ฤทธิ์ต้านแบคทีเรียของพลาสมาจากจระเข้น้ำจืดไทย (*CROCODYLUS SIAMENSIS*) ต่อแบคทีเรียดื้อยา

นางสาวนิตยา โรจน์ทินกร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ANTIBACTERIAL ACTIVITY OF PLASMA FROM SIAMESE CROCODILE (*CROCODYLUS SIAMENSIS*) ON DRUG RESISTANT BACTERIA

Nitaya Rojtinnakorn

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

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ANTIBACTERIAL ACTIVITY OF PLASMA FROM SIAMESE CROCODILE (*CROCODYLUS SIAMENSIS*) ON DRUG RESISTANT BACTERIA

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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นิตยา โรจน์ทินกร : ฤทธิ์ต้านแบคทีเรียของพลาสมาจากจระเข้น้ำจืดไทย (*CROCODYLUS SIAMENSIS*) ต่อแบคทีเรียดื้อยา (ANTIBACTERIAL ACTIVITY OF PLASMA FROM SIAMESE CROCODILE (*CROCODYLUS SIAMENSIS*) ON DRUG RESISTANT BACTERIA) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เกรียงศักดิ์ เอื้อมเก็บ, 124 หน้า.

การดื้อยาของเชื้อแบคทีเรียเป็นปัญหาที่สำคัญของโลกรวมถึงผู้ป่วยหนักที่โรงพยาบาลมหาราชนครราชสีมา ประเทศไทย ปัญหาดังกล่าวทำให้อัตราการเสียชีวิตและค่าใช้จ่ายในการรักษาพยาบาลเพิ่มขึ้น อีกทั้งต้องใช้ยาต้านแบคทีเรียขนานใหม่ที่มีราคาแพงและรุ่นที่สูงขึ้นตามมา ดังนั้นวัตถุประสงค์หลักของการศึกษาวิจัยในปัจจุบันจึงให้ความสำคัญไปที่สารต้านจุลชีพที่ได้จากธรรมชาติที่มีฤทธิ์ในการต้านแบคทีเรียดื้อยาหรือสารที่สามารถเพิ่มการออกฤทธิ์ของยาปฏิชีวนะดั้งเดิมให้มีประสิทธิภาพสามารถใช้ต้านเชื้อแบคทีเรียดื้อยาได้ ดังนั้น การศึกษาครั้งนี้ได้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์ในการต้านเชื้อแบคทีเรียดื้อยาของโปรตีนสกัดจากพลาสมาจระเข้น้ำจืดไทยเมื่อใช้เดี่ยว ๆ และใช้ร่วมกับยาปฏิชีวนะกลุ่มบีตาแลคแทม พลาสมาของจระเข้ถูกแยกเพื่อที่จะได้ 5 แฟรคชัน (พี 1 พี 2 พี 3 พี 4 และพี 5) โดยใช้คอลัมน์โครมาโทกราฟฟี ค่ายับยั้งต่ำสุดของ พี 1 และ พี 5 มีค่า 1024 สำหรับ พี 2 พี 3 และพี 4 มีค่า >1024 มิลลิกรัมต่อมิลลิลิตร ในการต้านเชื้อแบคทีเรีย เอนเทอโรแบคเตอร์ โคลเอเซ ที่ดื้อต่อยาเซฟตาซิดีม ดีเอ็มเอสที 21394 (ซีอาร์อีเอนซี 21394) ขณะที่ค่ายับยั้งต่ำสุดของทั้งห้าแฟรคชันในการต้านเชื้อสแตปฟิโลคอคคัส ออเรียสที่ดื้อต่อยาเมทิซิลิน ดีเอมเอสที 20651 (เอมอาร์เอสเอ 20651) คือ 1024 มิลลิกรัมต่อมิลลิลิตร ค่ายับยั้งต่ำสุดของยาเซฟตาซิดีมหรือยาออกซาซิลลินต้านเชื้อซีอาร์อีเอนซี 21394 และเอมอาร์เอสเอ 20651 พบว่ามีการดื้อยาในระดับที่สูง (ค่ายับยั้งต่ำสุดทั้งสอง >1024 ไมโครกรัมต่อมิลลิลิตร) ผลจากการศึกษาด้วยวิธีเชคเกอร์บอร์ดแสดงให้เห็นการเสริมฤทธิ์ที่ดัชนี เอฟไอซี 0.062 สำหรับสารผสม พี 1 หรือ พี 5 ผสมกับยาเซฟตาซิดีมต้านเชื้อซีอาร์อีเอนซี 21394 และที่ 0.375 สำหรับสารผสมพี 1 หรือ พี 5 กับยาออกซาซิลลินในการต้านเชื้อเอมอาร์เอสเอ 20651 กราฟการตายของเชื้อได้ยืนยันให้เห็นว่าเชื้อซีอาร์อีเอนซี 21394 และเชื้อเอมอาร์เอสเอ 20651 ที่มีชีวิตลดลงอย่างชัดเจนมากภายใน 6 ชั่วโมงและตลอดถึงชั่วโมงที่ 24 หลังจากได้รับสารผสมทั้งพี 1 และพี 5 ผสมกับทั้งยาเซฟตาซิดีมหรือยาคลอกซาซิลลิน ตามลำดับ จากการศึกษาด้วยกล้องจุลทรรศน์อิเลคตรอนแบบส่องผ่านพบว่าเชื้อซีอาร์อีเอนซีที่ได้รับสารผสมระหว่างยาเซฟตาซิดีมกับพี 1 หรือพี 5 มีขนาดของเซลล์เล็กลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับเซลล์ควบคุม (พีน้อยกว่า 0.01) อีกทั้งเซลล์ส่วนใหญ่มีรูปร่างบิดเบี้ยวและเยื่อหุ้มเซลล์ได้รับความเสียหาย นอกจากนั้นสารผสมระหว่าง พี 1 หรือ พี 5 กับยาเซฟตาซิดีม มีผลให้การซึมผ่านของเยื่อหุ้มเซลล์ชั้นนอกและชั้นในเพิ่มขึ้นอย่างชัดเจน (พีน้อยกว่า

0.01) ผลจากการศึกษาแถบโปรตีนของโปรตีนที่เกี่ยวข้องกับเยื่อหุ้มชั้นนอกเพปทิโดไกลแคน (โอเอมพีจี) ด้วยเอสดีเอส-เพจแสดงให้เห็นว่าที่น้ำหนักโมเลกุล 35 และ 45 กิโลดาลตันของแถบโปรตีนสารผสมระหว่างยาเซฟตาซิดีมผสมกับทั้งพี 1 หรือ พี 5 จางกว่าแถบโปรตีนควบคุมเล็กน้อย การทำงานของเอ็นไซม์บีตา แลคแทมเมส ชนิดที่ 4 ถูกยับยั้งโดยสารผสมระหว่างยาเซฟตาซิดีมผสมกับทั้งพี 1 หรือพี 5 เมื่อเทียบกับกลุ่มอื่น ๆ (พีน้อยกว่า 0.01) ผลการศึกษาดังกล่าวสามารถสรุปได้ว่าสารผสมทั้ง พี 1 หรือพี 5 ผสมกับยาเซฟตาซิดีมมีการเสริมฤทธิ์เสริมกันอย่างมากในการต้านเชื้อซีอาร์อีเอนซี และสามารถเปลี่ยนเชื้อที่ดื้อยาให้กลายเป็นเชื้อที่ไวต่อยาปฏิชีวนะที่เคยใช้ในการรักษา สำหรับกลไกการออกฤทธิ์ต้านแบคทีเรียเบื้องต้นนั้นอาจจะเกี่ยวข้องกับการยับยั้งการสังเคราะห์เปปทิโดไกลแคน การซึมผ่านของเยื่อหุ้มเซลล์ชั้นนอกและชั้นในของเชื้อชนิดนี้เพิ่มขึ้น และยับยั้งการทำงานของเอนไซม์บีตาแลคแทมเมส นอกจากนั้นอาจรบกวนการสังเคราะห์โปรตีนที่เกี่ยวข้องกับเพปทิโดไกลแคนและเยื่อหุ้มชั้นนอก ดังนั้นสารผสมดังกล่าวควรได้รับการพัฒนาไปเป็นเภสัช-ภัณฑ์ขนานใหม่ในการต้านเชื้ออี โคลเอเซ ซึ่งในปัจจุบันดื้อต่อยาปฏิชีวนะที่ใช้ในทางปฏิบัติเกือบทั้งหมด อย่างไรก็ตามการทดสอบความประสิทธิภาพและความเป็นพิษในสัตว์ทดลองหรือในมนุษย์ยังคงมีความจำเป็น

สาขาวิชาเภสัชวิทยา	ลายมือชื่อนักศึกษา ____________________
ปีการศึกษา 2556	ลายมือชื่ออาจารย์ที่ปรึกษา ____________________

NITAYA ROJTINNAKORN : ANTIBACTERIAL ACTIVITY OF PLASMA FROM SIAMESE CROCODILE (*CROCODYLUS SIAMENSIS*) ON DRUG RESISTANT BACTERIA. THESIS ADVISOR : ASST. PROF. GRIANGSAK EUMKEB, Ph.D., 124 PP.

*CROCODYLUS SIAMENSIS/*PLASMA/DRUG RESISTANT BACTERRIA/ *β*-LACTAM ANTIBIOTIC/SYNERGISTIC ACTIVITY

The resistance of bacteria is a major problem in the world, including intensive care patients at MaharatNakhonRatchasima Hospital, Thailand. This problem leads to increasing the morbidity, mortality and cost of medical care. The more expensive, newer and higher generation antibacterial agents have subsequently been rising. So, the current research is emphasized on naturally-derived antimicrobials against drug resistant bacteria or enhancing the effectiveness of existing antibiotics. Thus, this study aimed to investigate the activity of the separated fractions from Siamese crocodile (*Crocodylus siamensis*) plasma against drug resistant bacteria, when use alone and in combination with *β*-lactam antibiotics. The crocodile plasma was separated to give five fractions (P1, P2, P3, P4, and P5) using column chromatography. The MICs of P1 and P5 were 1024, and >1024 mg/mL for P2, P3, and P5 against clinical isolates of Ceftazidime-resistant *Enterobacter cloacae* DMST 21394 (CREnC 21394), whereas displayed at 1024 mg/mL for all fractions against Methicillin-resistant *Staphylococcus aureus* DMST 20651 (MRSA 20651). These strains, both CREnC 21394 and MRSA 20651, showed high resistance to both ceftazidime and cloxacillin with equal MICs at >1024 µg/mL. Checkerboard results revealed synergistic effects at FIC index 0.062 for either P1 or P5 plus ceftazidime against CREnC 21394 and at 0.375 for P1 or P5 plus

oxacillin against MRSA 20651. The killing curves exhibited marked decrease of CREnC 21394 and MRSA 20651 viability within 6 h and throughout 24 h after exposure to both P1 and P5 in combination with either ceftazidime or cloxacillin, respectively. The TEM study exhibited that the combination of ceftazidime plus either P1 or P5 on CREnC revealed a significantly smaller in cell size than the control cells (p<0.01), cell shape and cell envelope damage in most of these cells. In addition, either P1 or P5 in combination with ceftazidime showed steady increase the OM and CM permeability (p<0.01). The outer membrane and peptidoglycan (OMPG) associated protein band from SDS-PAGE of this strain revealed that the bands at 35 and 45 kDa of ceftazidime plus either P1 or P5 were slightly paler than control. Enzyme assay indicated that β-lactamase type IV activity were inhibited by P1 or P5 either alone or in combination with ceftazidime compared to controls (p<0.01). These results can be concluded that the combination of either P1 or P5 plus ceftazidime showed strong synergistic activity against CREnC 21394 and capable to reverse resistance to be susceptible to its primary antibiotic. The three elementary mechanisms of action may be involved; inhibition of peptidoglycan synthesis, increase OM and CM permeability, and β-lactamase inhibition. Moreover, the OMPG associated protein synthesis may be interfered. So, these combinations may be developed as a novel pharmaceutical agent against *E. cloacae*, which currently almost resistant to practically antibiotics. However, the confirmation of its efficacy and toxicity test *in vivo* and humans is required.

School of Pharmacology Student's Signature____________________

Academic Year 2013 Advisor's Signature____________________

มหาวิทยาลัยเทคโนโลยีสุรนารี

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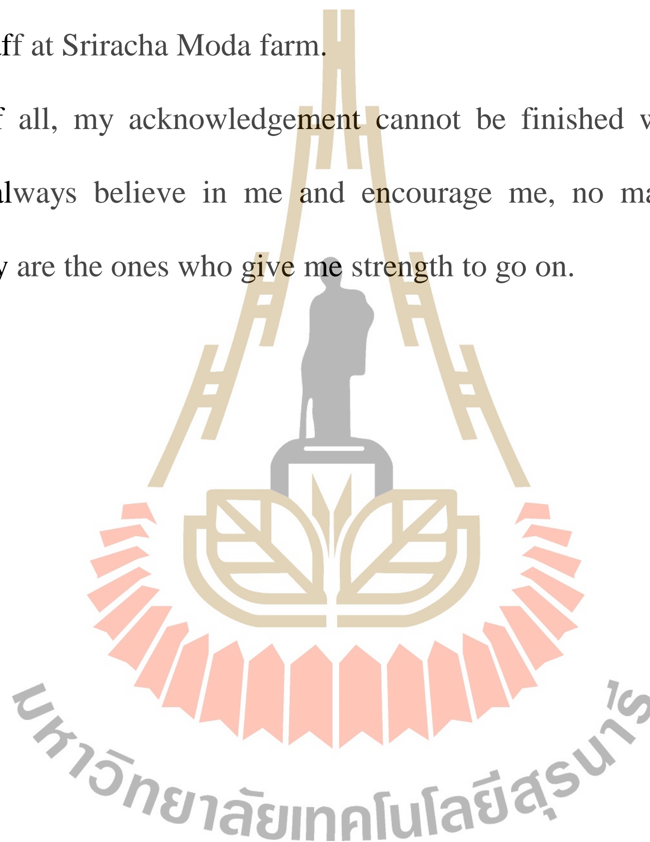

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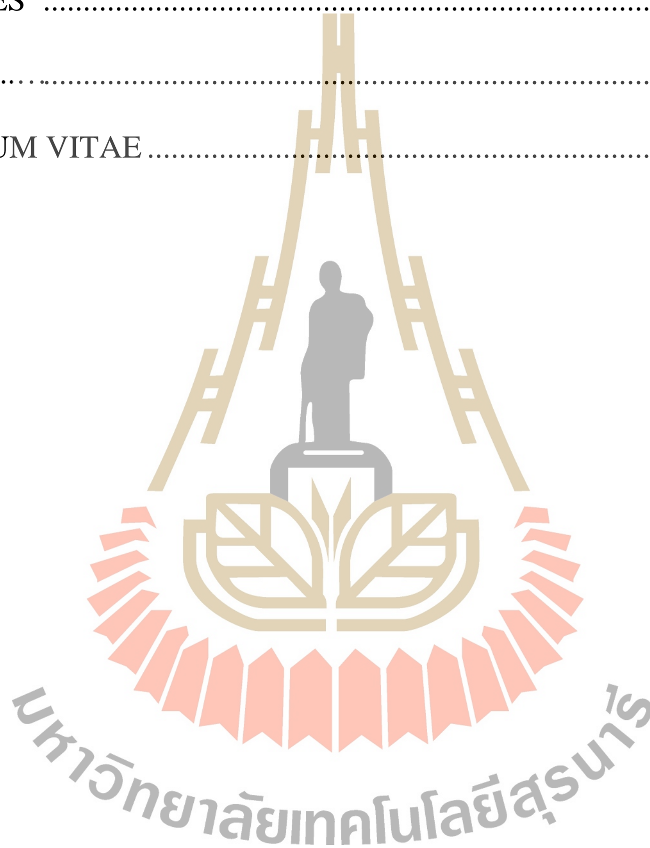

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LIST OF ABBREVIATIONS

ATCC = American Type Culture Collection

CFU = Colony Forming Unit

CH_2Cl_2 = Dichloromethane

CM = Cytoplasmic Membrane

CREC = Ceftazidime-Resistant *Escherichia coli*

CREnC = Ceftazidime-Resistant *Enterobacter cloacae*

CSEC = Ceftazidime-Sensitive *Escherichia coli*

Da = Dalton

DMST = Department of Medical Sciences Thailand

DNA = Deoxyribonucleic Acid

DNP = Dinitropyrene

EDTA = Ethylenediaminetetraacetic Acid

FIC = Fractional Inhibitory Concentration

g = Gram

h = Hour

HEPES = N-2-Hydroxyethyl Piperazine-N’-Ethanesulphonic Acid

HPLC = High-Performance Liquid Chromatography

HIV = Human Immunodeficiency Virus

HSVI = Herpes Virus Type I

IV = Intravenous

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LIST OF ABBREVIATIONS (Continued)

KDa	=	Kilo Dalton
KV	=	Kilo Volts
LPS	=	Lipopolysaccharide
MHA	=	Mueller Hueller-Hinton agar
MHB	=	Mueller Hueller-Hinton broth
MIC	=	Minimum Inhibitory Concentration
mL	=	Millilitre
µL	=	Microlitre
mM	=	Millimolar
MRSA	=	Methicillin-Resistant *Staphylococcus aureus*
MW	=	Molecular Weight
NaCl	=	Sodium Chloride
NAT	=	N-Acetyltransferase
NMR	=	Nuclear Magnetic Resonance
NO	=	Nitric Oxide
NT	=	Nitropyrene
OD	=	Optical Density
ODC	=	Ornithine Decarboxylase
OM	=	Outer Membrane
OM-PG	=	Outer Membrane and Peptidoglycan

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LIST OF ABBREVIATIONS (Continued)

OsO_4	=	Osmium Tetroxide
OSSA	=	Oxacillin-Sensitive *Staphylococcus aureus*
PMF	=	Protein Motive Force
PMSF	=	Phenyl Methyl Sulphonyl Fluoride
POX	=	Peroxidase
RNA	=	Ribonucleic Acid
s	=	Second
Sarkosyl	=	Sodium-N-Lauryl Sarcosinate
TEM	=	Transmission Electron Microscopy
TFA	=	Trifluoroacetic Acid
TNF	=	Tumor Necrosis Factor
Tris-HCl	=	Trizma Hydrochloride
TSS	=	Toxic Shock Syndrome
VSV	=	Vesicular Stomatitis Virus

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CHAPTER I

INTRODUCTION

1.1 Introduction

The widespread resistance of microorganisms to antibiotics is a global problem (Li et al., 2005). Highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), or extended β-lactamases (ESBL)-producing Gram-negative rods have emerged and well-known problem in healthcare systems and the emergence of these resistant strains have been increasing worldwide in recent years (Emori and Gaynes, 1993; Leclercq and Courvalin, 1997; Moellering, 1998; 2009; Vonberg et al., 2008). In addition, the current situation of the antimicrobial resistance in Thailand showed sustainable increase in the number of antimicrobial resistant microorganisms (Chokejindachai, 2007). Maharat Nakhon Ratchasima hospital is the largest hospital in Nakhon Ratchasima province, Northeast of Thailand. The problem of drug-resistant bacteria show high level in many sections of Maharat Nakhon Ratchasima hospital such as 90% in surgical intensive care unit (ICU), 80% in Pediatric ICU and Premature newborn (Maharat Nakhon Ratchasima Hospital, 2012).

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MRSA, *Escherichia coli* (*E. coli*), *Staphylococcus epidermidis*, *Enterobacter cloacae* and *Enterococcus faecium* are the most common organisms causing infections, MRSA is a major cause of the urinary tract infection (UTI), surgical

wounds, skin respiratory, and gastrointestinal tract infection (Isogai et al., 2001; Sundaram et al., 1983; Wang et al., 2003). *E. coli* are the predominant causative organisms of UTI, childhood enteritis, bacteria-related traveler's diarrhea and nosocomial infection in newborn (Thammasirirak et al., 2006). In the same way, the drugs-resistant bacteria have further complicated treatment in immunocompromised, acquired immune deficiency syndrome (AIDS) and cancer patients, especially in the case of nosocomial infections. The multi-drugs resistant bacteria have increased till now. Therefore, the infectious treatment costs are definitely expensive and have been increasing. The need for alternative antimicrobial drugs for treatment of resistant bacteria are research objectives of far researching importance. One approach is to find out new chemical substances such as from plant or animal that can be used as drug or chemical substance for treatment these resistant strains.

