Preliminary qualitative phytochemical screening of the *B. rotunda* extract

Phytochemical compounds	Result
Alkaloids	+
Flavonoids	+
Tannins	+
Saponin	-
Glycoside	+
Steroids	+
Coumarins	-

(+) = presence; (-) = absence

4.1.2 MIC determinations

The MIC values of BRE and cloxacillin (CLX), ampicillin (AMP), cefazolin (CFZ), ceftazidime (CAZ) and vancomycin were evaluated against the clinical isolates *S. aureus* DMST 20651, *S. aureus* DMST 20652, *S. epidermidis* DMST 14932, *E. coli* DMST 20662, *E. cloacae* DMST 19719, *A. baumanii* DMST 45378, *S. aureus* ATCC 29213 (a reference strain for Gram-positive bacteria) and *E. coli* ATCC 25922 (a reference strain for Gram-negative bacteria). MIC values of BRE were 16 μg/mL against all of the Gram-positive bacteria. *S. aureus* DMST 20651 exhibited high resistance to CLX, AMP, and CFZ with MIC values 512, 256 and 256 μg/mL, respectively. Similarly, *S. aureus* strain DMST 20652 had the MIC values for CLX, AMP and CFZ of 512, 64, and 256 μg/mL, respectively. *S. epiderdimis* DMST 14932 was also resistant to CLX (MIC = 2 μg/mL) and AMP (MIC = 32 μg/mL), except for CFZ (MIC = 4 μg/mL). All tested clinical isolates of Gram-positive bacteria were susceptible to VAN at MIC value of 0.5 μg/mL according to CLSI document (Table 4.2). The CLSI susceptibility breakpoints for CLX, AMP, CFZ and VAN against *Staphylococcus* spp. are ≤ 0.25, ≤ 0.25, ≤ 8 μg/mL and ≤ 4, respectively (Clinical Laboratory Standards Institute, 2014).

In Gram-negative bacteria, MIC values of BRE were higher than 1024 μg/mL against all test Gram-negative pathogens; *E. coli* DMST 20662, *E. cloacae* DMST 19719 and *A. baumanii* DMST 45378. MIC values of CAZ against these isolates were 512, 512 and > 1024 μg/mL, respectively. These findings indicated high resistance to CAZ antibiotic. However, the reference strain *E. coli* ATCC 25922 was still susceptible to this antibiotic (Table 4.2). The susceptibility breakpoint for CAZ

against Enterobacteriaceae is ≤ 4 μg/mL and against *Acinetobacter* spp. is ≤ 8 μg/mL (Clinical Laboratory Standards Institute, 2014).

Table 4.2 Minimum inhibitory concentration (MIC) of selected cell wall inhibitors antibiotic and BRE against important pathogenic Gram-positive bacteria and Gram-negative bacteria. The data expressed were obtained from three replications.

Bacterial Isolates	Minimum inhibitory concentration (μg/mL)					
	BRE	CLX	AMP	CFZ	CAZ	VAN
S. aureus DMST 20651	16^{ND}	512^{R}	256^{R}	256^{R}	-	0.5^{S}
S. aureus DMST 20652	16^{ND}	512^{R}	64^{R}	256^{R}	-	0.5^{S}
S. epidermidis DMST 14932	16^{ND}	2^{R}	32^{R}	4^{S}	-	0.5^{S}
E. coli DMST 20662	$>1024^{ND}$	-	-	-	512^{R}	-
E. cloacae DMST 19719	$>1024^{ND}$	-	-	-	512^{R}	-
A. baumanii DMST 45378	$>1024^{ND}$	-	-	-	$>1024^{R}$	-
S. aureus ATCC 29213*	16^{ND}	$\leq 0.25^{S}$	$\leq 0.25^{S}$	$\leq 0.25^{S}$	-	0.5^{S}
E. coli ATCC 25922**	$>1024^{ND}$	-	-	-	$\leq 0.25^{S}$	-

^{S} = Susceptible; ^{R} = resistant; ND = no data in CLSI breakpoint; BRE = *B. rotunda* extract; CLX = cloxacillin; AMP = ampicillin; CFZ = cefazolin; CAZ = ceftazidime; VAN = vancomycin;* A reference strain for Gram-positive bacteria: ** A reference strain for Gram-negative bacteria.

4.1.3 Chequerboard determinations

Drug combination therapy is a promising approach for the treatment of antimicrobial-resistant microorganisms and prevention or delay of antibiotic resistance development. The drug interaction is commonly determined by the chequerboard assay (Wagner, 2011). Table 4.3 illustrates the results of the interaction between BRE and CLX, AMP, CFZ or VAN against *Staphylococcus* spp., and the interaction between BRE and CAZ against clinical isolates of *E. coli*, *E. cloacae* and *A. baumanii*. The lowest FIC index (0.502) was observed in the combination of BRE and CLX against MRSA DMST 20651 and DMST 20652, while the combination of BRE plus AMP and BRE plus CFZ demonstrated similar results against these isolates (Table 4.3). MICs of CLX, AMP and CFZ were dramatically reduced when used in conjugation with the BRE. A half reduction in MIC was observed in BRE when used in the combination against all test Gram-positive bacteria. The combination of BRE and VAN exhibited no synergistic activity against all selected Gram-positive bacteria including a reference strain of *S. aureus* ATCC 29213. In *S. epidermidis*, a synergistic activity was only observed with the combination of BRE plus AMP and partial synergistic activity was seen in the combinations of BRE plus CLX and BRE. BRE in conjunction with VAN showed no synergistic interaction against this strain.

In Gram-negative bacteria, no synergistic activity between BRE and CAZ was observed against all selected Gram-negative isolates including the reference strain, *E. coli* ATCC 25922 (Table 4.3). CAZ showed no reduction in MIC when it was used in combination with BRE, while the MIC for BRE was reduced from 16 to ≤ 0.25 μg/mL. The antibacterial activity of these combinations may be as a result of CAZ alone.

Table 4.3 Drug interaction between BRE and selected antibiotics against test pathogenic bacteria.

Bacterial Isolates	Combination of agents	MIC (in combination)	FIC index	Type of Interaction
S. aureus DMST 20651	BRE CLX	8 1	0.502	Synergism
	BRE AMP	8 1	0.504	Synergism
	BRE CFZ	8 1	0.504	Synergism
	BRE VAN	8 ≤0.25	1.02	Addition
S. aureus DMST 20652	BRE CLX	8 1	0.502	Synergism
	BRE AMP	8 0.5	0.507	Synergism
	BRE CFZ	8 1	0.504	Synergism
	BRE VAN	8 ≤0.25	≤1	Addition
S. epidermidis DMST 14932	BRE CLX	8 ≤0.25	≤0.625	Partial Synergism
	BRE AMP	8 0.5	0.516	Synergism
	BRE CFZ	8 0.5	0.625	Partial Synergism
	BRE VAN	8 ≤0.25	≤1	Addition
E. coli DMST 20662	BRE CAZ	≤0.25 512	1.03	Addition
E. cloacae DMST 19719	BRE CAZ	≤0.25 512	1.03	Addition
A. baumanii DMST 45378	BRE CAZ	>1024 >1024	>2	Addition
S. aureus ATCC 29213	BRE CLX	≤0.25 ≤0.25	1.5	Addition
	BRE AMP	8 ≤0.25	1.5	Addition
	BRE CFZ	8 ≤0.25	1.5	Addition
	BRE VAN	8 ≤0.25	≤1	Addition
E. coli ATCC 25922	BRE CAZ	≤0.25 ≤0.25	≤1	Addition

4.1.4 Time-kill curve determination

To confirm the antibacterial activity and synergistic effect of BRE either used alone or in combination with antibiotics, the time-kill curve assay was determined by the combination showing the lowest FIC index from chequerboard assay. Therefore, the combination of BRE and CLX against MRSA 20651 was chosen to confirm its antibacterial and synergistic activity. Figure 4.1 demonstrates the effect of BRE and CLX, either alone or in combination, on cell viability of MRSA 20561 after exposure to the antimicrobial agents for 0, 0.5, 1, 2, 4, 6, 8 and 24 h. The results exhibited that untreated cells (control) grew exponentially after 2 h and grew up continuously until at 24 h, reaching approximately 10^{13} CFU/mL. In CLX (1 μg/mL)-treated cells, the viable count was steady until 6 h after treatment compared with starting inoculum, and they started growing after 6 h until 24 h of treatment. Similarly, no change in viable counts was observed until 8 hours of treatment cells treated with 8 μg/mL BRE alone. These treated cells grew, reaching 10^{9} CFU/mL at 24 h of exposure. In BRE (8 μg/mL) plus CLX (1 μg/mL)-treated group, a slight reduction in the viabilities of MRSA 20651 after 2 h of treatment was observed in this treated group. These cells were slightly regrown at 24 h, reaching approximately 10^{6} CFU/mL.

At 24 h of exposure, the combined-treated group (BRE+CLX) showed the substantial reduction in viable count ≥ 2-log10 CFU/mL compared with BRE and CLX alone. This finding indicated BRE worked synergistically with CLX in inhibiting the growth of MRSA 20651. Furthermore, all treated groups did not demonstrate bactericidal activity at any time point as no reduction in viable count ≥ 2-log10 CFU/mL was observed compared with starting inoculum.

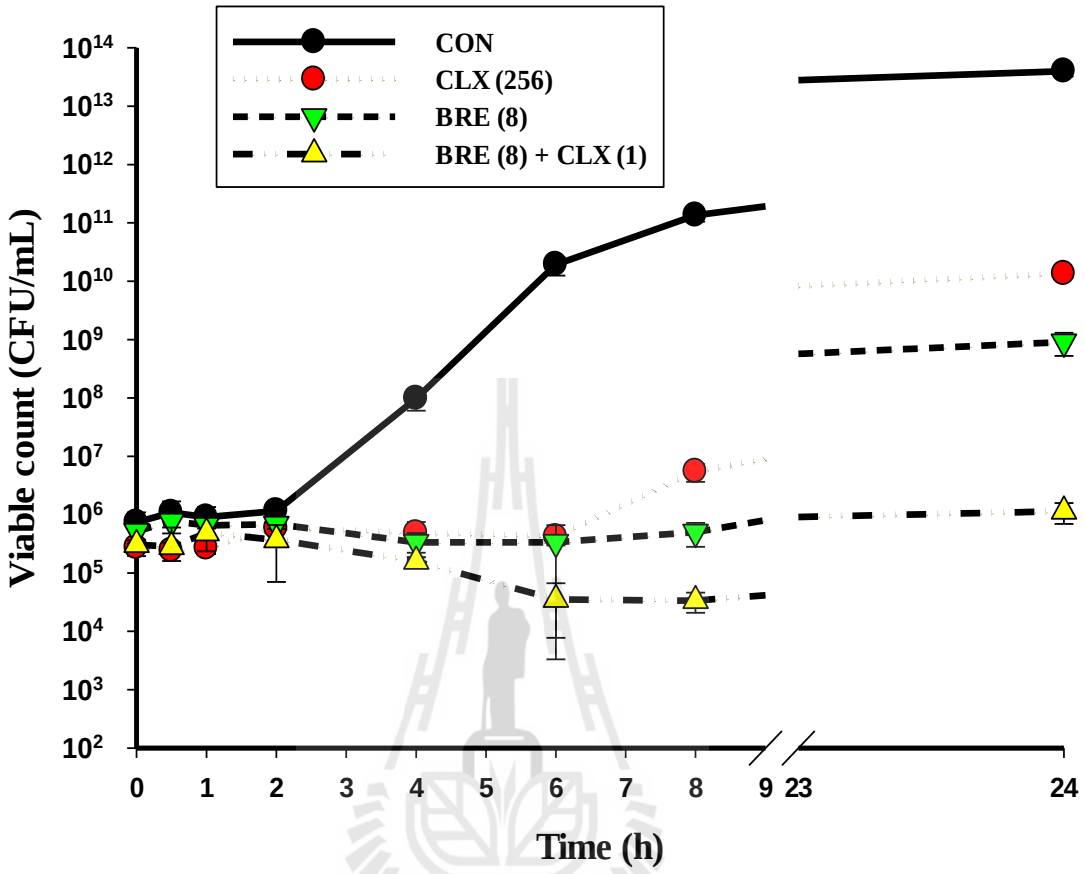


Figure 4.1 Viable counts of MRSA 20651 after they were challenged with *B. rotunda* extract (BRE) and cloxacillin (CLX) either separate or in combination at the different time points. The values were expressed as means ± the standard errors of the means (SEM) obtained from six replications. The vertical bars indicate the standard errors of the means. CON = control, CLX (256) = cloxacillin at a concentration of 256 μg/mL, BRE (8) = *B. rotunda* extract at a concentration of 8 μg/mL, BRE (8) + CLX (1) = BRE at a concentration of 8 μg/mL plus CLX at a concentration of 1 μg/mL.

4.1.5 Transmission electron microscope (TEM)

Ultracellular damages after exposure to BRE and antibiotic either used separately or in combination were elucidated by TEM. The electron microscopic investigation was performed with BRE (8 μg/mL), CLX (256 μg/mL), BRE (6 μg/mL) plus CLX (0.5 μg/mL) and untreated cells against MRSA 20651. The cell areas for each group were also measured to investigate the effect of the compounds on cell sizes.

The morphology of control cells grown in the absence of any antimicrobial agents is depicted in Figure 4.2. Untreated cells were round in shape. Their cytoplasmic membrane and cell wall of these cells were unambiguously identified and distinguished. In cells treated with CLX at a concentration of 256 μg/mL, there were disruptions of the cell wall of MRSA 20651. A distortion of these treated cells was also observed. It seemed that CLX inhibited cell division and induced cell lysis. CLX did not damage to the cytoplasmic membrane (Figure 4.3). TEM micrographs of cells treated with BRE alone at a concentration of 8 μg/mL are illustrated in Figure 4.4. Most cells in this treated group clearly exhibited collapse of the cytoplasmic membrane. It appeared that the cytoplasmic membrane was detached from the cell wall. This finding indicated that BRE inhibited the growth of MRSA 20651 by interacting with the cytoplasmic membrane and inducing damage to the cytoplasmic membrane. In cells treated with BRE (6 μg/mL) plus CLX (0.5 μg/mL), disruptions of both cytoplasmic membrane and cell wall were observed in this treated group. Many cells after exposure to the combination of BRE and CLX had lost their cytoplasmic components (Figure 4.5).

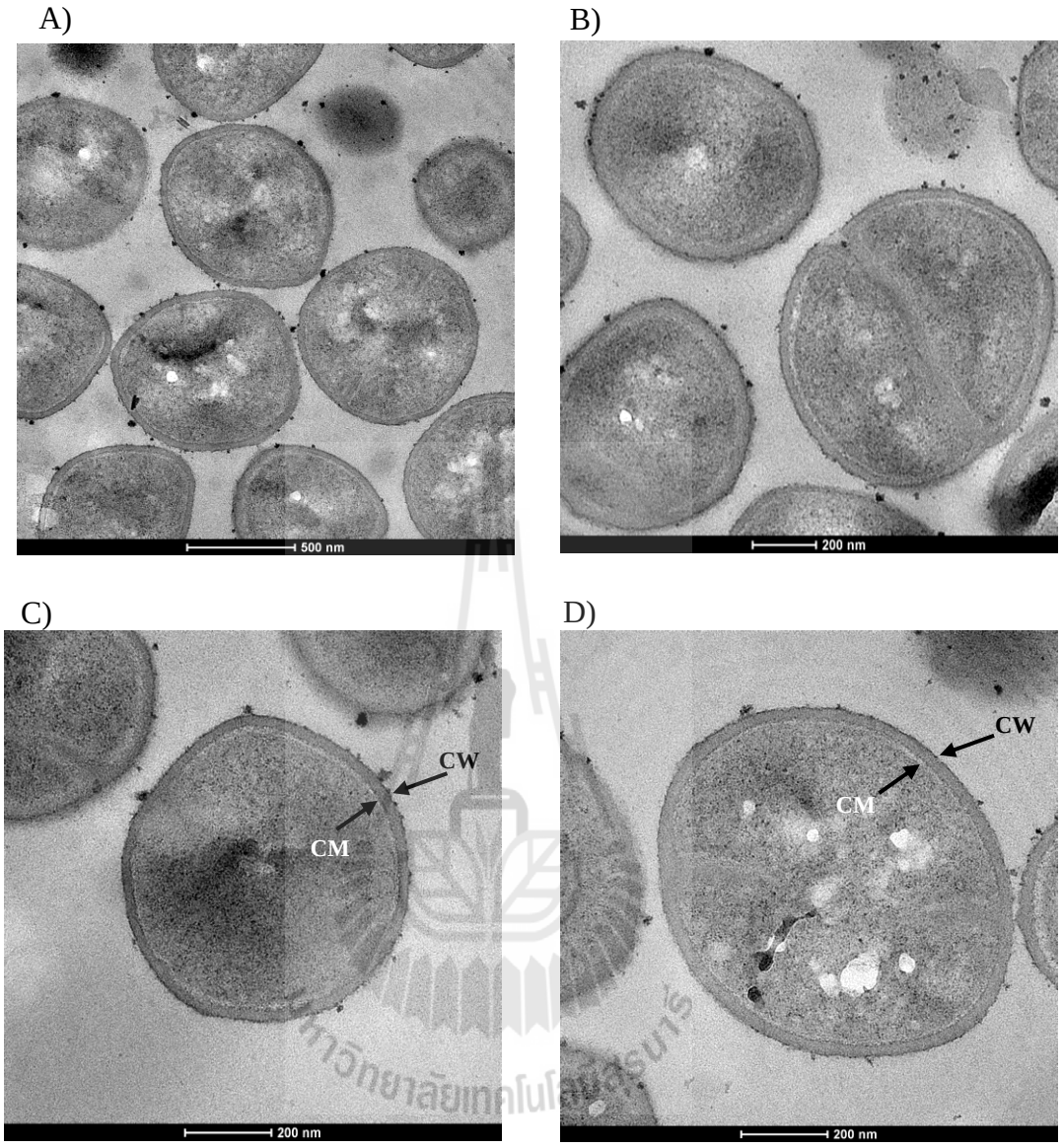


Figure 4.2 Ultrastructure of clinical isolates of MRSA 20651 grown in the Murller-Hinton broth with the absence of antimicrobial agent. A) bar = 500 nm and magnification 15,000x; B) bar = 200 nm and magnification 19,500x; C) bar = 200 nm and magnification 29,000x; D) bar = 200 nm and magnification 34,000x; CM = cytoplasmic membrane; CW = cell wall.

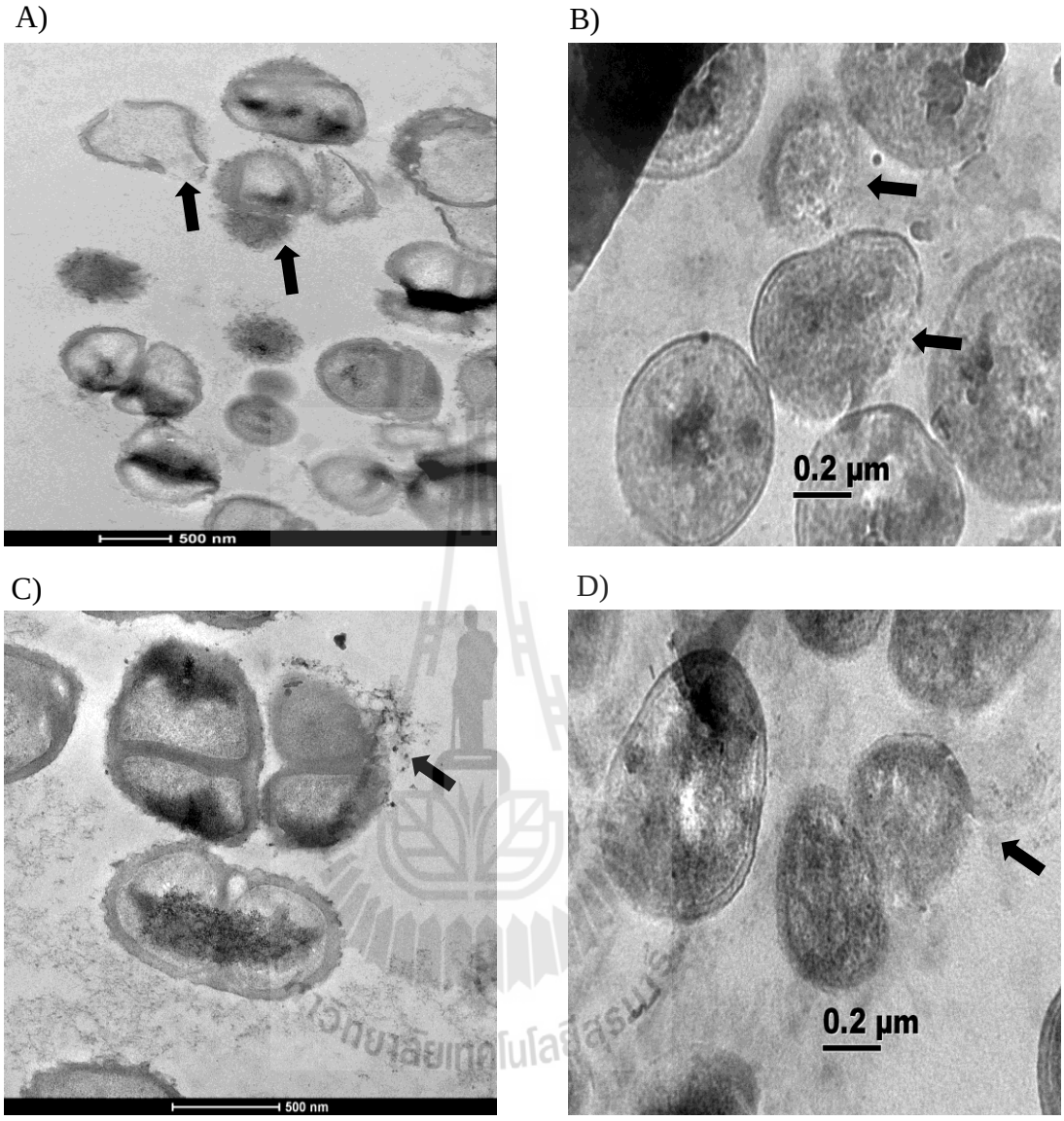


Figure 4.3 Ultrastructure of clinical isolates of MRSA 20651 grown in the presence of cloxacillin (CLX) at a concentration of 256 µg/mL. A) bar = 500 nm and magnification 9,900x; B) bar = 0.2 µm and magnification 19,500x; C) bar = 500 nm and magnification 19,500x; D) bar = 500 nm and magnification 29,000x. The black arrows indicate the sites of damage.

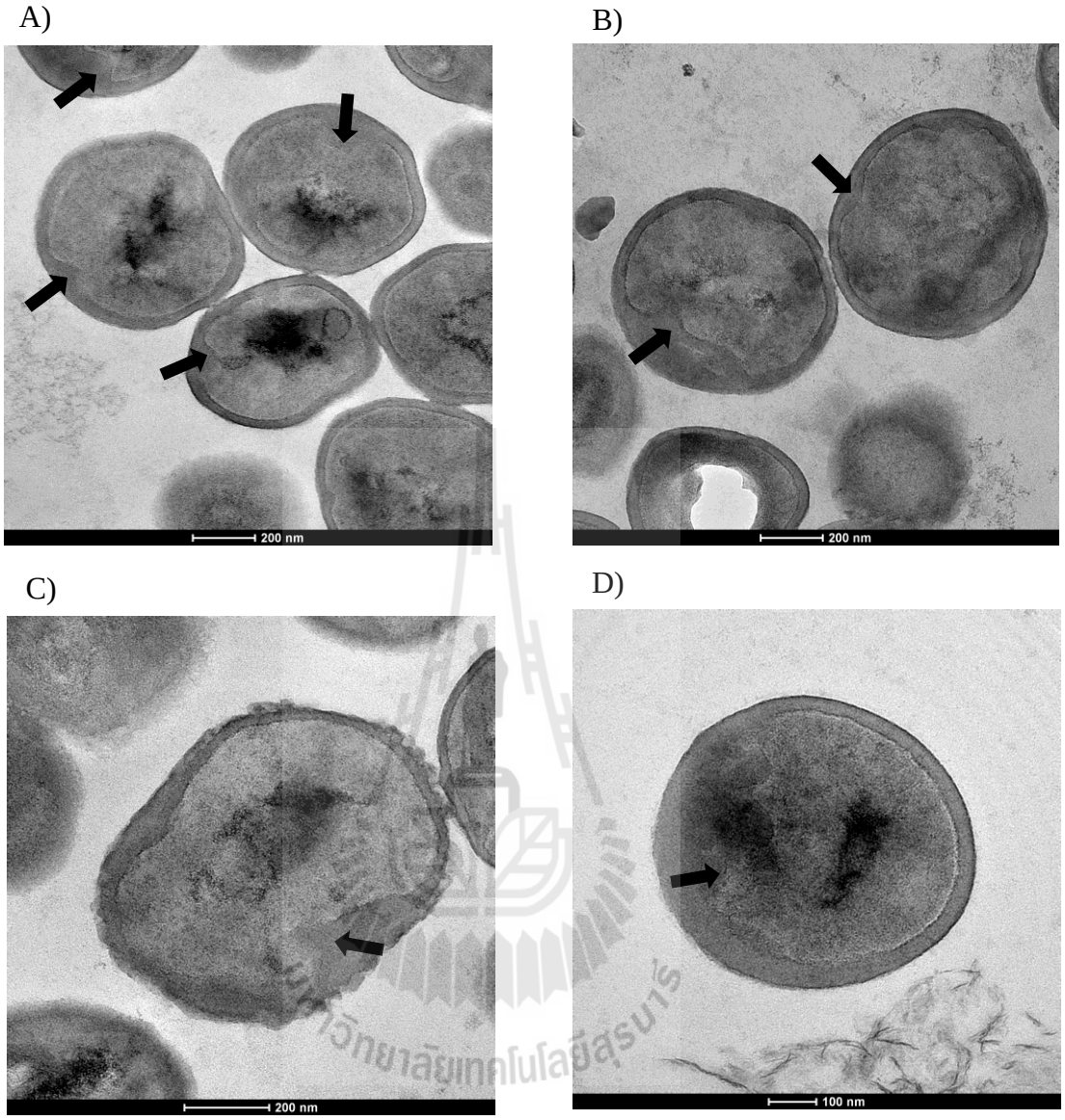


Figure 4.4 Ultrastructure of clinical isolates of MRSA 20651 grown in the presence of *B. rotunda* extract (BRE) at a concentration of 8 μg/mL. A) bar = 200 nm and magnification 15,000x; B) bar = 200 nm and magnification 19,500x; C) bar = 200 nm and magnification 29,000x; D) bar = 100 nm and magnification 29,000x. The black arrows indicate the sites of damage.