Wild crocodiles encounter high rates of injury, however, these animals rarely exhibit outward bacterial infection wound. The substances against infection in crocodilians were reported in many recent studies. Some research showed that crocodile tissues (*Crocodylus niloticus*) from lung or adrenal gland can inhibit *Micrococcus luteus* (Shaharabany et al., 1999). Also, Merchant et al. (2003) reported that serum from American alligator (*Alligator mississippiensis*) had much broader spectrum of antibacterial activity against gram positive and gram negative bacteria than human serum. Alligator serum also had moderate antiviral activity against herpes simplex virus type 1 (HSV-1), human immunodeficiency virus type 1 (HIV-1), West Nile virus (WNV) (Merchant et al., 2004). These antibacterial activities are partially due to the presence of a complement facilitated humoral immune response analogous to that described in mammalian systems (Merchant and Britton, 2006; Merchant,

et al., 2005). Although there have been reported that high concentrate of alligator serum caused sheep red blood cell hemolysis but the hemolysis activity was inhibited with EDTA, salicydoxime, ammonium hydroxide, methylamine (Merchant et al., 2005; 2009; 2010; Verret et al., 2005). The peptides from Siamese crocodile (*Crocodylus siamensis*) exhibited the antibacterial activities against *Salmonella typhi*, *Klebsiella pneumoniae*, *Staphylococcus aureus Staphylococcus epidermidis*, *E. coli*, *Psudomonas aeroginosa* and *Vibrio cholera* (Preecharram et al., 2010; Thammasirirak and Daduang, 2004). Moreover, the alligator leukocytes exhibited substantial antimycotic activities against six of eight species of *Candida* and ten of twelve bacterial species. This result was expressed cationic peptides that were responsible for their antimicrobial properties (Kommanee et al., 2012; Merchant et al., 2006; Pata, 2009) However, no work has been investigated on the effect of antibacterial activity of serum from the Siamese crocodile *(Crocodylus siamensis)* on *β*-lactam resistant bacteria such as MRSA, *Staphylococcus epidermidis*, *E. coli*, *Enterobactor cloacae* (EnC), and *Enterococcus faecium*. The purpose of this thesis was to investigate antibacterial activity of plasma fractions from the Siamese crocodile (*Crocodylus siamensis*) when used alone and in combination with *β*-lactam antibiotics. Thus, the development of a new antibacterial agent is urgently required.

1.2 Research objectives

1.2.1 To test the effectiveness of antibacterial characteristic from plasma fractions of the Siamese crocodiles (*Crocodylus siamensis*) alone on drug resistant bacteria.

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1.2.2 To test the effectiveness of antibacterial characteristic from plasma fractions of the Siamese crocodiles (*Crocodylus siamensis*) in combination with antibiotics on drug resistant bacteria.

1.2.3 To investigate the primary mechanism of action of antibacterial characteristic from plasma fractions of the Siamese crocodiles (*Crocodylus siamensis*) on drug resistant bacteria when used singly and in combination with antibiotic drugs by morphology examination with transmission electron microscopy (TEM), electrophoresis methods, outer membrane, cytoplasmic membrane permeability methods and enzyme assay.

1.3 Research hypothesis

1.3.1 The plasma fractions of Siamese crocodiles *(Crocodylus siamensis)* can show antibacterial activity against drug resistant bacteria.

1.3.2 The plasma fractions of Siamese crocodiles *(Crocodylus siamensis)* in combination with antibiotic drugs can show antibacterial activity against drug resistant bacteria.

1.3.3 The primary mechanism of action of antibacterial characteristics from plasma fractions of the Siamese crocodiles *(Crocodylus siamensis)* on drug resistant bacteria may be elucidated by morphology examination with TEM, outer and inner membrane permeability, electrophoresis and enzyme assay.

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1.4 Scope and limitations of the study

1.4.1 Clinical isolates of MRSA, *E. cloacae*, *E. coli* and *S. epidermidis* were obtained from Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand.

1.4.2 Plasma fractions of the Siamese crocodiles *(Crocodylus siamensis)* were obtained from Sriracha Moda Farm, Sriracha, Chonburi, Thailand.

1.4.3 Ceftazidime, Cloxacillin and Cephalexin were obtained from Sigma.

1.4.4 Checkerboard assay of combinations that show the lowest FIC index was selected to do further investigations such as viable counts, transmission electronmicroscopy (TEM), outer and cytoplasmic membrane permeability, electrophoresis and enzyme assay.

1.5 Expected results

1.5.1 Providing additional scientific data on synergism antimicrobial activity between the combination of peptide from plasma fractions of the Siamese crocodiles *(Crocodylus siamensis)* and antibiotics on drug resistant bacteria.

1.5.2 Providing novel knowledge for further investigations, such as investigate mechanism of action of peptide from plasma fractions of the Siamese crocodiles *(Crocodylus siamensis)* on drug resistant bacteria.

1.5.3 The results may useful for development of new drugs combination against resistant bacteria.

1.5.4 Providing benefit to physician and patient in case of tackle most dangerous resistant bacteria by using new antibacterial combination drugs.

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CHAPTER II

LITERATURE REVIEW

2.1 Overview of crocodiles

Crocodiles are large aquatic reptiles that live throughout the tropics in Africa, Asia, Americas and Australia. Crocodiles tend to congregate in freshwater habitats like rivers, lakes, wetlands, and sometimes in brackish water. They feed mostly on vertebrates like fish, reptiles, and mammals, sometimes on invertebrates like mollusks and crustaceans, depending on species. Scientific taxonomy of crocodile is presented on Table 2.1 (Teuber and Bader, 1976).

Table 2.1 Scientific classification of crocodile and alligator.

Classification	Crocodile/Alligator
Kingdom	Animalia
Phylum	Chordata
Class	Sauropsida
Order	Crocodilia
Family	Crocodylidae

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2.1.1 Classification of living crocodilians

There are 23 recognized species of extant crocodilians, divided into three Families-Alligatoridae (8 species; alligators and caimans), Crocodylidae (14 species; "true" crocodiles and Tomistoma) and Gavialidae (1 species; gharial).

Table 2.2 List of the species of crocodilians (King and Burke, 1989).

Taxon	Species
Class Reptilia	
Order Crocodilia	
Family Alligatoridae (alligators, caimans)	
American Alligator	*Alligator mississippiensis*
Black Caiman	*Melanosuchus niger*
Broad-snouted Caiman	*Caiman latirostris*
Chinese Alligator	*Alligator sinensis*
Spectacled Caiman	*Caiman crocodilus*
Yacare Caiman	*Caiman yacare*
Cuvier's Dwarf Caiman	*Paleosuchus palpebrosus*
Schneider's Smooth-fronted Caiman	*Paleosuchus trigonatus*

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Table 2.2 List of the species of crocodilians (King and Burke, 1989) (Continued).

Classification	Scientific name
Class Reptilia	
Order Crocodilia	
Family Crocodylidae	
Subfamily Crocodylinae ("True" crocodiles)	
African Dwarf Crocodile	*Osteolaemus tetraspis*
African Slender-snouted Crocodile	*Crocodylus cataphractus*
American Crocodile	*Crocodylus acutus*
Australian Freshwater Crocodile	*Crocodylus johnstoni*
Cuban Crocodile	*Crocodylus rhombifer*
Morelet's Crocodile	*Crocodylus moreletii*
Mugger Crocodile	*Crocodylus palustris*
New Guinea Freshwater Crocodile	*Crocodylus novaeguineae*
Nile Crocodile	*Crocodylus niloticus*
Orinoco Crocodile	*Crocodylus intermedius*
Philippine Crocodile	*Crocodylus mindorensis*
Saltwater Crocodile	*Crocodylus porosus*
Siamese Crocodile	*Crocodylus siamensis*
Subfamily Tomistominae	
False Gharial	*Tomistoma schlegelii*
Family Gavialidae (gharial)	
Indian Gharial	*Gavialis gangeticus*

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2.1.2 Siamese crocodile

The Siamese crocodile *(Crocodilus siamensis)* is a freshwater crocodile native to Indonesia (Borneo and possibly Java), Brunei, East Malaysia, Laos, Cambodia, Burma, Thailand, and Vietnam. The species is critically endangered and already extirpated from many regions. In the wild, they prefer slow moving waters like swamps, rivers, and some lakes. Most adults do not exceed 3 meters (10 feet) in length, although there are hybrids in captivity that can grow much larger. Due to excessive hunting and habitat loss, this crocodile is a critically endangered species. The total wild population is estimated to be less than 5,000 individuals. A number of captively held individuals are the result of hybridization with the saltwater crocodile, but several thousand "pure" individuals do exist in captivity and it is regularly bred at crocodile farms, especially in Thailand.

There are three types of crocodile in Thailand, but only two species of crocodile that usually farm raised culture in Thailand: Freshwater or Siamese crocodile *(Crocodylus siamensis)* (Figure 2.1) and Saltwater or Estuarine crocodile *(Crocodylus porosus)* (Figure 2.2).

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Figure 2.1 Freshwater crocodiles.

Source: Sriracha Moda Farm , Sriracha, Chonburi, Thailand

Figure 2.2 Saltwater crocodiles.

Source: (http://www.itsnature.org)

2.1.3 Hematological and chemistry values of Siamese crocodile serum

The hematological values are red blood cell counts 0.36-2.20x10^6 cell/mm^3, hematocrit 15.0-29.0%, hemoglobin 3.9-14.7 g/dL, white blood cell counts 2.5-32.0x10^3 cell/mm^3, thrombocyte counts 40.0-73.5x10^3 cell/mm^3, thrombocytes/WBC 100 cells were 52-425 cells, differential leukocyte cells counts : Heterophil 50-86%, lymphocyte 9-43%, monocyte 0-8%, basophil 0-3%, The size of erythrocyte cells length are as follows : 12.35-20.90 µm, width 5.70-11.40 µm, nucleus length 3.80-6.65 µm, width 2.85-5.70 µm. The diameter size of leukocyte heterophil is 10.45-15.20 µm, small lymphocyte 4.75-9.50 µm, large lymphocyte 8.55-14.73 µm, eosinophil 9.50-14.5 µm, monocyte 10.45-15.20 µm, basophil 9.03-12.83 µm, thrombocyte length 3.33-10.48 µm, width 2.85-7.60 µm. The biochemistry values are glucose 32.57-252.00 mg/L, uric acid 1.67-7.67 mg%, total protein 2.25-15.32 g%, albumin 1.04-2.02 g%, globulin 0.67-5.43 g%, cholesterol 163.78-350.17 mg%, triglyceride 49.67-443.71 mg%, BUN 0.58-19.61 mg%, calcium 7.2-26.2 mg/dL and phosphorus 1.5-5.9 mg/dL (Homswat, 1996).

2.1.4 The protein profiles of Siamese crocodile blood (*Crocodylus siamensis*)

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Threenet et al. (2011) studied protein profiles of Siamese crocodile blood by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). They found that protein profiles of crocodile blood were not difference in sexes, crocodile captive breeding locations and freeze-drying process. The whole blood revealed 7 bands of proteins at molecular weight of 119, 91, 67, 62, 59, 45 and 25 kDa, respectively. The serum protein represented six bands with molecule weight of 225, 121, 67, 62, 45 and 25 kDa, respectively. The blood cell fractions showed

2 bands of protein at molecular weight of 45 and 25 kDa, respectively. The highest concentration and broad band protein was albumin at 67-kDa in molecular weight.

2.1.5 Antimicrobial properties of crocodile blood

2.1.5.1 History

In 1998, Britton, a Darwin-based crocodile scientist, tested a hypothesis about crocodile blood that had major repercussion around the world. He observed that crocodiles could suffer horrific injuries while fighting, but that those injuries never seemed to become infected even in filthy water. Working with an American colleague, Diamond, they discovered that crocodiles have an extremely powerful immune system that capable of tackling even antibiotic-resistant like MRSA, and that it may have major medical implication for human (Britton et al., 2002).

2.1.5.2 Overview of reptile immunology

The immune system of vertebrates is generally divided into innate immunity and acquired immunity. The most important information on reptile immunity is summarized and mentioned below;

2.1.5.2.1 Innate immunity

Innate immunity is the first action in response to foreign organisms due to any additional activations do not require. This first defense mechanism is comprised of phagocytic cells that can process the antigen, and nonspecific effector molecules, such as the complement system, lysozyme, defensin and antimicrobial peptides, which can interact and neutralize foreign organisms directly (Brown, 2002). In response to different pathogens, the elements of innate defense mechanisms do not change themselves. Innate defense mechanism may have either a passive role, which played by natural surface barriers such as skin and

mucosal surface or an active role, which played by nonspecific humoral and cellular factors. The reptiles skin are thick keratin layer, which protect them from microbiological attack. In addition, The ability to replace new skin is the defense mechanism to prevent them from microorganism which using skin as a channel to enter the reptiles body. Reptilian mucosal are lack of the outer keratin layer, which make this surface more delicate and vulnerable to protect them from microorganism. Furthermore, Secretion, body fluid and mucosal surface are non adaptive immunity system against microorganism by neutralize invading pathogens and some are activated when detect the foreign organisms, such as **interferons,** which are a family of cytokines (type I and type II interferon) that play a dominant role as antiviral activity), In addition, transferrins, and together with albumin are found approximately the 95% of the small-molecular weight proteins in plasma of reptiles, extending from 70 and 90 kDa, lysozyme is produced by the monocyte/marcrophage cell line. It degrade peptidoglycan in bacterial cell wall. Apart from these**,** the reptiles complement system is comprise of multiple isoforms of C3 reptilian complement system plays an important role in response to or fighting invading microorganisms. (Origgi, 2007). There are the difference in innate immunity of the three families (Alligatoroidea, Crocodyloidea, and Gavialoidea) of Crocodylia by their species, The species within Alligatoroidea and Crocodyloidea exhibited remarkable immune activity similarities to others in their own family. In addition, The same genus are immune similarities to each other than the other within the same family. (Merchant et al., 2006). However, the immune system of crocodilians has not well characterized, but the innate immunity, especially complement system is thought to combat bacteria, viruses and amoeba (Merchant, 2003; 2005; Kommanee, 2012).

2.1.5.2.2 Adaptive immunity

Many organisms are neutralized and eliminated by innate immunity which act to limit the spread of infections. But, more virulent microorganisms can be able to escape its control, adaptive immunity becomes activated and a series of complex cell-to-cell interactions occurs to defense specific pathogens (Origgi, 2007; Zimmerman, 2010). Adaptive immunity are widely divided into cell-mediated immunity and humoral immunity. Cell-mediated immunity; cell-mediated immune response involves a class of lymphocytes theoretically known as T cells. These effector cells can target and kill infected cell through specific signals that expose on their membranes, recognizing the presence of invasive pathogen in host cells using their specific T-cell receptor by engaging the specific major histocompatibility (MHC) molecules, especially MHC class I for T-cells. MHCs play a critical role in presenting the specific portion of invading microorganisms. Humoral immunity; humoral immune response involves another class of lymphocytes, B cells, which can differentiate to plasma cells to produce antibodies (immunoglobulins) when stimulate by antigen. There are 5 distinct classes of immunoglobulins in mammals, including IgG, IgA, IgM, IgD, and IgE (Origgi, 2007). As previously report, reptilian immunoglobulins have not been well characterized and much of limited information available, suggested there are at least two classaes, IgM and IgY (Zimmerman, 2010). Recently, classes of immunoglobulins of crocodilian American alligator and saltwater crocodile have been identified that comprised of 4 different classes, IgM, IgD, IgA and IgY (Magadán-Mompó, 2013).

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2.1.5.3 Evidence of antimicrobial activity from crocodile blood

Merchant et al. (2003) found that the antibacterial spectrum of alligator serum was shown to be much broader than that of human serum. In addition, Siruntawineti et al. (2003) found that the complement activity in crocodile serum was effective against gram negative bacteria. Besides, Merchant et al. (2004) found that the alligator leukocyte extracts exhibited antimycotic, antiviral and antibacterial activity. Moreover, Merchant et al. (2005a) found that the alligator serum exhibited potent anti HIV virus, HSV virus and West Nile virus. Also, Merchant et al. (2005b) found that the alternative pathway was primarily response for antibacterial activity.

In addition, Leelawongtawon et al. (2010) studied that fresh crocodile serum and freeze dried serum were showed the inhibited activities with gram negative bacteria including *E. auroginosa*, *E. coli*, *K. pneuniae*, *S. tpimurium* and *P. aeruginosa*. Furthermore, Preecharram et al. (2010) reported that the spectrum of antibacterial activity of Crocosin VI., that was purified from crocodile plasma by membrane filter and reverse phase high performance liquid chromatography (RP-HPLC). It potentially inhibited growth of *Salmonella typhi* and *Staphylococcus aureus*. Apart from this, Pata et al. (2007) found that the leukocyte extracts from crocodile *(Crocodylus siamensis)* showed antibacterial and antifungal activity. As well as, Preecharram et al. (2008) and Thammasirirak and Daduang (2004) showed that the peptides from Siamese crocodile (*Crocodile siamensis*) exhibited antibacterial activity against *Salmonella typhi*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *E. coli*, *Pseudomonas*

aeroginosa, and *Vibrio cholerae*. These results were thought that the peptides at MW ranged from 5-75 kDa had antibacterial activity (Thammasirirak and Daduang, 2004; Preecharram et al., 2008)

2.2 Overview of microorganisms

2.2.1 Bacterial structure

Bacteria are divided into gram-positive and gram-negative organisms, depending on their staining characteristics with certain vital chemicals. Both types of organisms possess a cytoplasmic (or plasma) membrane, which contains the cytoplasm and serves as a permeability barrier across which a limited range of solutes actively are transported. Directly embedded in the cytoplasmic membrane are a series of transpeptidase and carboxypeptidase enzymes, which function to crosslink peptidoglycan precursors in the synthesis of the cell wall. These enzymes serve as the target for all β-lactam antimicrobials and, hence, are known as “penicillin-binding proteins” (PBPs). Inactivation of these enzymes leads to cessation of cell wall synthesis and, ultimately, cell death. In gram-positive bacteria, this plasma membrane is surrounded only by a mechanically rigid and rather porous cell wall made of peptidoglycan (Figure 2.3).

There is no barrier for diffusion in gram-positive bacteria; β-lactamases are released into the surrounding environment, requiring high concentrations or very high potency of the enzymes to be effective. In contrast, the cell envelope of gram-negative bacteria is much more complex, consisting of the innermost cytoplasmic membrane, the periplasm, the peptidoglycan layer, the outer membrane and in many cases, additional appendages, such as capsules, extracellular polysaccharides,

fimbriae, and flagella. In enteric bacteria, such as *E. coli*, this outer membrane is a very effective barrier that protects the organism from a number of potentially harmful

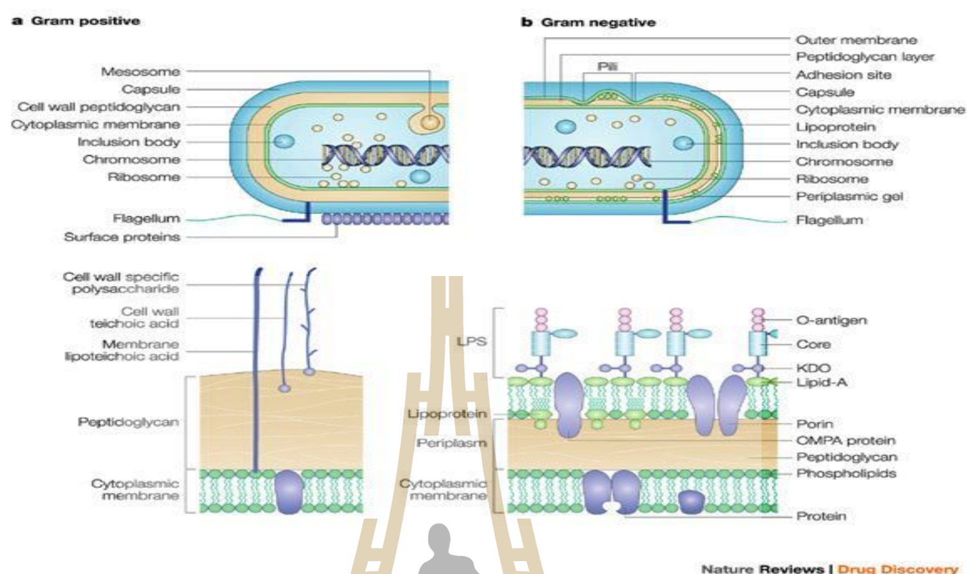

Figure 2.3 The cell membrane of Gram-positive (a) and Gram-negative (b) bacteria.

Source: http://www.nature.com/nrd/journal/v2/n8/fig_tab/nrd1153_F1.html

compounds, including antibiotics, disinfectants and detergents such as bile salts (Nikaido, 1985). The effectiveness of such a barrier depends, at least in the case of enteric bacteria, on the unusually low permeability of the lipid bilayer region of the outer membrane to hydrophobic solutes. This property is thought to be a result of the asymmetric construction of the bilayer, whose outer leaflet contains only the lipopolysaccharide (LPS) molecules. In contrast, to classical membrane phospholipid membranes, the LPS in the outer leaflet of the outer membrane contains multiple highly saturated fatty acid chains attached to each head group in the LPS molecule with strong covalent bonds between them. Such an arrangement decreases the fluidity, of the interior of the LPS and increases the hydrophobic properties of the inner leaflet of the LPS. Hydrophobic molecules have been shown to partition poorly into the

hydrophobic portion of LPS and to permeate across the outer membrane bilayer at about one fiftieth to one hundredth the rate through the usual phospholipid bilayer (Nikaido, 1988). This provides a fairly rigid and impermeable barrier to hydrophobic molecules. Most clinically important antibiotics show some hydrophobicity, which allows them to diffuse across most lipid bilayer. However, the LPS-containing asymmetric bilayer in gram-negative bacteria serves as an efficient barrier against the rapid penetration by such antibiotics and chemotherapeutic agents (Nikaido, 1994).

2.2.2 *β*-Lactamases and Transpeptidases

The former enzymes are responsible for resistance to *β*-lactam antibiotics since they catalyze the hydrolytic opening of the lactam ring, often very efficiently (Figure 2.4).

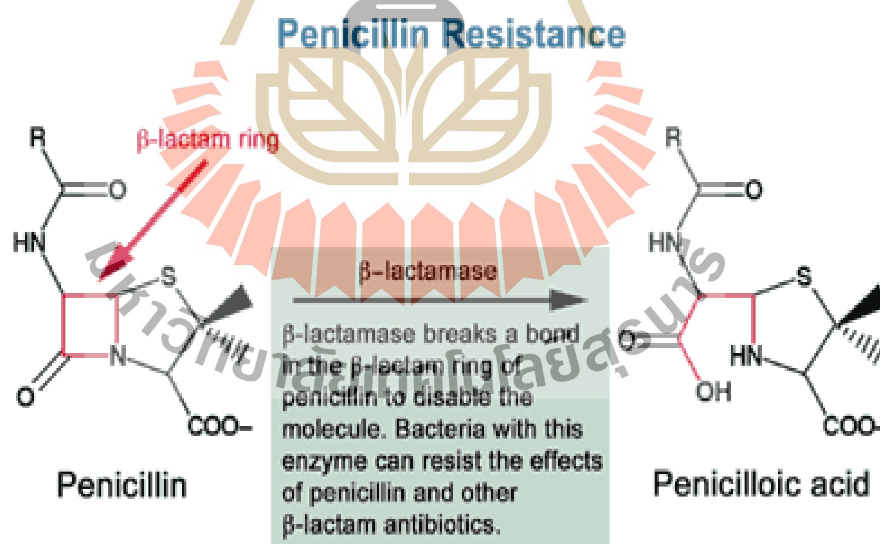

Figure 2.4 *β*-lactamase break down *β*–lactam ring of antibiotic.

Source: http://www.wiley.com/college/pratt/0471393878/student/activities/bacterial_drug_resistance/index.html

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Transpeptidase is a bacterial enzyme that cross-links the peptidoglycan chains to form rigid cell walls. This enzyme is also known by several other names including DD-peptidase, DD-transpeptidase, D-alanyl-D-alanine carboxypeptidase and serine-type D-Ala-D-Ala carboxypeptidase. The protein transpeptidase is necessary for the final step of peptidoglycan formation, and is inhibited by penicillin. The antibiotic penicillin irreversibly binds to and inhibits the activity of the transpeptidase enzyme by forming a highly stable penicilloyl-enzyme intermediate. Because of the interaction between penicillin and transpeptidase, this enzyme is also known as "penicillin-binding protein" (Pratt and Govardhan, 1984) (Figure 2.5).

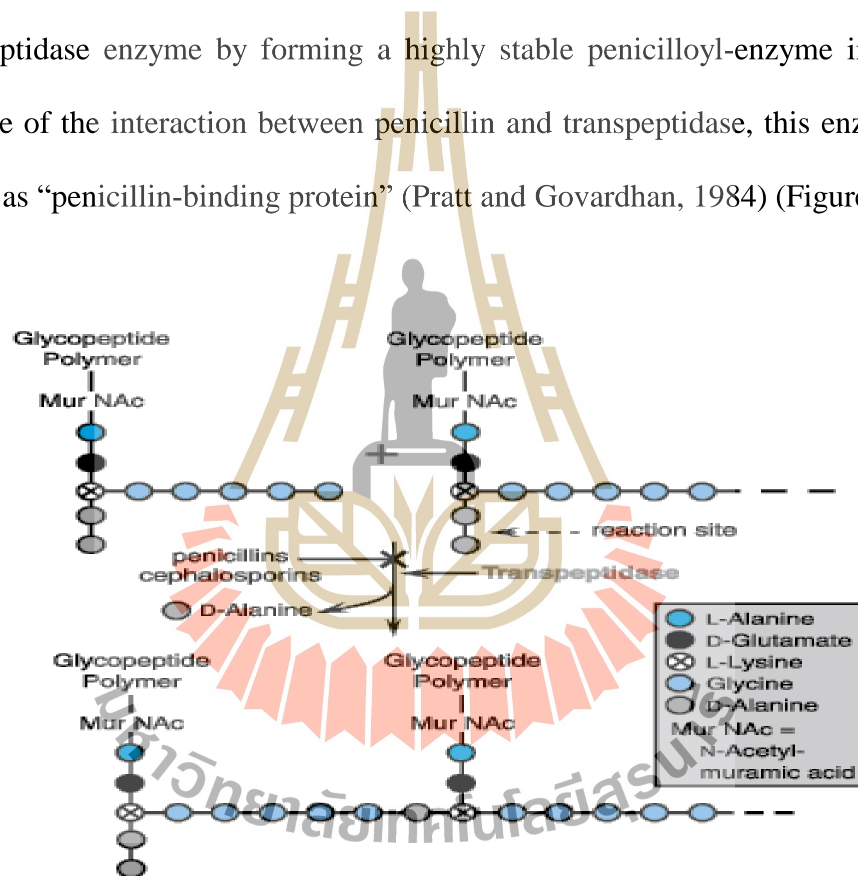


Figure 2.5 Reaction site to peptidoglycan synthesis (Brunton et al., 2011).

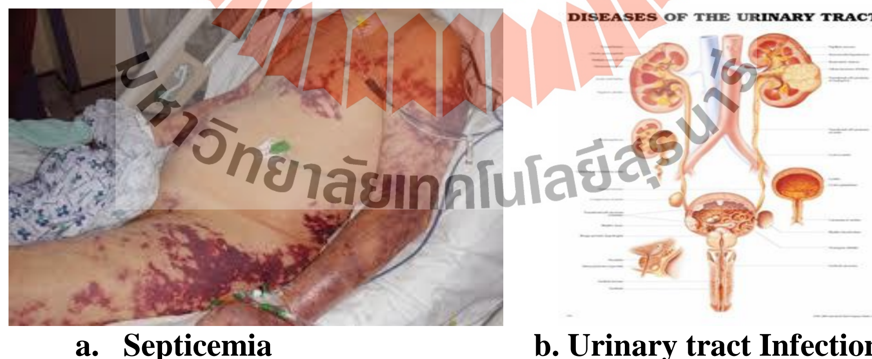
2.2.3 Bacterial types

2.2.3.1 *Enterobacter cloacae*

Family: Enterobacteriaceae

General characteristics

E. cloacae is a gram-negative, rod-shaped bacterium that has peritrichous flagella, measures 0.3-0.6 x 0.8-2.0 µm, is oxidase-negative, catalase-positive, and is facultative anaerobic. An *E. cloacae* is an important nosocomial pathogen whose incidence in hospitals appears to be rising. *E. cloacae* significantly cause the infections in immunocompromised or otherwise debilitated patient. This species is an opportunistic pathogen causing burn, wound and urinary tract infections and occasionally septicemia and meningitis (Shimeld and Rodgers, 1999) (Figure 2.6a and 2.6b). Introduction of piperacillin in hospital provided the emergence of *E. cloacae* strains resistant to this antibiotic (Jang and Nishijima, 1990; Namavar et al., 1997).



a. Septicemia **b. Urinary tract Infection**

Figure 2.6 Disease from *E. cloacae* infection.

Source: a. https://online.epocrates.comu2911245SepsisSummaryHighlights

b. http://www.papermasters.com/images/urinary-tract-disease.jpg

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Clinical significant

The degree to which cell-wall-deficient bacteria (CWDB) are involved in the generation of β-lactamase derepressed mutants (DM) was measured using *E. cloacae*. The chromosome of *E. cloacae* encodes an ampC gene that is regulated by an ampR and an ampD regions. Mutations in the ampD region result in "derepressed" mutants (DM) that constitutively produce high levels of β-lactamase. Due to high levels of β-lactamase, DM becomes resistant to most β-lactam antibiotics. Most wild-type to β-lactam resistant mutants in *Enterobacter* occur as a result of mutation in the genes that regulate the amount of enzyme produced (Huber, 2002).

2.2.3.2 *Staphylococcus aureus*

Family: Staphylococcaceae

General characteristics

S. aureus are gram positive cocci bacteria which have long been recognized as one of the most important agent of food poisoning worldwide. The primary habitat of *S. aureus* is the mucous membranes of the human nasopharynx and animal skin (Genigeorgis, 1989). The micro-organism can exist as a persistent or a transient member of the normal flora without causing any symptoms in humans. The presence of *S. aureus* in foods is often related to improper manipulation by personnel, who are frequently contaminated with these micro-organisms (Hatakka et al., 2000). *Staphylococcus aureus* causes a variety of suppurative (pus-forming) infections and toxinoses in humans. It causes superficial skin lesions such as boils, styesand furuncules; more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis. *S. aureus* is a major cause of hospital acquired

(nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* causes food poisoning by releasing enterotoxins into food, and toxic shock syndrome by release of superantigens into the blood stream (http://textbookofbacteriology.net/staph_2.html) (Figure 2.7).

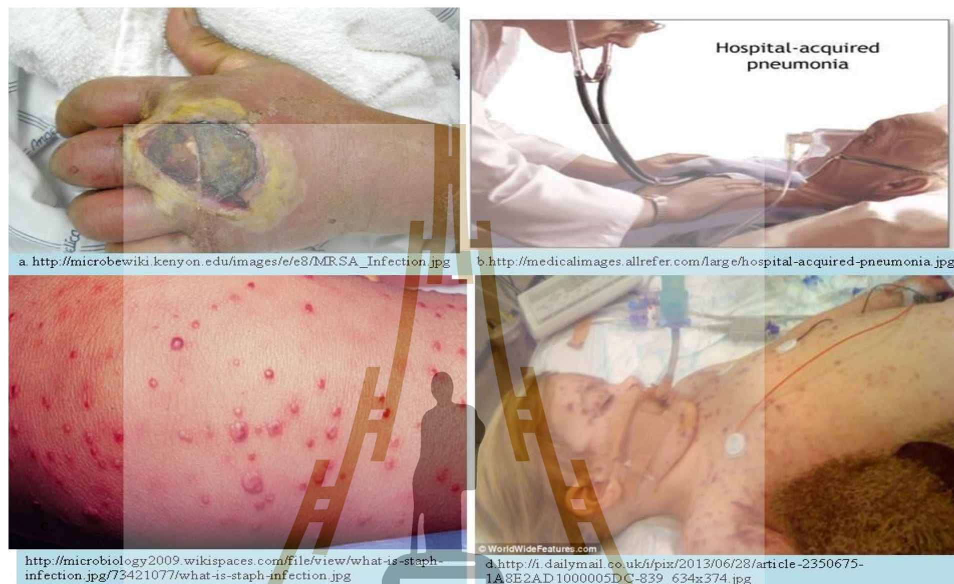

Figure 2.7 Disease from *S. aureus* infection.

Source: a. http://microbewiki.kenyon.edu/images/e/e8/MRSA_Infection.jpg

b. http://medicalimages.allrefer.com/large/hospital-acquired-pneumonia.jpg

c. http://microbiology2009.widispaces.com/file/view/what-is-staph-infection jpg/73421077/what-is-staph-infection.jpg

d. http://i.dailymail.co.uk/i/pix/2013/06/28/article-2350675-1A8E2AD100 0005DC-839_634x374.jpg

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Clinical significant

When the majority of nosocomial *Staphylococcal* isolates were already penicillin-resistant, the introduction of semi-synthetic β-lactamase resistant

penicillins was a major therapeutically breakthrough (Voss and Doebbeling, 1995). Methicillin resi.5stance in *S. aureus* mediated by the *mec*A gene encoding a penicillin binding protein with reduced affinity to β-lactam antibiotics (Hiramatsu et al., 2001).

Treatment of *S. aureus* infections has been complicated by the persistent rise in rates of *S. aureus* isolates with methicillin resistance. MRSA was first described in 1961 and has since become endemic in many hospitals. In some institutions, MRSA accounts for more than 50% of all *S. aureus* infections (Pfaller et al., 1999).

Many clinicians believe that vancomycin is inferior to that of the β-lactams and this belief stems from *in vitro* data that demonstrates the slower bactericidal activity of vancomycin compared with β-lactams against *S. aureus* (Cantoni et al., 1990). Some clinical evidence suggested that glycopeptides are inferior to β-lactam antibiotics as therapy for serious *Staphylococcal* infections (Hartstein et al., 1992).

2.2.3.3 *Staphylococcus epidermidis*

Family: Staphylococcaceae

General characteristics

S. epidermidis is primarily a normal inhabitant of the healthy human skin and mucosal microflora and has emerged as a common cause of numerous nosocomial infections. *S. epidermidis* as one of the most often isolated bacterial pathogens in hospitals in general and as the most important pathogen involved in nosocomial bloodstream infections, cardiovascular infections, and infections of the eye, ear, nose, and throat. *S. epidermidis* very often becomes the major infective agent in compromised patients, such as drug abusers and immuno-

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compromised patients (patients under immunosuppressive therapy, AIDS patients, and premature newborns). The port of entry into the human body in all of these infections is usually an intravascular catheter (Lim and Webb, 2005).

Clinical significant

S. epidermidis was isolated from device-associated infections are resistant to methicillin. Nosocomial *S. epidermidis* isolated are characterized by their pronounced resistance against many of today's commonly used antibiotics including methicillin. The *mecA* gene and its regulators are located on large DNA elements that are termed *staphylococcal* cassette chromosome *mec* (SCC*mec*). In addition, the methicillin resistance determinant, SCC*mec* carry a set of recombinases and a wide variety of mobile DNA elements such as transposons, insertion sequences or integrated plasmids (Kozitskaya et al., 2004). To date, five major SCC*mec* types have been identified, ranging in size from 21-67 KDa 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* have been shown to be transferable among *Staphylococcal* species. Genome sequencing of the methicillin-resistant *S. epidermidis* RP62A revealed recently the presence of a SCC*mec* type II cassette (Hanssen et al., 2004). Antibiotic resistance and the ability of many nosocomial *S. epidermidis* isolate to form biofilms on inert surfaces made these infections hard to treat (Ziebuhr et al., 2006).

2.2.3.4 *Escherichia coli*

Family: Enterobacteriaceae

General characteristics

The bacterium *E. coli* is a facultatively anaerobic, Gram negative short rod shape that is one of the best and most thoroughly studied free-

living organisms. It is also a remarkably diverse species because some *E. coli* strains live as harmless commensals in animal intestines, whereas other distinct genotypes including the enteropathogenic, enterohemorrhagic, enteroinvasive, enterotoxigenic, and enteroaggregative *E. coli* causes significant morbidity and mortality as human intestinal pathogens. *E. coli* can cause diarrhea, urinary tract infections, respiratory illness, bloodstream infections, and other illnesses. Other kinds of *E. coli* are used as markers for water contamination. Extraintestinal *E. coli* are another varied group of life-threatening pathogens of this manifestly versatile species (Hooton and Stamm, 1997) (Figure 2.8).

a. ICU Maharat Nakhonratchasima Hospital, 2013
b. http://media.kimatv.com/images/660*491/Brody+Cropped.png
c.http://en.ria.ru/infographics/20110603/164419834.html
d. http://www.featurepics.com/FI/Thumb300/20090428/Foley-Bag-1166380.jpg

Figure 2.8 Disease from *E.coli* infection.

Source: a. ICU Maharat Nakhonratchasima Hospital, 2013

b. http://media.kimatv.com/images/660*491/Brody+Cropped.png

c. http://en.ria.ru/infographics/20110603/164419834.html

d. http://www.featurepics.com/FI/Thumb300/20090428/Foley-Bag-1166380.jpg

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Clinical significant

The emerging resistance to fluoroquinolones and the production of extended-spectrum β-lactamases (ESBL) by multidrug resistant *E. coli* strains has caused increasing concern over the last decade due to the limited therapeutic options if infections with these strains occur (Garau et al., 1999).

2.3 Overview of antibiotics

2.3.1 Overview

The word antibiotic comes from the Greek *anti* meaning "against" and *bios* meaning "life" (a bacterium is a life form). Antibiotics are also known as antibacterials, and they are drugs used to treat infections caused by bacteria. The first antibiotic was penicillin. Such penicillin-related antibiotics as ampicillin, amoxicillin and benzylpenicilllin are widely used today to treat a variety of infections-these antibiotics have been around for a long time. There are several different types of modern antibiotics and they are only available with a doctor's prescription in industrialized countries. A broad-spectrum antibiotic can be used to treat a wide range of infections. A narrow-spectrum antibiotic is only effective against a few types of bacteria. There are antibiotics that attack aerobic bacteria, while others work against anaerobic bacteria. Aerobic bacteria need oxygen, while anaerobic bacteria do not. Antibiotics may have a killing effect or an inhibitory effect on a range of microbes. The range of bacteria or other microorganisms that is affected by a certain antibiotic is expressed as its spectrum of action. Antibiotics effective against prokaryotes that kill or inhibit a wide range of Gram-positive and Gram-negative bacteria are said to be broad spectrum. If effective mainly against Gram-positive or Gram-negative bacteria,

they are narrow spectrum. If effective against a single organism or disease, they are referred to as limited spectrum.

Most antimicrobial agents used for the treatment of bacterial infections may be categorized according to their principal mechanism of action. There are 5 major modes of action (Harvey et al., 2012; Tenover, 2006).

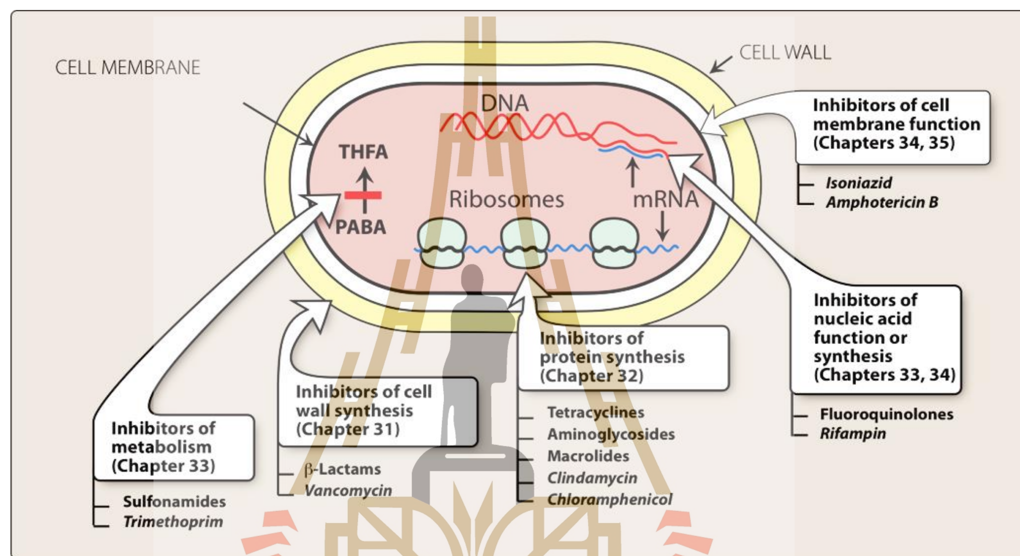

Figure 2.9 Mechanisms of antibiotic drugs (Harvey et al., 2012).

2.3.1.1 Cell wall synthesis inhibitors

Cell wall synthesis inhibitors generally inhibit some step in the synthesis of bacterial peptidoglycan and attack bacterial cell wall synthesis. Bacteria have murein in their cell walls and murein (peptidoglycan) is essential to the viability of the bacterial cell. They exert their selective toxicity against bacteria because human cells lack cell walls. Antibacterial drugs that work by inhibiting bacterial cell wall synthesis include the β-lactams, such as the penicillins, β-Lactam agents inhibit synthesis of the bacterial cell wall by interfering with the enzymes required for the

synthesis of the peptidoglycan layer (Figure 2.10) (Harvey et al., 2012; Tenover, 2006).

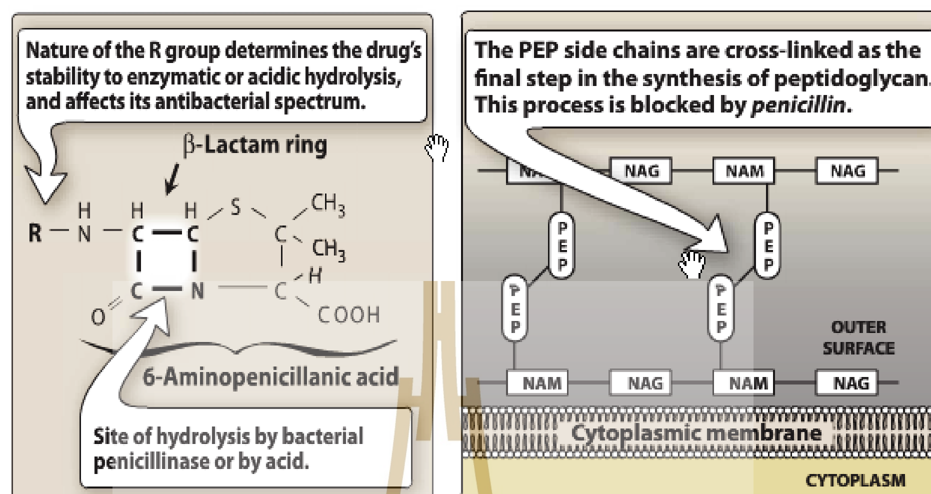

Figure 2.10 Peptidoglycan synthesis inhibitor mechanism of action (Harvey et al., 2012).