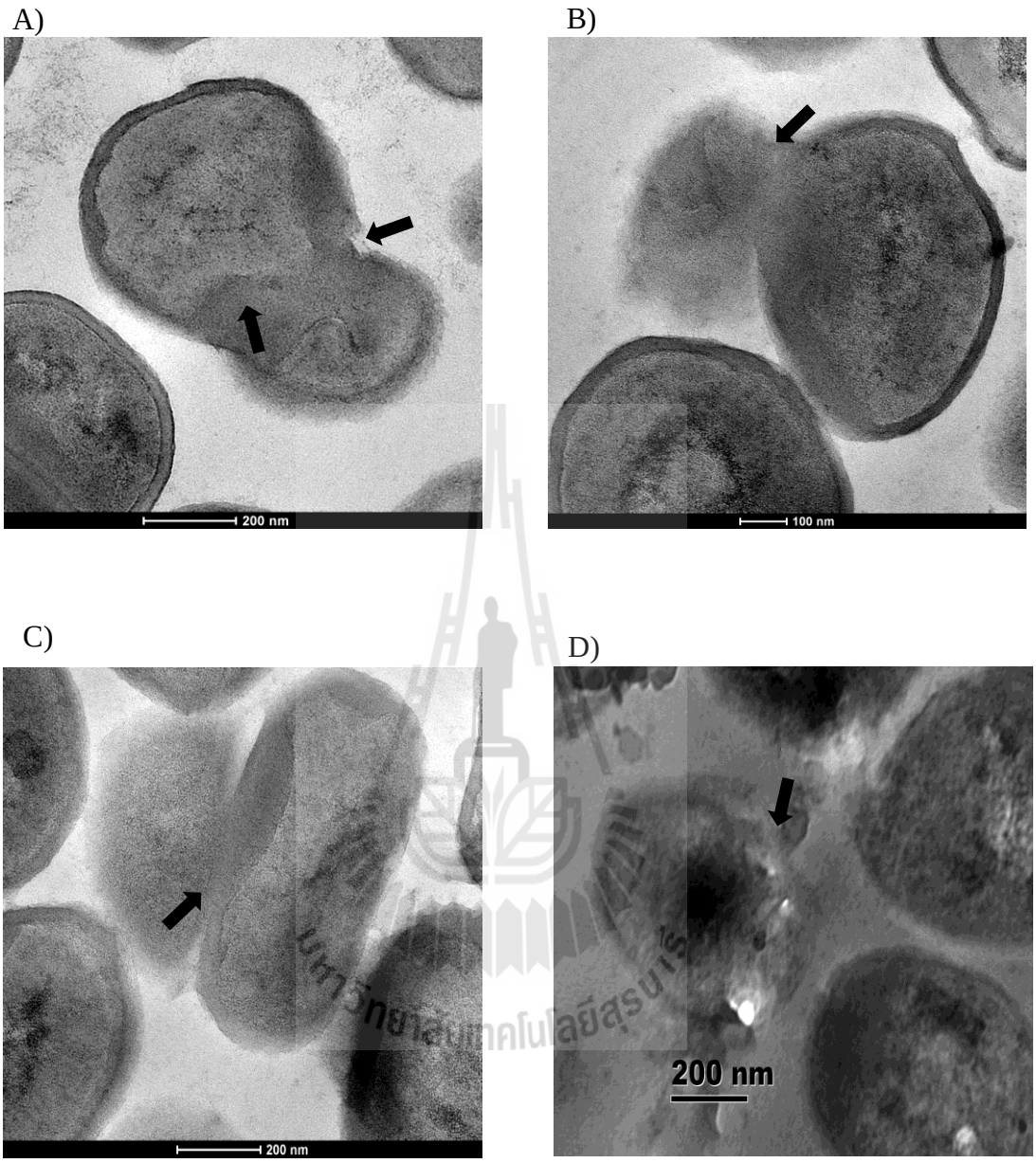


Figure 4.5 Ultrastructure of clinical isolates of MRSA 20651 grown in the presence of *B. rotunda* extract (BRE) at a concentration of 6 μg/mL plus cloxacillin (CLX) at a concentration of 0.5 μg/mL. A) bar = 200 nm and magnification 29,000x; B) bar = 100 nm and magnification 29,000x; C) bar = 200 nm and magnification 29,000x; D) bar = 200 nm and magnification 29,000x. The black arrows indicate the sites of damage.

The cell areas of a clinical isolate of MRSA 20651 after treatment with and without BRE or CLX, either alone or in combination were measured and calculated in order to examine the effect of each treatment on cell size, as displayed in Figure 4.6. The cell area of untreated clinical isolates of MRSA 20651 was approximately $5.06 \times 10^5 \pm 3.47 \times 10^4$ nm^2. This result revealed a significant difference (higher) in cell area compared with the cells treated with CLX alone, BRE alone and CLX plus BRE ($p < 0.05$). CLX-treated cells exhibited the lowest cell area ($3.22 \times 10^5 \pm 4.66 \times 10^4$ nm^2), which was not significantly different compared to the treatment with BRE alone ($3.84 \times 10^5 \pm 2.26 \times 10^4$ nm^2) and CLX plus BRE ($3.68 \times 10^5 \pm 2.49 \times 10^4$ nm^2). These findings suggest that exposure to CLX and BRE either alone or in combination resulted in smaller in cell size of clinical isolates of MRSA 20651 cell size.

4.1.6 Cytoplasmic membrane (CM) permeability

The effect of 256 μg/mL CLX, 8 μg/mL BRE alone and the combination of 1 μg/mL CLX plus 8 μg/mL BRE on CM permeability were determined by measuring the loss of OD_{260}-absorbing materials. Nisin was used as a positive control. The results of this assay are illustrated in Figure 4.7. The CLX-treated group exhibited no leakage of OD_{260}-absorbing materials in comparison with control group throughout 4 h of treatment. Nisin, a positive control, successfully permeabilised the cytoplasmic membrane of MRSA 20651. In cells treated with BRE alone, Nisin alone and the combination of BRE plus CLX, there were significant differences in optical density at a wavelength of 260 nm compared with cells treated with CLX and control ($p < 0.01$). However, no significant differences among cells treated with BRE, nisin, and BRE plus CLX were observed.

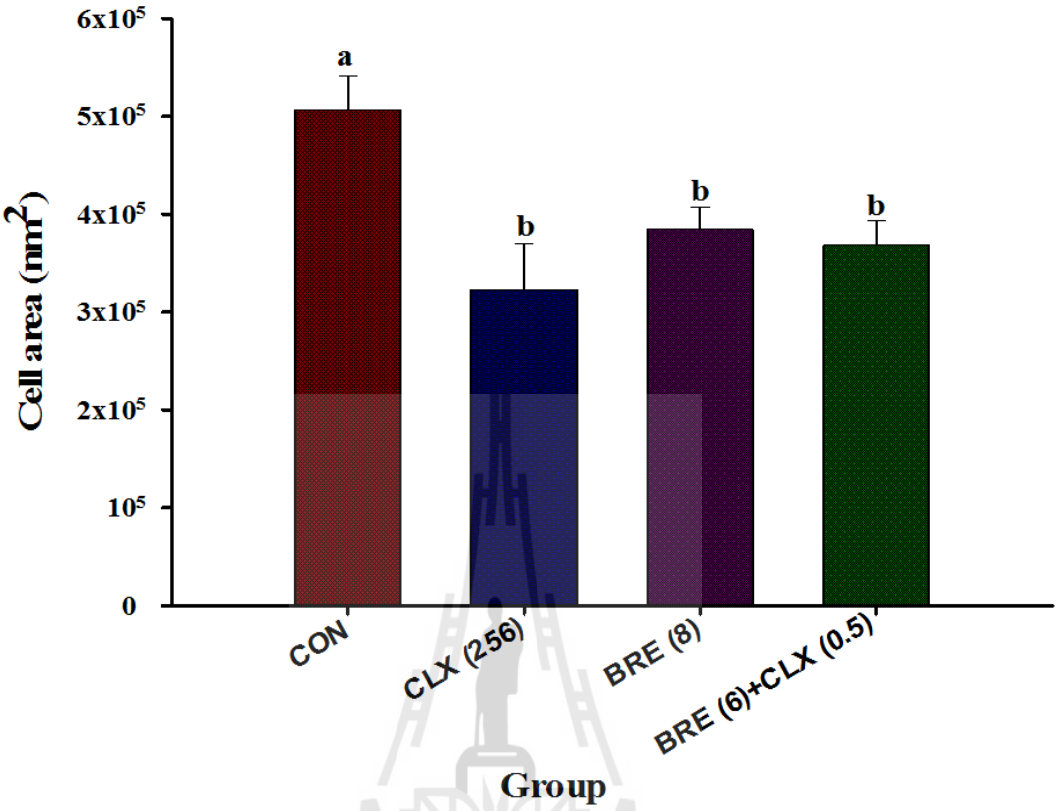


Figure 4.6 Effect of BRE and CLX either used alone or in combination on the cell area of MRSA 20651. CON= control (n=10), CLX (256) = cells treated with cloxacillin at a concentration of 256 µg/mL (n=7), BRE (8) = cells treated with *B. rotunda* extract at a concentration of 8 µg/mL (n=11), BRE (6) + CLX (0.5) = cells treated with *B. rotunda* extract at a concentration of 6 µg/mL plus cloxacillin at a concentration of 0.5 µg/mL. The area of the cell was determined by cell width cell length (nm^2). The different superscript alphabet represents a statistically significant difference using one-way ANOVA with Tukey's HSD ($p < 0.05$). The data were expressed as mean ± standard error of the mean (SEM).

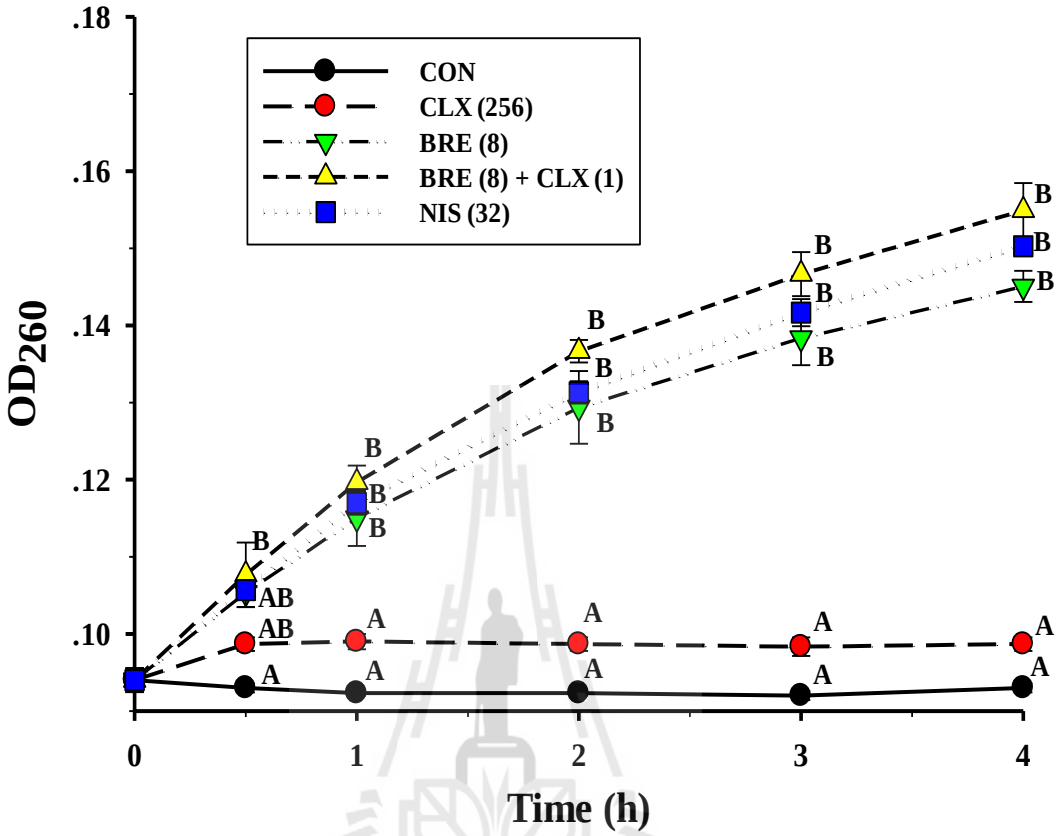


Figure 4.7 Cytoplasmic membrane permeabilisation of a clinical isolate of MRSA 20651. CON = control, CLX (256) = cloxacillin at a concentration of 256 µg/mL, BRE (8) = *B. rotunda* extract at a concentration of 8 µg/mL, BRE (8) + CLX (1) = *B. rotunda* extract at a concentration of 8 µg/mL plus cloxacillin at a concentration of 1 µg/mL. Data were expressed as mean ± standard error of the mean (SEM) from 6 repetitions. The results were analysed and compared by One-way ANOVA followed by Tukey's HSD Post-hoc test. The different alphabetical characters represent a statistically significant difference between each group ($p < 0.01$).

4.1.7 Quantification of biofilm formation inhibition

The antibiofilm activity of CLX and BRE either used singly or in combination against MRSA 20651 using a standard quantitative biofilm assay is demonstrated in Figure 4.8. After 48 of treatment, MRSA 20651 grown in the absence of any antimicrobial agent showed the highest biofilm biomass. All treated groups significantly dislodged the biofilm of MRSA 20651 compared with a control group, $p \leq 0.01$. CLX (256 μg/mL)-treated group exhibited lowest biofilm formation, but no significant difference compared with BRE (8 μg/mL) alone and BRE (8 μg/mL) plus CLX (1 μg/mL), $p > 0.01$.

4.1.8 Microscopic analysis of biofilm formation

The effect of 256 μg/mL CLX, 8 μg/mL BRE alone and the combination of 1 μg/mL CLX plus 8 μg/mL BRE on biofilm formation of MRSA 20651 were also qualitatively examined under a light microscope. The results of this assay are shown in Figure 4.9. The crystal violet staining in a control group exhibited a thick coating of biofilm (Figure 4.9A), whereas the explicit reduction in biofilm formation was observed in cells treated with BRE and CLX either alone or in combination. The CLX-treated group showed the greatest activity in the eradication of biofilm formation compared with BRE alone and BRE plus CLX. MRSA 20651cells were observed under light microscope.

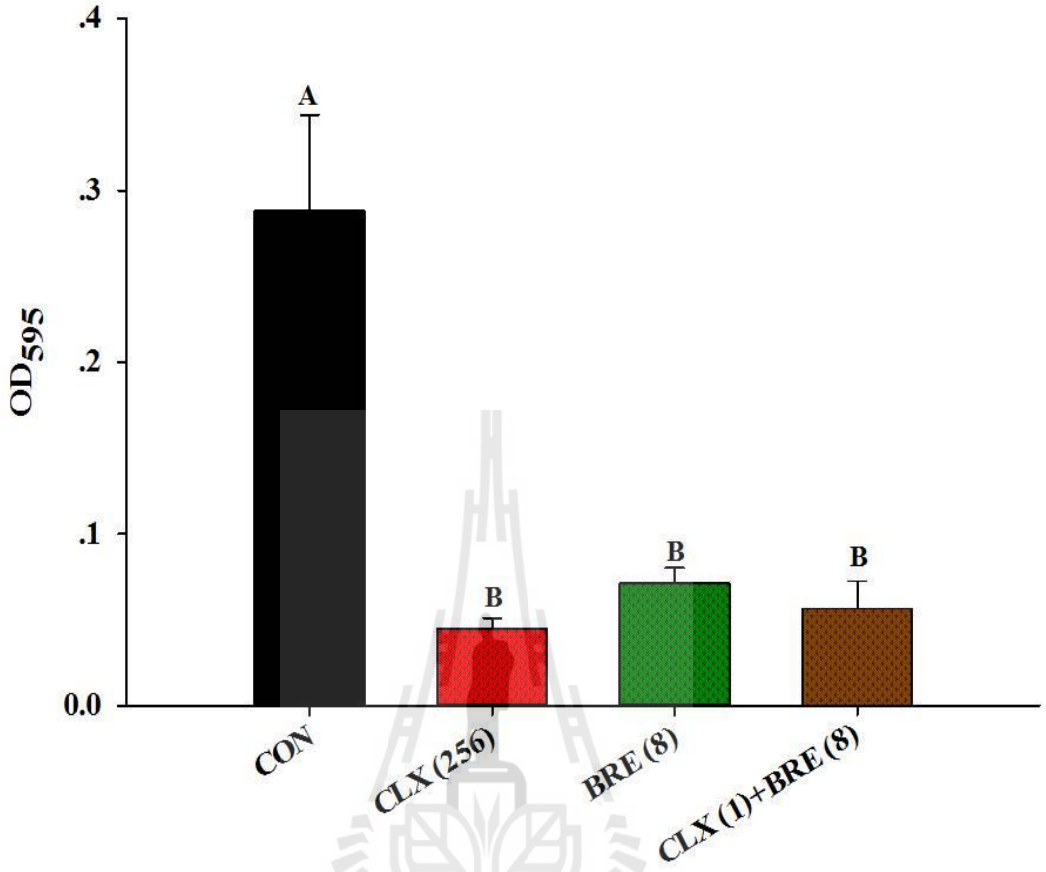


Figure 4.8 Quantitative analysis of biofilm formation of MRSA 20651 after exposure to no antibacterial agents, *B. rotunda* extract and cloxacillin either used alone or in combination. CON = control, CLX (256) = cloxacillin at a concentration of 256 µg/mL, BRE (8) = *B. rotunda* extract at a concentration of 8 µg/mL, CLX (1) + BRE (8) = cloxacillin at a concentration of 1 µg/mL and *B. rotunda* extract at a concentration of 8 µg/mL. The values were expressed as mean ± standard error of the mean (SEM) from six replications. The results were analysed and compared by One-way ANOVA followed by Tukey's HSD Post-hoc test. The different alphabetical characters represent a statistically significant difference between each group ($p < 0.01$).

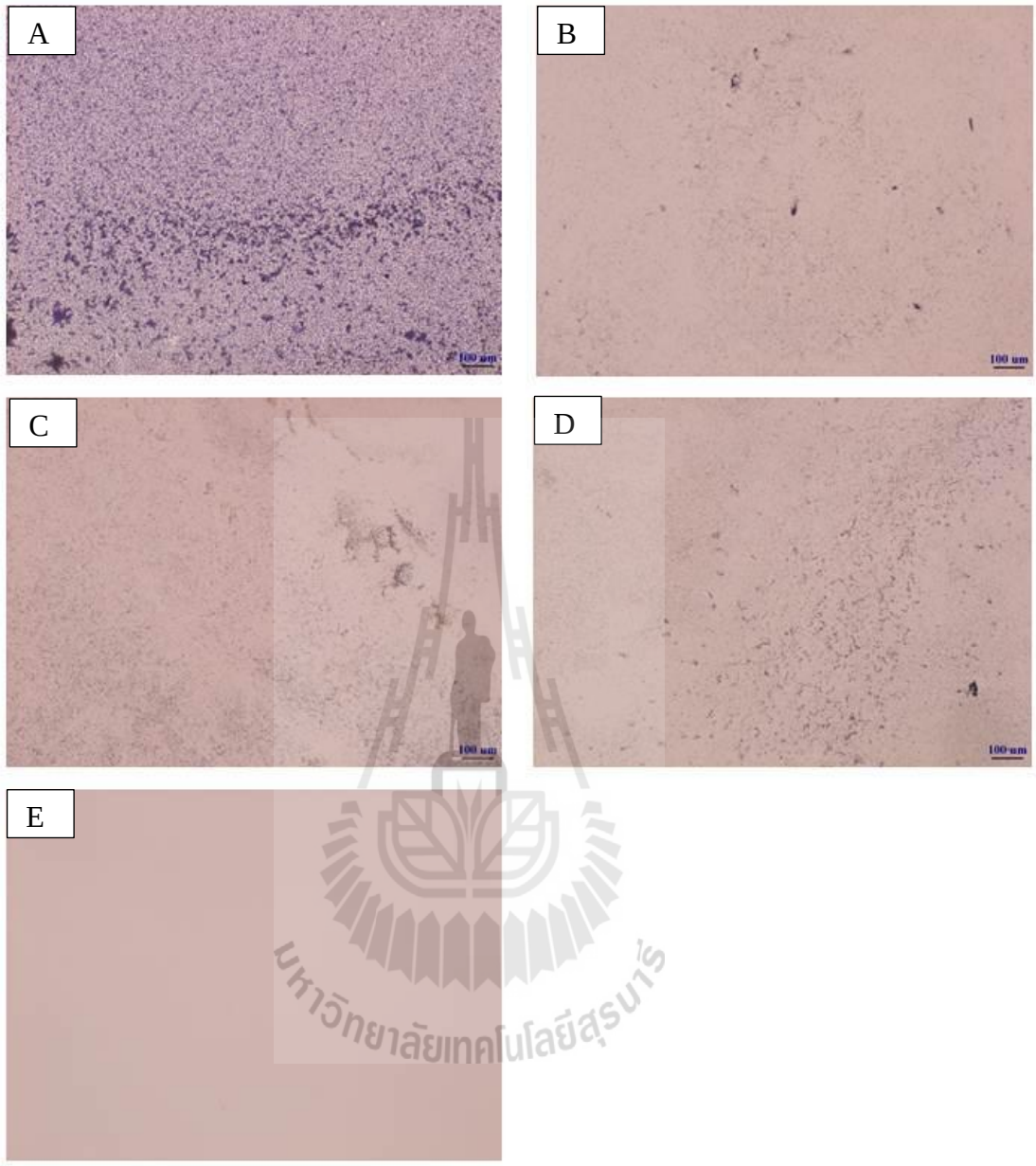


Figure 4.9 Qualitative microscopic analysis of biofilm formation using a light microscope. A= positive control, B = CLX (256 μg/mL)-treated group, C = BRE (8 μg/mL), D = CLX (1 μg/mL) plus BRE (8 μg/mL)-treated group, E = a negative control. Bar = 100 μm and magnification = 40x.

4.2 Detection and differentiation of β-lactamases

4.2.1 Genotypic test using multiplex PCR and sequencing

For the detection of carbapenemase-producing isolates using multiplex PCR, the primers used successfully amplified and differentiated the different types of carbapenemase genes. Figure 4.10 and 4.11 illustrate the results of the ethidium bromide stained agarose gel of multiplex PCR products. A sum of 30 carbapenemase-producing Enterobacteriaceae was identified, including 8 *Klebsiella pneumoniae* carbapenemases (KPCs), 11 metallo-β-lactamases (MBLs) and 11 OXA-48 carbapenemases. Following gene sequencing, the KPCs were identified as KPC-2 (n =2), KPC-3 (n= 4), KPC-4 (n=1) and KPC (n=1), while the molecular types of MBLs were NDM-1 (n =6), IMP-1 (n =1) and VIM-1 (n=4).

For the detection of ESBL and AmpC β-lactmases using multiplex PCR, 2 multiplexes were used to identify bla_{TEM} and bla_{SHV} (multiplex I) and $bla_{CTX\text{-}M}$ (multiplex II) and 6 primer sets were employed to amplify 6 important bla_{ampC} genes. The results of multiplex PCR for the detection of bla_{TEM} and bla_{SHV} and $bla_{CTX\text{-}M}$ are demonstrated in Figures 4.12 and 4.13, respectively. The representative results of multiplex PCR for the detection of bla_{ampC} are illustrated in Figure 4.14. The negative controls (no primers and no template DNA) are not shown in this specific gel. The multiplex PCR successfully identified ESBL-mediated resistance in 15 isolates, including 5 CTX-Ms, 3 SHV family genes, 1 CTX-M + SHV, 3 SHV+TEM, 1 CTX-M+SHV+TEM. A total of 32 AmpC-producing isolates, including 6 DHA –family genes, 7 CIT family genes, 2 MOX family genes, 11 EBC (ACT-type) family genes and 6 FOX family genes, were identified. Nine strains were co-producers of ESBL and AmpC (1 TEM+ACT, 4 CTX-M+ACT, 1 TEM+SHV+ACT, 1 TEM + CTX-

M+ACT, 1 SHV + ACT and 1 SHV+CTX-M+ACT). The results are summarised in Table 4.4. The molecular types of each strain are detailed in Appendix B.

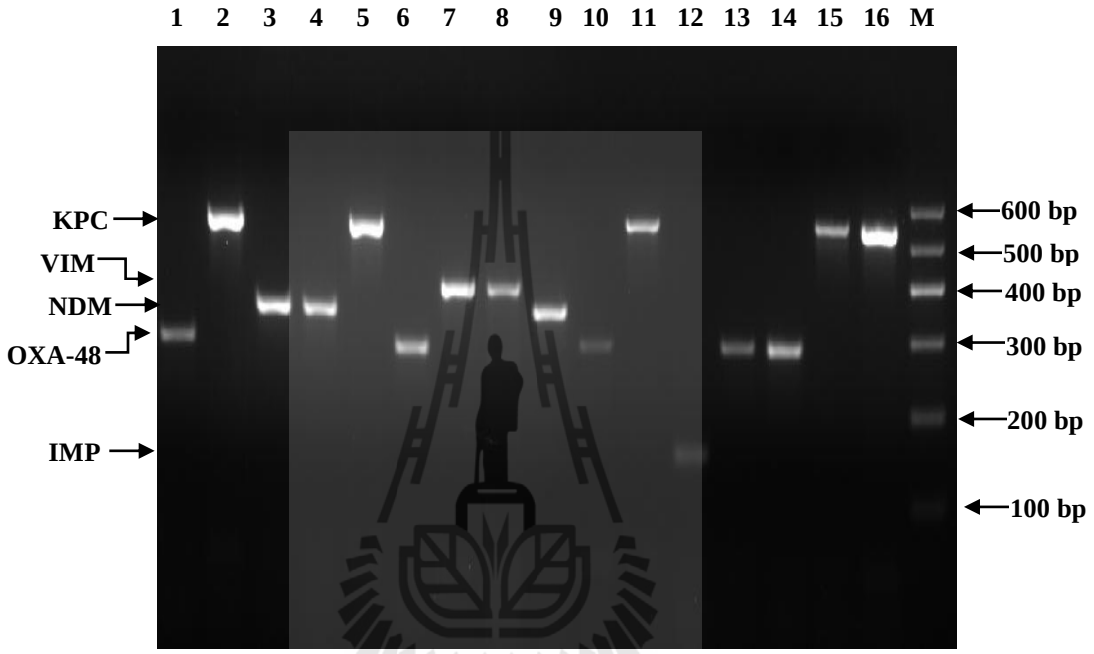


Figure 4.10 Multiplex PCR for the detection of carbapenemases family genes (*bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$ and *bla*$_{OXA-48}$). 1 = *E. cloacae* JMU 4002, 2 = *K. pneumoniae* JMU 4014, 3 = *K. pneumoniae* JMU 4036, 4 = *E. cloacae* JMU 4005, 5 = *K. oxytoca* JMU 4032, 6 = *E. cloacae* JMU 4003, 7 = *K. pneumoniae* JMU 4034, 8 = *K. pneumoniae* JMU 4033, 9 = *E. cloacae* JMU 4004, 10 = *E. coli* JMU 4007, 11 = *E. coli* JMU 4042, 12 = *K. pneumoniae* JMU 4031, 13 = *E. coli* JMU 4008, 14 = *E. coli* JMU 4009, 15 = *E. coli* JMU 4006, 16 = *K. pneumoniae* JMU 4040. M = Marker (GelPilot 100 bp, QIGEN, Germany).

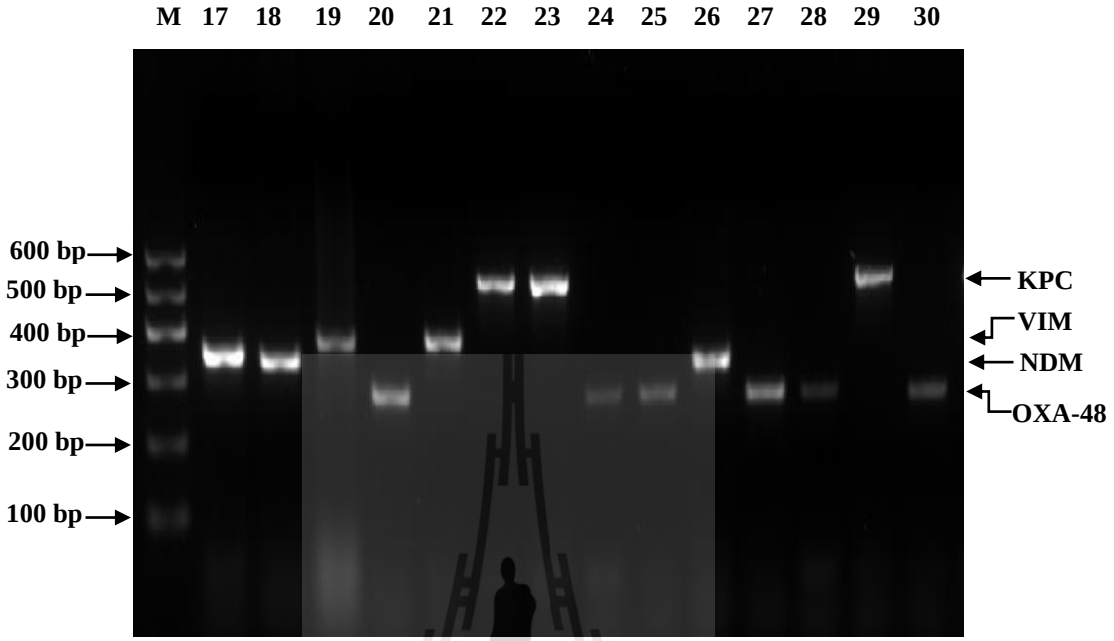


Figure 4.11 Multiplex PCR for the detection of carbapenemases family genes (continue). 17 = *E. coli* JMU 4011, 18 = *K. pneumoniae* JMU 4023, 19 = *K. pneumoniae* JMU 4017, 20 = *E. coli* JMU 4010, 21 = *K. pneumoniae* JMU 4025, 22 = *K. pneumoniae* JMU 4015, 23 = *K. pneumoniae* JMU 4016, 24 = *K. pneumoniae* JMU 4012, 25 = *K. pneumoniae* JMU 4019, 26 = *K. pneumoniae* JMU 4024, 27 = *K. pneumoniae* JMU 4020, 28 = *K. pneumoniae* JMU 4021, 29 = *K. pneumoniae* JMU 4022, 30 = *K. pneumoniae* JMU 4026. M = Marker (GelPilot 100 bp, QIAGEN, Germany)

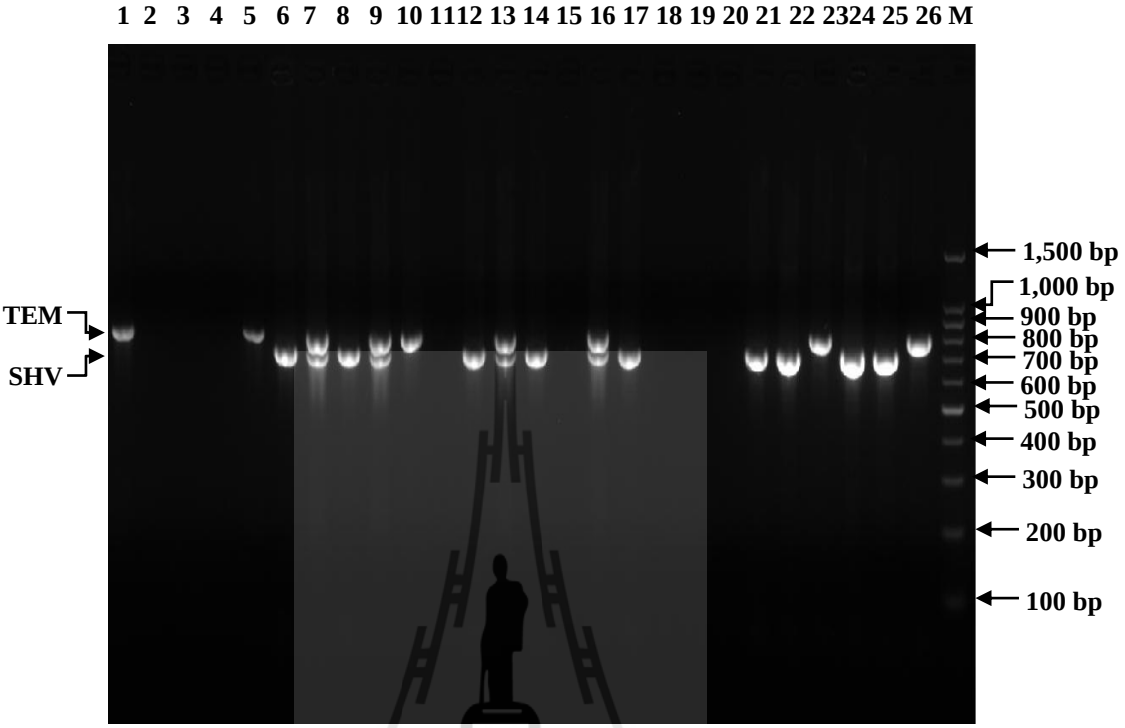


Figure 4.12 Multiplex PCR for detection of bla_{TEM} and bla_{SHV}. 1 = *E. coli* JMU 1004, 2= *E. coli* JMU 1005, 3 = *E. coli* JMU 1006, 4 = *E. coli* JMU 1008, 5 = *E. coli* JMU 1009, 6 = *K. pneumoniae* JMU 1010, 7 = *K. pneumoniae* JMU 1012, 8 = *K. pneumoniae* JMU 1013, 9 = *K. pneumoniae* JMU 1015, 10 = *E. coli* NTCC 13352, 11 = *E. coli* NTCC 13353, 12 = *K. pneumoniae* JMU 1021, 13 = *K. pneumoniae* JMU 1022, 14 = *K. pneumoniae* JMU 1023, 15 = *E. cloacae* JMU 3007, 16 = *E. cloacae* JMU 3009, 17 = *E. aerogenes* JMU 3002, 18 = *E. coli* JMU 3003, 19 = *C. freundii* JMU 3004, 20 = *C. freundii* JMU 3005, 21 = *K. pneumoniae* JMU 4033, 22 = *K. pneumoniae* JMU 4034, 23 = *E. aerogenes* JMU 3010, 24 = *E. aerogenes* JMU 3008, 25 = *E. cloacae* JMU 1019, 26 = *E. cloacae* JMU 30016. M = Marker (GelPilot 100 bp plus, QIAGEN, Germany)

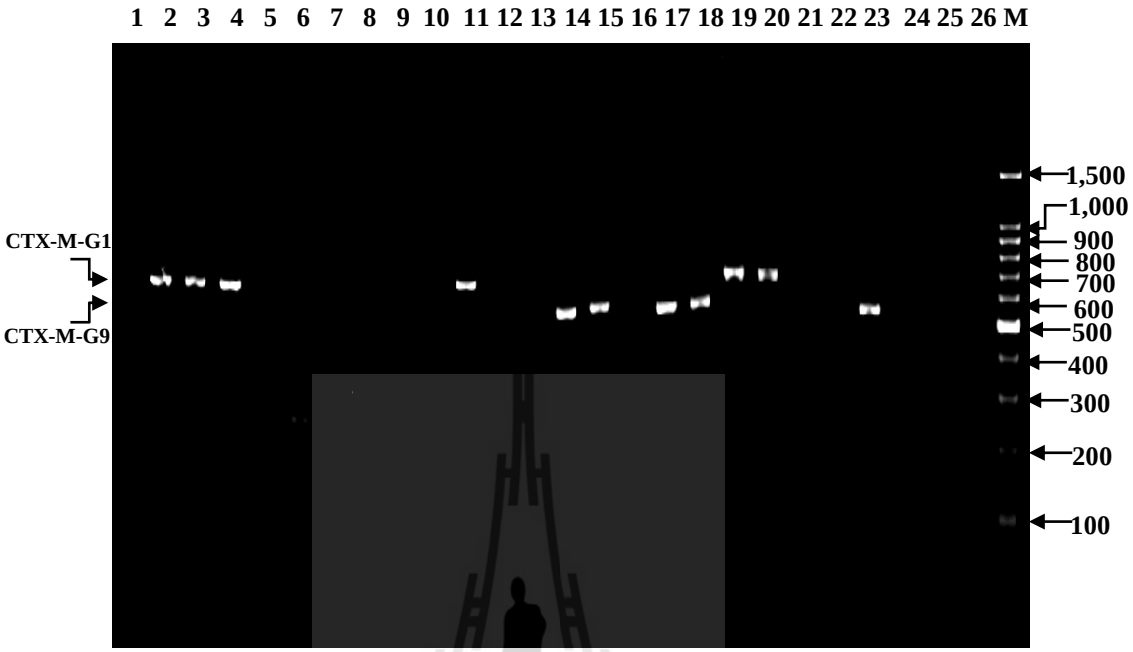


Figure 4.13 Multiplex PCR for detection of bla_{CTM} family genes. 1 = *E. coli* JMU 1004, 2 = *E. coli* JMU 1005, 3 = *E. coli* JMU 1006, 4 = *E. coli* JMU 1008, 5 = *E. coli* JMU 1009, 6 = *K. pneumoniae* JMU 1010, 7 = *K. pneumoniae* JMU 1012, 8 = *K. pneumoniae* JMU 1013, 9 = *K. pneumoniae* JMU 1015, 10 = *E. coli* NTCC 13352, 11 = *E. coli* NTCC 13353, 12 = *K. pneumoniae* JMU 1021, 13 = *K. pneumoniae* JMU 1022, 14 = *K. pneumoniae* JMU 1023, 15 = *E. cloacae* JMU 3007, 16 = *E. cloacae* JMU 3009, 17 = *E. aerogenes* JMU 3002, 18 = *E. coli* JMU 3003, 19 = *C. freundii* JMU 3004, 20 = *C. freundii* JMU 3005, 21 = *K. pneumoniae* JMU 4033, 22 = *K. pneumoniae* JMU 4034, 23 = *E. aerogenes* JMU 3010, 24 = *E. aerogenes* JMU 3008, 25 = *E. cloacae* JMU 1019, 26 = *E. cloacae* JMU 30016. M = Marker (GelPilot 100 bp plus, QIAGEN, Germany)

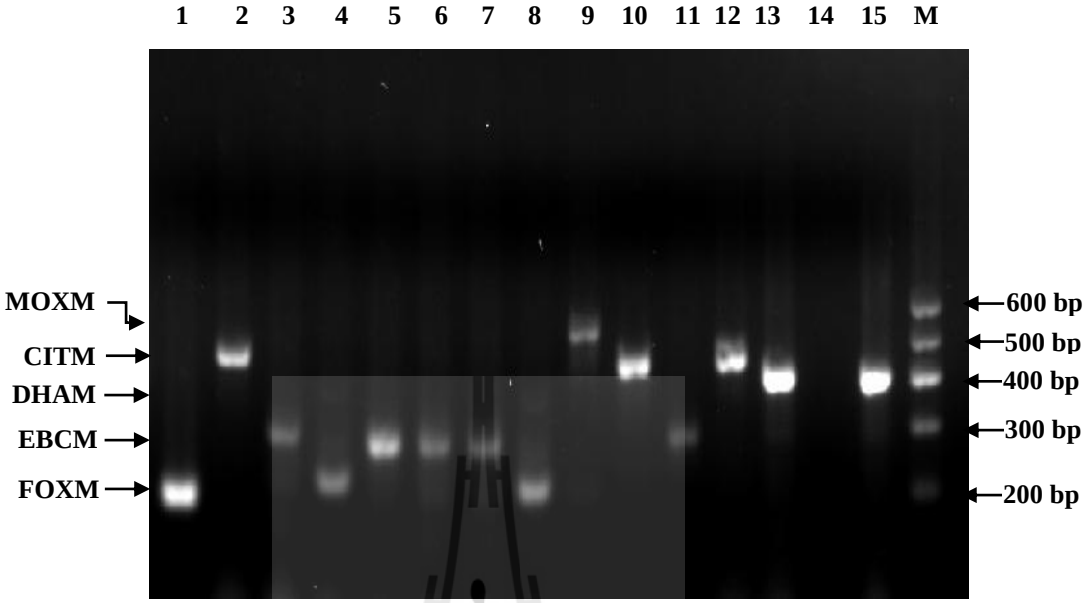


Figure 4.14 Multiplex PCR for detection of bla_{ampC} family genes. 1 = *E. aerogenes* JMU 2039, 2 = *E. aerogenes* JMU 2040, 3 = *E. cloacae* JMU 2041, 4 = *E. aerogenes* JMU 2042, 5 = *E. aerogenes* JMU 2043, 6 = *E. aerogenes* JMU 2044, 7 = *E. cloacae* JMU 2045, 8 = *E. aerogenes* JMU 2046, 9 = *E. coli* JMU 2052, 10 = *E. coli* JMU 2053, 11 = *E. coli* JMU 2054, 12 = *E. coli* JMU 2055, 13 = *K. pneumoniae* JMU 2057, 14 = *K. pneumoniae* JMU 2058, 15 = *M. morganii* JMU 2059. M = Marker (GelPilot 100 bp, QIAGEN, Germany)

Table 4.4 Summary of ESBL, AmpC, ESBL+AmpC and carbapenemase-producing isolates used in the present study.

Group of β-lactamases	*EC*	*KP*	*EA*	ECL	*MM*	*CF*	*KOX*	*KOZ*
Ambler class A ESBL (n =15)								
CTX-M family (n=4)	4	-	-	-	-	-	-	
SHV family (n=3)	-	2	-	1	-	-	-	-
TEM-family (n=3)	3	-	-	-	-	-	-	-
CTX-M + SHV (n=1)	-	1	-	-	-	-	-	-
SHV + TEM (n=3)	-	3	-	-	-	-	-	-
CTX-M + SHV+TEM (n=1)	-	1	-	-	-	-	-	-
Ambler class C AmpC (n =32)								
DHA family (n=6)	2	2	-	-	2	-	-	-
CIT family (n= 7)	4	1	1	-	-	1	-	-
MOX family (n=2)	1	-	-	-	-	1	-	-
EBC family (n=11)	1	1	3	6	-	-	-	-
FOX family (n=6)	2	-	3	1	-	-	-	-
Class A + Class C (n=9)								
TEM+ACT (n=1)	-	-	-	1	-	-	-	-
CTX-M + ACT (n=4)	1	-	1	-	-	2	-	-
TEM+SHV+ACT (n=1)	-	-	1	-	-	-	-	-
TEM+CTX-M+ACT (n=1)	-	-	1	-	-	-	-	-
SHV+ACT (n=1)	-	-	1	-	-	-	-	-
SHV+CTX-M+ACT (n=1)	-	-	1	-	-	-	-	-
Carbapenemase producers (n=30)								
class A KPC (n=8)	2	5	-	-	-	-	1	-
class A MBL (n=11)*	1	7	-	2	-	-	-	1
Ambler class D OXA-48 (n=11)	4	5	-	2	-	-	-	-
Total (number of isolates)	**24**	**28**	**12**	**14**	**2**	**4**	**1**	**1**

EC = *E. coli*, KP = *K. pnuemoniae*, EA = *E. aerogenes*, ECL = *E. cloacae*, MM = *M. morganii*, CF = *C. freundii*, KOX = *K. oxytoca*, KOZ = *K. ozaenae.* ESBL = extended-spectrum-β-lactamase, KPC = *Klebsiella pneumoniae* carbapenemase, MBL = metallo-β-lactamase. * Two of them are VIM-1 + SHV-12 and VIM-1 + SHV-102.

4.2.2 Phenotypic test for carbapenemases

The colorimetric phenotypic method using an RCA plate with a combined disc test for early detection and differentiation of MBL, KPC, and OXA-48 carbapenemases explicitly demonstrated zone diameters within seven hours (Figure 4.15). The distribution of the zone diameters of MER with and without PBA or EDTA and TMC alone against carbapenemase and non-carbapenemase-producing Enterobacteriaceae are shown in Figure 4.16. In MBL producers, the range of zone diameters of MER and MER+PBA was 6.5-20 mm and 6.5-19 mm, respectively. The median diameters for these discs were equal at 17 mm. Discs containing EDTA (median = 26 mm and range = 23-27 mm) resulted in increased zone diameters compared with the discs without EDTA. The range of TMC zone diameters varied from 6.5-17 mm (median = 6.5 mm), as shown in Figure 4.16A. The combined disc test using MER and EDTA successfully detected all MBL producers without false positive results in non-MBL isolates (sensitivity 100%, specificity 100%; Table 4.5).

For detection of KPC-producing Enterobacteriaceae, an increase in zone diameters was observed in MER+PBA (median = 22 mm and range = 19-27 mm) and MER+PBA+EDTA (median = 22.5 mm and range = 19-27 mm) compared with MER alone (median = 15 mm and range = 8-20 mm) or MER+EDTA (median = 16 mm and range = 7-21 mm). Synergistic effects of PBA were found only in KPC strains. The median zone diameter of TMC was 14 mm (range = 11-20 mm) as shown in Figure 4.16B. The sensitivity and specificity values of the PBA synergy test along with RCA assay for detection of KPC-producing Enterobacteriaceae were 100% (Table 4.5).

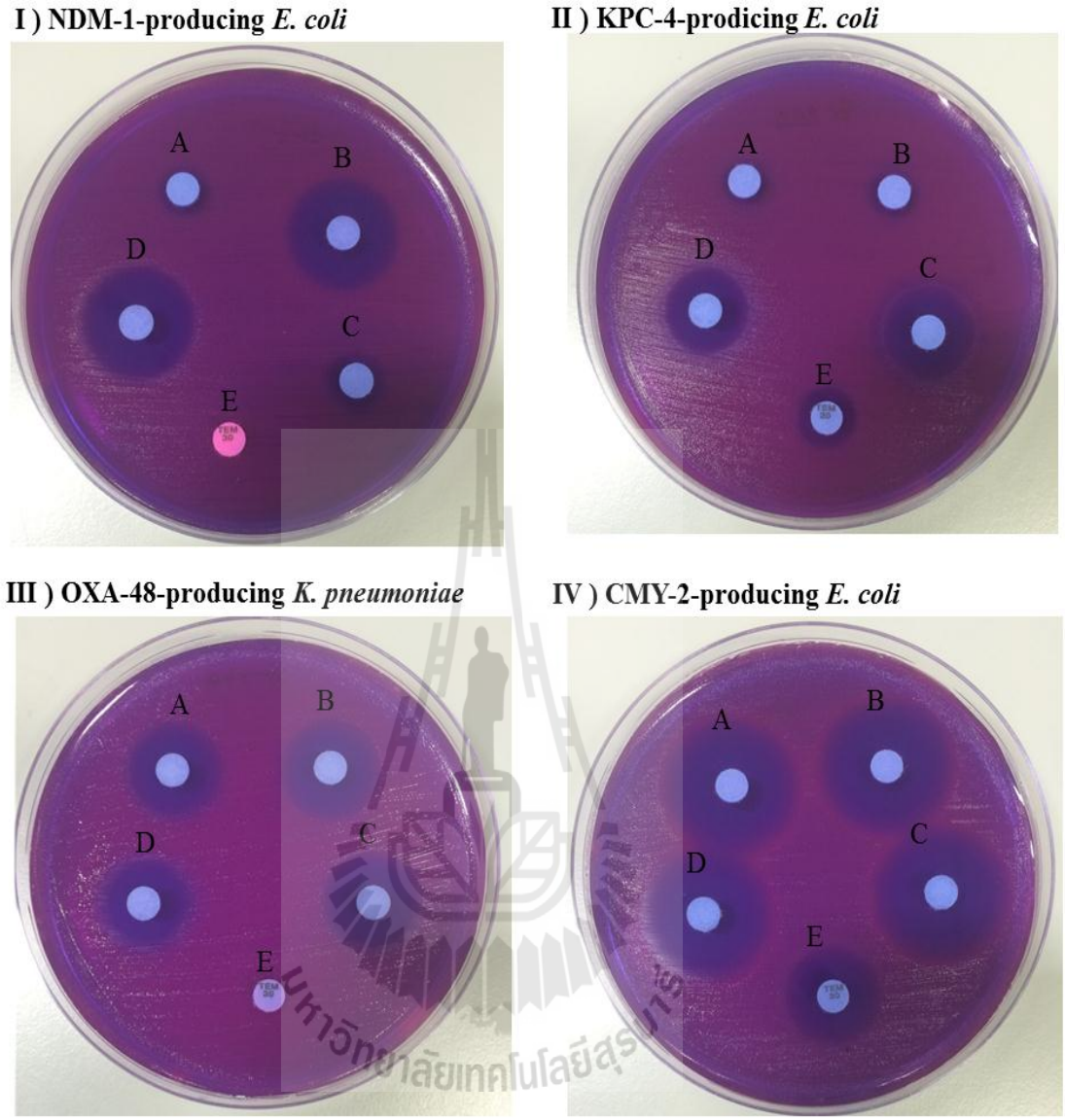


Figure 4.15 Phenotypic results from a combined disc method along with RCA plate assay at 7 h. A = meropenem (10 µg); B = meropenem (10 µg) + EDTA (750 µg); C = meropenem (10 µg) + phenylboronic acid (400 µg); D = meropenem (10 µg) + EDTA (750 µg)+ phenylboronic acid (400 µg); E = temocillin (30 µg). I) *E. coli* JMU 4011; II) *E. coli* JMU 4042; III) *K. pneumoniae* JMU 4026; IV) *E. coli* JMU 2004.

No significant differences in zone diameters of MER disc alone (median = 21 mm and range = 8-25 mm) and with PBA (median = 21 mm and range = 11-26 mm), EDTA (median = 21 mm and range = 12-25 mm), or PBA+EDTA (median = 22 mm and range = 12-25 mm) were observed in OXA-48 producers. These findings indicated no synergistic effect between PBA or EDTA and MER. TMC inhibition zone diameters were ≤ 10 mm (median = 6.5 mm and range = 6.5-9 mm) for all tested OXA-48 producers (Figure 4.16C). The zone diameters of TMC considered together with an absence synergistic effect of MER and PBA, or EDTA were good indicators for identification of OXA-48-producing isolates (sensitivity 100% and specificity 100%; Table 4.5). In non-carbapenemase producers, there was no significant difference in zone diameters between MER alone and PBA or EDTA. The median diameter for TMC was 19 mm (range = 13-26 mm) against these isolates (Figure 4.16D). In addition, 10 µg MER clearly inhibited the growth of reference strain *E. coli* ATCC 25992. The zone diameter against this strain was 29 mm, which was in the quality control range (28- 34 mm) for non-fastidious organisms in the CLSI document (Clinical Laboratory Standards Institute 2014). The results of the present investigation using a combined disc method with RCA clearly discriminated different types of carbapenemases without discrepancy.