2.3.1.2 Inhibition of metabolic pathway

Sulfonamides and trimethoprim (TMP) block the pathway for folic acid synthesis, which ultimately inhibits DNA synthesis. The common antibacterial drug combination of TMP, a folic acid analogue, plus sulfamethoxazole (SMX) (a sulfonamide) inhibits 2 steps in the enzymatic pathway for bacterial folate synthesis (Harvey et al., 2012; Tenover, 2006).

2.3.1.3 Protein synthesis inhibitors

Bacterial ribosomes differ in structure from their counterparts in eukaryotic cells. Antibacterial agents take advantage of these differences to selectively inhibit bacterial growth. Macrolides, aminoglycosides, and tetracyclines bind to the 30S subunit of the ribosome, whereas chloramphenicol binds to the 50S subunit (Harvey et al., 2012; Tenover, 2006).

2.3.1.4 Effect on nucleic acids

Some antibiotics and chemotherapeutic agents affect the synthesis of DNA or RNA, or can bind to DNA or RNA so that their messages cannot be read. Fluoroquinolones exert their antibacterial effects by disrupting DNA synthesis and causing lethal double-strand DNA breaks during DNA replication (Harvey et al., 2012; Tenover, 2006).

2.3.1.5 Cell membrane function inhibitor

Cytoplasm of each living cell is bounded by cytoplasmic membrane, which serves as a selective permeability barrier and performs active transport functions. By performing such vital functions, cell membrane controls the internal composition of the cell. If the functional integrity of cell membrane is interrupted, macromolecules and ions escape from the interiors of the cells resulting in damage or death of the cell.

The chemotherapeutic agents and some natural antibacterial compounds are due to effect that cytoplasmic membrane of certain bacteria and fungi can be more readily disrupted by some agents than cell membrane of humans and animal cells. Example of this mechanism is that polymyxins are active against Gram-negative bacteria and Polyenes antibiotics (Amphotericin) are acting of fungi. However polymyxins are inactive against fungi and Polyenes are inactive against bacteria. This is because ergosterol is present in the cell membrane of fungi and absent in bacterial cell membrane. Polyenes (drugs acting against fungi) require ergosterol to be present in cell membrane to exert their effects that's why they are active against fungi and not active against bacteria (http://thepharmacistpharma.blogspot.com/2009/03/2-inhibition-of-cell-membrane-fucntion.html.).

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2.3.2 Beta-lactam antibiotics

2.3.2.1 Beta-lactam

β-lactam antibiotics are antibiotics widely used in clinical practice because of their high antibacterial activity. Basic structure of *β*-lactam antibiotics consist of five-membered thiazolidine ring fused to the *β*-lactam portion (Figure 2.11). Different types of antibiotics are determined side chain (R-group) (Tenover, 2006).

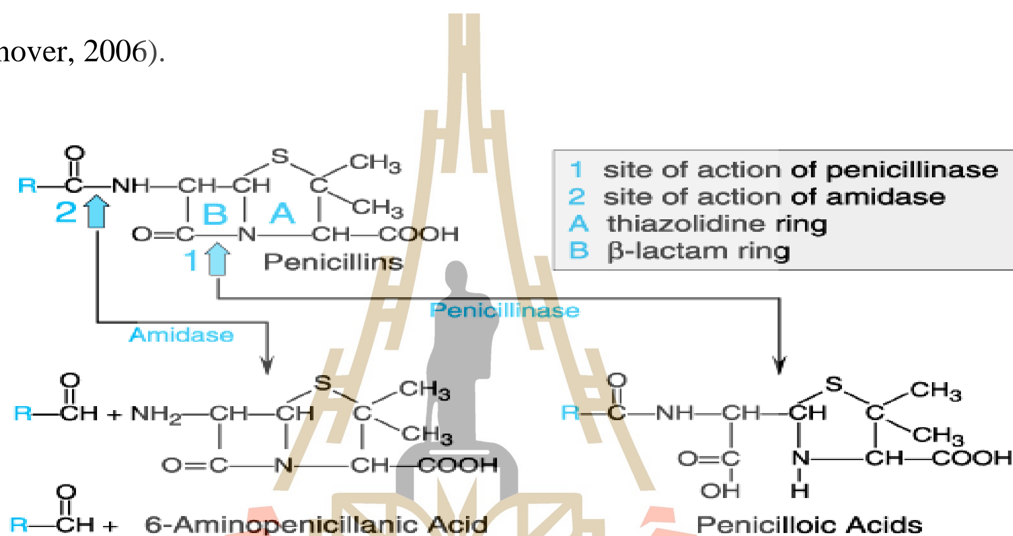

Figure 2.11 Common structure of penicillin and site of cleavage by penicillinase (Brunton et al., 2011).

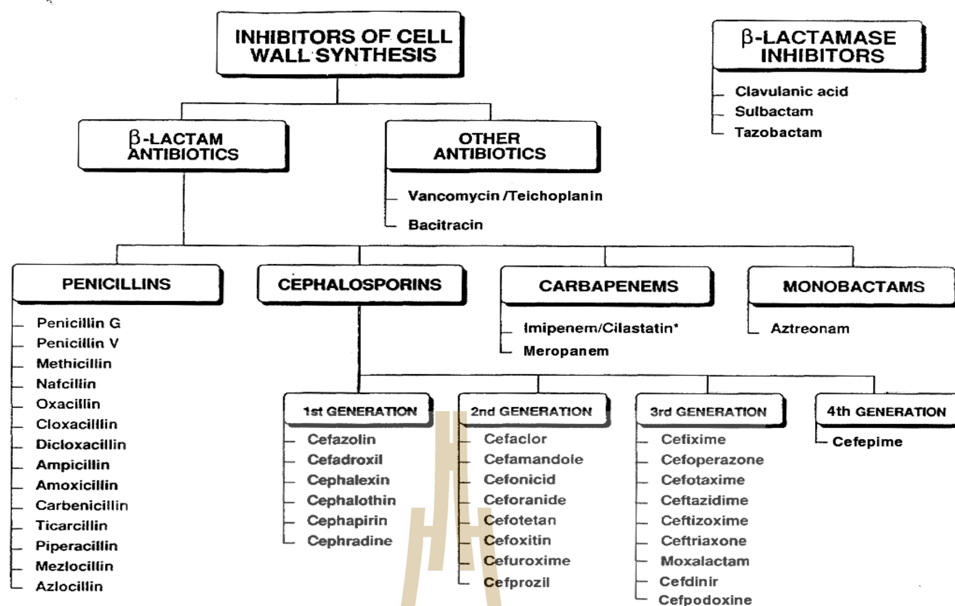

Figure 2.12 Summary of antimicrobial agents affecting cell wall synthesis (Harvey et al., 2012).

2.3.2.2 Beta-lactam compounds

2.3.2.2.1 Ceftazidime

Ceftazidime is a semisynthetic, broad-spectrum, β-lactam antibiotic for parenteral administration. Ceftazidime is bactericidal in action exerting its effect by inhibition of enzymes responsible for cell-wall synthesis. A wide range of Gram-negative organisms is susceptible to ceftazidime in vitro, including strains resistant to gentamicin and other aminoglycosides. In addition, ceftazidime has been shown to be active against Gram-positive organisms. It is highly stable to most clinically important β-lactamases, plasmid or chromosomal, which are produced by both Gram-negative and Gram-positive organisms and, consequently, is active against many strains resistant to ampicillin and other cephalosporins. Ceftazidime has activity against the gram-negative organisms *Pseudomonas* and Enterobacteriaceae. Its activity against *Pseudomonas* is a distinguishing feature of ceftazidime among the

cephalosporins. The bactericidal activity of ceftazidime results from the inhibition of cell wall synthesis via affinity for penicillin-binding proteins (PBPs) (Brunton et al., 2011).

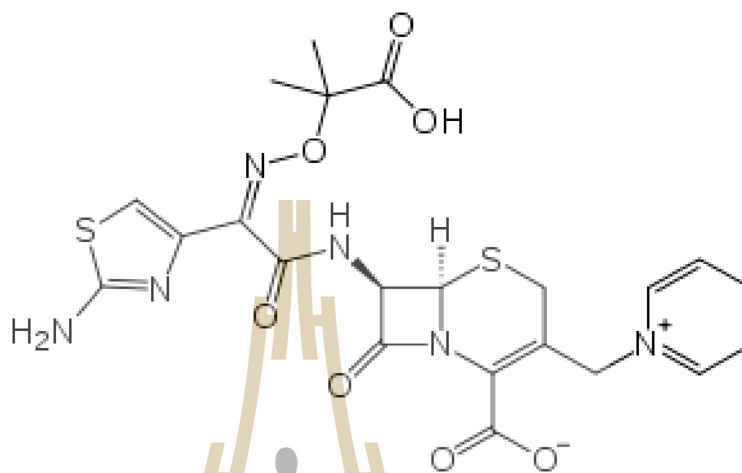

Figure 2.13 Common structure of Ceftazidime.

Source: http://www.theodora.com/drugs/images

2.3.2.2.2 Cloxacillin

Cloxacillin is a semisynthetic antibiotic in the same class as penicillin. Cloxacillin is for use against *Staphylococci* that produce *β*-lactamase. By binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall, cloxacillin inhibits the third and last stage of bacterial cell wall synthesis. Cell lysis is then mediated by bacterial cell wall autolytic enzymes such as autolysins; it is possible that cloxacillin interferes with an autolysin inhibitor (Brunton et al., 2011).

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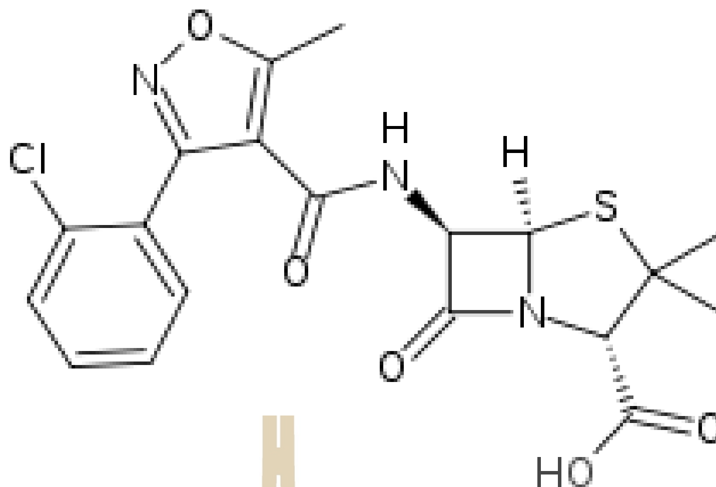

Figure 2.14 Common structure of Cloxacillin.

Source: http://www.onlinepharmacycatalog.com/category/common-drugs- and-medications/antibiotics/cloxacillin-cloxapen-orbenin/

2.3.2.2.3 Cefalexin

Cefalexin/Cephalexin is the first generation cephalosporin antibiotic. It is one of the most widely prescribed antibiotics, often used for the treatment of superficial infections that result as complications of minor wounds or lacerations. It is effective against most gram-positive bacteria.

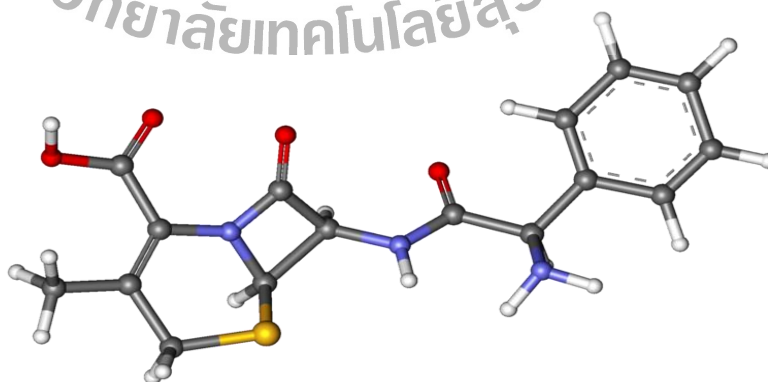

Figure 2.15 Structure of Cefalexin.

Source: http://en.wikipedia.org/wiki/Cefalexin

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2.3.3 Mechanism of antimicrobial resistant

Bacteria either have preexisting resistance to drugs, or they develop resistance. Human activity has contributed greatly to the increase in resistant strains of bacteria. Often, when bacteria acquire resistance to a certain drug from a particular class (e.g. the penicillin), the bacteria also acquire resistance to all other drugs in that class. Some of the many mechanisms of resistance are indicated schematically in the following diagram (Roe, 2008).

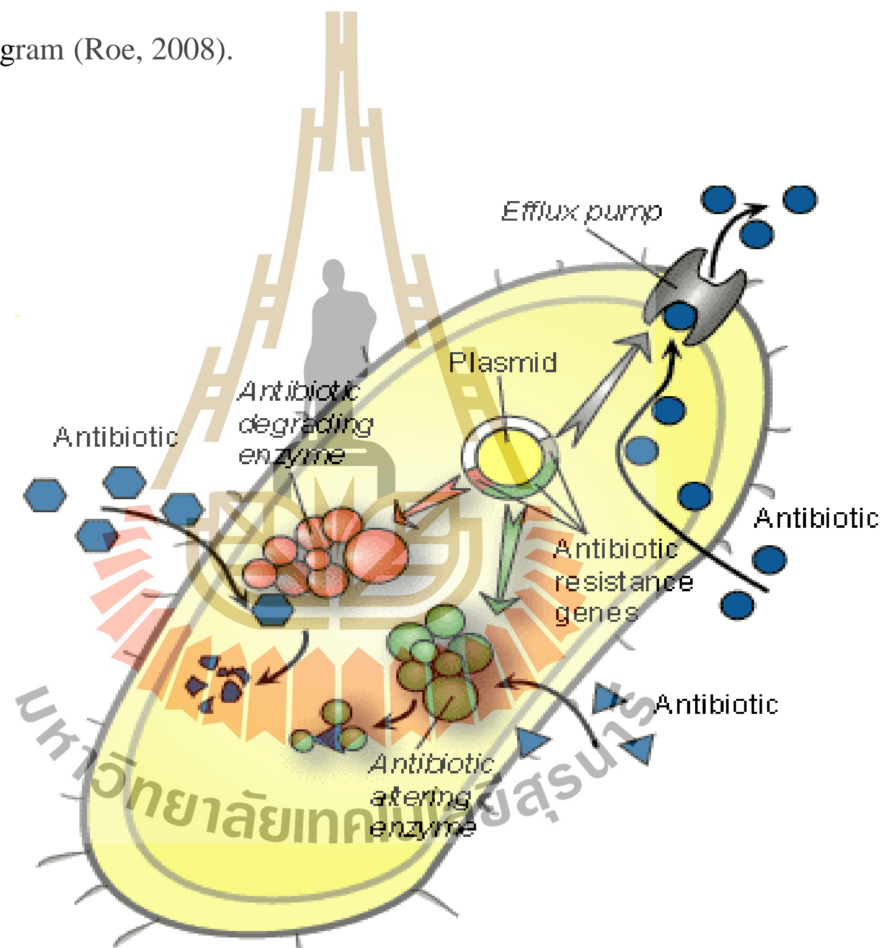

Figure 2.16 Mechanisms of antibiotic resistance (Roe, 2008).

2.3.3.1 Enzyme-based resistance

Bacterial resistance to antibiotics is most often mediated by the bacterial synthesis of β-lactamases enzyme. These are the main cause of bacterial

resistance to penicillins and cephalosporins. Definitive identification of these enzymes is only possible by gene or protein sequencing (Livermore and Brown, 2001).

2.3.3.2 Ribosomal modifications

The ribosome can be methylated so that an antibiotic cannot bind to it (Tenover, 2006).

2.3.3.3 Protein modifications

For antibiotics that target DNA gyrase, the enzyme that unwinds DNA for replication, random mutations in the bacterial DNA may alter the gyrase and make it unrecognizable to antibiotics while still leaving it functional (Tenover, 2006).

The penicillin-binding protein (PBPs) occurs in the bacterial cell wall and has an enzymatic role in the synthesis of peptidoglycan. PBPs normally possess a high affinity for *β*-lactam antibiotics, in MRSA this affinity is reduced resulting in antibiotic resistance. MRSA carry the mecA gene which encodes affinity penicillin binding protein, known as PBP2a (Cook, 1998).

2.3.3.4 Metabolic resistance

In the case of sulfonamides, which operate by mimicking PABA and competing for an enzyme that synthesizes folic acid, an increase in the amount of PABA can out complete the sulfonamide and render it ineffective; or an alteration in the code for the enzyme itself can prevent its sulfonamide binding (Tenover, 2006).

2.3.3.5 Effluxing the toxin

Antibiotic efflux pumps are nowadays believed to significantly contribute to acquire bacterial resistance because of the very broad variety of

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substrates they recognize, their expression in important pathogens, and their cooperation with other mechanisms of resistance. Their existence also explains many situations of apparent intrinsic resistance of specific organisms (Seral et al., 2003). For example, a strain of Enterococcal bacteria can pump out tetracycline. This type of pumping is called an "efflux phenomenon" (Tenover, 2006).

2.3.3.6 Acquired resistance

Bacteria also develop resistance through the acquisition of new genetic material from other resistant organisms. This is termed *horizontal evolution*, and may occur between strains of the same species or between different bacterial species or genera. Mechanisms of genetic exchange include conjugation, transduction, and transformation. Through genetic exchange mechanisms, many bacteria have become resistant to multiple classes of antibacterial agent (Roe, 2008; Tenover, 2006).

Table 2.3 How Bacteria Transfer Genetic Material (Roe, 2008).

How Bacteria Transfer Genetic Material	
Transduction	Viruses carry genetic material from one bacterium to another
Transformation	Bacteria engulf genetic material from dead bacteria in the environment
Conjugation (most common)	Two bacteria join cells and transfer genetic material via plasmids

2.3.3.6.1 Transduction

A virus (bacteriophage) serves as the agent of transfer DNA segment between bacterial strains (McManus, 1997). When a phage is

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being replicated inside a host cell, the new viruses self-assemble from proteins and viral nucleic acid (genetic material) that the host cell has produced. Sometimes some of the DNA of the host, which had been chopped up during the lytic replication process, gets inside a new virus during viral self-assembly. When that phage then infects another cell, the new host may incorporate the donated DNA into its chromosome by recombination (Bauman, 2005).

2.3.3.6.2 Transformation

This is the process in which a recipient cell takes up DNA from the environment (such as DNA released from a dead organism) (Port, 2008). After the new DNA is introduced via transformation it is incorporated DNA segments into the cell and results in the emergence of a new, resistant genotype (McManus, 1997).

2.3.3.6.3 Conjugation

Transmission of resistance genes via plasmid exchange. Bacteria have circles of DNA called plasmids that they can pass to other bacteria during conjugation. Plasmids is the key players in conjugation, are even referred to as resistance transfer factors. During conjugation, a gram-negative bacterium transfers plasmid-containing resistance genes to an adjacent bacterium, often via an elongated protein aqueous structure termed a *pilus*, which joins the 2 organisms. Conjugation among gram-positive bacteria is usually initiated by production of sex pheromones by the mating pair, which facilitate the clumping of donor and recipient organisms, allowing the exchange of DNA. This type of acquisition allows resistance to spread among a population of bacterial cells much faster than simple mutation and vertical evolution would permit (McManus, 1997).

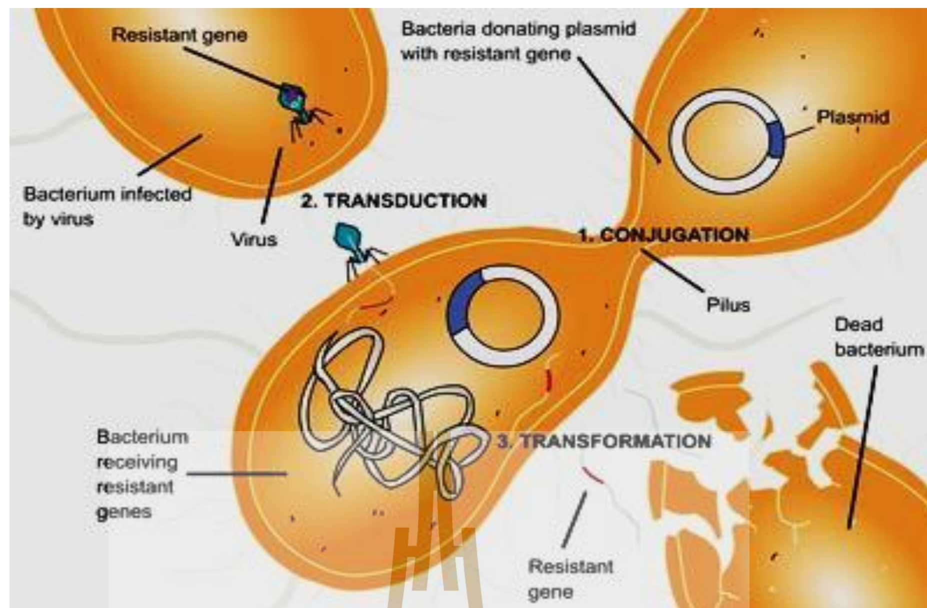

Figure 2.17 Bacteria transfer genetic material.