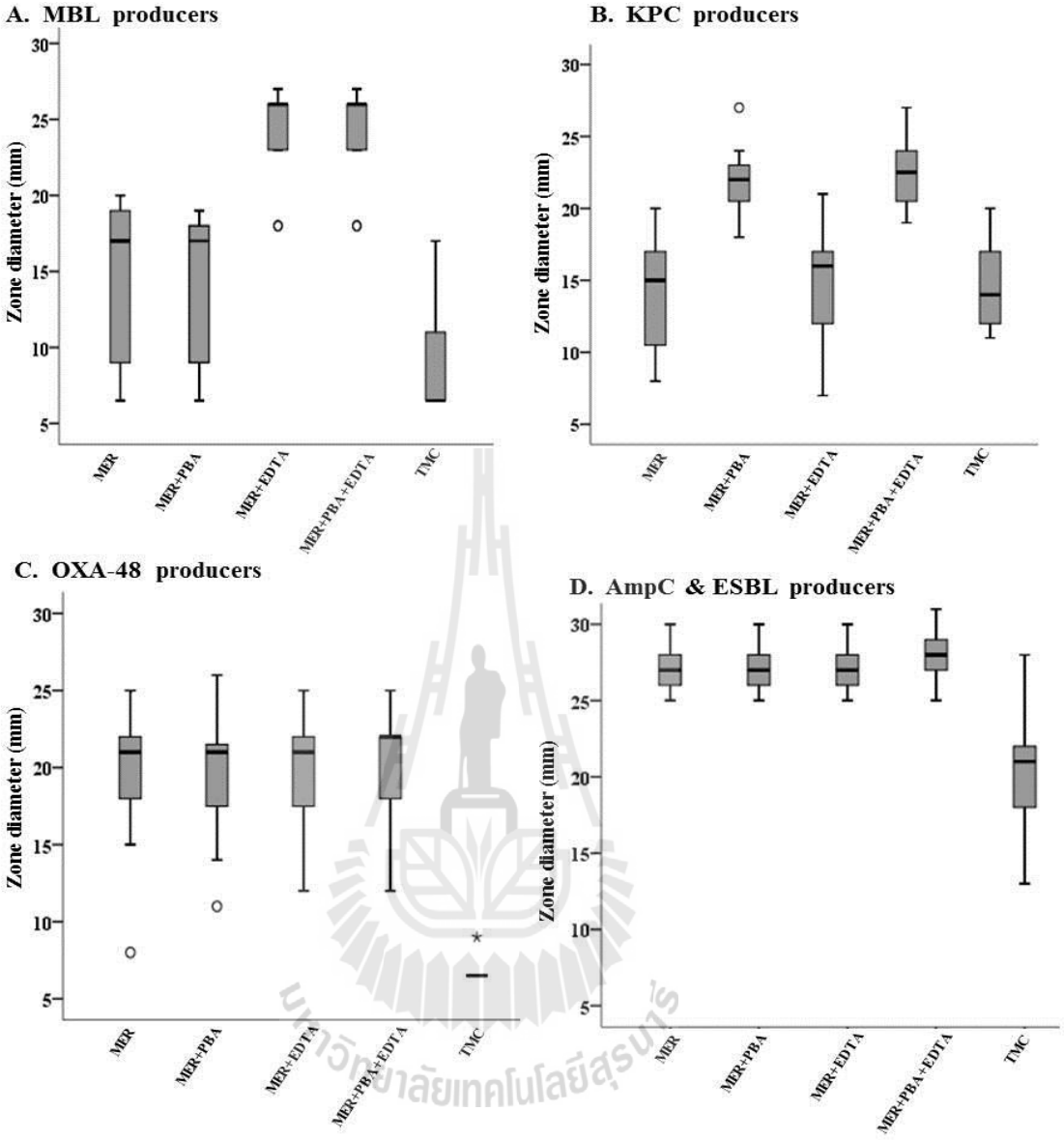


Figure 4.16 Distribution of Zone diameters of meropenem (MER) alone and with phenylboronic acid (PBA) and EDTA and temocillin (TMC) alone.

A = MBL producers (n=11); B = KPC producers (n=8); C = OXA-48 producers (n=11); D = non-carbapenemase producers (n=57). º = mild outlier; * extreme outlier.

Table 4.5. The performance of a combined disc test along with RCA assay for the early detection of carbapenemase-producing Enterobacteriaceae.

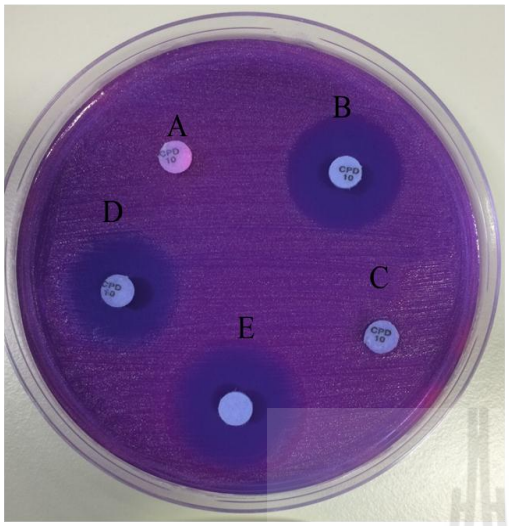
Test		Sensitivity	Specificity
EDTA synergy	MER+EDTA vs MER ≥ 5 mm and MER+EDTA+PBA vs MER+PBA ≥ 5 mm	100 (11/11)	100 (76/76)
PBA synergy	MER+PBA vs MER ≥ 5 mm and MER+EDTA+PBA vs MER+EDTA ≥ 5 mm	100 (8/8)	100 (79/79)
TMC zone ≤ 10 mm*	Considered when the absence of EDTA, PBA, and EDTA+PBA synergy tests	100 (11/11)	100 (76/76)

MER = meropenem disc (10 μg), PBA = phenylboronic acid disc (400 μg) TMC = temocillin disc (30 μg). EDTA = Ethylenediaminetetraacetic acid (750 μg)

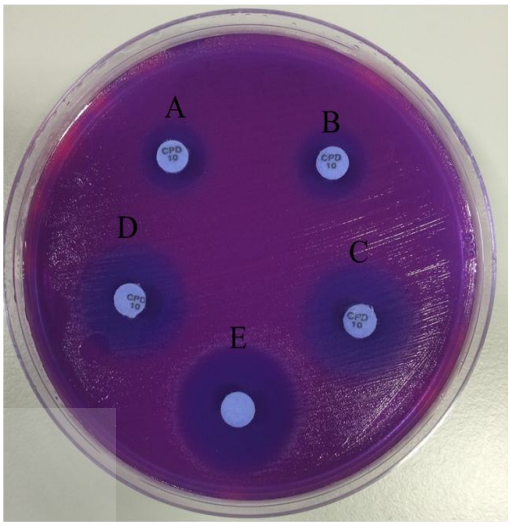
4.2.3 Phenotypic test for ESBL and AmpC β-lactamases

RCA assay along with CPD combined disc synergy test was phenotypically able to screen and differentiate ESBL, AmpC, and co-expression of ESBL plus AmpC within 7 h (Figure 4.17). The distribution of the zone diameters of CPD impregnated with and without CA, CX, or CA plus CX, and MER alone against ESBL, AmpC, co-existence of ESBL and AmpC, and carbapenemase-producing Enterobacteriaceae is illustrated in Figure 4.18.

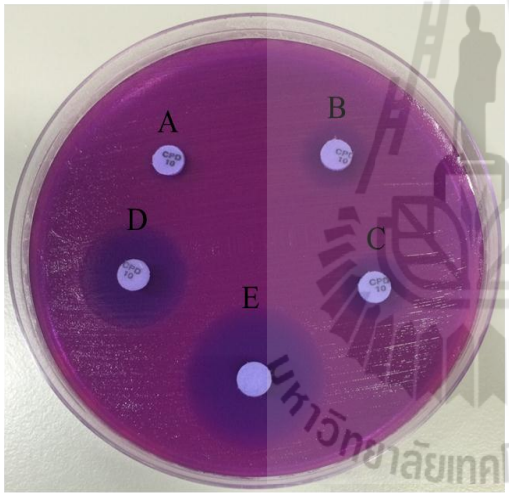
I) SHV-27+TEM-115-producing *K. pneumoniae*



II) Fox-3-producing *E. coli*



III) CTX-M-3+ACT-1-producing *C. freundii*



IV) KPC-2-*K. pneumoniae*

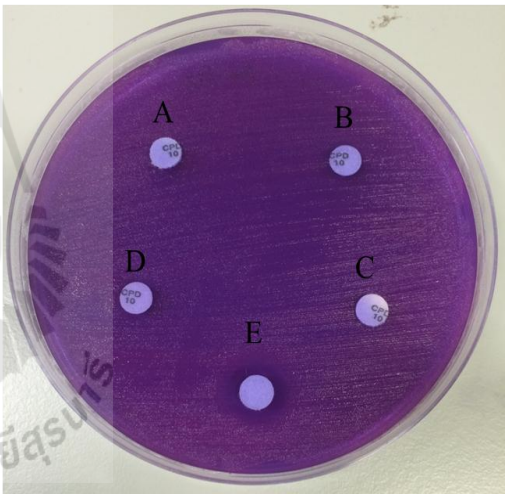


Figure 4.17 Phenotypic results from RCA plate assay with a combined disc method. A = cefpodoxime (10 µg); B = cefpodoxime (10 µg) + clavulanic acid (10 µg); C = cefpodoxime (10 µg) + cloxacillin (500 µg); D = cefpodoxime (10 µg) + clavulanic acid (10 µg) + cloxacillin (500 µg); E = meropenem (10 µg). I) *K. pneumoniae* JMU 1022; II) *E. coli* JMU 2042; III) *C. freundii* JMU 3005; *K. pneumoniae* JMU 4016.

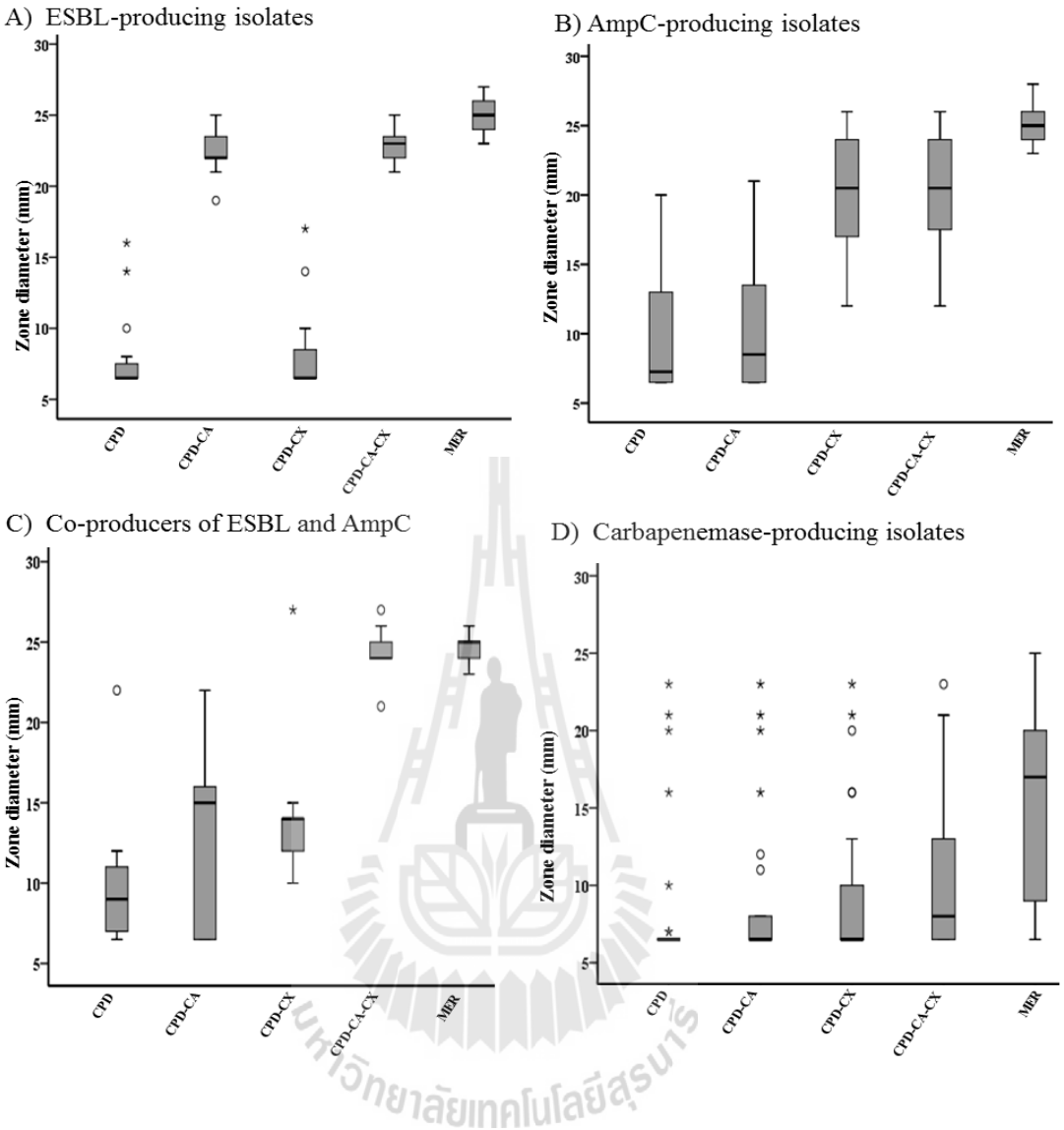


Figure 4.18 Distribution of zone diameters of cepodoxime alone and with clavulanic acid, cloxacillin, and meropenem alone. A = ESBL producers (n=15); B = AmpC producers (n=32); C = co-producers AmpC and ESBL (n=9); D = carbapenemase producers (n=30). CPD = cefpodoxime (10 µg); CA = clavulanic acid (10 µg); CX = cloxacillin (500 µg); MER = meropenem (10 µg). º = mild outlier;

* extreme outlier.

Table 4.6 Sensitivity and specificity of a combined disc synergy method along with RCA assay for rapid screening of ESBL, AmpC, and co-producers of ESBL and AmpC among Enterobacteriaceae

Synergy test	Definition of the test	Sensitivity	Specificity
CA synergy test	CPD+CA vs CPD ≥ 5 mm and CPD+CA+CX vs CPD+CX ≥ 5 mm	100 (15/15)	98.6 (71/72)
CX synergy test	CPD+CX vs CPD ≥ 5 mm and CPD+CA+CX vs CPD+CA ≥ 5mm	100 (32/32)	96.36 (53/55)
CA+CX synergy test	Both CPD+CA+CX vs CPD+CX ≥ 5 mm and CPD+CA+CX vs CPD+CA ≥ 5 mm	88.89 (8/9)	100 (78/78)

CPD = cefpodxime (10 µg); CA = clavulanic acid (10 µg); CX = cloxacillin (500 µg).

For screening of ESBL-producing isolates, the median zone diameter of CPD alone was 6.5 mm (range = 6.5-16 mm) and diameters of CPD supplemented with CA, CX, or CA plus CX were 22 mm (range = 19-25 mm), 6.5 mm (range = 6.5-17 mm), and 23 mm (range = 21-25 mm), respectively. MER discs exhibited potent activity in the inhibition of ESBL producers with median zone diameter 25 mm (range = 23-27 mm) (Figure 4.18A). A substantial increase in zone diameters of CA-containing discs compared with the discs without CA was only observed in ESBL-producing isolates. The mean zone increase of CPD plus CA compared with CPD alone was 14.60 mm (range = 5-17.50 mm). No marked increase in zone diameter was observed in AmpC producers (mean = 0.48 mm and range = 1.5-3.5 mm), co-producers of ESBL and AmpC (mean = 2.72 mm and range 3.57 mm), as well as carbapenemase-producing isolates (mean = 0.37 mm and range = 0-55 mm). The RCA assay with combined disc

method successfully detected all test ESBL producers with 100% sensitivity and 98.6% specificity (Table 4.6). A false-positive result was observed in OXA-48-producing *E. coli.*

In AmpC producers, an increase in median zone diameters was seen in CX-containing discs. CPD plus CX and CPD plus CX plus CA had equal median zone diameters (20 mm) and range 12-26 mm. The Median zone diameter of CPD against these isolates was 7.25 mm (range = 6.5-20 mm), which was similar to CPD plus CA (median = 8 mm and range = 6.25-21 mm). MER discs inhibited the growth of AmpC-producing isolates at a median zone diameter of 25 mm and range 23-28 mm (Figure 4.18B). The mean difference of zone diameter of CPD plus CX versus CPD alone was 10.1 mm (range = 5.5 -16.5 mm) against AmpC producers, whilst no dramatic difference in mean zone increased was observed in ESBL producers (mean = 0.33 mm and range = 0-2 mm), co-producers of ESBL and AmpC (mean = 4.33 mm and range = 1-7.5 mm), and in carbapenemase producers (mean = 0.88 mm and range = 0-9.5 mm). The RCA assay demonstrated excellent performance in the screening of AmpC-producing strains by detecting all AmpC producers (100% sensitivity), but there were two false-positives in the KPC-3-producing *K. pnuemoniae* and OXA-48-producing *E. coli* (96.36% specificity; Table 4.6).

For screening of ESBL and AmpC-co-producing Enterobacteriaceae, CPD discs alone had median zone diameters of 9 mm (range = 6.5-22 mm). CPD plus CA (median = 15 mm and range = 6.5-22 mm) and CPD plus CX (median = 14 mm and range = 10-27 mm) showed a slight increase in median zone diameter compared with CPD alone. CPD plus CA plus CX demonstrated excellent activity in inhibiting the growth of ESBL and AmpC co-producers. The median zone was significantly

increased (median = 24 mm and range = 21-27 mm) in comparison with those of CPD alone, CPD plus CA, and CPD plus CX. The median zone diameter and zone range of MER against these isolates were 25 mm and 23-26 mm, respectively (Figure 4.18C). The mean difference in zone diameter of CPD plus CA and CX versus CPD plus CA, or versus CPD plus CX was also calculated. The mean zone increase of CPD plus CA and CX versus CPD plus CA was 11. 6 mm (range=5-19.5 mm). A similar result was observed in CPD plus CA and CX versus CPD plus CX. The sensitivity and specificity of the RCA assay with a combined disc method were 88.9% and 100%, respectively (Table 4.6). The assay failed to detect ESBL activity in an SHV plus ACT-producing *E. aerogenes*. Furthermore, in carbapenemase-producing isolates, the median zone diameters of CPD with and without CA, CX, or CA and CX were not markedly different, while the ranges were varied. The MER disc alone had a median zone diameter of 17 mm and range 6.5-25 mm (Figure 4.18D). A reference strain *E. coli* ATCC 25922 was inhibited by a CPD disc alone with zone diameter 25 mm which was in the susceptible range according to the CLSI breakpoint (≥21 mm) (Clinical Laboratory Standards Institute, 2014). The findings of this study demonstrated that the RCA assay with CPD combined discs showed an excellent performance in screening and differentiation of ESBL, AmpC, and co-production of ESBL and AmpC in Enterobacteriaceae.

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Discussion

5.1.1 Antibacterial activity of *B. rotunda* extract

The resistance of microorganisms to practically-prescribed antibiotics is becoming one of the greatest issues threatening human health. This problem motivates scientists to develop novel antimicrobial agents (Page and Bush, 2014). Plant-derived antimicrobials are an interesting source for new antimicrobial agents (Savoia, 2012). To prevent and delay the development of antibiotic resistance, the use of a drug combination therapy between natural-occurring antimicrobials and well-tried agents which have lost their original effectiveness is an area of far-reaching importance. Using a phytochemical and antibiotic combination approach is of interesting avenue for combating multidrug-resistant (Eumkeb and Chukrathok, 2013; Wagner, 2011; Worthington and Melander, 2013; Wright and Sutherland, 2007). Cell wall inhibitor antibiotics are potent antibiotics that are commonly used for the treatment of the infections caused by Gram-positive bacteria and Gram-negative bacteria (Brenner and Stevens, 2010). Resistance to this group of antibiotics has been previously reported in such pathogenic bacteria (Maharat Nakhon Ratchasima Hospital, 2015; Nikolaidis et al., 2014). This study described the effect of BRE and cell wall inhibitor antibiotics when they were used singly or in combination. The

following preliminary mechanisms of action were also investigated: ultrastructure damage (using TEM), cytoplasmic membrane permeability and antibiofilm activity.

MIC determination using a resazurin microtitre plate showed that BRE potentially inhibited the growth of all tested Gram-positive bacteria at low MIC values. MRSA 20651 and MRSA 20652 were highly resistant to CLX, AMP, and CFZ, but they were still susceptible to VAN. A clinical isolate of *S. epidermidis* DMST 14919 was also resistant to CLX and AMP, whereas this bacterium was susceptible to CFZ and VAN. The MICs of these antibiotics against *S. epidermidis* were lower than MRSA strains. CLX treated MRSA strains had highest MIC values against both MRSA strains. The resistance of *Staphylococcus* spp. to penicillin antibiotics is mainly mediated by acquisition of *mecA*-encoded penicillin-binding protein 2a (PBP2a) and *blaZ*-encoded penicillinase (Arede and Oliveira, 2013; Zhang et al., 2001). CLX has been reported to be stable to staphylococcal penicillinases (Knox and Smith, 1963). Therefore, it is possible that two of the resistance mechanisms of the MRSA strains tested in the present study are mainly mediated by *mecA*-encoded PBP2a. This could also be the major cause of β-lactam resistance in *S. epidermidis*. In Gram-negative bacteria, no inhibitory effects of BRE at a concentration of 1024 μg/mL were observed. However, a previous study reported that the growth of *E .coli* strains was inhibited by BRE at a concentration of 2.5 mg/mL (Zainin et al., 2013).

Drug interaction from the chequerboard assay exhibited synergistic activity between BRE plus CLX, BRE plus AMP and BRE plus CFZ against MRSA isolates. The combination of BRE and CLX showed a lowest FIC index. For *S. epidermidis*, only BRE plus AMP combination showed synergistic activity. BRE is

enriched with flavonoids and their derivatives (Chahyadi et al., 2014; Tan et al., 2015). There were some studies reporting that flavonoids showed, such as flavonoid galangin, quercetin, and baicalein isolated from *Alpinia officinarum* Hance, demonstrate synergistic interaction with antibiotics against clinical isolates of *S. aureus* (Eumkeb et al., 2010). Similarly, apigenin in combination with ceftazidime was synergistic against clinical isolates of ceftazidime-resistant *E. cloacae* (Eumkeb and Chukrathok, 2013).

The time-kill curve assay confirmed synergistic activity of the BRE and CLX combination against MRSA 20651. This combination acted bacteriostatically against MRSA 20651. A previous study by Limsuwan and Voravuthikunchai (2013) found that *Streptococcus pyogenes* was grown steady at a concentration of 1/2 MIC, while was inhibited bactericidally at MIC, 2x MIC and 4x MIC in a dose-dependent manner. The present study investigated at a half-MIC of BRE and CLX against MRSA 20651, at this concentration, this bacterium grew continuously. It is speculated that the activity of BRE or BRE plus CLX may achieve bactericidal activity as previously reported when a concentration of MIC or higher is used.

The micrographs from the transmission electron microscope (TEM) explicitly illustrated ultrastructure damage in BRE and CLX either treated alone or in combination. Disruption of cell wall was seen in CLX-treated cells. It has been well-established that β-lactams inhibit peptidoglycan synthesis by binding covalently to PBP resulting in cell lysis (Brenner and Stevens, 2010). In cells treated with BRE, destruction of the cytoplasmic membrane without damage to cell wall was observed. This result suggests that the mechanism of action of BRE is destroying the bacterial

cytoplasmic membrane accompanied by the leakage of intracellular constituents. In cells treated with CLX plus BRE, damage of both the cytoplasmic membrane and the cell wall was observed in this treated group. Some bacteria were losing their cytoplasmic materials. The findings indicate that CLX and BRE inhibit the growth of MRSA and target different mechanisms of action. Therefore, they work together synergistically. The synergistic interactions occur when two compounds target different mechanisms, while additively happens when different compounds target the same mechanism of action against the target (Basri and Sandra, 2016).

BRE alone and in combination with CLX efficiently induced hyperpermeability of the cytoplasmic membrane of MRSA 20651. The combination of BRE and CLX showed the highest amount of OD_{260}-absorbing materials, but no significant difference compared with BRE alone and nisin alone. No marked increase in OD was observed in CLX alone and the control group. This finding may be due to the effect of the interaction between BRE and the cytoplasmic membrane. How BRE exactly interacts with the cytoplasmic membrane is unknown. Nisin, a positive membrane permeable control, primarily kills Gram-positive bacteria by forming pores in the cytoplasmic membrane and also by inhibiting peptidoglycan synthesis (Zhou et al., 2014).

It was also shown that BRE prevents the development of biofilm. The MRSA 20651 biofilm was significantly minimised following exposure to BRE either used alone or in combination. This finding agrees with Yanti and colleagues who reported that Panduratin A isolated from *Kaempferia pandurata* Roxb (synonym *B. rotuda* extract) has the ability to disperse biofilm formed by multispecies oral bacteria (Yanti et al., 2009). Also, flavonoids from *Moringa oleifera* seed extract have

inhibitory effects on biofilms of *S. aureus*, *P. aeruginosa* and *C. albicans* (Onsare and Arora, 2015). In addition, other flavonoids, such as naringenin, kaempferol, quercetin, and apigenin have also been reported to have the ability to eradicate *Vibrio harveyi* and *E. coli* biofilms (Vikram et al., 2010).

5.1.2 Development of diagnostic methods for detection of β-lactamases

5.1.2.1 Detection of carbapenemase-producing Enterobacteriaceae

Carbapenem resistance in Enterobacteriaceae poses a challenging issue for treatment and infection control. The rapid diagnostic test plays an important role in guiding clinicians to appropriate antibiotic administration and minimising treatment failure (Nordmann et al., 2012a). Molecular-based methods for characterisation of carbapenemase-producing Enterobacteriaceae are restrictive due to high cost, the requirement of skilled and experienced technicians and more importantly the inability to detect novel carbapenemase-encoding genes (Picao et al., 2008). Current combined disc synergy tests with β-lactamase inhibitors are simple, inexpensive and able to discriminate the different types of carbapenemases effectively, but they are limited in time as results require at least 18 h incubation (Osei Sekyere et al., 2015). To improve turnaround time, in the present study we conducted RCA plate along with combined disc method for early screening of clinically-important carbapenemases including MBL, KPC, and OXA-48.