Source: http://www.wiley.com/college/

2.4 Laboratory methods used for antimicrobial susceptibility testing

The inhibitory activity of an antimicrobial agent is determined by dilution testing, which produces a quantitative result. The decision concerning which method to used is based on several factors including cost, ease of use, flexibility, and degree of automation (Swan and Manivannan, 2000).

2.4.1 Susceptibility test method

Dilution Testing: Broth dilution test

This procedure involved preparing two-fold dilutions of antibiotics (eg, 1, 2, 4, 8, and 16 μg/mL) in a liquid growth medium dispensed in test tubes. The antibiotic-containing tubes were inoculated with a standardized bacterial suspension of 1-5x10^5 CFU/mL. Following overnight incubation at 37°C, the tubes were examined for visible bacterial growth as evidenced by turbidity. The lowest

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concentration of antibiotic that prevented growth represented the minimal inhibitory concentration (MIC). The precision of this method was considered to be plus or minus 1 two-fold concentration, due in large part to the practice of manually preparing serial dilutions of the antibiotics. The advantage of this technique was the generation of a quantitative result (ie, the MIC). The principal disadvantages of the macrodilution method were the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test.

Dilution susceptibility tests determine the minimal concentration of an antimicrobial agent needed to inhibit growth of the microorganism being tested. For most dilution tests, antimicrobial agents are tested at log 2 (two-fold) serial dilutions. The lowest concentration at which there is no visible growth is called the minimal inhibitory concentration (MIC) (Swan and Manivannan, 2000).

2.4.2 Checkerboard results

When two antimicrobial agents act simultaneously on a homogeneous microbial population, the effect may be one of following.

1. No interaction; The combined action is equivalent to the sum of the actions of each drug when used alone (FIC index > 0.5-4.0).

2. Synergism; The combined action is significantly greater than the sum of both effects (FIC index ≤ 0.5).

3. Antagonism; The combined action is less than that of more effective agent when used alone (FIC index >4.0) (Johnson et al., 2004b; Odds, 2003a).

The most popular method used to detect antimicrobial interaction is checkerboard or chessboard titration, in which two drugs are cross-titrated against

each other (Sawan and Manivanna, 2000). After incubation, the isobologram is constructed by plotting the inhibition of growth observed at each drug concentration on an arithmetic scale. The line of additive joins the MICs of the individual drugs acting alone, a deviation of this line towards the axes of the graph suggests synergy; a deviation away from the axes is often taken to indicate antagonism, although indifference may also produce this result (Sawan and Manivannan, 2000).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Crocodile plasma

Blood of 40 Siamese crocodiles *(Crocodylus siamensis)*, both males and females, good health status, weighed approximately 25 kilograms, aged between 2-4 years old were collected from Sriracha Moda Farm, Sriracha, Chonburi, Thailand. The *Crocodylus siamensis* was authenticated by expertise veterinarian at Sriracha Moda Farm, Sriracha, Chonburi, Thailand. This farm was registered as commercial captive breeding institution with Convention on International Trade in Endangered Species (CITES) to breed *Crocodylus siamensis*. The experimental protocol used in this study was approved in according to guideline for the care and use of laboratory animal by animal care and use committee (ACUC), Suranaree University of Technology (The approved serial number 30/2553). The separation of crocodile plasma fractions were mentioned in this chapter.

3.1.2 Test organisms

3.1.2.1 Bacterial strains

Clinical isolates of ceftazidime-resistant *Enterobacter cloacae* DMST 21394 (CREnC), clinical isolates of Ceftazidime-Resistant *Escherichia coli* DMST 20662 (CREC), clinical isolates of *Escherichia coli* ATCC 25922 (*E. coli*), Clinical isolates of Methicillin-Resistant *Staphylococcus aureus* DMST 20651

(MRSA), Clinical isolates of *Staphylococcus aureus* ATCC 29213 (*S. aureus*), and *Staphylococcus epidermidis* (*S. epidermidis*) DMST 15505 were obtained from Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand and the American Type Culture Collection (ATCC), USA.

3.1.2.2 Preparation and maintenance of stock cultures

The clinical isolates of bacteria were inoculated on nutrient agar slopes and incubated overnight at 37°C. These cultures were stored in a refrigerator at 4°C. Fresh slope cultures were refreshed every 3-4 weeks (Eumkeb, 1999).

3.1.3 *β*-lactam antibiotics

Ceftazidime, Cloxacillin and Cefalexin were obtained from Sigma, Bristol-Myers.

3.1.4 Culture media

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

3.1.5 Chemicals

All chemicals used were laboratory grades and the detail were in the appendix.

3.1.6 Equipment

All equipments used were detailed in appendix.

3.2 Methods

3.2.1 Separated peptide from crocodile plasma

Crocodile blood was collected by drawing blood from paravertebral vein from anterior dorsal sinus (40 mL) and were transferred to EDTA tube and kept

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at 4ºC overnight and then centrifuged at 4000 rpm for 10 minutes to obtain the plasma and kept at -70ºC until tested.

Ion exchange chromatography: The plasma was diluted (1:3) in 25 mM Tris-HCl, pH 8.1 and filtrated with 0.45 µM membrane filter. Separation of plasma was performed by Econo-Column Chromatography 1x50 cm over Q Sepharose fast flow column previously equilibrated with 25 mM Tris-HCl, pH 8.1. Elution was achieved with a linear NaCl gradient in 25 mM Tris-HCl, pH 8.1, the salt was eliminated by dialysis membrane (pore 6, flat width 38 mm, diameter 24 mm) to obtain protein fractions, and the eluted fractions were monitored spectrophotometrically at 280 nm and determined the molecular weight by SDS-PAGE.

Gel Filtration Chromatography: The antibacterial fraction protein from ion exchange chromatography was determined molecular weight. The protein peak containing antibacterial activity was pooled and then lyophilized. The lyophilized sample was re-suspended in 1 mL water and applied to a Sephadex G-50 (Superfine, Amersham Bio-sciences, 2.5x100 cm) gel infiltration column equilibrated with 0.1% trifluoroacetic acid (TFA). Elution was performed with the 60% acetronitile in 0.1% TFA. Applied blue dextran and bromophenol blue for indicator to select separated fraction with small size of the elute and the absorbance was monitored at 280 nm. The salt was eliminated by dialysis membrane (pore 6, flat width 38 mm, diameter 24 mm) to obtain separated fractions (Thammasirirak and Daduang, 2004).

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3.2.2 Preparation of test solution and inoculums

Antibiotics used were prepared by dissolving Ceftazidime, Cloxacillin and Cephalexin in steriled water.

A separated solution of fraction P1, P2, P3, P4 and P5 from crocodile plasma alone and in combination with selected drugs were prepared by the doubling dilution method with sterilized water and adjusted to give the required test concentrations.

Test organisms were incubated in 100 mL nutrient broth for 18 h at 37ºC. The cell cultures were centrifuged at 4,000 rpm for 10 minute (mins). The cell pellets were washed with saline, recentrifuged and resuspended in saline. The cell concentrations were adjusted with saline to give $5x10^8$ CFU/mL using a predetermined calibration curve of absorbance at 500 nm (Liu et al., 2000). The minimum inhibitory concentrations (MICs) of separated solution of fraction P1, P2, P3, P4, P5 and the selected antibiotics alone and each fraction in combination with each antibiotic were examined.

3.2.3 Bacterial suspension standard curve

To select bacterial suspensions with a known viable count the following steps were followed:

A separate loopful of each bacterium was used to inoculate in 100 mL of mueller hinton broth. The cultures were incubated at 37°C for 18 h. The bacterial cells were pelletted by centrifugation at 4,000 r.p.m. for 10 mins. The cells were washed twice by resuspending and centrifuging at 4,000 r.p.m/min for 10 mins in 10 mL of 0.9% NaCl. The cells were resuspended in 50 mL of sterile 0.9% NaCl. The cell suspensions were diluted so that 5-6 spectrophotometer readings could obtained over

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the absorbance range of approximately 0.05-0.25 at a wavelength of 500 nm. Viable counts for each absorbance reading were determined in triplicates using over dried agar plate counting method (Eumkeb, 1999; Richards et al., 1993).

3.2.4 MIC determinations

Conventional broth dilution tests were used when only a few strains of bacteria need to be tested or when an accurate MIC estimation was required. A series of two-fold dilutions of the antibiotic under study was prepared in a volume of a suitable medium and a standard inoculum of the test strain (commonly 100,000 bacteria) was introduced into each tube. The culture was incubated at 37°C overnight and the end-point was read that concentration of antibiotic in which no turbidity can be seen. Uninoculated tubes containing broth plus antibiotic and broth alone act as sterility controls an antibiotic-free tube inoculated with the test organism indicated that the organism is viable in case the end-point was missed (Greenwood, 2000).

MICs were determined using broth microdilution method. The sterile wire loop test organism from a slope culture was inoculated into Mueller Hinton broth and was incubated for 18 h at 37°C. Then, preparation of a bacterial suspension, the density of the bacterial suspension in normal saline was adjusted to approximately $1x10^8$ CFU/mL by using the absorption of bacterial suspension viable count standard curve.

The susceptibility panel in 96-well microplates (Fisher Scientific, Illinois, IL) were prepared by dispensing 200 µL of separated fraction solutions with the highest concentrations into the first column wells and 50 µL of CAMHB (pH 5.9) into the test wells. Then, the two-fold serial dilutions of separated fraction solutions were carried out by aliquoting 100 µL of separated fraction solutions in the first

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column wells into the second column and next columns to achieve the final concentrations, respectively. Aliquots (100 μL) of each bacterial suspension were inoculated into wells of the microplates to obtain a final volume of 200 μL in each well of the plate. The well containing without antibacterial agent was used as positive control while the well without inoculation was used as negative control. The inoculum was adjusted to give a final concentration approximately 10^5 CFU/well. The 96-microwell plates were sealed using a perforated plate seal (TREK Diagnostic Systems Inc., Cleveland, OH) and incubated at 37°C for 18-24 h. The MICs of separated fraction solutions were recorded as the lowest concentration where no visible growth was observed in the wells of 96-microwell plates after incubation for 18-24 h (Clinical Laboratory Standards Institute, 2013; Jiang, 2011).

3.2.5 Checkerboard determination

Antimicrobial combinations were selected for various reasons including minimize drug toxicity by using the lowest possible doses of two or more agents that have additive efficacies but independent toxicities, or to reduce the potential for development of resistance to one agent (Swan and Manivannan, 2000). Checkerboard titrations are relatively simple to perform and allow the assessment synergy at 24 h only. Dilution of antimicrobial agents may reduce to concentrations tested to a level at which synergy cannot be detected (Eumkeb, 1999; Lorian, 1999).

Checkerboard determinations in antimicrobial combinations were performed following the method of Sabath (1967) with slight modification (Eumkeb, 1999). Antibacterial agent “A” and antibacterial agent “B” were diluted to 1/2 of their MICs along the ordinate and abscissa respectively. The checkerboard assays were done using microdilution method as follows. An 18 h culture of each of the test

bacteria was prepared. The test bacterial suspensions were adjusted to $1x10^8$ CFU/mL using the absorption of bacterial suspension from the previously determine standard curve.

The susceptibility panel in 96-well microplates (Fisher Scientific, Illinois, IL) were prepared by dispensing 100 µL of separated fraction solutions with the highest concentrations into the first column wells and 100 µL of CAMHB (pH 5.9) into the test wells. Then, the two-fold serial dilutions of separated fraction solutions were performed by aliqouting 100 µL of separated fraction solutions in the first column wells into the second column and then move on to the next column to achieve the final concentrations. Add 50 µL of various concentration of antibiotic in each microplate wells. Aliquots (50 µL) each bacterial suspension were inoculated into wells of the microplates to obtain a total volume of 200 µL in each well. The last two wells were used as positive and negative controls, respectively. The positive control was inoculated with bacterial suspension only, while the negative well was left blank without inoculation. The final inoculum on the microplates were approximately 10^5 CFU/well. The 96-microwell plates were sealed using a perforated plate seal (TREK Diagnostic Systems Inc., Cleveland, OH) and incubated at 37°C for 18-24 h. The test was carried out in triplicate. MICs were determined for each antibacterial combination and the isobolograms were plotted. The calculation of the FIC (Fractional Inhibition Concentration) index for each antibacterial combination was undertaken as follows:

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

FIC (A+B)	≤ 0.5	Synergy
FIC (A+B)	$> 0.5\text{-}4.0$	No interaction
FIC (A+B)	> 4.0	Antagonism

Source: Johnson et al., 2004b; Odds, 2003a.

3.2.6 Killing curve determinations

Viable counts for the determination of killing curve was performed as previously described (Richards et al., 1993) with slight modification (Eumkeb, 1999) using a culture medium volume of 100 mL. Inocula of 5×10^6 CFU/mL of drug resistant bacteria was exposed to the antibacterials either singly or in combination with antibiotic drugs at concentrations ¼ of their MICs of separated fractions and incubation temperature of 37°C. After exposed to antibacterial agents at 0, 0.5, 1, 2, 4, 6 and 24 h, subsequent dilution plating on overdried Mueller Hinton agar plates in quadruplicate and incubation at 37°C for 18 h was allowed counting of growing colonies. The lowest detectable limit for counting is 10^3 CFU/mL. Positive controls were used containing similar cell and solvent concentrations (Iain et al., 2000).

3.2.7 Transmission electronmicroscopy (TEM) method

Preparation of cultures

To examine the effect of drugs, selected purified fraction (P1 and P5) on the cell structure of CREnC 21394 the following methods were performed.

CREnC 21394 was incubated in 10 mL Mueller Hinton broth for 18 h at 37°C. A 2.0 mL volume of 18 h culture was inoculated into a 250 mL conical flask

containing 98 mL Mueller Hinton broth which was placed in a water bath shaking at 100 oscillations/min for 4 h at 37°C. The cells were then, washed two times by suspending and centrifuging at 4,000 r.p.m. for 10 mins in 0.9% NaCl. Volume of 10 mL of $5x10^7$ CFU/mL in 0.9% NaCl was inoculated into 250 mL conical flasks each containing 90 mL Mueller Hinton broth plus antibiotic drugs at concentrations ¼ of their MICs of separated fractions : P1, P5 alone, and separated fractions; P1, P5 plus selected antibacterial to give approximately $5x10^6$ CFU/mL final concentration. A flask containing 90 mL MHB for *E. cloacae* without any antibiotics was used as the control. The cultures together with either the separated fractions; P1, P5 alone, and separated fractions; or in combination with selected antibiotic and control cultures were incubated for 4 h shaking at 100 oscillations/min in a water bath at 37°C (Richards et al., 1993; Xing, 1994).

The CREnC 21394 cultures were centrifuged at 6,000 g for 15 min at 4°C, and the supernate was removed. The pellets were fixed in glutaraldehyde 8% v/v in 0.1 M phosphate buffer (pH 7.2) for 1 h at 4°C and then were fixed in 4%v/v glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h at 4°C. After washing in the buffer, the bacteria was suspension in osmium tetroxide (OsO_4) (Emscope, Watford) 1% w/v for 1 h at room temperature. They were then washed three times by centrifugation and resuspended in distilled water. The final pellets were resuspended in a small volume of warm agarose 2% w/v, poured on to a glass slide and were allowed to cool. When set, small pieces of gel containing suspended bacteria were cut out and dehydrated through a grade series of ethanol solutions. After embedding in Resin, thin sections were cut with a diamond knife on a RMC ultramicrotome model

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MTX, stained with uranyl acetate and lead citrate, and examined in a TecnaiG20 electron microscope at 80-100 kV (Eumkeb, 1999; Richards et al., 1993).

3.2.8 Outer and cytoplasmic membrane permeability

3.2.8.1 Outer membrane (OM) permeability

To examine the effect of antibacterial characteristic from separated fractions either P1, P5 alone or in combination with antibiotics drugs on the function of the OM as a permeability barrier. The following method was performed.

The separated fractions either P1, P5 or in combination with antibacterials induced permeabilization of the OM of CREnC 21394 was determined essentially as recently described. The cells culture were incubated in 100 mL MHB for 18 h at 37°C. Inocula of 1 mL of quantities of 18 h culture were added to 20 mL centrifuged containing 9 mL MHB in shaking water bath at 37°C and shaking at 100 oscillations/min for 4 h. Inocula of $5x10^6$ of CFU/mL for 1 mL of the 4 h log phase culture were added to 20 mL conical flasks each containing 9 mL MHB plus selected separated fraction either P1, P5 alone or in combination with antibiotic drugs at concentrations ¼ of their MICs against this strain to give final concentration approximately $5x10^5$ CFU/mL, rinsed twice using centrifugation at 4700 r.p.m. for 1 min and suspended in HEPES buffer to measure OD 600 of 0.3. The separated fractions were dissolved in HEPES buffer. An NCF stock solution was prepared by dissolving 1 mg NCF in DMSO and diluting with HEPES buffer at a concentration of 60 µg/ml. ONPG was dissolved in HEPES buffer to get 300 µg/mL. Membrane permeabilization was assayed in 96-well microtitre plates. The OM permeabilization assay was carried out with wells filled with 50 µL selected separated fraction P1 or P5 alone or in combination with antibiotic drugs. NCF, a substrate of β-lactamase

localized within the periplasmic space, is normally excluded from CREnC 21394 by the outer LPS layer. The concentration of half-maximal membrane permeabilization (EC50) was derived from dose–response curves giving the difference in the absorption values measured for peptide-exposed cells and peptide-free wells at 500 nm after 5 min (OM permeabilization) (Eumkeb and Chukrathok, 2013; Junkes et al., 2008).

3.2.8.2 Cytoplasmic membrane (CM) permeability

Cytoplasmic membrane permeability was determined by the ability of the peptides to unmask cytoplasmic *β*-galactosidase activity in bacteria by using ortho-nitrophenylgalactoside (ONPG) as the substrate. The method of sample preparation was prepared the same as for the OM permeability determinations. To assay CM permeabilization, the wells contained 50 µL selected separated fraction either P1, P5 alone or in combination with antibiotic drugs and 50 µL ONPG solution. ONPG can be cleaved by *β*-galactosidase localized within the cytoplasm, but it is blocked from cell entry by the CM since the strain lacks *lac* permease. The plates were prepared shortly before the experiment. Finally, 50 µL of cell suspension (OD 0.3) was added to the wells to give a final concentration of 20 µg/mL NCF or 100 µg/mL ONPG. Depending on the MIC of the samples, the concentrations ranged from 1 to 200 µM. After warming to 37°C the plates were positioned in the plate reader at 37°C. NCF entry and cleavage by *β*-lactamase was followed by optical density measurement at 500 nm over 10 mins, and ONPG uptake and cleavage by *β*-galactosidase within the cytoplasm was characterized by monitoring absorption over a period of 60 min at 420 nm. Complete permeabilization was induced in the presence of 5 µM PMX as a positive control and wells lacking separated fraction and drugs

served as negative control. The concentration of half-maximal membrane permeabilization (EC50) was derived from dose–response curves giving the difference in the absorption values measured for peptide-exposed cells and peptide-free wells at 420 nm after 40 mins.

3.2.9 Electrophoresis

3.2.9.1 Extraction of outer membrane and peptidoglycan (OM-PG)-associated protein

To examine the effect of antibacterial characteristic from separated fractions either P1, P5 alone or in combination with antibiotics drugs on the outer membrane and peptidoglycan associated protein (OM-PG). The following method was practiced.

CREnC 21394 was incubated in 100 mL quantities of Mueller Hinton broth for 18 h at 37°C. An 8.0 mL volume of 18 h culture was inoculated into a 250 mL conical flask containing 192 mL Mueller Hinton broth which was place in a water bath shaking at 100 oscillations/min for 4 h at 37°C. Volumes of 100 mL of $1x10^6$ CFU/mL for the 4 h log phase culture were inoculated into 250 mL conical flasks each containing 100 mL Mueller Hinton broth plus concentrations ¼ of their MICs of separated fractions either P1, P5 alone or in combination with antibiotics. A flask containing 100 mL Mueller Hinton broth without any antibacterials was used as the control. The log phase cultures together with the concentrations ¼ of their MICs of separated fractions either P1, P5 alone or in combination with antibiotics and log phase control culture was incubated for 4 h shaking at 100 oscillations/min in a water bath at 37°C. The separated fractions either P1, P5 and drugs alone or in combination

were used at ¼ MIC for clinical isolates of CREnC 21394 (Eumkeb, 1999; Richards et al., 1993; Xing, 1994).

A 200 mL of bacterial culture was harvested by centrifugation (15 min, 6,000 g, 4°C) and washed twice with N-2-hydroxyethyl piperazine-N,-ethanesulphonic acid (HEPES) buffer (10 mM, pH 6.8). The bacteria were resuspended in 10 mL diluted water and disintegrated by sonication (3x60s with a 30 s cooling period between each burst) at 4°C. Unbroken cells were removed by centrifugation at 5,000 g, 4°C for 5 min and the pellet was discarded.

The bacterial membrane and peptidoglycan complex was recovered by centrifugation at 40,000 g for 60 min, washed twice in distilled water containing 2 mg/mL phenyl methyl sulphonyl fluoride (PMSF). Then, the same precise weight (25 mg) of OM-PG extract of each sample from CREnC 21394 was resuspended in 0.5 mL of (distilled water + 2 mL/mL PMSF). Therefore, the same quatity of OM-PG extract of each sample (50 mg/mL) from CREnC 21394 could be investigated. These extract proteins were then stored frozen at -70°C (Eumkeb, 1999; Gledhill et al., 1991; Richards and Xing, 1994).

Bovine serum albumin (Sigma) was used as the protein standard. The extract was stored at -70°C and re-diluted in sample buffer before SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separation. The extract was shown to be stable for over 2 months under these conditions (Eumkeb, 1999).

3.2.9.2 SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used a gel system having a 4% stacking gel and a 15% separating gel. A volume of the OM-PG extract was mixed with a volume of sample buffer containing 0.125 M Tris-

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HCl buffer, pH 6.8, containing 0.04 M Na_2EDTA (Sigma), 4% (w/v) SDS, 10% (w/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.1% (w/v) bromophenol blue (Sigma) and boiled for 5 mins. The electrophoresis was performed at 8 mA per gel for stacking gel and 15 mA per gel for separating gel to maximise the resolution at the important subtyping areas of the gel. After electrophoresis, the separating gel was stained with a Coomassie Brilliant Blue stain for 2 h at room temperature with gentle mixing. The gel was initially destained with 45% ethanol (v/v), 10% acetic acid solution followed by final destaining with 7% acetic acid solution. The following standard proteins (BDH) was used as molecular mass markers: myoglobin (17,200), carbonic anhydrase (30,000), ovalbumin (42,700), albumin (66,250) and ovotransferrin (76,000-78,000) (Eumkeb, 1999).

3.2.10 Enzyme assay

β-lactamases type IV of *Enterobacter cloacae* were obtained from Sigma (Poole, England). Enzyme activities were adjusted to the sufficient concentration to hydrolyse 50-60% of substrate in 5 minute. Selected purified fraction either P1, P5 alone or in combination with antibiotic drugs were pre-incubated with enzyme in 50 mM sodium phosphate buffer (pH 7.0) at 37°C for 5 minutes prior to substrate addition. Time-course assays were carried out using methanol/acetic acid (100:1) as stopping reagent. The analyses of the remaining substrate were determined by reverse-phase HPLC using acetronitrile/ammonium acetate as a mobile phase (Reading and Farmer, 1983).

The concentration of ceftazidime which reduced either the rate of substrate hydrolysis by 50% alternatively reduces loss of substrate by 50% after a given enzymation period was termed the I_{50} value of the inhibitor. I_{50} values are not

kinetic constants and depend entirely on the test conditions under which they were determined. Assays with pre-incubation were carried out by reacting enzyme with β-lactam antibiotics for a set time period and then adding the substrate to measure the residual enzyme activity. β-lactamase had been used at a concentration sufficient to hydrolyse 100 µg/mL of benzyl penicillin within 5 mins.

Enzyme activity and hence its inhibition was measured by following the decrease the U.V. maximum for β-lactam antibiotics which occurs on hydrolysis by β-lactamase (O'Callaghan et al., 1968; Reading and Farmer, 1983). High performance liquid chromatography (HPLC) provides a further technique which was used to measure to stability of an antibiotic to β-lactamase in the presence of an enzyme inhibitor. Reaction samples (20 µL) were injected at various times on to Waters Bio-Sil C_{18} HL 90-5s reverse phase column eluted at 1.5 mL/min with 50 mM sodium phosphate buffer (pH 7.0). The eluent was monitored at 214 nm (Reading and Farmer, 1983).

3.2.11 Statistic analysis

OM and CM permeability and enzyme assay were carried out in triplicate. At least ten cells in each treated cells from TEM study were measured. The data were expressed as mean ± SEM. Significant differences between these groups were examined using one-way ANOVA. $p<0.05$ and $p<0.01$ of Tukey's HSD post hoc test were considered as a statistically significant difference.

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CHAPTER IV

RESULTS AND DISCUSSION

4.1 Fractions separation from crocodile plasma

4.1.1 Ion exchange chromatography: The 5 peaks of pooled fraction from crocodile plasma were separated by ion exchange chromatography with Q Sepharose as shown in Figure 4.1.

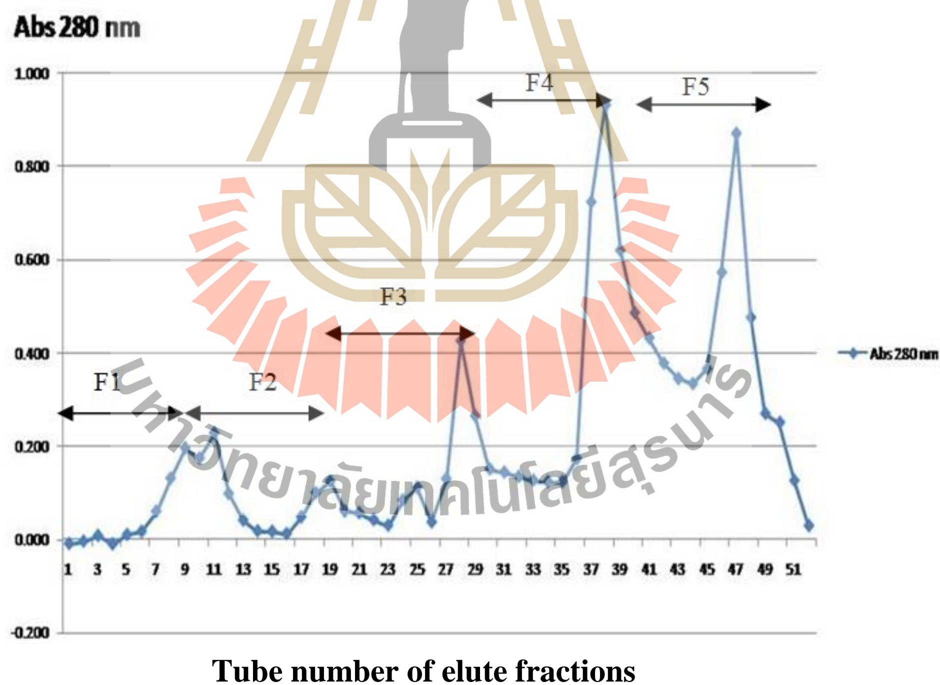

Figure 4.1 Ion exchange chromatography of crocodile plasma on Q Sepharose column. There were 5 pooled fractions. Fraction 1-5 were achieved with elution buffer containing 25 mM of Tris-HCl and vary concentrations of NaCl at pH 8.1 (0.1, 0.2, 0.3, 0.4 and 0.5 M for fraction 1, 2, 3, 4 and 5, respectively).

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Crocodile plasma was separated to select cationic proteins by ion exchange chromatography with Q Sepharose column, and 25 mM of Tris-HCl buffer was used as a mobile phase. The sequence of concentrations of NaCl at pH 8.1 (0.1, 0.2, 0.3, 0.4 and 0.5 M) were used as an elution buffer. The result showed that the optical density value (O.D.) of cationic proteins of each pooled fraction were varied as a result of difference of NaCl concentrations. The elution solution of cationic peptides from tube number one to ten were fraction 1 (F1), eleven to twenty were fraction 2 (F2), twenty one to thirty were fraction 3 (F3), thirty one to forty were fraction 4 (F4) and forty one to fifty were fraction 5 (F5).

4.1.2 Gel filtration chromatography: The 5 pooled fractions from ion exchange chromatography were further separated by gel filtration chromatography with Sephadex G-50 (Superfine, Amersham Bio-sciences, 2.5x100 cm) gel infiltration column, this method was performed to separate and select the 0-250 kDa of cationic proteins. The 5 separated fractions; P1, P2, P3, P4 and P5 were confirmed their molecular weight using SDS-PAGE. The results are illustrated in Figure 4.2A and 4.2B. The results showed that separated P1 displays two protein bands at 67 and 80 kDa, P2 presented four protein bands at 23, 67, 70 and 160 kDa, P3 revealed two protein bands at 67 and 75 kDa, P4 exhibited three protein bands at 23, 67 and 75 kDa, and P5 showed four protein bands at 23, 67, 75 and 160 kDa (Figure 4.2A). Most of represented protein bands in this study are likely different from previous study. Threenet et al. (2011) reported that the protein profiles on SDS-PAGE of Siamese crocodile serum presented 6 bands at MW at 225, 121, 67, 62, 45 and 25 kDa respectively. These results can be explained by assuming that our study use separated

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plasma fractions, whereas previous study used hold serum. However, the range of MW, 23-160 kDa compared to 25-225 kDa, is presumably similar.

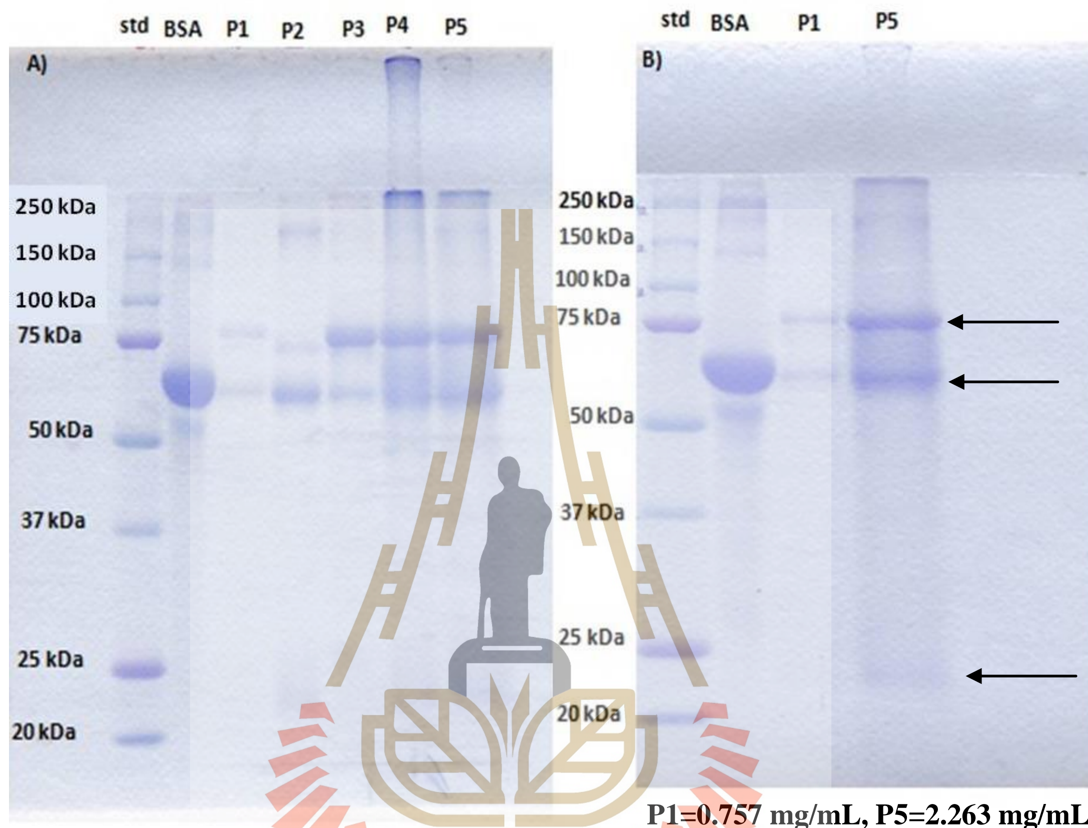

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Figure 4.2A, B Confirmation of molecular weight of separated P1, P2, P3, P4 and P5 P1 = separated fraction P1 (0.757 mg/mL), P2 = separated fraction P2 (1.757 mg/mL), P3 = separated fraction P3 (1.958 mg/mL), P4 = separated fraction P4 (3.914 mg/mL), P5 = separated fraction P5 (2.613 mg/mL), Loading volume = 10 µL, std; molecular weight marker proteins (kDa) and BSA; bovine serum albumin.

4.2 Bacterial suspensions viable count absorption standard curve

The bacterial suspensions standard curves were carried out in order to select bacterial suspensions with a known viable count. The results of the bacterial

suspensions viable count standard curve for Ceftazidime-resistant *Enterobactor Cloacae*DMST 21394 (CREnC 21394), Ceftazidime–resistant *Escherichia coli* DMST 20662(CREC 20662), Methicillin-resistant *Staphylococcus aureus* DMST 20651 (MRSA 20651), Methicillin-sensitive *Staphylococcus aureus* ATCC 29213 (MSSA 29213), Ceftazidime-sensitive *Escherichia coli* ATCC 25922 (CSEC 25922) and *S. epidermidis* DMST 15505 (*S. epidermidis*) are shown in Figure 4.3 to 4.8.

Figure 4.3 to 4.8 illustrate that the absorptions of CREnC 21394, CREC 20662, CSEC 25922, MRSA 20651, MSSA 29213 and *S. epidermidis* at 500 nm were approximately $1x10^8$ CFU/mL at the absorption of 0.10, 0.15, 0.15, 0.08, 0.08 and 0.22 respectively.

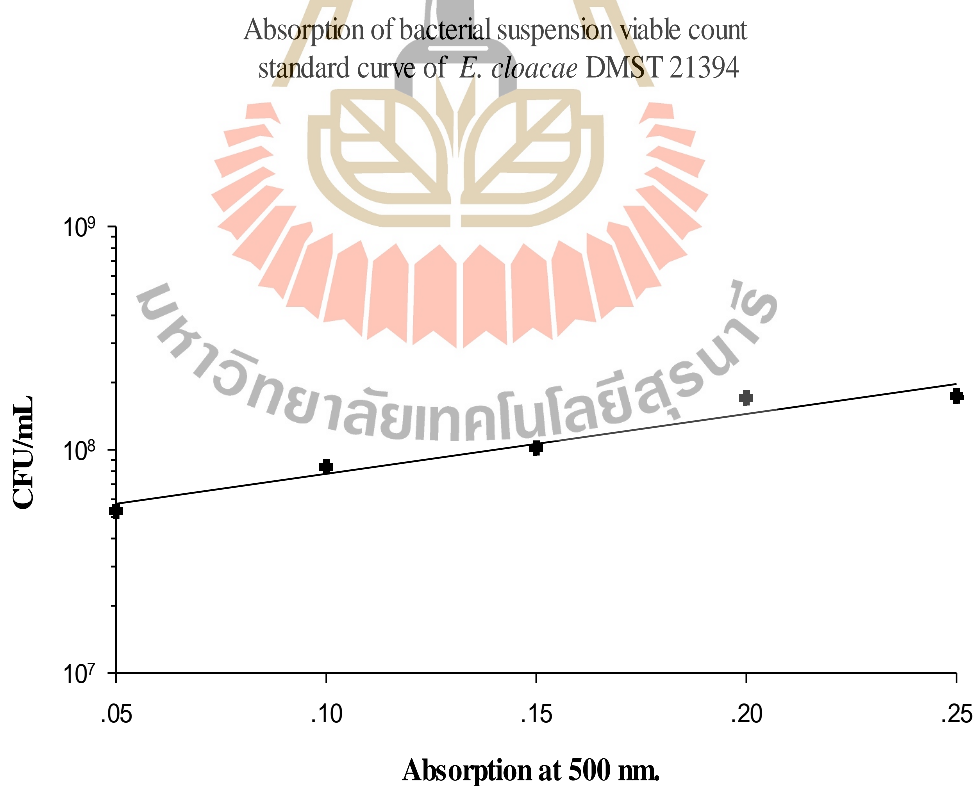

Figure 4.3 Standard curve for suspensions of ceftazidime-resistant *Enterobacter cloacae* DMST 21394.

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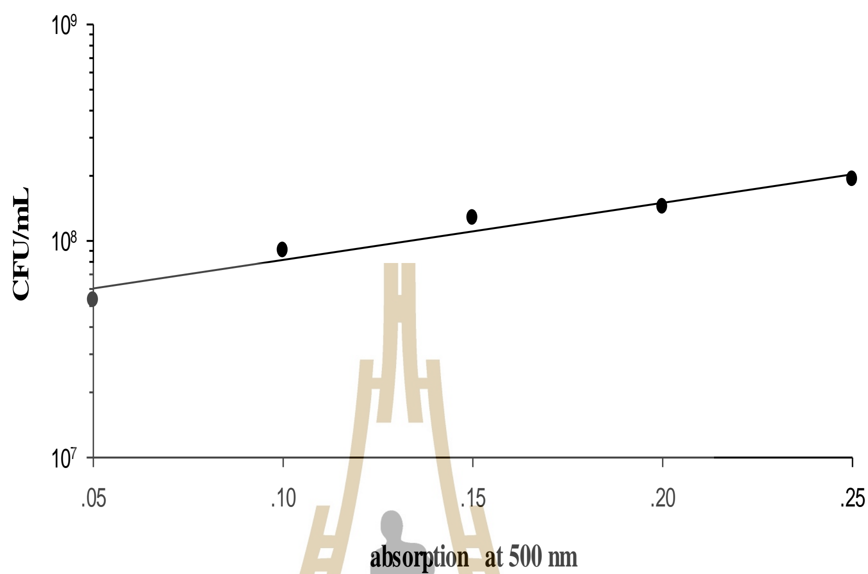

Figure 4.4 Standard curve for suspensions of ceftazidime-resistant *Escherichia coli* DMST 20662.

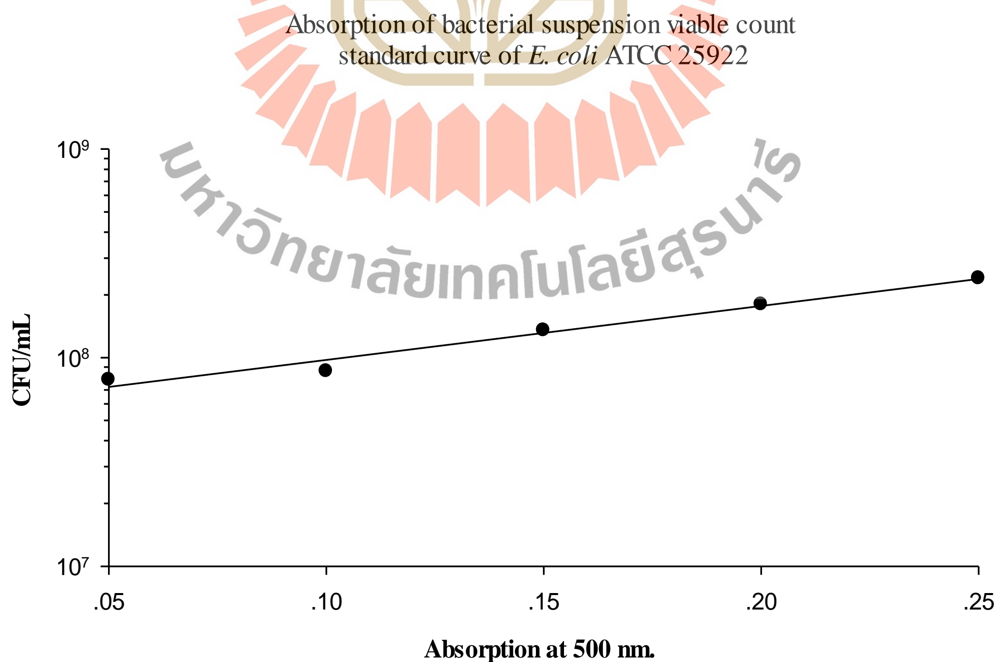

Figure 4.5 Standard curve for suspensions of ceftazidime-sensitive *Escherichia coli* ATCC 25922.

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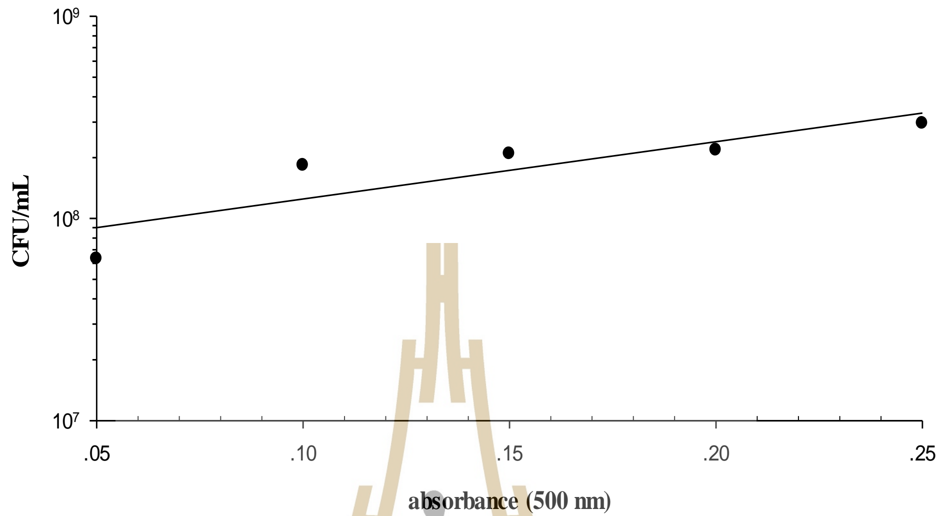

Figure 4.6 Standard curve for suspensions of methicillin-resistant *S. aureus* DMST 20651.