For detection of MBL-producing Enterobacteriaceae, EDTA synergy test was able to detect all tested MBL producers with 100% sensitivity and specificity. This result agrees with a study previously reported by Tsakris et al. (2010). They found that the combined disc method containing EDTA successfully detected all clinical isolates of VIM-producing Enterobacteriaceae. Surprisingly, some

studies reported using EDTA as an MBL inhibitor and MER as a substrate gave some false-positive results in non-MBL-producing *K. pneumoniae* (Giske et al., 2011). Similarly, a combined disc method supplemented with imipenem and DPA showed better activity than EDTA against *Pseudomonas* spp. and *Acinetobacter* spp. producing IMP-1-like, VIM-2-like, and SIM-1-type MBLs (Yong et al., 2012). Giske *et al.* (2011) found that DPA synergy test with MER as a substrate had both 100% sensitivity and specificity, which was a superior performance in detection of MBL producers compared with EDTA. A good sensitivity (90%) and specificity (96%) of DPA synergy test were also previously reported by van Dijk et al. (2014) for detection of MBL-producing Enterobacteriaceae. Nevertheless, a study performed by Pitout et al. (2005) found that MER was more effective than imipenem and suggested the use of MER in combination with EDTA for detection of MBL-producing *Pseudomonas aeruginosa*.

The result of the PBA synergy test in the present study using 400 µg PBA was reliably able to detect all KPC-producing Enterobacteriaceae clinical isolates (100% sensitivity). Tsakris et al. (2011) reported a few false-positive results were observed (97.6% specificity) and also PBA was more effective than aminophenylboronic acid in the detection of KPC-producing isolates. A similar sensitivity and specificity of the PBA synergy test for detecting KPC-producing Enterobacteriaceae were also reported by several studies (Tsakris et al., 2010; van Dijk et al., 2014). The PBA synergy test was not only positive in KPC producers, but it was also observed in producers of AmpC plus porin loss. It has been suggested that using cloxacillin synergy test can potentially discriminate between KPC and AmpC plus porin loss (Giske et al., 2011).

TMC zone diameter ≤10 mm considered together with absence in a synergy of MER and EDTA or PBA was able to detect all OXA-48-producing Enterobacteriaceae. The results of the present study were consistent with several studies reporting excellent sensitivity and specificity of TMC disc in the detection of OXA-48-like enzymes (OXA-48, OXA-162, OXA-181 and OXA-204) (Hartl et al., 2013; Oueslati et al., 2015; van Dijk et al., 2014). The zone diameters of MER discs against all non-carbapenemase-producing Enterobacteriaceae including ESBL and AmpC isolates were in the susceptible range in the CLSI guideline (Clinical Laboratory Standards Institute 2014).

5.1.2.2 Detection of ESBL and AmpC-producing isolates

Resistance to β-lactams, the most potent bactericidal antibiotics commonly used for the treatment of bacterial infection, has been continuously documented throughout the world. β-Lactamase-mediated resistance is a major mechanism that can potentially render β-lactams inactive by hydrolytically cleaving the amide bond of the β-lactam ring (Wright, 2005). To guide clinicians to prescribe antibiotic therapy appropriately, development of rapid diagnostic methods is necessary. The detection of the presence of β-lactamase enzymes in Gram-negative bacteria at phenotypic level is a promising method because it is not costly compared with genotypic tests (PCR and sequencing). Also, a phenotypic method does not require skilled and experienced technicians (Picao et al., 2008). The principle of the phenotypic test is fundamentally based upon synergistic effect between antibiotics and β-lactamase inhibitors (Coudron, 2005). Several phenotypic tests for the detection of β-lactamase enzymes in Gram-negative bacteria have currently been proposed, including disc diffusion assays and broth microdilution methods (Derbyshire et al.,

2009; Jeong et al., 2009; Nourrisson et al., 2015; Yagi et al., 2005). In the present study, the rapid screening method using an RCA assay along with a CPD combined disc method to detect and discriminate the presence of β-lactamases within 7 h has been proposed.

CA synergy test using the RCA assay with CPD combined discs to screen the presence of ESBL production in Enterobacteriaceae was capable of detecting all test ESBL-producing isolates. There was only one false-positive found in an OXA-48-producing isolate. This finding agrees with a previous report published by (Derbyshire et al., 2009). They found that CA synergy test using CPD was able to detect all 117 ESBL producers indicated by a ≥ 5 mm increase in zone diameter of CPD plus CA in comparison with CPD alone. This synergy test could not detect ESBLs in the co-presence with AmpCs. This diagnostic tool was not able to discriminate the strains that produced, especially, complex mutant TEM and inhibitor-resistant TEM, such as TEM-18, TEM-29, TEM-50, TEM-109, TEM-151, TEM-125, TEM-126, TEM-152, PER-1, GES-6, and GES-7 (Nourrisson et al., 2015). In our investigation, these particular β-lactamase-producing isolates were not available for validation. Therefore, the difference in sensitivity and specificity of the phenotypic test from several studies may result from using distinct types of β-lactamases.

For screening of AmpC-producing isolates using CX synergy test, the assay was able to detect all AmpC producers and two false positive results (100% Sensitivity and 96.36% specificity). This result is consistent with many previous works reporting a good performance of CPD and CX synergy test in detection of these enzymes. MAST® D68C successfully detected almost all AmpC producers, and few false-positive results were reported (96.7% sensitivity and 96.9%

specificity). It could not detect the presence of AmpC β-lactamases in naturally producing AmpC species (Nourrisson et al., 2015). A similar result was reported by Ingram et al. (2011), they found that MAST® D68C exhibited a sensitivity and specificity above 90% in detection of the presence of AmpC β-lactamase in Enterobacteriaceae. Likewise, a previous study by Mansour et al. (2013) reported that MAST-4 disc demonstrated good sensitivity (92%) and specificity (86.7%) in the detection of AmpC-producing nosocomial *Klebsiella* isolates. All test AmpC producers used in the present study exhibited high resistance to CPD, indicating hyperproduction of AmpC β-lactamases.

Combined activity of ESBL and AmpC in the same strain can result in phenotypic detection failure (Goossens and Grabein, 2005). Co-production with AmpC β-lactamases can mask ESBL production with CLSI confirmatory tests leading to false-negative results (Munier et al., 2010). Therefore, adding two or more specific β-lactamase inhibitors could exclude different types of β-lactamase in the same strain. The present study used CA plus CX synergy test to discriminate co-producers of ESBL and AmpC. The assay was able to detect 8 out of 9 co-producers of ESBL and AmpC. A false-positive isolate detected was susceptible to CPD according to the CLSI breakpoint (Clinical Laboratory Standards Institute, 2014). The finding from this study is similar to the result from MAST® D68C which can successfully detect all 8 ESBL and AmpC-co-producing isolates (Nourrisson et al., 2015). To screen carbapenemase-producing isolates, it has been recommended to use a cutoff point at lower than 25 mm for MER disc because the zone diameter of MER in some OXA-48 like-producing is still in the susceptible range (≥ 23 mm) (Giske et al., 2011; van Dijk et al., 2014). The current study found that MER zone diameters

against ESBL, AmpC, and Co-ESBL and AmpC ranged from 23-28 mm, while in carbapenemase-producing isolates zone diameters ranged from 6.5-25 mm. Only one OXA-48 producing isolate had a zone diameter of 25 mm. Thus, the isolates showing zone diameters < 25 mm for the 10 μg MER disc should be further investigated to detect the distinct type of carbapenemase (MBL, KPC, and OXA-48 like carbapenemases) or AmpC plus porin loss.

5.2 Conclusion

BRE possess an excellent inhibitory activity towards MRSA and *S. epidermidis*. Its mechanism of action is by interacting with the bacterial cytoplasmic membrane of bacteria resulting in the destruction of the cytoplasmic membrane, leading to leakage of the intracellular materials. Synergistic activity of BRE in combination with CLX against MRSA strains may involve co-inhibition of cell wall synthesis and cytoplasmic membrane damage as a result of CLX and BRE, respectively. BRE alone and in combination with CLX potentially permeabilised the cytoplasmic membrane leading to loss of OD_{260}-absorbing materials, including DNAs. Furthermore, they also exhibit a good activity against biofilm formation. *B. rotunda* has a high potential for the development of novel adjunct phytopharmaceutical to cloxacillin for the treatment of the infections caused by MRSA, which is highly resistant to cloxacillin. The efficacy and toxicity of this combination in an animal test or humans should be further investigated and confirmed.

The combined disc method is effectively applicable in any microbiological laboratory. Using this test along with RCA assay is very simple and provides a faster result compared with the combined disc method alone. The result of

RCA assay is visually easy to interpret. It also demonstrates excellent sensitivity and specificity for differentiation of MBL, KPC, and OXA-48-producing Enterobacteriaceae, as well as in discrimination of ESBL, AmpC and co-production of ESBL and AmpC among Enterobacteriaceae. The RCA assay can be applied to commercially available discs, such as MAST-CDS (Mast Group, UK) and Rosco Diagnostica A/S (Denmark). However, further studies should be performed against a larger sample size of clinical isolates with co-producers of class A and class B or AmpC plus porin loss to establish the robustness of this assay. The early detection of carbapenemase would aid healthcare professionals to manage patients, control the spread of infection and achieve epidemiological surveillance.

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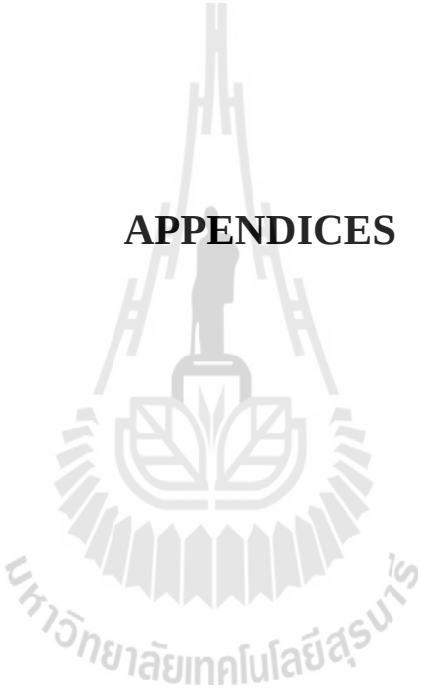
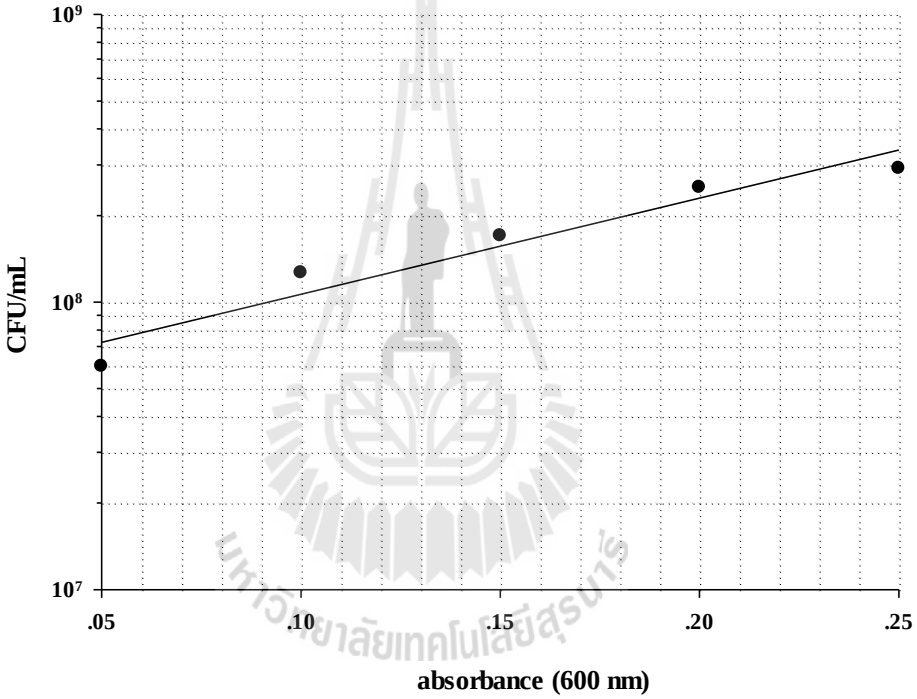
APPENDICES



APPENDIX A

BACTERIAL SUSPENSION STANDARD CURVE

Bacterial suspention standard curve of *S. aureus* DMST 20651



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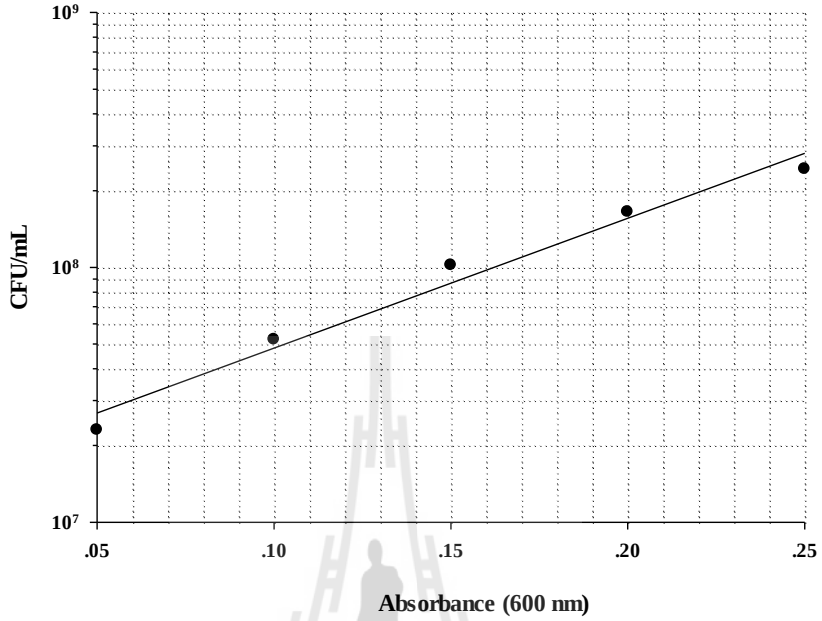

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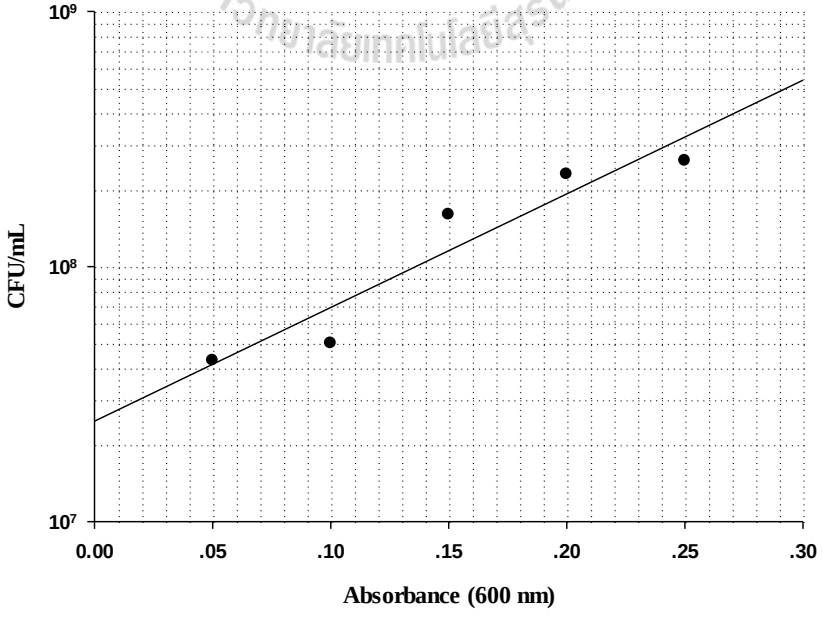
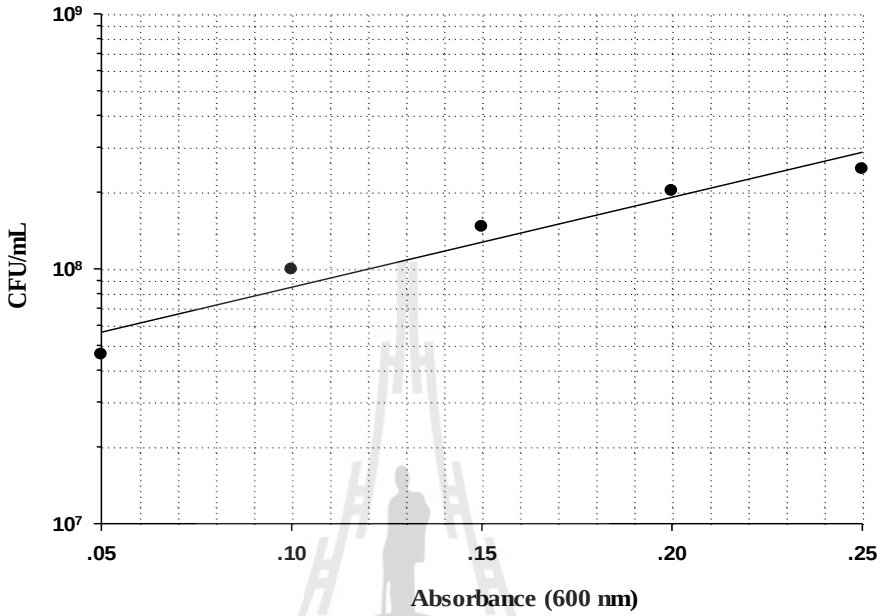
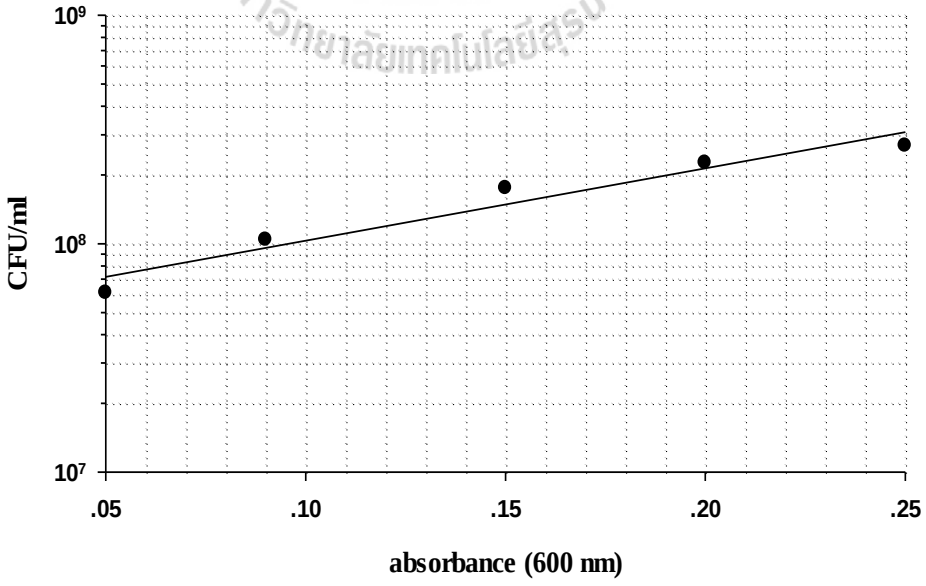


Bacterial suspension Standard curve *S. aureus* ATCC 29213



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



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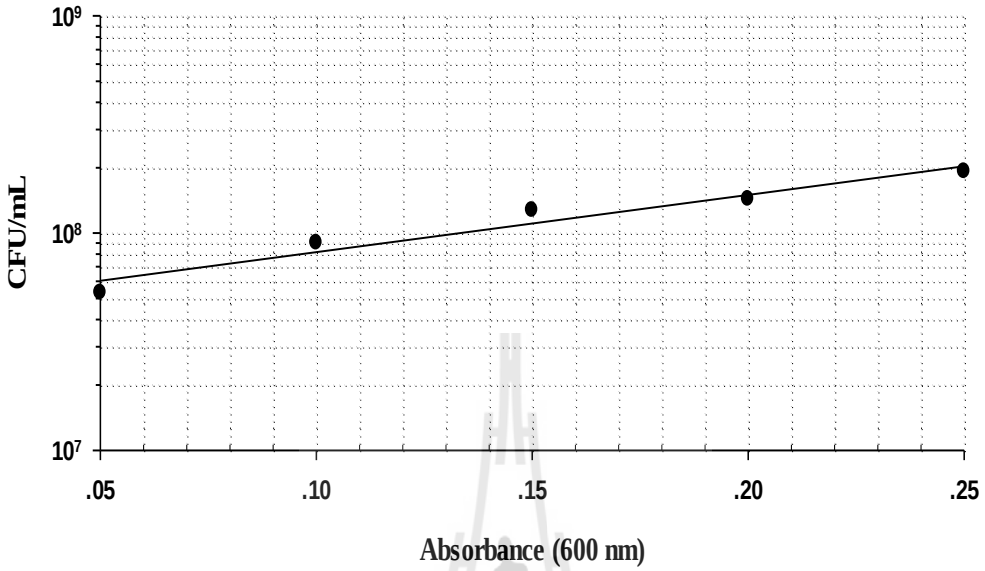
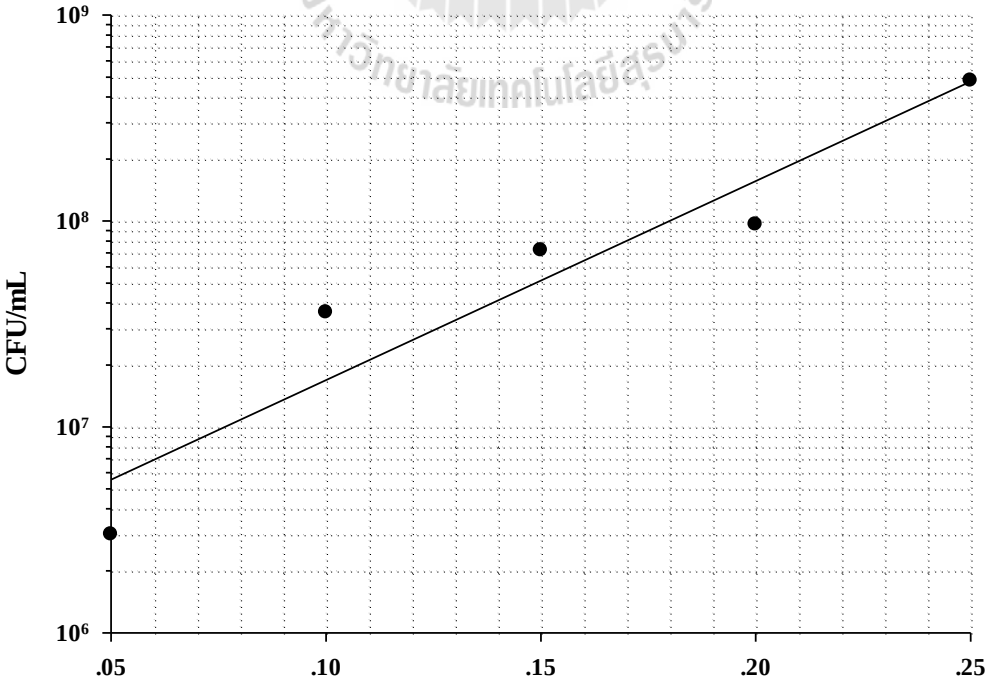


Bacterial suspension standard curve of *A.baumanii* DMST 45378



APPENDIX B

MOLECULAR TYPES OF ENTEROBACTERIACEAE

Table B Molecular types of β-lactmases-producing Enterobacteriaceae used in the present study.

No.	Bacterial isolates	JMU code	Molecular type
1	*K. pneumoniae*	4034	VIM-1+SHV-12
2	*K. pneumoniae*	4033	VIM-1+SHV-102
3	*K. pneumoniae*	4036	NDM-1
4	*K. ozaenae*	4031	IMP-1
5	*E. cloacae*	4005	NDM-1
6	*E. cloacae*	4004	NDM-1
7	*E. coli*	4011	NDM-1
8	K. *pneumoniae*	4017	VIM-1
9	K. *pneumoniae*	4023	NDM-1
10	K. *pneumoniae*	4024	NDM-1
11	K. *pneumoniae*	4025	VIM-1
12	*K. pneumoniae*	4014	KPC-3
13	*K. oxytoca*	4032	KPC-2
14	*E. coli*	4042	KPC-4
15	*E. coli*	4006	KPC-3
16	*K. pneumoniae*	4040	KPC-3
17	*K. pneumoniae*	4015	KPC-3
18	*K. pneumoniae*	4016	KPC-2
19	*K. pneumoniae*	4022	KPC
20	*E. cloacae*	4002	OXA-48
21	*E. cloacae*	4003	OXA-48
22	*E. coli*	4007	OXA-48
23	*E. coli*	4008	OXA-48
24	*E. coli*	4009	OXA-48
25	*E. coli*	4010	OXA-48
26	*K. pneumoniae*	4012	OXA-48
27	*K. pneumoniae*	4019	OXA-48
28	*K. pneumoniae*	4020	OXA-48
29	*K. pneumoniae*	4021	OXA-48
30	*K. pneumoniae*	4026	OXA-48
31	*K. pneuminiae*	2001	DHA-1

Table B Molecular types of β-lactmases-producing Enterobacteriaceae used in the present study (Continued).