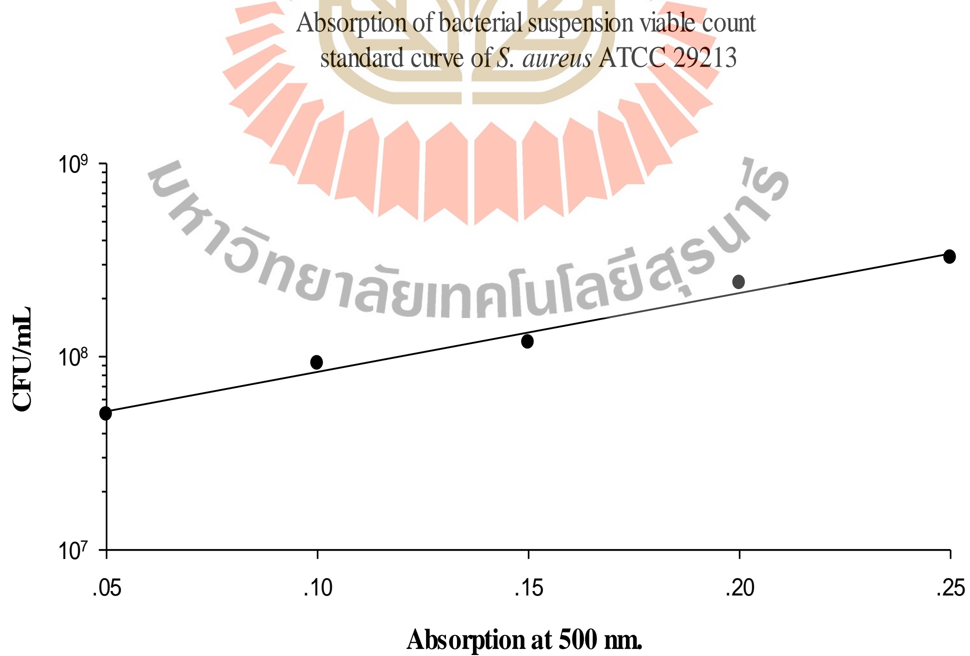

Figure 4.7 Standard curve for suspensions of Methicillin-sensitive *Staphylococcus aureus* ATCC 29213.

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Absorption of bacterial suspension viable count
Standard curve *S. epidermidis* DMST 15505

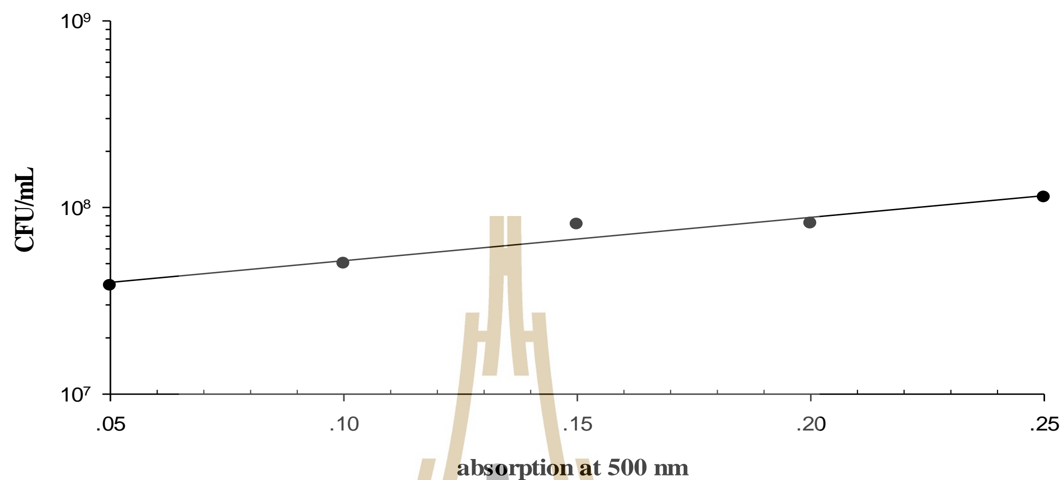

Figure 4.8 Standard curve for suspensions of *Staphylococcus epidermidis* DMST 15505.

4.3 MIC determinations

The MIC determinations for fraction 1 (P1), fraction 2 (P2), fraction 3 (P3), fraction 4 (P4) and fraction 5 (P5) of separated crocodile plasma and selected antibiotics (ceftazidime, cloxacillin and cephalexin) against clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 (CREnC 21394), ceftazidime–resistant *E. coli* DMST 20662 (CREC 20662), ceftazidime-sensitive *E. coli* ATCC 25922 (CSEC 25922), methicillin-resistant *S. aureus* DMST 20651 (MRSA 20651), methicillin-sensitive *S. aureus* ATCC 29213 (MSSA 29213) and *S. epidermidis* DMST 15505 were determined using microdilution method. The results are shown in Table 4.1.

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Table 4.1 Minimum inhibitory concentration (MIC) of *β*-lactams (µg/mL) and separated fraction from crocodile plasma P1, P2, P3, P4 and P5 (mg/mL) against clinical isolates of ceftazidime-resistant *E. cloacae*, ceftazidime–resistant *E. coli*, ceftazidime-sensitive *E. coli*, methicillin-resistant *S. aureus*, methicillin-sensitive *S. aures* and *S. epidermidis* determined by microdilution method. All data was performed in triplicate.

Separated fraction or *β*-lactam	**Minimum inhibitory concentration (MIC)**					
	E. cloacae DMST 21394	*E. coli* DMST 20662	*E. coli* ATCC 25922	*S. aureus* DMST 20651 (MRSA)	*S. aureus* ATCC 29213	*S.epidermidis* DMST 15505
P1 (mg/mL)	1024	512	512	1024	8	512
P2 (mg/mL)	>1024	512	256	1024	64	512
P3 (mg/mL)	>1024	512	1024	1024	128	512
P4 (mg/mL)	>1024	512	512	1024	128	512
P5 (mg/mL)	1024	512	512	1024	256	512
Ceftazidime (µg/mL)	>1024	32	8	-	-	512
Cloxacillin (µg/mL)	-	-	-	>1024	32	-
Cephalexin (µg/mL)	-	-	-	-	-	32

The MIC determinations for fraction 1 (P1), fraction 2 (P2), fraction 3 (P3), fraction 4 (P4) and fraction 5 (P5) of separated crocodile plasma and selected antibiotics (ceftazidime, cloxacillin and cephalexin) against clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 (CREnC 21394), ceftazidime–resistant

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E. coli DMST 20662 (CREC 20662), ceftazidime-sensitive *E. coliI* ATCC 25922 (CSEC 25922), methicillin-resistant *S. aureus* DMST 20651 (MRSA 20651), methicillin-sensitive *S. aureus* ATCC 29213 (MSSA 29213) and *S. epidermidis* DMST 15505 were determined using microdilution method. The results are shown in Table 4.1.

Ceftazidime alone showed no antibacterial activity against clinical isolates of CREnC 21394 at MIC >1024 mg/mL, whereas exhibited antibacterial activity against clinical isolates of CREC 20662, CSEC 25922 and *S. epidermidis* DMST 15505 at MICs 32, 8 and 512 µg/mL, respectively. Similarly, no antibacterial activity was observed in cloxacillin against MRSA 20651 at MIC >1024 µg/mL, while this drug showed some antibacterial activity against MSSA 29213 at MIC 32 µg/mL. In according to CLSI guideline, clinical isolate of *E. cloacae* used in this study was proven to be a resistant strain to ceftazidime, while the reference strain CSEC 25922 was susceptible to ceftazidime at MIC 8 µg/mL (Clinical Laboratory Standards Institute, 2013)

In addition, the MIC values of separated fraction P1, P2, P3, P4 and P5 against clinical isolates of CREnC 21394 were at 1024, >1024, >1024, 1024 and 1024 mg/mL, respectively. While the MICs for all separated fractions against clinical isolates CREC 20662 and *S. epidermidis* DMST 15505 were all equal at 512 mg/mL. Furthermore, the MICs of the separated fractions against clinical isolates of CSEC 25922 were 512, 256, 1024, 512 and 512 mg/mL for P1, P2, P3, P4 and P5 fractions, respectively. Additionally, The MIC values of separated fractions P1, P2, P3, P4 and P5 against MRSA 20651 were all equal at 1024 mg/mL and 8, 64, 128, 128 and 256 mg/mL for MSSA 29213, respectively. These results indicated that the separated

fractions P1, P2, P3, P4 and P5 alone exhibited weak antibacterial activity against those of CREC 20662 and *S. epidermidis* 15505. Whereas, all separated fractions alone did not inhibit both MRSA 20651 and CREnC 21394. Interestingly, the separated protein P1 showed stronger antibacterial activity than cloxacillin against MSSA 29213.

The crude plasma from Siamese crocodile (*Crocodylus siamensis*) has been reported to possess antibacterial activities against *Salmonella typhi*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *E. coli*, *Psudomonas aeroginosa* and *Vibrio cholerae* (Thammasirirak and Daduang, 2004; Preecharram et al., 2008; Kommanee et al., 2012). Similarly, the antibacterial component from crude plasma of Siamese crocodile, Crocosin, has been reported its antibacterial activity against susceptible strains of *S. typhi* and *S. aureus* (Preecharram et al., 2010). In addition, antibacterial activity of serum from American alligator (*Alligator mississippiensis*) toward *E. coli* has been reported that its displayed the diameter of the clear zone about 5 mm against susceptible strains of *E. coli*, *S. aureus* and *S. epidermidis*. Besides, the amino acid composition of hydrolyzed and non-hydrolyzed crocosin, MW between 525-796 Da, showed slightly inhibited the growth of *S. aureus* at 10 h (Preecharram et al., 2010). In addition, antibacterial activity of serum from American alligator (*Alligator mississippiensis*) exhibited 10-fold lower bacterial survival rates than human serum against *E. coli.* These findings suggested that an active serum complement system produced antibacterial properties (Merchant et al., 2003). In addition, previous research showed that antibacterial peptide, Leucrocin I-IV, isolated from white blood cells of *C. siamensis* exhibited MICs between 0.66-25 µg/mL against *S. epidermidis* (Pata et al., 2011). In the same way,

the present findings also found that separated fractions P1, P2, P3, P4 and P5 of plasma from Siamese crocodile exhibited weak antibacterial activity against those of CREC 20662, CSEC 25922, MSSA 29213 and *S. epidermidis* DMST 15505. Obviously, MICs of these fractions against these sensitive strains were rather lower than those of resistant strains. Our findings lend support to the assumption that antibacterial activity of separated fractions of Siamese crocodile plasma may presumably caused by the amino acid composition of polypeptide that heavier or longer than crocosin (Preecharram et al., 2010).

4.4 Checkerboard determination

Combinations of antibacterial agents were performed using checkerboard assay in order to examine synergistic antibacterial activity of separated fractions from crocodile plasma and antibiotics. The fraction P1 and P5 were selected to evaluate synergistic antibacterial activity when used in combinations with ceftazidime and cloxacillin against CREnC 21394, CREC 20662 and MRSA 20651. The isobolograms of each combination that showed synergistic effect were plotted as shown in Figure 4.9 to 4.12. The data from checkerboard determinations are summarized in Table 4.2. The interactions between separated fraction P1, P5 from crocodile plasma plus either ceftazidime or cloxacillin were determined by the fractional inhibitory concentration (FIC). The FIC index (FICI) was calculated and interpreted in accordance with Odds's description as follows; FICI ≤ 0.5 denoting synergistic; FICI > 0.5-4.0 denoting no interaction; FICI > 4.0 denoting antagonism (Johnson, 2004; Odds, 2003). Figure 4.9 and 4.10 exhibited that the synergistic activity of combination of separated fraction either P1 or P5 from crocodile plasma plus cloxacillin against

clinical isolate of MRSA 20651 was occurred (FIC index ≤ 0.5). Also, the synergistic antibacterial effect of separated fraction either P1 or P5 from crocodile serum plus ceftazidime against clinical isolates of CREnC 21394 are plotted in Figure 4.11 and 4.12 (FIC ≤ 0.5).

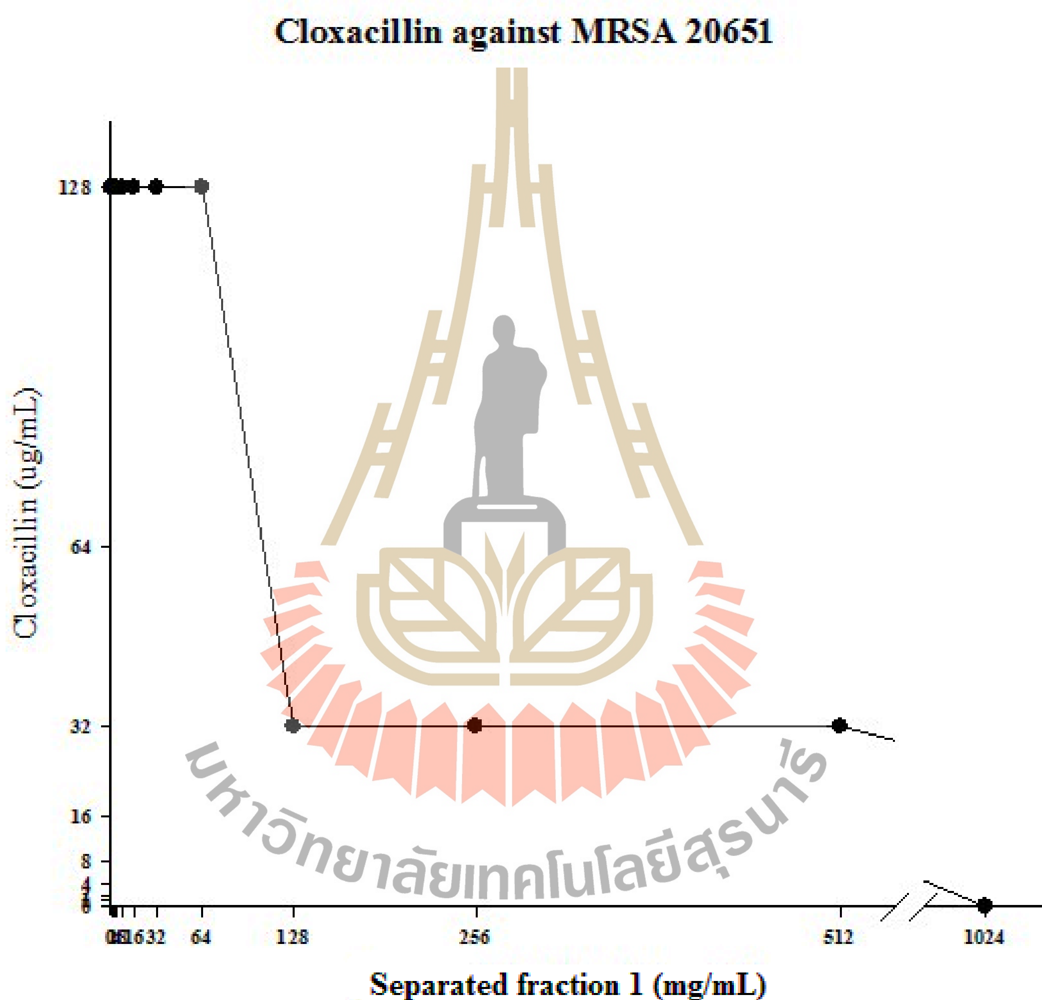

Figure 4.9 Isobologram constructed from checkerboard MIC data showing antibacterial combination of Cloxacillin plus separated fraction 1 against clinical isolates of methicillin-resistant *S. aureus* DMST 20651.

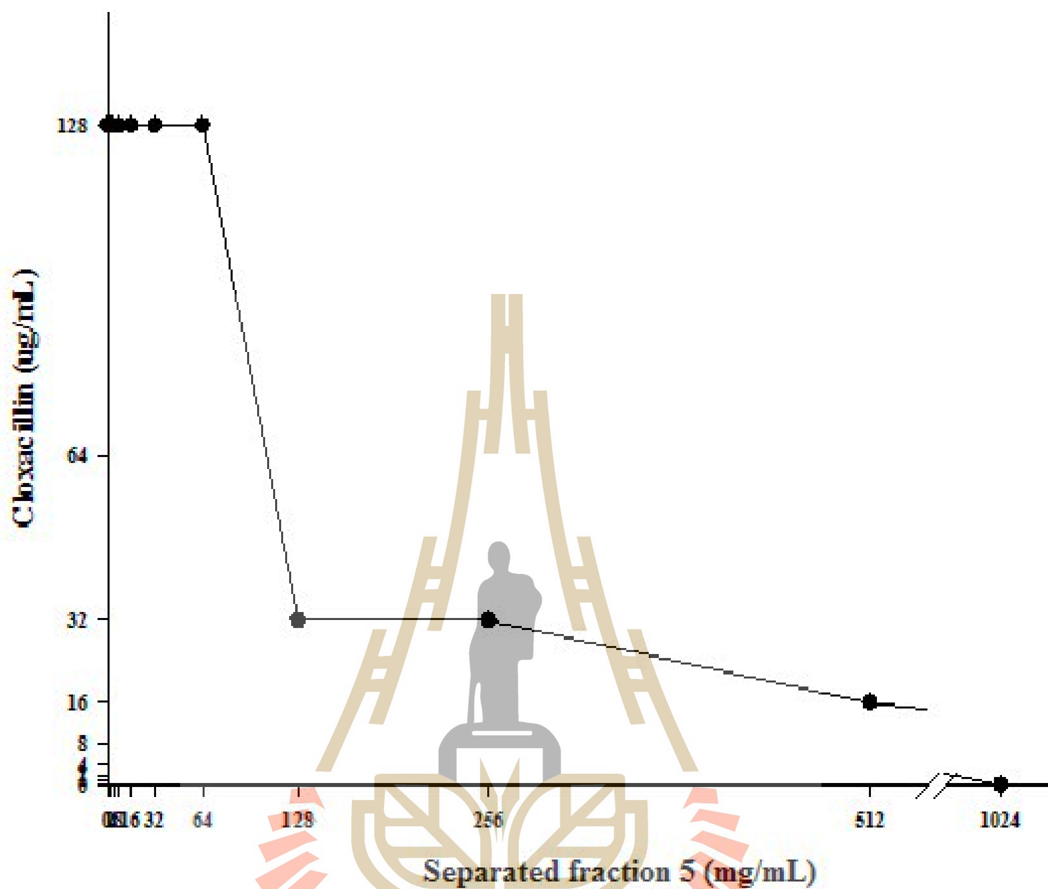

Figure 4.10 Isobologram constructed from checkerboard MIC data showing antibacterial combination of Cloxacillin plus separated fraction 5 against clinical isolates of methicillin-resistant *S. aureus* DMST 20651.

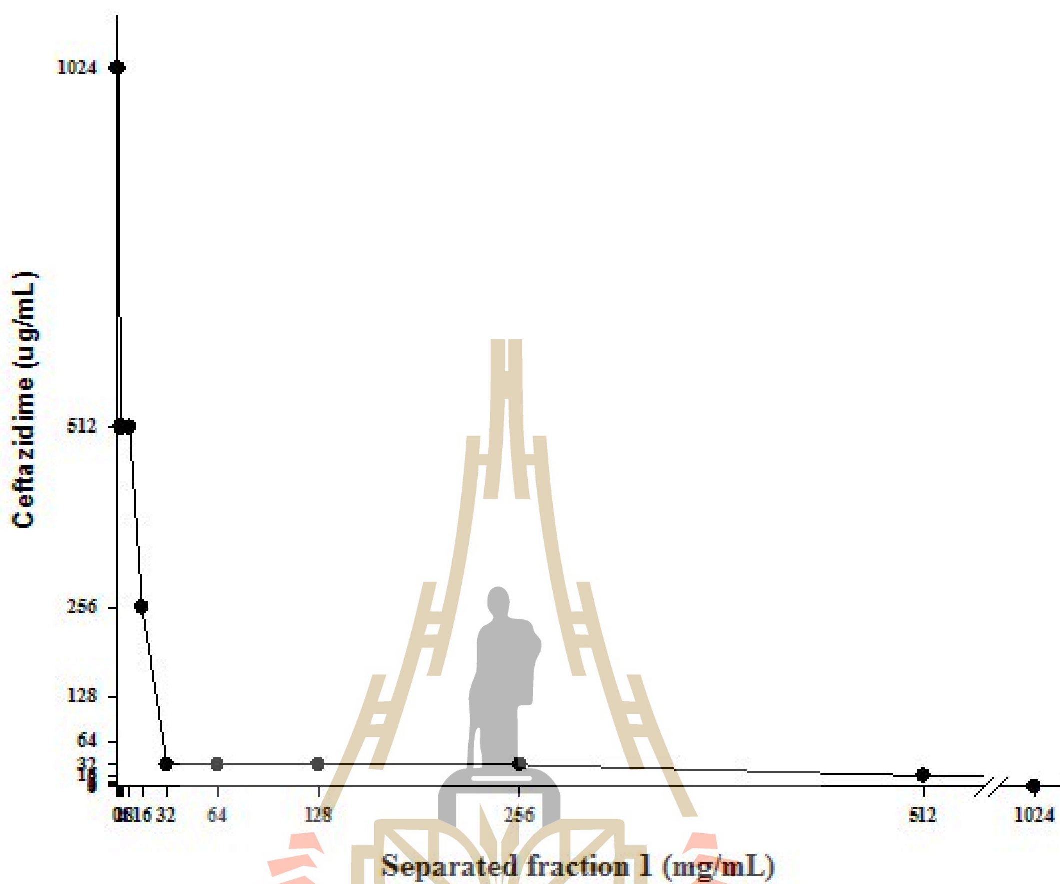

Figure 4.11 Isobologram constructed from checkerboard MIC data showing antibacterial combination of Ceftazidime plus separated fraction 1 against clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394.