No.	Bacterial isolates	JMU code	Molecular type
32	*E. coli*	2002	DHA-1
33	*E. coli*	2003	DHA-1
34	*E. coli*	2004	CMY-2
35	*E. coli*	2005	FOX
36	*E.coli*	2006	CMY-2
37	*E. aerogenes*	2007	ACT-31
38	*En. cloacae*	2008	FOX
39	*En. cloacae*	2009	ACT32
40	*M. morganii*	2010	DHA-1
41	*En. cloacae*	2011	ACT
42	*C. freundii*	2026	CMY-112
43	*K. pneuminiae*	2020	CMY-2
44	*E. coli*	2021	FOX-3
45	*E. cloacae*	2022	ACT-32
46	*E. cloacae* ATCC BAA-1143*	-	ACT-32
47	*E. coli*	1004	TEM-214
48	*E. coli*	1005	CTX-M-3
49	*E. coli*	1006	CTX-M-3
50	*E. coli*	1008	CTX-M-3
51	*E. coli*	1009	TEM-70
52	*K. pneumoniae*	1010	CTX-M-15+SHV-27
53	*K. pneumoniae*	1012	SHV-27+TEM-53
54	*K. pneumoniae*	1013	SHV-27
55	*K. pneumoniae*	1015	SHV-27+TEM-71
56	*E. coli* NCTC 13352*	-	TEM-10
57	*E. coli* NCTC 13353*	-	CTX-M-15
58	*E. cloacae*	3001	TEM-95+ACT-17
59	*E. aerogenes*	3002	SHV-12+CTX-M-9+ACT-32
60	*E. coli*	3003	CTX-M-9+ACT-18
61	*C. freundii*	3004	CTX-M-3+ACT-23
62	*C. freundii*	3005	CTX-M-3+ACT-1
63	*C. freundii*	2038	MOX-1 MOX-2 CMY-1 CMY-8 to CMY-11
64	*E. aerogenes*	3007	CTX-M-9+ ACT
65	*E. aerogenes*	3008	SHV+ACT

Table B Molecular types of β-lactmases-producing Enterobacteriaceae used in the present study (Continued).

No.	Bacterial isolates	JMU code	Molecular type
66	*E. aerogenes*	3009	TEM-214+ SHV-12+ACT-36
67	*E. aerogenes*	3010	TEM+ CTX-M+ACT-27
68	*E. aerogenes*	2039	FOX
69	*E. aerogenes*	2040	CMY-86
70	*E. cloacae*	2041	ACT-36
71	*E. aerogenes*	2042	FOX
72	*E. aerogenes*	2043	ACT
73	*E. aerogenes*	2044	ACT
74	*E. cloacae*	2045	ACT
75	*E. aerogenes*	2046	FOX
76	*E. cloacae*	1019	SHV
77	*E. coli*	2052	MOX-1 MOX-2 CMY-1 CMY-8 TO CMY-11
78	*E. coli*	2053	CMY-2
79	*E. coli*	2054	ACT
80	*E. coli*	2055	CMY-2
81	*K. pneumoniae*	2057	DHA-1
82	*K. pneumoniae*	2058	ACT-17
83	*K. pneumoniae*	1021	SHV-18
84	*K. pneumoniae*	1022	SHV-110+TEM-84
85	*K. pneumoniae*	1023	TEM SHV CTX-M
86	*M. morganii*	2059	DHA-1

APPENDIX C

CULTURE MEDIA, CHEMICALS AND EQUIPMENT

1. Culture media

Nutrient agar, Mueller-Hinton broth and agar were obtained from Oxoid. Approximate formula per liter of each medium was as following:

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 Ca^{2+}20 mg/l and Mg^{2+}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
Casein acid hydrolysate	17.5
Solubel 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
Cloxacillin	AR grade
Cefalexin	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 D

PUBLICATIONS, PROCEEDINGS AND CONFERENCES

D. 1 Publications

Teethaisong, Y., Autarkool, N., Sirichaiwetchakoon, K., Krubphachaya, P., Kupittayanant, S., and Eumkeb, G. (2014). Synergistic activity and mechanism of action of Stephania suberosa Forman extract and ampicillin combination against ampicillin-resistant *Staphylococcus aureus*. **Journal of Biomedical Science**. 21(1): 90.

Teethaisong, Y., Eumkeb, G., Nakouti, I., Evans, K., and Hobbs, G. (2016). A combined disc method with resazurin agar plate assay for early phenotypic screening of KPC, MBL and OXA-48 carbapenemases among Enterobacteriaceae. **Journal of Applied Microbiology**. 121(2): 408-414.

Siriwong, S., **Teethaisong, Y.**, Thumanu, K., Dunkhunthod, B., and Eumkeb, G. (2016). The synergy and mode of action of quercetin plus amoxicillin against amoxicillin-resistant Staphylococcus epidermidis. **BMC Pharmacology and Toxicology**. 17(1): 39.

D. 2 Proceedings and academic conferences

1. **Teethaisong, Y**.; Hobbs, G.; Evans, K; Ismini, N and. Eumkeb, G. A Disc Diffusion Method Along with a Resazurin Agar Plate Assay for the Early Characterisation of Carbapenemase-Producing Enterobacteriaceae. RGJ congress 17, 8-11 June 2016, Jomtien Palm Beach Hotel And Resort, Pattaya, Thailand (*oral presentation*).

2. **Teethaisong, Y**.; Eumkeb, G.;Nakouti, I.; Hobson, J.; Evans, K, and Hobbs, G. (2016). Evaluation of resazurin microtitre plate assay for early phenotypic characterisation of ESBL, AmpC and MBL β-lactamase-producing Enterobacteriaceae. Abstract In: The Microbiology Society Annual Conference 2016, 21–24 March at the Arena and Convention Centre (ACC), Liverpool, UK (Poster presentation).

3. The 2nd Course on Principles of Molecular Microbiological Diagnostics, 20 - 22 January 2016, Maastricht, the Netherlands.

4. The ICMMAAC 2015 17th: International Conference on Medical Microbiology, Antimicrobial Agents and Chemotherapy, 25-26 May 2015, London, UK (Listener)

5. **Teethaisong, Y**.; Autarkool, N., and Eumkeb, G. (2014). Synergistic antibacterial activity of *Boesenbergia rotunda* extract and β-lactam antibiotic combination against multidrug-resistant bacteria. Proceeding In: the The Fifth international conference of natural products for health and beauty (NATPRO5). 6-8 May, Phuket, Thailand. pp. 226-230.

6. Autarkool, N.; **Teethaisong, Y**.; Kupittayanant, S., and Eumkeb, G. (2014). Antibacterial activity of *Stephania suberosa* extract against methicillin-resistant

Staphylococcus aureus. Proceeding In: the The Fifth international conference of natural products for health and beauty (NATPRO5). 6-8 May, Phuket, Thailand. pp. 123-127.

7. Eumkeb, G.; Phitaktim, S.and **Teethaisong, Y.** (2013). Antibacterial activity of α-mangostin from the pericarp extract of *Garcinia mangostana L*. Against drug resistant bacteria. *Thai Journal of Pharmaceutical Sciences*. 38(SUPPL.): 83-87.

8. **Teethaisong, Y.**; Eumkeb, G.; Nakouti, I.; Evans, K. and Hobbs, G. (2016) Development of a novel, simple and rapid chromogenic method to detect the presence of carbapenemase-producing Enterobacteriaceae IDweek 2016 (Infectious disease society of America), 26-30 October 2016, New Orleans, USA (*poster presentation*)

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ORIGINAL ARTICLE

A combined disc method with resazurin agar plate assay for early phenotypic screening of KPC, MBL and OXA-48 carbapenemases among Enterobacteriaceae

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Abstract

Aim: To validate a combined disc method along with resazurin chromogenic agar for early screening and differentiation of *Klebsiella pneumoniae* carbapenemase, metallo-β-lactamase and OXA-48 carbapenemase-producing Enterobacteriaceae.

Methods and Results: The combined disc test comprising of meropenem alone and with EDTA, phenylboronic acid or both EDTA and phenylboronic acid, and temocillin alone were evaluated with the resazurin chromogenic agar plate assay against a total of 86 molecularly confirmed Enterobacteriaceae clinical isolates (11 metallo-β-lactamases, eight *Kl. pneumoniae* carbapenemases, 11 OXA-48, 32 AmpC and 15 extended-spectrum-β-lactamase producers and nine co-producers of extended-spectrum-β-lactamase and AmpC). The inhibition zone diameters were measured and interpreted at 7 h for the presence of carbapenemase. All carbapenemase producers were phenotypically distinguished by this assay with 100% sensitivity and specificity.

Conclusions: This early phenotypic method is very simple, inexpensive, and reliable in the detection and differentiation of carbapenemase-producing Enterobacteriaceae. It could be exploited in any microbiological laboratory for diagnosis of these recalcitrant bacteria.

Significance and Impact of the study: This assay poses excellent performance in discrimination of *Kl. pneumoniae* carbapenemase, metallo-β-lactamase and OXA-48 carbapenemases within 7 h, which is much faster than conventional disc diffusion methods. The rapid detection could help clinicians screen patients, control infection and provide epidemiological surveillance.

Introduction

Resistance to carbapenems, a 'last resort' β-lactam antibiotic for the treatment of Gram-negative bacteria, in Enterobacteriaceae, is increasing at an alarming rate and becoming one of the most serious concerns in public-health worldwide (Tangden and Giske 2015). Carbapenemases have been recognized since imipenem was approved for clinical use in the 1980s (Walsh 2010). The first carbapenemase (NmcA) was identified in the clinical isolate of *Enterobacter cloacae* in 1993 and since then numerous carbapenemase-encoded genes in Enterobacteriaceae have been identified (Naas and Nordmann 1994). Three major classes of clinically important carbapenemases have molecularly been classified; Ambler class A (mostly *Klebsiella pneumoniae* carbapenemase (KPC)), class B metallo-β-lactamase (MBL: IMP, VIM and NDM) and class D enzymes with carbapenemase activity (mostly OXA-48 and OXA-181) (Patel and Bonomo 2013). Currently, the USA, Israel, Greece and Italy are endemic for KPC, while OXA-48- producing *Kl. pneumoniae* and *Escherichia coli* have extensively been identified in North Africa and Turkey. The Indian subcontinent is an important reservoir of NDM-producing-Enterobacteriaceae,

especially in *Kl. pneumoniae* and *E. coli*, as well as KPC and OXA-48 like-producing isolates (mostly OXA-181) (Nordmann and Poirel 2014).

Detection of carbapenemase production among Enterobacteriaceae and other Gram-negative bacteria is more challenging as a result of multiple resistance mechanisms in the same strain (Nordmann *et al.* 2012). Several carbapenemase- identifying assays have been developed and can be grouped as phenotypic (based on synergistic effect between antibiotics and carbapenemase inhibitors), genotypic (PCR-based detection), biochemical-based methods (e.g. Carba NP test and Blue-carba test) (Miriagou *et al.* 2013; Pires *et al.* 2013; Dortet *et al.* 2015). Furthermore, MALDI-TOF and immunochromatography detection methods are also available (Vogne *et al.* 2014; Glupczynski *et al.* 2016). Earlier carbapenemase screening methods relied upon the antimicrobial susceptibility profile determined by disc diffusion test and minimum inhibitory concentration (MIC) value from broth dilution method or by automated system (Miriagou *et al.* 2010). The cloverleaf test or the Modified Hodge Test (MHT) has been recommended by the Clinical and Laboratory Standards Institute (CLSI) in 2009 as a confirmatory test for carbapenemase production in isolates demonstrating reduced susceptibility to carbapenem antibiotics. There are several shortcomings of these tests as they have poor specificity and sensitivity and relatively slow turnaround times (Carvalhaes *et al.* 2010).

Combined disc-inhibitor synergy tests have been used widely to discriminate different classes of carbapenemases. Boronic acids, particularly phenylbornonic acid (PBA), have been used to inhibit class A KPC activity, while metal chelating agents such as EDTA and dipicolinic acid (DPA) have been used to inhibit MBL activity (Giske *et al.* 2011; Tsakris *et al.* 2011; Nordmann *et al.* 2012). The temocillin (TEM) resistance profile (MIC≥ 128 μg ml^{-1} or zone diameter of 30 μg disc ≤ 10 mm) has been suggested as a phenotypic marker of OXA-48-producing Gram-negative bacteria where there is a decrease in carbapenem susceptibility and absence of synergistic effect of carbapenem plus KPC or MBL inhibitors (Hartl *et al.* 2013; van Dijk *et al.* 2014; Woodford *et al.* 2014). A time to result of a disc diffusion method usually takes at least 18 h or overnight. The colorimetric plate containing resazurin showed excellent performance and reproducibility for disc diffusion susceptibility testing in *E. coli* isolates (Sener *et al.* 2011). The resazurin reduction assay is a colorimetric method that has extensively been used as an indicator for cell growth, cell viability, toxicity and indirect antimicrobial susceptibility testing. This dye is nontoxic to cells and stable in culture media. A blue coloured resazurin is irreversibly converted to a pink coloured resorufin by active cells (O'Brien *et al.* 2000; Sarker *et al.* 2007). No studies have been reported on phenotypic detection for β-lactamases using this colorimetric assay. This study describes the resazurin chromogenic agar (RCA) plate along with combined disc-inhibitor synergy test for early screening and differentiation of KPC, MBL and OXA-48 carbapenemases.

Materials and methods

Bacterial isolates and RCA plate preparation

A sum of 86 β-lactamase-producing Enterobacteriaceae UK clinical isolates (collected between 2012–2015), including 11 MBL producers, (five NDM-1s, one NDM, one IMP-1 and one VIM-1 + SHV-102, one VIM-1 + SHV-12 and two VIM-types) eight KPC producers (two KPC-2s, four KPC-3s, one KPC-4 and one KPC-type), 11 OXA-48 producers and 56 noncarbapenemase-producing strains (32 AmpCs, 15 ESBLs and nine co-producers of ESBL and AmpC), were used to validate RCA plate assay in the present study. The bacterial strains employed were *E. coli* (n = 25), *Kl. pneumoniae* (n = 28), *Ent. aerogenes* (n = 12), *Ent. cloacae* (n = 13), *Morganella morganii* (n = 2), *Citrobacter freundii* (n = 4), *Klebsiella oxytoca* (n = 1) and *Klebsiella ozaenae* (n = 1) (Table S1). Non-β-lactamase-producing *E. coli* ATCC 25922 was used as a negative control strain. The isolates were biochemically and molecularly identified by PCR and sequencing following previous reports (Ellington *et al.* 2007; Dallenne *et al.* 2010; Poirel *et al.* 2011).

The RCA plates were prepared according to Sener and colleagues (Sener *et al.* 2011). Briefly, 25 mg of resazurin sodium salt (Sigma-Aldrich, Dorset, UK) was dissolved in 10 ml of sterile water and sterilised by filtration through 0·2 μm syringe filter. The sterile resazurin solution was added to 990 ml sterile Mueller-Hinton (MH) agar (Oxoid, Basingstoke, UK) when the temperature of the medium reached approx. 45–50°C (to a final concentration of 25 μg ml^{-1}). The resazurin-containing MH agar was gently mixed prior to pouring 25 ml of the solution or approx. 4 mm depth into 90 mm circular Petri dishes. Uninoculated RCA plates were stored in the fridge (4°C) for up to a week and kept away from the light.

Disc preparation and experiment procedure

Meropenem (MER) discs (10 μg) were prepared by supplementing blank discs (6·5 mm, MAST Diagnostic Group, UK) with 10 μl of 1 mg ml^{-1} MER (Sigma-Aldrich). Dried MER discs were then impregnated with 10 μl of PBA (Sigma-Aldrich) at a concentration of 40 mg ml^{-1} and EDTA at 75 mg ml^{-1} to obtain final amount of 400 and 750 μg disc^{-1} respectively. PBA was

dissolved in dimethylsulphoxide (Sigma-Aldrich) and sterile water as previously recommended (Coudron 2005). EDTA and MER were dissolved in sterile water. Discs were air-dried in the cabinet for 1 h prior to use.

The disc susceptibility testing was performed in accordance with the CLSI guideline (Clinical Laboratory Standards Institute 2010). A sterile swab was dipped in a 0·5 McFarland standard suspension of test bacteria and spread thoroughly on entire RCA's surface. Five discs including MER, MER + PBA, MER + EDTA, MER + PBA + EDTA and 30 μg TEM (MAST Diagnostic Group, UK) were firmly placed at equidistant points on the surface of the RCA plate. Following incubation at 37°C for 7 h, the diameters of the blue zones of inhibition were measured. A change in the colour of medium from blue (resazurin) to pink (resorufin) was visually observed in live bacteria. No colorimetric change was indicative of bacterial growth inhibition. The results were interpreted according to previously described assays with additional modifications (Table 1) (Miriagou *et al.* 2013; van Dijk *et al.* 2014). Synergy between MER and EDTA and PBA was considered as positive results for MBL and KPC respectively. Absence of synergy between MER and EDTA or PBA with TEM zone diameter (≤10 mm) was denoted a positive result for OXA-48-producing isolates (Table 1). Sensitivity and specificity values were calculated by comparing results from RCA assay to molecular characterisation results. SPSS statistical analysis software (SPSS Inc, Chicago, IL, USA) was used to analyse the data and create box-and-whisker plot.

Results

The colorimetric phenotypic method using RCA plate with combined disc test for early detection and differentiation of MBL, KPC and OXA-48 carbapenemases explicitly demonstrated zone diameters within 7 h (Fig. 1). Distribution of the zone diameters of MER with and without PBA or EDTA and TEM alone against carbapenemase and noncarbapenemase-producing Enterobacteriaceae are shown in Fig. 2. In MBL producers, the range of zone diameters of MER and MER + PBA was 6·5–20 mm and 6·5–19 mm respectively. The median diameters for these discs were equally 17 mm. Discs containing EDTA (median = 26 mm and range = 23–27 mm) resulted in increased zone diameters compared with the discs without EDTA. The range of TEM zone diameters varied from 6·5 to 17 mm (median = 6·5 mm) as shown in Fig. 2a. The combined disc test using MER and EDTA successfully detected all MBL producers without false positive results in non-MBL isolates (sensitivity 100%, specificity 100%; Table 1).

For detection of KPC-producing Enterobacteriaceae, an increase in zone diameters was observed in MER + PBA (median = 22 mm and range = 19–27 mm) and MER + PBA + EDTA (median = 22·5 mm and range = 19–27 mm) compared with MER alone (median = 15 mm and range = 8–20 mm) or MER + EDTA (median = 16 mm and range = 7–21 mm). Synergistic effects of PBA were found only in KPC strains. The median zone diameter of TEM was 14 mm (range = 11–20 mm) as shown in Fig. 2b. The sensitivity and specificity values of PBA synergy test along with RCA assay for detection of KPC-producing Enterobacteriaceae were 100% (Table 1).

No difference in zone diameters of MER disc alone (median = 21 mm and range = 8–25 mm) and with PBA (median = 21 mm and range = 11–26 mm), EDTA (median = 21 mm and range = 12–25 mm) or PBA + EDTA (median = 22 mm and range = 12–25 mm) was observed in OXA-48 producers. These findings indicated no synergistic effect between PBA or EDTA and MER. TEM inhibition zone diameters were ≤10 mm (median = 6·5 mm and range = 6·5–9 mm) for all tested OXA-48 producers (Fig. 2c). The zone diameters of TEM considered together with an absent synergistic effect of MER and PBA or EDTA were good indicators for identification of OXA-48-producing isolates (sensitivity 100% and specificity 100%; Table 1). In noncarbapenemase producers, there was no significant difference in zone diameters

Table 1 Interpretation criteria for combined disc synergy test and performance of combined disc test along with RCA assay for the early detection of carbapenemase-producing Enterobacteriaceae

Test	MBL	KPC	OXA-48	Sensitivity	Specificity
B-A and D-C (EDTA synergy) ≥ 5 mm	+	–	–	100 (11/11)	100 (76/76)
C-A and D-B (PBA synergy) ≥ 5 mm	–	+	–	100 (8/8)	100 (79/79)
E ≤ 10 mm*	–	–	+	100 (11/11)	100 (76/76)

A = meropenem disc; B = meropenem + EDTA disc; C = meropenem + phenylboronic acid (PBA) disc; D = meropenem + EDTA + PBA disc; E = temocillin disc.

*Considered when absence of EDTA, PBA and EDTA + PBA synergy tests.

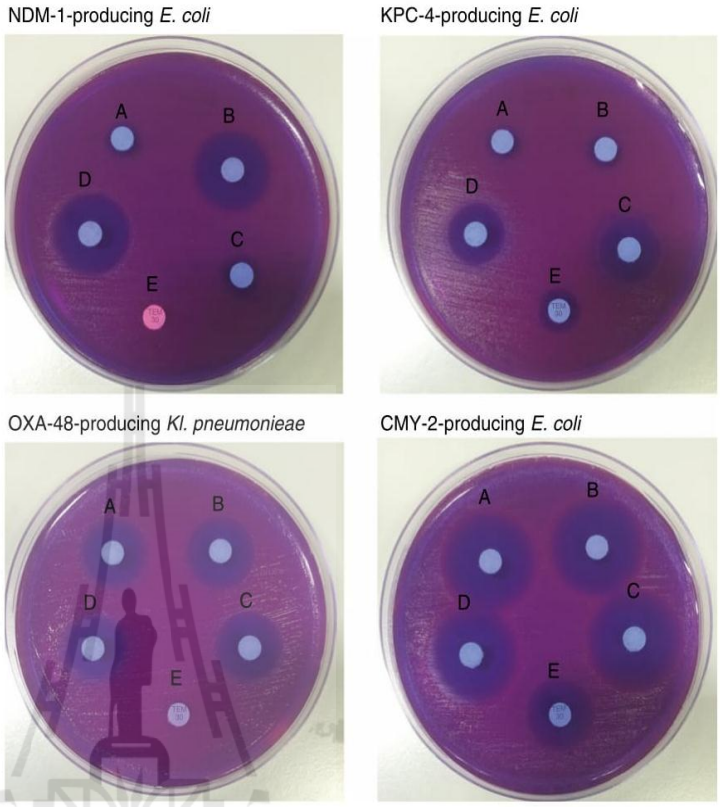

Figure 1 Phenotypic results from combined disc method along with RCA plate assay at 7 h. A = meropenem (10 μg); B = meropenem (10 μg) + EDTA (750 μg); C = meropenem (10 μg) + phenylboronic acid (400 μg); D = meropenem (10 μg) + EDTA (750 μg) + phenylboronic acid (400 μg); E = temocillin (30 μg).

between MER alone and PBA or EDTA. The median diameter for TEM was 19 mm (range = 13–26) against these isolates (Fig. 2d). In addition, 10 μg MER clearly inhibited the growth of reference strain *E. coli* ATCC 25992. The zone diameter against this strain was 29 mm, which was in the quality control range (28–34 mm) for nonfastidious organisms in CLSI document (Clinical Laboratory Standards Institute 2014). The results in the present investigation using combined disc method with RCA clearly discriminated different types of carbapenemases without discrepancy.

Discussion

Carbapenem resistance in Enterobacteriaceae poses a challenging issue for treatment and infection control. The rapid diagnostic test plays an important role in guiding clinicians to appropriate antibiotic administration and minimising treatment failure (Nordmann *et al.* 2012). Molecular-based methods for characterisation of carbapenemase-producing Enterobacteriaceae are restrictive due to high cost, requirement of skilled and experienced technicians and more importantly, the inability to detect novel carbapenemase-encoding genes (Picao *et al.* 2008). Current combined disc synergy tests with β-lactamase inhibitors are simple, inexpensive and able to discriminate the different types of carbapenemases effectively, but they are limited in time as results require at least 18 h incubation (Osei Sekyere *et al.* 2015). To improve the turnaround time, in this study, we conducted RCA plate along with combined disc method for early screening of clinically important carbapenemases including MBL, KPC and OXA-48.

For detection of MBL-producing Enterobacteriaceae, EDTA synergy test was able to detect all test MBL producers with 100% sensitivity and specificity. This result agrees with a study previously reported by Tsakris *et al.* (2010). They found that the combined disc method containing EDTA successfully detected all clinical isolates of VIM-producing Enterobacteriaceae. Surprisingly, some studies reported using EDTA as a MBL inhibitor and MER as a substrate gave some false-positive results in non-MBL-producing *Kl. pneumoniae* (Giske *et al.* 2011). Similarly, a combined disc method supplemented with imipenem and DPA showed better activity than EDTA against *Pseudomonas* spp. and *Acinetobacter* spp. producing IMP-1-like, VIM-2-like and SIM-1-type MBLs (Yong *et al.* 2012). Giske *et al.* (2011) found that DPA synergy test with MER as a substrate had 100% in both sensitivity and specificity, which had superior performance in

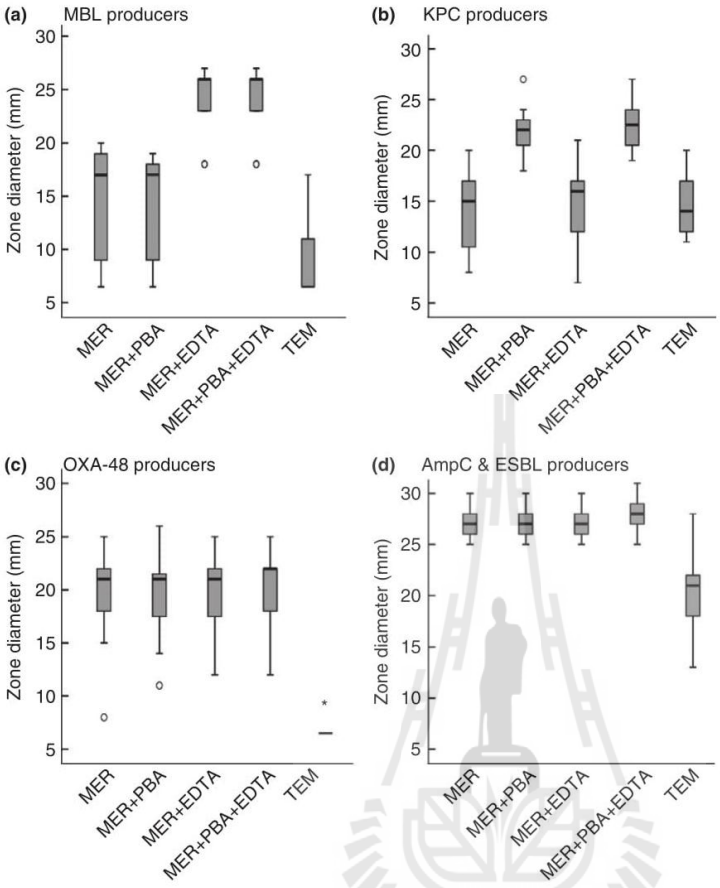

Figure 2 Zone diameters of meropenem (MER) alone and with phenylboronic acic (PBA) and EDTA and temocillin alone. (a) MBL producers (n = 11); (b) KPC producers (n = 8); (c) OXA-48 producers (n = 11); (d) noncarbapenemase producers (n = 57). °Mild outlier; *extreme outlier.

detection of MBL producers compared with EDTA. A good sensitivity (90%) and specificity (96%) of DPA synergy test was also previously reported by van Dijk *et al.* (2014) for detection of MBL-producing Enterobacteriaceae. Nevertheless, a study performed by Pitout *et al.* (2005) found that MER was more effective than imipenem and suggested the use of MER in combination with EDTA for detection of MBL-producing *Pseudomonas aeruginosa*.

The result of PBA synergy test in this study using 400 μg PBA was reliably able to detect all KPC-producing Enterobacteriaceae clinical isolates (100% sensitivity). Tsakris *et al.* (2011) reported a few false-positive results were observed (97·6% specificity) and also PBA was more effective than aminophenylboronic acid in detection of KPC-producing isolates. A similar sensitivity and specificity from PBA synergy test for detecting KPC-producing Enterobacteriaceae was also reported by several studies (Tsakris *et al.* 2010; van Dijk *et al.* 2014). PBA synergy test was not only positive in KPC producers, but it was also observed in producers of AmpC plus porin loss. It has been suggested that using cloxacillin synergy test can potentially discriminate between KPC and AmpC plus porin loss (Giske *et al.* 2011).

TEM zone diameter ≤10 mm considered together with absence in synergy of MER and EDTA or PBA was able to detect all OXA-48-producing Enterobacteriaceae. The results from this study were consistent with several studies reporting excellent sensitivity and specificity of TEM disc in detection of OXA-48-like enzymes (OXA-48, OXA-162, OXA-181 and OXA-204) (Hartl *et al.* 2013; van Dijk *et al.* 2014; Oueslati *et al.* 2015). The zone diameters of MER discs against all noncarbapenemase-producing Enterobacteriaceae including ESBL and AmpC isolates were in the susceptible range in CLSI guideline (Clinical Laboratory Standards Institute 2014).

In conclusion, the combined disc method is effectively applicable in any microbiological laboratory. Using this

test along with RCA assay is very simple and provides a faster result compared with the combined disc method alone. The result from RCA assay is visually easy to interpret. It also demonstrates excellent sensitivity and specificity for differentiation of MBL, KPC and OXA-48-producing Enterobacteriaceae. The RCA assay can be applied to commercially available discs, such as MAST-CDS (Mast Group, UK) and Rosco Diagnostica A/S (Denmark). However, further studies should be performed against a larger sample size of clinical isolates with co-producers of class A and class B or AmpC plus porin loss to establish the robustness of this assay. The early detection of carbapenemase would aid healthcare professionals to manage patients, control the spread of infection and for epidemiological surveillance purpose.

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Conflict of Interest

The authors have no conflict of interest to declare.

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Supporting Information

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

Table S1. Carbapenemase-producing and noncarbapenemase-producing isolates used in this study.

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Synergistic activity and mechanism of action of *Stephania suberosa* Forman extract and ampicillin combination against ampicillin-resistant *Staphylococcus aureus*

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Abstract

Background: Ampicillin-resistant *S. aureus* (ARSA) now poses a serious problem for hospitalized patients, and their care providers. Plant-derived antibacterial that can reverse the resistance to well-tried agents which have lost their original effectiveness are the research objectives of far reaching importance. To this aim, the present study investigated antibacterial and synergistic activities of *Stephania suberosa* extracts (SSE) against ARSA when used singly and in combination with ampicillin.

Results: The majority chemical compounds of SSE were alkaloid (526.27 ± 47.27 mg/1 g of dried extract). The Minimum inhibitory concentration (MICs) for ampicillin and SSE against all ARSA strains were >512 μg/ml and 4 mg/ml, respectively. Checkerboard assay revealed synergistic activity in the combination of ampicillin (0.15 μg/ml) and SSE (2 mg/ml) at fractional inhibitory concentration index (FICI) <0.5. The killing curve assay had confirmed that the viability of ARSA was dramatically reduced from $5x10^5$ cfu/ml to 10^3 cfu/ml within 6 h after exposure to SSE (2 mg/ml) plus ampicillin (0.15 μg/ml) combination. Electron microscopic study clearly revealed that these ARSA cells treated with this combination caused marked morphological damage, peptidoglycan and cytoplasmic membrane damage, and average cell areas significant smaller than control. Obviously, Immunofluorescence staining and confocal microscopic images confirmed that the peptidoglycan of these cells were undoubtedly disrupted by this combination. Furthermore, the CM permeability of ARSA was also increased by this combination. Enzyme assay demonstrated that SSE had an inhibitory activity against β-lactamase in concentrations manner.

Conclusions: So, these findings provide evidence that SSE has the high potential to reverse bacterial resistance to originate traditional drug susceptibility of it and may relate to three modes of actions of SSE: (1) inhibits peptidoglycan synthesis, resulting in morphological damage, (2) inhibits β-lactamases activity, and (3) increases CM permeability. It is widely recognized that many types of drugs are derived from alkaloids. So, this SSE offers the prominent potential to develop a novel adjunct phytopharmaceutical to ampicillin for the treatment of ARSA. Further active ingredients study, toxicity of it, and the synergistic effect on blood and tissue should be performed and confirmed in an animal test or in humans.

Keywords: β-lactam antibiotics, Ampicillin-resistant *S. aureus* (ARSA) *Stephania suberosa* Forman, Synergistic activity, Ampicillin

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Background

Since Alexander Fleming discovered the first antibiotic, an increased emergence of multidrug resistance in pathogenic bacteria has been globally documented. In London, 38% of *Staphylococcus aureus* strains were resistant to penicillin and increased to approximately 90% in the UK and USA in the recent year [1]. Similarly, 70-80% of *S. aureus* strains in most of Asian countries were resistant to methicillin [2]. *S. aureus* infections are notorious etiological pathogens for several human ailments, including pneumonia, meningitis, toxic shock syndrome, bacteremia, wound sepsis, osteomyelitis, and endocarditis [3-5]. This strain is also the second most common cause of bloodstream infection with case fatality rates of approximately 15-25% [6]. Methicillin-resistant *S. aureus* (MRSA) is notably one of the greatest threats to human health, and is the major causes of both hospital and community-acquired MRSA infection that affects both hospitalized patients and their health care providers [7,8]. Methicillin, like ampicillin, is the one member of penicillins. So, the resistance pattern of *S. aureus* against both drugs are almost the same [9]. β-lactam antibiotics are the most common and effective agents for treating staphylococcal infections, but current higher resistance levels were reported in these strains. A raised incidence of β-lactam resistance in MRSA has rapidly emerged due to mediated by acquisition of *mecA* encoded an additional penicillin-binding protein 2a (PBP2a) resulting in reducing the ability of β-lactam in binding to its target site. In summation, the production of *blaZ* encoded β-lactamase that can hydrolyze and inactivate β-lactam antibiotic activity, in particular penicillins. This enzyme is also one of the most important resistant mechanisms to β-lactam in this strain [10-12]. For these problems, searching and development of novel antibacterial compounds or new strategies are urgently required. Plants are well known and being interested sources for new antibacterial agents because they produce an enormous variety compounds to protect themselves from plant pathogens and environmental pathogens [13]. Thus, plant-derived antibacterials are often a source of novel therapeutics, but they have weaker antibacterial activity compared to that of antibiotic generated by bacteria or fungi. Therefore, it should be considered to use in a different paradigm, including combination with conventional antibiotic that has been resisted by bacteria to achieve synergism in treatment of bacterial infections [14].

Phytochemical and antibiotic combination approach has been recommended and tested by several reports for combating multidrug-resistant bacteria by achieving multiply synergistic drug targets, interacting with drug-resistant mechanisms of bacteria, and neutralizing and eliminating adverse effects [15-18]. Previous research found that the hasubanalactam alkaloid (glabradine) isolated from *Stephania glabra* showed antimicrobial activity against *Staphylococcus aureus*, *S. mutans*, *Microsporum gypseum*, *M. canis* and *Trichophyton rubrum* greater than those of novobiocin and erythromycin with IZD values of 19–27 cm [19]. To develop novel plant-derived antibacterials, the *Stephania suberosa* was selected to investigate its antibacterial property. *S. suberosa* belongs to Genus *Stephania* and family Menisperamaceae. Plants in this genus have been traditionally used for treatments of asthma, tuberculosis, dysentery, hyperglycemia, cancer, fever, intestinal discomforts, sleep disturbances and inflammation. Also, previous research found that the chemical compounds in this plant were alkaloids such as, (+)-Cepharanthine and its derivative, stephasubimine and etc. [20]. Recently, however, limited study is available on antibacterial activity of *S. suberosa*. Hence, the purpose of this investigation was to test the antibacterial activity and synergism with ampicillin of this plant against ARSA. Some elementary mechanism of actions, such as cytoplasmic membrane (CM) permeabilization, enzyme assay, and transmission electron microscopy, was also studied.

Methods

Plant preparation and extraction

Roots of *S. suberosa* were purchased from Lamtakhong Research Station, NakhonRatchasima Province, Thailand. The plant specimens were deposited at the Thailand National Herbarium and authenticated in comparison with the voucher specimen no. BK257189 by Dr. Paul J. Grote, a lecturer and plant biologist at Institute of Science, Suranaree University of Technology, Thailand. The roots of *S. suberosa* were cleaned thoroughly and dried under a hot air oven at the 50°C until dry. The dried samples were powdered using a grinder. Then, dried powder was extracted with 95% ethanol using Soxhlet extractor at 75°C for 8 h. The extracts were concentrated using a rotary evaporator and were lyophilized to yield a brown powder and a dark brown sticky oil of ethanolic extract.

Preliminary qualitative phytochemical screening analysis was proceeded for notable bioactive compounds, such as alkaloids, tannin, flavonoids, saponins, glycosides, steroids, terpeniods, coumarins and anthocyanin. The methods were achieved in accordance with previous description and slightly modifications [21-24]. Screening for the presence of the total alkaloid content in the extract was also accordingly carried out with previously reported [25]. In brief, 1 g of the dried extract was alkalinized with NH_4OH, partitioned with chloroform, and evaporated to dryness on a water bath at 60°C. The net mass of triplicate remaining solid was accurately weighed and expressed as milligrams per 1 g of dried

extract. After weighing, the solids were redissolved by 95% ethanol and confirmed in the presence of alkaloids.

Bacterial strains, antibiotics, chemicals, and antibodies

Clinical isolates of ampicillin-resistant *S. aureus* (ARSA) DMST 20651, 20652 and 20653 were obtained from the Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand. The reference strain of *S. aureus* ATCC 29213 was obtained from the American Type Culture Collection (ATCC). Ampicillin, *o*-nitrophenol-β-D-galactoside (ONPG), nisin, NH_4OH, chloroform and β-lactamase type IV from *Enterobacter cloacae* were obtained from Sigma-Aldrich, UK. Mueller-Hinton broth was purchased from Oxoid (Basingstoke, UK). Primary mouse monoclonal anti-*S. aureus* peptidoglycan antibody (ab20002), secondary goat polyclonal anti-mouse IgG conjugated with Alexa Fluor®488 (ab150117), and fluoroshield mounting medium with DAPI (ab104139) were purchased from Abcam, UK.

Bacterial suspension standard curve

In order to obtain a known viable count of bacterial suspension, the method of Liu and company was followed as previously described with some modifications [26]. Mueller-Hinton agar and Cation-adjusted Mueller-Hinton broth (CAMHB) were practiced as a culture medium [27].

Minimum inhibitory concentration (MIC) determinations

MIC determinations of ampicillin, nisin, and SSE against ARSA strains were performed following the method of Liu et al. [26]; Eumkeb et al. [28] and Clinical and Laboratory Standards Institute [27]. Briefly, bacterial suspension was adjusted to approximately 1×10^8 cfu/ml and serial tenfold dilution was performed to achieve 5×10^6 cfu/ml. The diluted inoculum (0.1 ml) of each stain was added to 0.9 ml of CAMHB plus serial dilutions of the antibacterial agents, to give a final concentration approximately 5×10^5 cfu/ml. Antibiotics used and SSE was prepared by dissolving in sterile distilled water to obtain stock solutions at 1024 μg/ml for antibiotics or 1024 mg/ml of the extract. Then, the stock was serially twofold diluted to achieve respective concentration. The lowest concentration that showed no visible growth after incubated at 35°C for 18 h was recorded as the MIC. *S. aureus* ATCC 29213 was used as a reference strain.

Checkerboard determination

The interaction between SSE and ampicillin against ARSA was assayed using checkerboard determination following Eumkeb et al. [28] and Bonapace et al. [29]. Briefly, the cultured and antibacterial agents were prepared and performed similarly with MIC determination. Otherwise, the SSE and ampicillin were combined and incubated at 35°C for 18 h. The MICs were determined as the lowest concentration SSE in combination with ampicillin. The FIC index (FICI) was calculated to determine drug interaction, and interpreted as follows:

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

Where, FICI ≤ 0.5 denoting synergistic; FICI > 0.5–4.0 denoting no interaction; FICI > 4.0 denoting antagonism [30].

Killing curve determination

Killing curve determination was carried out in order to confirm antibacterial and synergistic activities of SSE when used singly and in combination with ampicillin. The viabilities of drug resistant bacteria after exposure to these agents alone and in combination at nine distinct times (0, 0.5, 1, 2, 3, 4, 5, 6 and 24 h) were counted. The assay was followed the previously described with some modifications [28,31]. Concisely, inocula (5×10^5 cfu/ml) were exposed to SSE either singly or in combination with ampicillin. Aliquots (0.1 ml) of each exposed time were removed and diluted in normal saline as needed to enumerate 30–300 colonies. The diluted cultures were platted and spread thoroughly on plates containing MHA. After incubating at 35°C for 18 h, the growing colonies were counted. The lowest detectable limit for counting is 10^3 cfu/ml. The experiment was performed in triplicate; data are shown as mean ± SEM.

The preliminary mechanism of action was performed in duplicate methods for confirmation except for enzyme assay that clearly determine by one method.

Transmission electron microscopy (TEM)

Ultrastructure damages of ARSA treated with SSE either alone or in combination with ampicillin were examined using TEM. TEM preparations were performed in accordance with previously reported with slight modifications [32]. After preincubated at 35°C for 18 h, ARSA strains were adjusted spectrophotometrically to give a final concentration approximately 5×10^5 cfu/ml. The cultured were grown in the absence of antibacterial agent (control), in SSE alone, ampicillin alone, and SSE plus ampicillin combination, for 4 h with shaking 110 oscillations/min in a water bath at 37°C. Then, the cultured were harvested by centrifugation at 6000 ×g for 15 min at 4°C and the pellets were fixed in 2.5% glutaraldehyde (Electron Microscope Sciences; EMS) in 0.1 M phosphate buffer (pH 7.2) for 12 h. The samples were then carefully washed twice with 0.1 M phosphate buffer. Post-fixation was carried out with 1% osmium tetroxide (EMS) in 0.1 M phosphate buffer (pH 7.2) for 2 h

at room temperature. After washing in the buffer, the samples were gently dehydrated with graded ethanol (20%, 40%, 60%, 80% and 100%, respectively) for 15 min. Then, infiltration and embedding were performed using Spurr's resin (EMS). The samples were sectioned using an ultramicrotome with a diamond knife and were then mounted on copper grids. Ultimately, the ultrathin sectioned were counterstained with 2% (w/v) uranyl acetate for 3 min and then 0.25% (w/v) lead citrate for 2 min. After staining, the specimens were viewed in a Tecnai G2 electron microscope (FEI, USA), operating at 120 kV.

In addition, the cell area of these cells from micrographs were calculated by measuring cell width multiplied by cell length (nm^2) in order to confirm the effects of SEE either used singly and in combination on cell size.

Immunofluorescence staining and confocal microscopy

Disruption of peptidoglycan after exposure to SSE either used singly or in adjunction with ampicillin was carried out by the immunofluorescence and visualized under a confocal laser scanning microscope. The 18 h cultured of ARSA was challenged with distinct agents; ampicillin (256 μg/ml), SSE (2 mg/ml), ampicillin (0.11 μg/ml) plus SEE (1.5 mg/ml) for 4 h. The cell grown without antibacterial agent was used as a control. After incubation, the cells were harvested by centrifugation and subsequently fixed with 2.6% paraformaldehyde and 0.04% glutaraldehyde mixture for 10 min at room temperature, and 50 min on ice. Fixed cells were washed and resuspended in PBS, smeared directly to poly-L-lysine coated slides, and air-dried. Nonspecific antibody binding in the samples was blocked with 5% BSA for 30 min at room temperature. The specimens were consecutively incubated with the primary antibody (1:800 dilution with PBS containing 2% BSA), a mouse anti-*S. aureus* peptidoglycan antibody, in a moist chamber for 1 h. The cells were washed thoroughly with PBS containing 0.1% Tween 20. The secondary antibody (Alexa 488-conjugated goat anti-mouse IgG) was prepared by diluting with PBS plus 2% BSA solution (1:1000) and incubated with the samples for 1 h in the dark at room temperature, several washed with PBS + 0.1% Tween 20. To reduce photobleaching and to counterstain bacterial DNA, the slides were mounted with a few drops of fluoroshield mounting medium containing 4′,6-Diamidino-2-Phenylindole (DAPI) [33]. Images were captured and performed with a confocal laser scanning microscope (Nikon 90i A1R) equipped with 100x NA 1.40 oil objective (Nikon), Intensilight fiber illuminator (Nikon) and NIS Elements 4.11 AR/BR B871 (Nikon). DAPI and Alexa 488 were excited at 360 nm and 488 nm, respectively. The background cell fluorescence was subtracted. An Adobe Photoshop CS5 was used for the figure preparation.

Cytoplasmic membrane (CM) permeability

SSE used either singly or in combination with ampicillin induced CM permeability were examined by the ability of these antimicrobial agents to disclose cytoplasmic β-galactosidase activity in bacteria using ONPG as a substrate. ONPG can be cleaved by β-galactosidase localized within the cytoplasm. The products of β-galactosidase-ONPG reaction were galactose (colorless) and *o*-nitrophenol (yellow). The assays were prepared in according to the methods of Marri et al. and Eumkeb et al. with slight modification [34,35]. Shortly, 18 h ARSA cultured was adjusted to $5x10^5$ cfu/ml and grown in CAMHB without antibacterial agents (control), 2 mg/ml SSE, 256 μg/ml ampicillin and 1.5 mg/ml SSE plus 0.11 μg/ml ampicillin in 110 oscillations/min in shaking water bath at 37°C. These bacterial cells were then compiled to analyze cytoplasmic membrane alteration at six different interval times (0, 1, 2, 3, 4 and 5 h). Nisin (8 μg/ml) was applied as a positive command. Each sample 2 ml aliquots at each time were transferred to tubes containing ONPG (4 mg/ml) plus Phosphate buffered saline (PBS). Observed yellow was recorded as positive β-galactosidase activity (increased CM permeability), while appearing colorless was recorded as negative β-galactosidase activity (no effect on CM permeability).

Apart from this, the second cytoplasmic membrane permeabilization experiment was executed to confirm as previously described by Shen et al. [36] and Zhou et al. [37] with some modifications. This method was performed by measurement the release of UV-absorbing material concentrations using UV–VIS spectrophotometer. In brief, the ARSA cultures were prepared on CAMHB for 18 h at 35°C. Inocula of 2.0 ml of culture were added into 98.0 ml CAMHB and shaking at 100 r.p.m. at 37°C for 4 h to give log phase. Bacterial cultures were adjusted in saline to give 5×10^6 cfu/ml. Log phase of the adjusted cultures 1.0 ml was added to 9.0 ml of 2.5 mmol/l sodium HEPES buffer (pH 7.0) supplemented with 100 mmol/l glucose plus 256 μg/ml ampicillin, 2 mg/ml SSE (½ MICs), and 0.11 μg/ml ampicillin plus 1.5 mg/ml SSE (¾ FIC) in each flask to give a final concentration at 5×10^5 cfu/ml. The flasks of cell suspensions without antibacterial agent were used as the negative control and with 8 (μg/ml) nisin (½ MIC) was applied as positive control. The bacterial suspensions were incubated at 37°C in the shaker water bath. CM permeability was determined after a contact time of 0, 0.5, 1.0, 2.0, 3.0 and 4.0 h. After treatment, samples (1.0 ml) were taken every contact time and filtered through a sterile nitrate cellulose membrane (0.22 μm), and OD_{260} value of the supernatant was taken as a percentage of the extracellular UV-absorbing materials released by cells. All the measurements were done in triplicates in Varian Cary 1E UV/VIS spectrophotometer [28].

Enzyme assay

The ability of SSE when used alone to deteriorate β-lactamase type IV activity of *E. cloacae* was performed following the method as previously described by Richards et al. [32] with little modifications. Concisely, benzylpenicillin, a substrate for β-lactamase type IV, was adjusted to concentrations sufficient to hydrolyze 50-60% substrate within 5 min. SSE at 1, 2, and 4 mg/ml were preincubated with enzyme in 50 mM sodium phosphate buffer (pH 7.0) at 37°C for 5 min prior to adding a substrate. Time-course assay were performed in 0, 5, 10, 15 and 20 min using methanol/acetic acid (100:1) as a stopping agent. 10 μl of each sample was injected to reverse-phase HPLC to analyze the remaining benzylpenicillin. A mobile phase employed was 10 mM ammonium acetate (pH 4.5 acetic acid): acetronitrile (75:25) with flow rate 1 ml/min, UV detector at 200 nm, Ascentis C18 column, and 35°C for column temperature [9]. The quantity of remaining benzylpenicillin was calculated by comparing the area under the chromatographic curve.

Statistical analysis of experimental data

All experiments were carried out in triplicate; data were expressed as mean ± standard error of the mean (SEM) due to it takes into account sample size. Significant differences of cell area in each treated group from TEM, CM permeability and enzyme assay among each treated group at the same interval times were analyzed by one-way ANOVA. A p valve <0.01 of Tukey's HSD post-hoc test was considered as statistically significant difference.

Results

Phytochemical screening, MIC, and checkerboard determinations

The preliminary phytochemical characteristics results of SSE indicated that the presence of alkaloids, tannins, glycosides, steroids, and anthocyanin were detected. The total alkaloid containing in the extract was 526.27 ± 47.27 mg/1 g of dried extract. The MIC results of SSE, ampicillin, and nisin against ARSA were 4 mg/ml, >512 μg/ml, and 16 μg/ml respectively, while these agents against susceptible *S. aureus* strain was 4 mg/ml, 0.25 μg/ml, and 0.5 μg/ml respectively (Table 1). According to CLSI, these outcomes suggested that ARSA used in this study revealed high resistant to ampicillin and nisin, but susceptible to reference strain *S. aureus* ATCC 29213. SSE exhibited little inhibitory effect against these strains. In checkerboard assay, based upon FICI calculation, the combination of SSE and ampicillin exhibited synergistic activity at FICI <0.5 (Table 1). Obviously, the concentration of ampicillin that can inhibit ARSA growth had considerably reduced from >512 μg/ml to 0.15 μg/ml in combination with SSE.

Killing curve determinations

The viable counts for ARSA after exposure to antimicrobial agents at different times are shown in Figure 1. The control cells revealed no reduction in viable counts and steady growth in log phase viable counts throughout 24 h. Whereas, no significant change was observed in cells treated with the SSE and ampicillin alone. Interestingly, the combination of the SSE plus ampicillin exhibited a steady reduction of 5×10^5 cfu/ml to 10^3 cfu/ml within 6 h and did not recover within 24 h. These results had also been confirmed antibacterial and synergistic activity of MIC and checkerboard determinations.

TEM

The electron microscope images were chosen to present from triplicate samples in each group. Electron microscopic investigation clearly exhibited that the cytoplasmic membrane and cell wall of ARSA grown in the absence of antibacterial agent (control) can be undoubtedly distinguished and no damage to ultrastructure was observed (Figure 2a). ARSA treated with ampicillin 256 μg/ml alone showed slight peptidoglycan damage to a minority of these cells (Figure 2b). A number of these cells treated with SSE 2 mg/ml caused somewhat peptidoglycan damage (Figure 2c). Besides, these average cell areas were somewhat smaller than the control and ampicillin groups, but not a significant difference ($p > 0.01$) (Figure 3). These findings indicate that the SSE treated cells cause rather higher peptidoglycan damage than ampicillin treated cells. Obviously, the synergistic effect was observed with the combination of ampicillin plus SSE that these cells demonstrated a lot of these cells exhibited marked morphological damage, noticeable peptidoglycan damage (Figure 2d). Obviously, these average cell areas were significantly smaller than control and others ($p < 0.01$) (Figure 3).

Immunofluorescence staining and confocal microscopy

Confocal laser scanning images of peptidoglycan-labeled ARSA unambiguously revealed intact coccus-shaped and no damage was observed in control cell (Figure 4). Cells treated with ampicillin and SSE alone showed a slight damage to peptidoglycan, but SSE alone seemed to have more damage than ampicillin alone. Substantial peptidoglycan disruption was seen in cell received ampicillin plus SSE combination. The bright field image of this treated bacterium demonstrated distortion in cell shape (a white arrow). Data from this experiment had ratified damage of ARSA's peptidoglycan after treatment with SSE in adjacent with ampicillin. These results support a predominant mechanism of action of this combination probably be inhibiting peptidoglycan synthesis.

Table 1 MICs and FICI of SSE, AMP when used either alone or in combination against ARSA

Strains	MIC			FIC	FIC index
	AMP (μg/ml)	SSE (mg/ml)	NIS (μg/ml)	AMP + SSE (μg/ml + mg/ml)	
S. aureus DMST 20651	$>512^{R}$	4.0^{ND}	16	0.15 + 2.0	<0.5
S. aureus DMST 20652	$>512^{R}$	4.0^{ND}	16	0.15 + 2.0	<0.5
S. aureus DMST 20653	$>512^{R}$	4.0^{ND}	16	0.15 + 2.0	<0.5
**S. aureus* ATCC 29213	0.25^{S}	4.0^{ND}	0.5	NT	NT

^{S} = Susceptible; ^{R} = resistant; ND = No data in CLSI; NT = not test; AMP = Ampicillin; SSE = *S. suberosa* crude extract; NIS = Nisin; FICI ≤ 0.5 denoting synergistic; FICI > 0.5–4.0 denoting no interaction; FICI > 4.0 denoting antagonism; **S. aureus* ATCC 29213 was used as a reference strain.

CM permeability

The effect of 256 μg/ml ampicillin, 2 mg/ml SSE alone and the combination of 0.11 μg/ml ampicillin plus 1.5 mg/ml SSE on CM permeability determined by cytoplasmic β-galactosidase activity is illustrated in Table 2. The result showed that there was no activity of β- galactosidase with increasing time in cells grown without antibacterial agent (control), with ampicillin and SSE alone. Whereas, cell treated with SSE plus ampicillin combination and nisin exhibited β-galactosidase activity (observed yellow) after 1 h exposure time. These results indicated that the combination of SSE plus ampicillin revealed the ability to increase CM permeability of ARSA.

Furthermore, the cytoplasmic membrane permeability was measured by UV-absorbing release materials as presented in Figure 5. After treatment ARSA cells with 8 μg/ml nisin, and 0.11 μg/ml ampicillin plus 1.5 mg/ml SSE could induce the release of 260 nm absorbing material, which we interpret to be mostly DNA, RNA, metabolites and ions significantly higher than controls, ampicillin, and SSE alone within 0.5 h and throughout the 4 h ($p < 0.01$). These results imply that the synergistic activity of SSE plus ampicillin increased cytoplasmic membrane permeability of this strain [36,37].

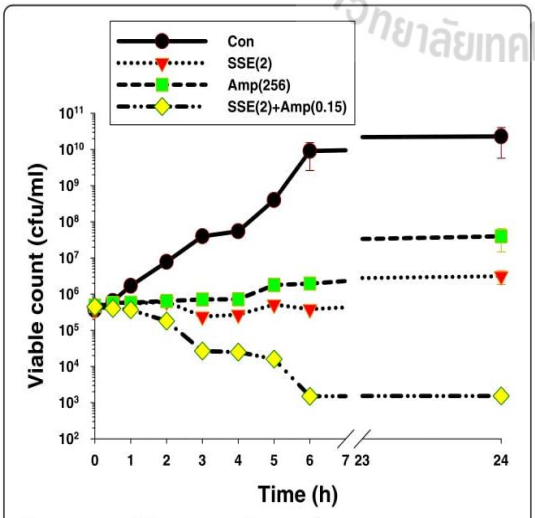

Figure 1 Time killing-curve of ARSA after exposure to SSE, ampicillin either alone or in combination. Con = control (drug free); SSE (2) = SSE at 2 mg/ml; Amp(256) = ampicillin at 256 μg/ml; SSE(2) + Amp(0.15) = SSE at 2 mg/ml plus ampicillin at 0.15 μg/ml; the values plotted are the means of 4 observations, and the vertical bars indicate the standard errors of the means.

Enzyme assay

The ability of SSE to inhibit activity of β-lactamase type IV isolated from *E. cloacae* was assayed by determining the amount of remaining benzylpenicillin using reverse-phase HPLC. As shown in Figure 6, the result displayed that benzylpenicillin treated with SSE was significantly higher than control starting from 5 minutes ($p < 0.01$). The benzylpenicillin remainder was significantly increased by an increase in SSE as a concentration-dependent manner. These results suggest that one activity of SSE against ARSA may involve in β-lactamase inhibition [9].

Discussion

The present investigation is the first report of antibacterial and synergistic activities of *S. suberosa* extract when used singly and in combination with ampicillin against clinical isolated ARSA. The preliminary mechanisms of action of those agents were also evaluated in this study. Practically-prescribed antibiotic resistance in MRSA due to drug target-site alteration, enzyme modification and changes in membrane permeability, has increasingly emerged. Therefore, the selection of antibiotic to treat multidrug resistant MRSA has been daily decreasing. So, the research approach to find out new anti-MRSA agents are still necessary [4]. The MIC results revealed that these testing *S. aureus* strains were highly resistant to ampicillin alone because of the standard value of the sensitivity of ampicillin against these strains are ≤ 0.25 μg/ml [27]. As well as, SSE demonstrated little bacteriostatic effect against these strains while the reference *S. aureus* strain exhibits susceptible to ampicillin. Likewise, these results are in substantial agreement with those of Eumkeb et al. [9] that the MIC of ampicillin against *S. aureus* DMST 20651 was > 1,000 μg/ml. Also, the MIC result of nisin against MRSA strains seem consistent with previous finding that 90% MIC of nisin against MRSA was 16 μg/ml [38]. The checkerboard

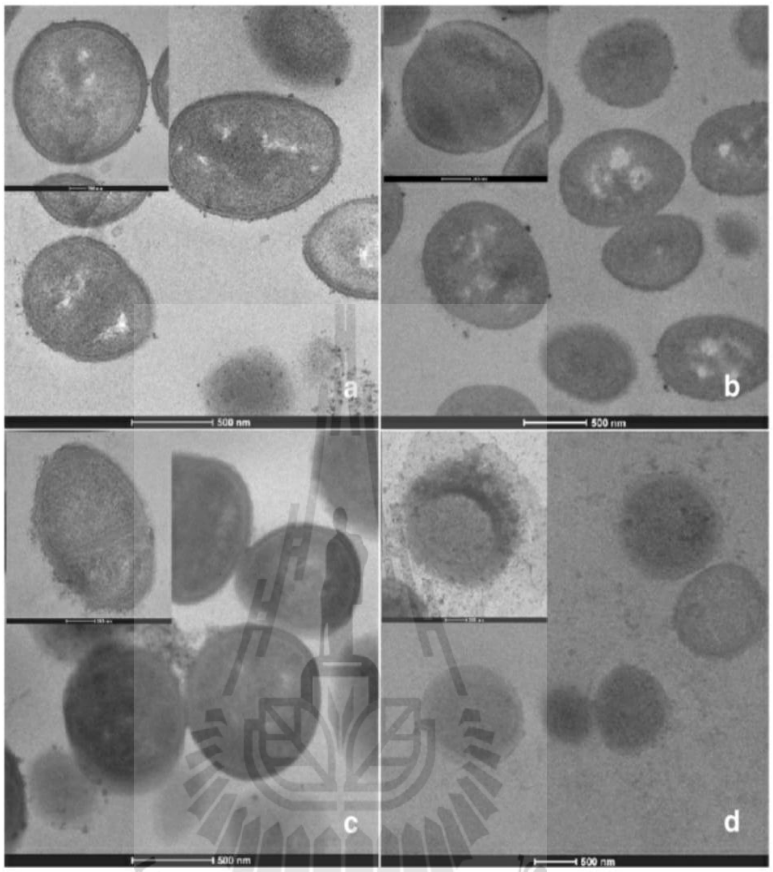

Figure 2 Ultrathin sections of log phase of ARSA DMST 20651 grown in MHB: a = Control (bar = 500 nm, x19500; *inset*: bar = 100 nm, x43000); b = 256 μg/ml ampicillin (bar = 500 nm, x15000; inset: bar = 200 nm, x38000); c = 2 mg/ml SSE (bar = 500 nm, x19500; *inset*: bar = 200 nm, x38000); d = 1.5 mg/ml SSE plus 0.11 μg/ml ampicillin (bar = 500 nm, x8700; *inset*: bar = 200 nm, x29000).

determination revealed synergistic effects of ampicillin plus SSE against all of tested *S. aureus* strains with FIC index at <0.50 [39]. These results are in substantial correspondence with those of Eumkeb et al. that galangin, quercetin or baicalein plus ampicillin exhibited synergistic activity against penicillins-resistant *S. aureus* strains at FIC indices < 0.03 [9]. Besides, previous studies reported that a synergistic effect between quercetin and oxacillin against vancomycin-intermediate *S. aureus* displayed the lowest FIC index value of 0.0417 [40]. Apart from this, the antibacterial activity of quercetin plus ampicillin or vancomycin against the sensitive MRSA strain were significantly increased compared to control (no any testing agent) ($p < 0.01$) [41]. As previously documented, drug combination approach by achieving a synergistic effect can eliminate and neutralize the adverse effects [16]. The killing curve determination can confirm MIC and checkerboard determinations that synergistic effect of SSE plus ampicillin caused marked reduction in viable counts of ARSA cells from 6 h and throughout 24 h. These results appear consistent with previous findings that galangin, quercetin or baicalein plus ceftazidime exhibited synergistic activity against MRSA result in a large decrease from 6 to 24 h [9], Apart from this, the combinations of baicalin and β-lactam antibiotics showed that the killing of MRSA and beta-lactam-resistant *S. aureus* cells were dramatic reduction by these combinations [26]. Clearly, the synergistic effect of SSE plus ampicillin against ARSA was observed. For this reason, the elementary mechanism of action such as TEM, CM permeability, and enzyme assay were more investigated.

TEM results of SSE plus ampicillin treated cells demonstrated that ARSA cells exhibited marked morphological damage, clear peptidoglycan and cytoplasmic membrane damage, and average cell areas significant smaller than control. These results seem consistent with previous findings that the combination of ceftazidime plus galangin caused damage to the ultrastructures of the cells, affected the integrity of the cell walls and led

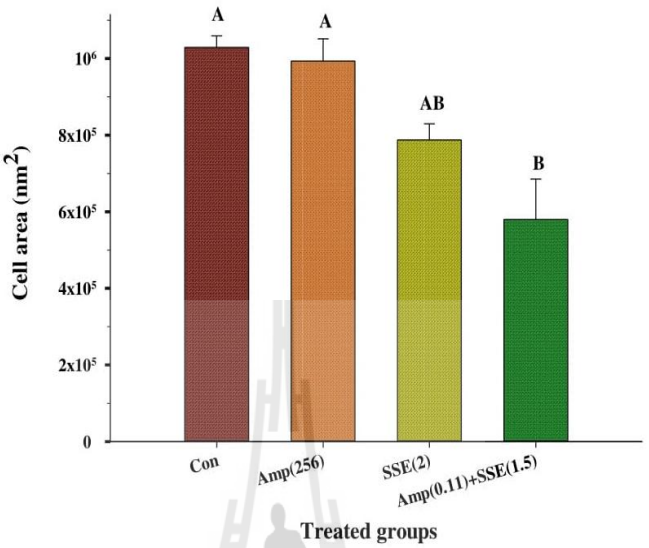

Figure 3 The cell area of ARSA after treatment with SSE, ampicillin either alone or in combination: Con = control (drug free); Amp (256) = ampicillin at 256 μg/ml; SSE (2) = SSE at 2 mg/ml; Am (0.11) + SSE (1.5) = ampicillin at 0.11 μg/ml plus SSE at 1.5 mg/ml; The graph shows an area of cell determined by cell width x cell length (nm^2). The different superscript alphabets are significantly different from each other. Each treated group was compared using one-way ANOVA and Tukey's HSD Post-hoc test, $p < 0.01$ are presented.

to an increase in cell size of ceftazidime-resistant *S. aureus* [9]. The TEM results have been confirmed by confocal microscopic images that the peptidoglycan of this combination treated cells was undoubtedly destroyed. These effects can be explained by assuming that SSE may insert synergistic action with ampicillin to inhibit peptidoglycan synthesis leads to marked morphological damage and delay cell division.

The CM permeability revealed that SSE in combination with ampicillin increased cytoplasmic membrane

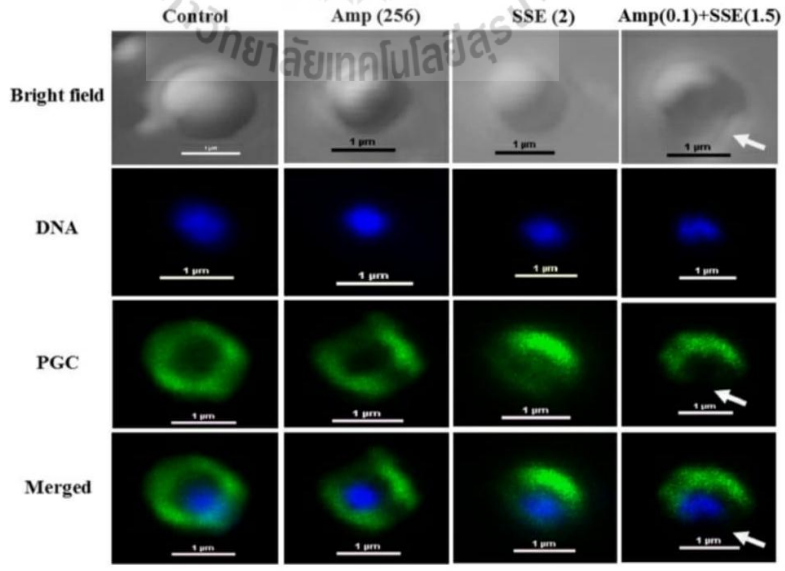

Figure 4 Schematic representation of the results from immunofluorescence and a confocal laser scanning microscope; Samples of ARSA after treatment for 4 h with Ampicillin, SSE, either alone or in combination. Amp (256), ampicillin at 256 μg/ml; SSE (2), *Stephania suberosa* extract at 2 mg/ml; Amp (0.1) + SSE (1.5) = Ampicillin 0.11 μg/ml plus SSE 1.5 mg/ml. The cells were stained for DNA with DAPI (blue) and labeled for peptidoglycan (PGC) (green) using respective antibodies. DNA in all groups was localized in the central of cell and surrounded by a peptidoglycan layer (merged images). The white arrows showed explicit disruption of peptidoglycan. Scale bar = 1 μm.

Table 2 β-galactosidase activity results of ARSA after treatment with ampicillin, SSE alone or in combination

Time	Control (no drug)	Amp (256)	SSE (2)	Amp + SSE (0.11 + 1.5)	NIS (8) (positive control)
0	Neg	Neg	Neg	Neg	Neg
1	Neg	Neg	Neg	Pos	Pos
2	Neg	Neg	Neg	Pos	Pos
3	Neg	Neg	Neg	Pos	Pos
4	Neg	Neg	Neg	Pos	Pos
5	Neg	Neg	Neg	Pos	Pos

Neg, no evidence of activity; Pos, have evidence of activity; Amp (256), ampicillin at 256 μg/ml; SSE (2), *Stephania suberosa* extract at 2 mg/ml; Amp + SSE (0.11 + 1.5) = Ampicillin 0.11 μg/ml plus SSE 1.5 mg/ml; NIS (8), Nisin at 8 μg/ml was used as a positive control. The experiment was carried out in triplicate observations.

permeability of this strain. The β-galactosidase activity result was virtually the same as UV-absorbing material concentrations result that CM permeability was significantly increased from 1 h onward (Table 2 and Figure 5). These results are in substantial agreement with previous findings that luteolin either alone or combined with amoxicillin and apigenin alone and in combination with ceftazidime increased CM permeability of amoxicillin-resistant *E. coli* and ceftazidime-resistant *E. cloacae* respectively [15,28]. In general, nisin incorporates into the membrane and makes the membrane permeable for ions. So that, both the membrane potential and pH gradient are dissipated [42]. Apart from this, nisin inhibits peptidoglycan synthesis and forms highly specific pores through interaction with the membrane-bound cell wall precursor lipid II [43]. The increase in CM permeability may be one of the synergistic activity of this combination against ARSA strain. The more SSE concentrations results in more benzylpenicillin remainder of enzyme assay results are in substantial agreement with previous findings that galangin inhibits β-lactamase in a concentration-dependent manner [9]. Noteworthy that the SSE alone could show β-lactamase inhibitory activity results in very high MIC value. Whereas, its combination with ampicillin showed a synergistic effect by peptidoglycan synthesis inhibition and increase CM permeability. Six new protoberberines and ten known alkaloids were found in *Stephama suberosa* root extracts [44]. However, the bioactive compounds of *S. suberosa* extract that showed antibacterial effect in this study have not been well characterized. Although, there is devoid of the report has been documented for toxicity of *S. suberosa*, but some plant in the genus *Stephania*, for instance, previous studies found that the LD_{50} of oral feeding of aqueous extract of *Stephania cepharantha* wet and dry root tuber in mice were 41.4 g/kg and 22.9 g/kg respectively [45]. In addition, the acute toxicities of protoberberine alkaloids, berberine, coptisine, palmatine and epiberberine, from *Rhizoma coptidis* (RC)

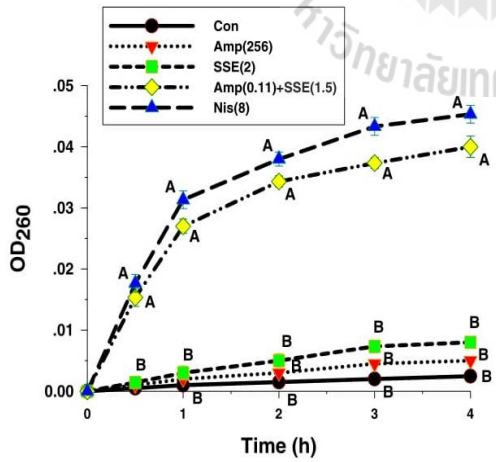

Figure 5 Presence of 260 nm absorbing material in the supernatants ARSA treated with Ampicillin, SSE, either alone or in combination. Amp (256), ampicillin at 256 μg/ml; SSE (2), *Stephania suberosa* extract at 2 mg/ml; Amp (0.11 + 1.5) + SSE (1.5) = Ampicillin 0.11 μg/ml plus SSE 1.5 mg/ml; NIS (8), Nisin at 8 μg/ml was used as a positive control and untreated cells were used as negative control. The mean ± SEM for three replicates are illustrated. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, $p < 0.01$).

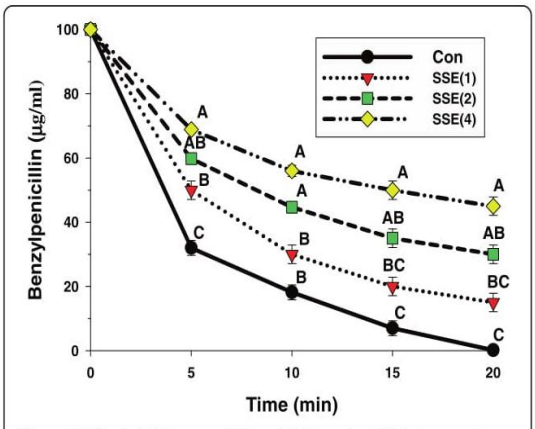

Figure 6 The inhibitory activity of SSE against β-lactamase type IV from *E. cloacae* in hydrolyzing benzylpenicillin; Con = control (no testing agent); SSE(1) = SSE at 1 mg/ml. The graph shows the remaining benzylpenicillin at the same time. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, $p < 0.01$).

were evaluated, the LD_{50} value of four alkaloids were 713.57, 852.12, 1533.68 and 1360 mg/kg, respectively. The sub-chronic toxicity study in rats treated with the RC alkaloids at a dose of 156 mg/kg/day for 90 days revealed that no abnormality in clinical signs, body weights, organ weights, urinalysis, hematological parameters, gross necropsy, histopathology, no mortality and morbidity were observed in any of the animals [46]. Likewise, the acute oral LD_{50} of lupanine from *Lupinus angustifolius* in rats was 1464 mg/kg [47]. Our finding found that the total alkaloid content in SSE was approximately 526.27 mg/1 g of dried extract. The FIC for SSE in combination with ampicillin against ARSA was 2 mg/ml, possibly therefore the total alkaloids of 1.05 mg may roughly presented in 2 mg/ml. In this case, the in vitro determination of starting dose for in vivo tests was used to predict starting doses for subsequent in vivo acute lethality assays. The results lend support to the assumption that if most of these alkaloids are Cepharanthine, the estimated LD_{50} was > 5,000 mg/kg, which is classified as practically nontoxic [48,49]. Hence, SSE when used in combination with ampicillin at this concentration may have a sufficient margin of safety for therapeutic use. Obviously, many alkaloids have been used as modern medicine, for example colchicine (anti-gout), quinine (anti-malaria), morphine and codeine (analgesics), reserpine (anti-hypertension), vinblastine and vincristine (anti-cancer), theophylline (anti-asthma) [50,51]. However, further investigation should be focused on active ingredients of SSE that play an important role on antibacterial effect, as well as toxicity confirmation is still necessary.

Conclusions

In summary, our study provides evidence that SSE has the extraordinary potential to reverse bacterial resistance to originate traditional drug susceptibility of it. This is the first report of the mechanism of synergistic action of SSE plus ampicillin combination against ampicillin-resistant *S. aureus*. Three modes of actions would be implied that this combination inhibit peptidoglycan synthesis, inhibit β-lactamases activity, and increase CM permeability. So, this *Stephania suberosa* proposes the high potential to develop a useful of novel adjunct phytopharmaceutical to ampicillin for the treatment of ARSA. Future studies should be investigated and confirmed the efficacy and toxicity of this combination in an animal test or in humans, Also, The synergistic effect on blood and tissue would be evaluated and achieved.

Abbreviations

ARSA: Ampicillin-resistant *Staphylococcus aureus* DMST 20651; SSE: *Stephania suberosa* Forman extract; MIC: Minimal inhibitory concentration; FIC: Fraction inhibitory concentration; ATCC: American Type Culture Collection; CM: Cytoplasmic membrane; cfu: Colony forming unit; CAMHB: Cation-adjusted Mueller-Hinton broth; OD: Optical density; MHA: Mueller-Hinton agar; HPLC: High performance liquid chromatography; PBS: Phosphate buffer solution; BSA: Bovine serum albumin; DAPI: 4′,6-Diamidino-2-Phenylindole; CLSI: Clinical and Laboratory Standards Institute; DMSO: Dimethyl sulfoxide.

Competing interests

The authors have declared that they have no competing interests.

Authors' contributions

YT and NA, performed the experiments, analyzed data. KS, PK and SK also analyzed data and gave comments. GE designed the project, supervised the experiments and wrote the report. All authors have read and approved the final manuscript.

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extraction and isolation of date syrup phenolic compounds detected 3-*O*-caffeoylshikimic acid, caffeic acid, hydrocaffeic acid and *P*-coumaric acid. The antioxidant potential was dependent on date syrup polyphenol extract concentration. In biological assays, results demonstrated that phenolic compounds are inhibitory against *S. aureus* and *E. coli* with an MIC of 30 mg/mL. Isolated phenolic fractions demonstrates significant antibacterial activity in particular hydrocaffeic acid and 3-*O*-caffeoylshikimic acid with mean zones of inhibition at 23.19 mm (± 0.15) and 12.93 mm (± 0.15) for *E. coli* and 21.09 mm (± 0.32) and 13.43 mm (±0.35) for *S. aureus* respectively. However, the antimicrobial activity was more prominent in the whole phenolic extract rather than isolated fractions suggesting the combination of phenolic compounds act synergistically as antimicrobials.

S19/P17

The Role of IQGAP1 in Actin-Based Motility of Burkholderia pseudomallei

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Burkholderia pseudomallei is a Gram-negative bacterium that causes melioidosis, a serious disease of animals and humans in tropical countries, southeast Asia and northern Australia. This pathogen can invade many cell types. It lyses and escapes from the endocytic vacuole after cellular uptake. In the cytosol, it exploits the host cell actin cytoskeleton by a process known as actin-based motility to promote its movement within and between cells. BimA (*Burkholderia* intracellular motility A) is required for this process and is located at the pole of the bacterial cell where actin polymerisation takes place. The mechanism by which BimA subverts the cellular actin machinery is ill-defined. We have used proteomics to identify cellular factors associated with bacteria expressing BimA. IQGAP1 (IQ motif containing GTPase activating protein 1), a scaffold protein that interacts with many different cellular proteins, was detected. Confocal microscopy confirmed that it is recruited to the actin tails of *B. pseudomallei* in infected cells. IQGAP1 plays a role in regulating the actin cytoskeleton and is targeted by several other pathogens. For example, it promotes *Salmonella* invasion into epithelial cells and supports cell attachment and pedestal formation in Enteropathogenic *Escherichia coli*. In this study, we tested whether BimA and IQGAP1 are interacting partners by yeast two-hybrid assay. We found that IQGAP1 does not interact with BimA directly. Using siRNA knockdown we found that the bacterium can still form actin tails in IQGAP1-knockdown cells, however the tails are statistically longer tail with a lower actin density.

S19/P18

Evaluation of Resazurin microtitre plate assay for early phenotypic characterisation of ESBL, AmpC and MBL β-lactamases-producing Enterobacteriaceae

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A rapid dissemination of pathogenic bacteria resistant to multiple antibiotics has become one of the most significant global problems, affecting both patients and health care providers. The effective antibiotics to treat these recalcitrant infections have been decreasing daily resulting in difficulty in selecting appropriate antibiotic treatments. Development of rapid, simple and inexpensive methodology to identify the various resistance mechanisms present in clinical isolates is necessary to inform chemotherapeutic decisions. In the present study a 96-well plate-based resazurin assay, incorporating cefotaxime (CTX) or ceftazidime (CAZ) with specific □-lactamase inhibitors was developed. A total of 42 clinical Enterobacteriaceae producing ESBL, AmpC, MBL □-lactamases, and co-□-lactamase producers were subjected to the resazurin microtiter plate (RMP) assay. The MIC ratios were calculated and interpreted with6 h of incubation. The assay positively detected all 11 ESBLs, 16 AmpCs, 10 MBL, 4 AmpC-ESBL co-producers and 1 ESBL-MBL co-producer. CAZ-based showed better performance in detection of AmpC singly compared to CTX-based, while no difference in characterising ESBL alone. This assay is a promising approach that could be utilised in any laboratories. It could also inform effective treatment and minimise and control the spread of resistant bacterial infections.

S19/P19

Selective conditions for a multidrug resistance plasmid depend on the sociality of antibiotic resistance

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Multiple antibiotic resistance genes (ARG) are frequently clustered on conjugative plasmids and often confer qualitatively different mechanisms of resistance. These multidrug resistance (MDR) plasmids are an important source of clinical resistance. It is critical therefore to understand the selective conditions promoting the spread of these MDR plasmids. Here, we tested how the antibiotic conditions required to select for a multidrug resistant plasmid, RK2, in *Escherichia coli* depended on the mechanism of resistance, specifically whether drug resistance was selfish or cooperative. We observed highly contrasting selective conditions depending upon the sociality of resistance: A selfish drug resistance, efflux of tetracycline, was selected for at

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