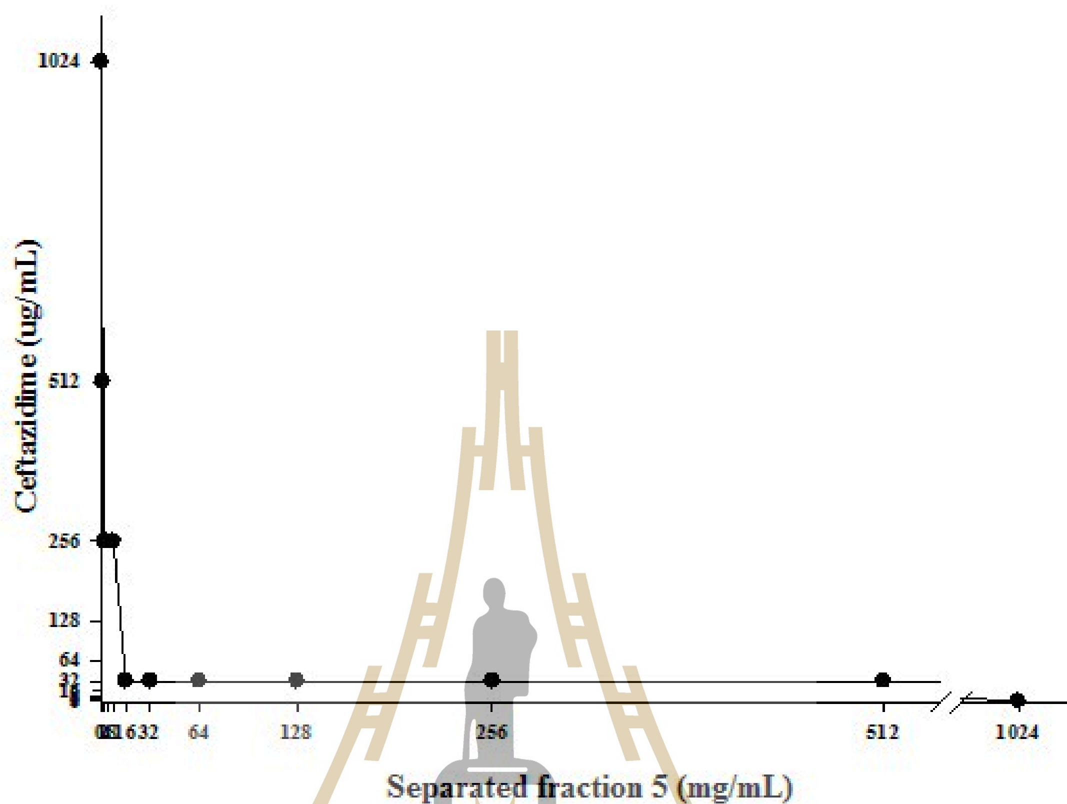

Figure 4.12 Isobologram constructed from checkerboard MIC data showing antibacterial combination of Ceftazidime plus separated fraction 5 against clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394.

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The MICs of separated fraction both P1 and P5 plus ceftazidime were substantially reduced from both equal MICs at >1024 μg/mL plus 1024 μg/mL to both equal MICs at 32 (1/32) μg/mL plus 32 (1/32 MIC) μg/mL respectively, against CREnC 21394. In addition, the MICs of separated fraction both P1 and P5 plus cloxacillin were also dramatically decreased from both equal MICs at >1024 μg/mL plus 128 μg/mL to both equal MICs at 128 (1/8 MIC) μg/mL plus 32 (1/4 MIC) respectively, against MRSA 20651. The reduction of MICs of separated fraction both

P1 and P5 in combination with ceftazidime were also observed against clinical isolate of CREC 20662. The MICs of P1 and P2 were reduced both equal MICs at 512 to 256 µg/mL and 128 µg/mL, respectively. Besides, the MICs of ceftazidime were reduced from 32 µg/mL to 1 µg/mL when used in combination with P1, but no change of MIC was observed when combined with P5.

The synergistic activity of combinations of separated fraction both P1 and P5 plus tested β-lactams against both clinical isolates of CREnC 21394 and MRSA 20651 were determined with equal FIC index values at 0.062 for both P1 and P5 plus ceftazidime against CREnC 21394, and both FIC index values at 0.375 for both P1 and P5 plus cloxacillin against MRSA 20651. However, no synergistic activities were observed for combination of both P1 and P5 plus ceftazidime against EREC 20662 with FIC index 0.531 and 1.25, respectively (Table 4.2). The FIC index values below 0.5 are generally accepted as synergism between 2 antimicrobial agents (American Society for Microbiology, 2004; Johnson, 2004; Odds, 2003). Therefore, the present findings provide evidence that strong synergistic antibacterial activity of the purified fraction P1 and P5 in combination with ceftazidime or cloxacillin against CREnC 21394 and MRSA 20651, respectively has taken place.

Table 4.2 Summarizes the FICs for checkerboard assay of *β*-lactams used alone and in combination with separated fraction P1 and P5 from crocodile plasma against drug resistant bacteria.

Test bacteria	Combination of agents	MIC (/mL)	MIC (A+B)	FIC index	Type of interaction
CREnC 21394	Ceftazidime (µg)	>1024	32	0.062	synergism
	P1 (mg)	>1024	32		
	Ceftazidime (µg)	>1024	32	0.062	synergism
	P5 (mg)	>1024	32		
MRSA 20651	Cloxacillin (µg)	128	32	0.375	synergism
	P1 (mg)	1024	128		
	Cloxacillin (µg)	128	32	0.375	synergism
	P5 (mg)	1024	128		
E. coli 20662	Ceftazidime (µg)	32	1	0.531	no interaction
	P1 (mg)	512	256		
	Ceftazidime (µg)	32	32	1.25	no interaction
	P5 (mg)	512	128		

One approach to treating drug resistant bacteria is combination using two or more antimicrobial agents during a treatment regimen. This approach helps to decrease the emergence of resistant strain, dose-related toxicity as a result of reduced dosage and treated polymicrobial infection (Lorian, 1999). The mechanisms of drug combinations exert via following four mechanisms based on pharmacokinetic, molecular-biological and clinical investigations; 1) synergistic multi-target effect, 2) pharmacokinetic or physicochemical effects based on improved solubility, resorption rate and enhanced bioavailability, 3) interactions of agents with resistance mechanisms of bacteria, and 4) The respective elimination or neutralization of adverse effects by agents contained in the extract (Wagner and Ulrich-Merzenich, 2009). Cationic peptides have been reported the ability to inhibit both gram positive and negative bacteria. The synergistic interactions between peptides were reported (Yan and Hancock, 2001). Additionally, previous work has been studied antibiotic plus

antibacterial peptides combinations against *Pseudomonas fluorescens* and antibiotic-resistant variants, suggested that a synergistic inhibitory effect (FICI $\leq$ 0.5) was observed when resistant variants were treated with peptide plus antibiotic combinations (Naghmouchi et al., 2012). Crocodile plasma used in present study comprises of cationic peptides. Likewise, the synergistic antibacterial activity of separated fractions from crocodile plasma and β-lactam antibiotics combination were observed in this study, which are consistent with those of previously reported.

4.5 Killing curve determinations

Viable counts for the determination of killing curves were performed in order to confirm antibacterial activity and synergism of separated fraction P1 and P5 from crocodile plasma when used singly and in combination with ceftazidime against CREnC 21394 and with cloxacillin against MRSA 20651. Figure 4.13 illustrates the effect of separated fractions either P1 (512 mg/mL), P5 (512 mg/mL) and ceftazidme (32 µg/mL) alone or in combination on the clinical isolates of CREnC 21394. While the effect of either cloxacillin (64 µg/mL), P1 (512 mg/mL) and P5 (512 mg/mL) alone or in combination on the clinical isolates of MRSA 20651 is demonstrated in Figure 4.14.

The CREnC 21394 with antibacterial free (control) showed steadily increased in the counts of CFU throughout 24 h. Similarly, the growth of this strain after exposed to separated fractions both P1, P2 and ceftazidime alone had gradually increased within 24 h, but these results were rather lower in CFU count than control. Surprisingly, the combination of ceftazidime plus separated fraction either P1 or P5 caused dramatic reduction of $5x10^5$ CFU/mL of CREnC 21394 to 10^3 CFU/mL within

6 h and throughout the remainder of 24 h (Figure 4.13). These results establish evidence that separated fraction of either P1 (32 mg/mL) or P5 (32 mg/mL) from crocodile plasma in combination with ceftazidime (32 µg/mL) show strong synergistic activity. These results seem consistent with earlier findings that ceftazidime in combination with tested flavonoids reduced the CFU/mL of MRSA strain by $5x10^3$ over 6 h and did not recover in 24 h (Eumkeb and Chukrathok, 2013).

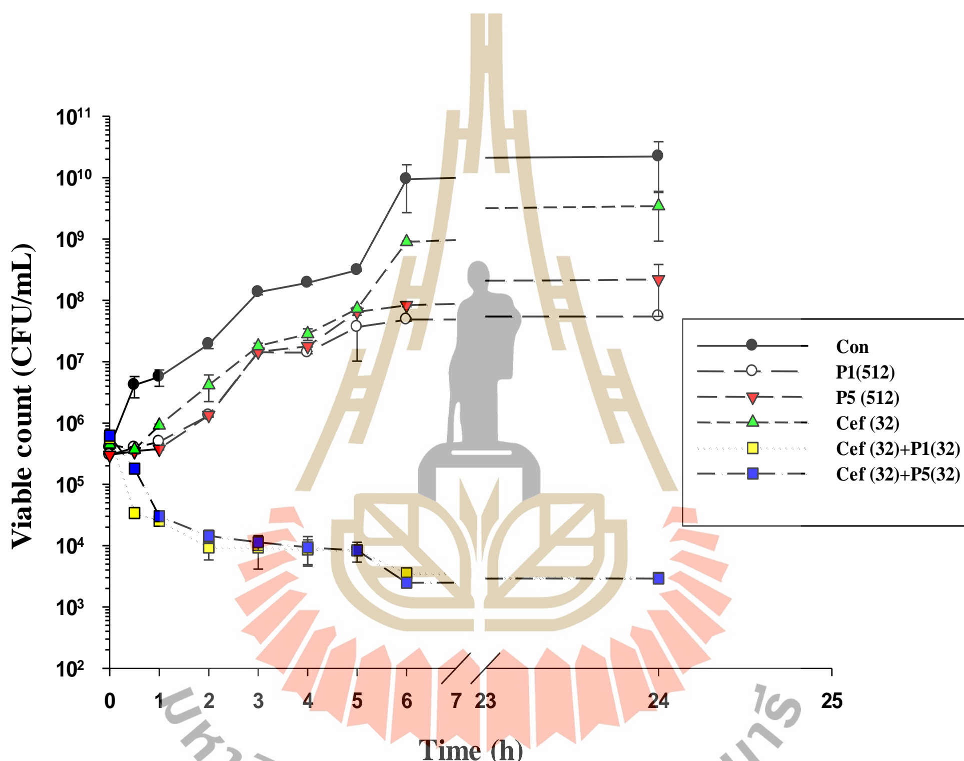

Figure 4.13 The effect of separated fraction either P1 (512 mg/mL), P5 (512 mg/mL) and ceftazidime (32 µg/mL) alone or in combination on the clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394. The values plotted are the means of three observations, and the vertical bars indicate the standard errors of the means. Con = control, P1(512) = P1 at 512 mg/mL, P5(512) = P5 at 512 mg/mL, Cef(32) = ceftazidime at 32 µg/mL, P1(32)+Cef(32) = P1 at 32 mg/mL + ceftazidime at 32 µg/mL, P5(32)+Cef(32) = P5 at 32 mg/mL + ceftazidime at 32 µg/mL. The values plotted are the means of 3 observations, and the vertical bars indicate the standard errors of the means.

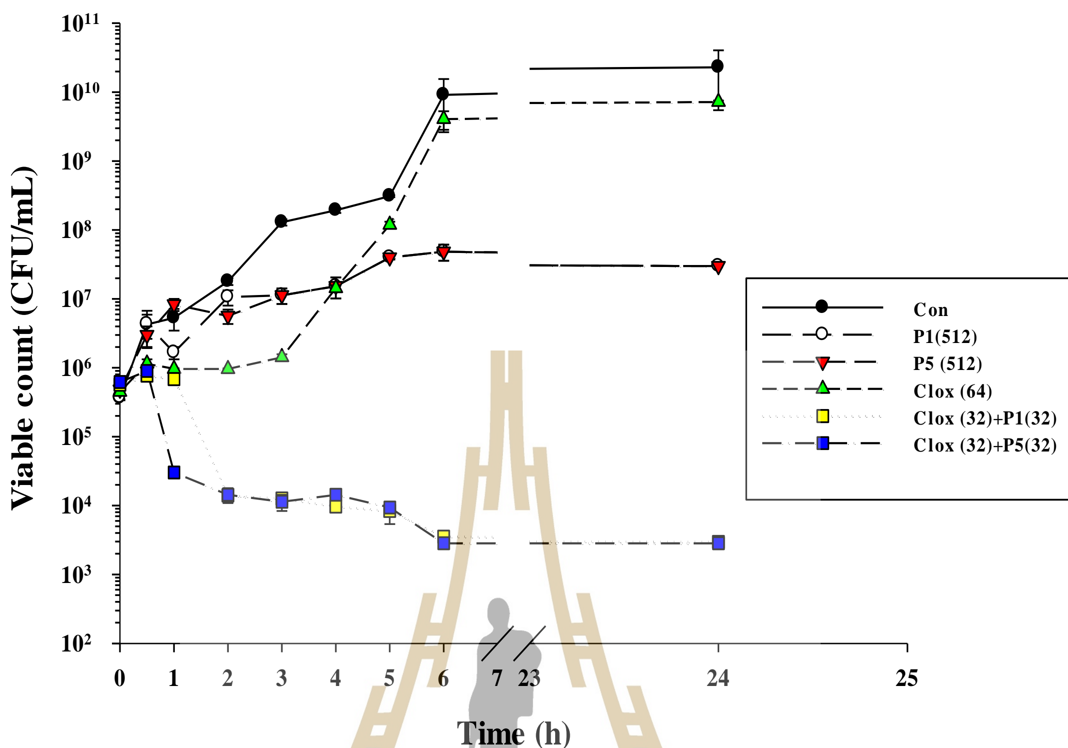

Figure 4.14 The effect of cloxacillin combined with separated fraction either P1 (512 mg/mL), P5 (512 mg/mL) and cloxacillin (32 µg/mL) alone or in combination on the clinical isolates of methicillin-resistant *S. aureus* DMST 20651. The values plotted are the means of three observations, and the vertical bars indicate the standard errors of the means.

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Con = control, P1(512) = P1 at 512 mg/mL, P5(512) = P5 at 512 mg/mL, Clox(64) = cloxacillin at 64 µg/mL, P1(32)+Clox(32) = P1 at 32 mg/mL + cloxacillin at 32 µg/mL, P5(32)+Clox(32) = P5 at 32 mg/mL + cloxacillin at 32 µg/mL. The values plotted are the means of 3 observations, and the vertical bars indicate the standard errors of the means.

Sampling killing curves resulting from separated fraction P1 and P5 from crocodile plasma alone and in combination with cloxacillin against clinical isolates of MRSA 20651. The culture was grown in absence of any antibacterial agents (control) showed no reduction in the counts of CFU from start till 24 h. The results indicated that the combination of both separated fraction P1 (32 mg/mL) plus cloxacillin (32 µg/mL) and P5 (32 mg/mL) plus cloxacillin (32 µg/mL) caused a gradual reduction of $5x10^5$ CFU/mL of clinical isolates of MRSA 20651 to 10^3 CFU/mL within 6 h and throughout the remainder of a 24 h period. These results suggest that separated fraction P1 or P5 from crocodile plasma plus cloxacillin combinations have powerful synergistic activity against clinical isolates of MRSA 20651. In fact, these results are also confirmed the synergistic effects finding in checkerboard assay.

4.6 Transmission electronmicroscopy (TEM)

The lowest FIC index, combinations of separated fraction P1 or P5 plus ceftazidime against CREnC 21394 were chosen for transmission electronmicroscopy study. The electronmicroscopic investigations clearly showed that the combination of ceftazidime plus separated fraction P1 or P5 caused damage to ultrastructures of clinical isolates of CREnC 21394. The results of electronmicroscopic study are shown in Figure 4.15-4.20 and the comparison of cell size, n ≥10 cells, was calculated by cell width x cell length (nm^2) is presented in Figure 4.21.

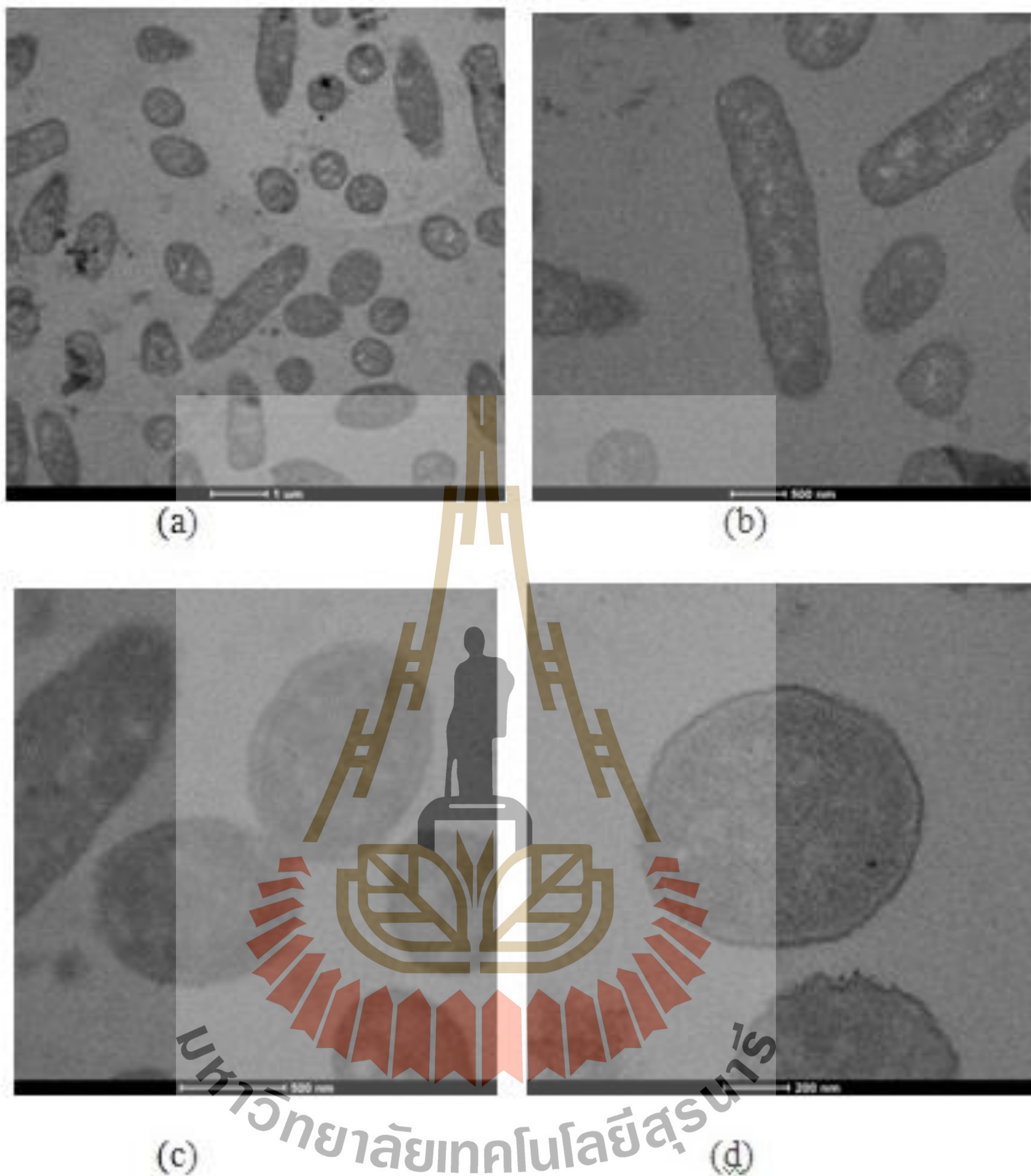

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Figure 4.15 Ultrathin sections of log phase of clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 grown for 4 h in Mueller–Hinton broth: (a), (b), (c) and (d) represent control (no antibacterial agent). x5,000, bar = 1 μm (a); x 9,900, bar = 500 nm (b); x19,500, bar = 500 nm (c); x29,900, bar = 200 nm (d).

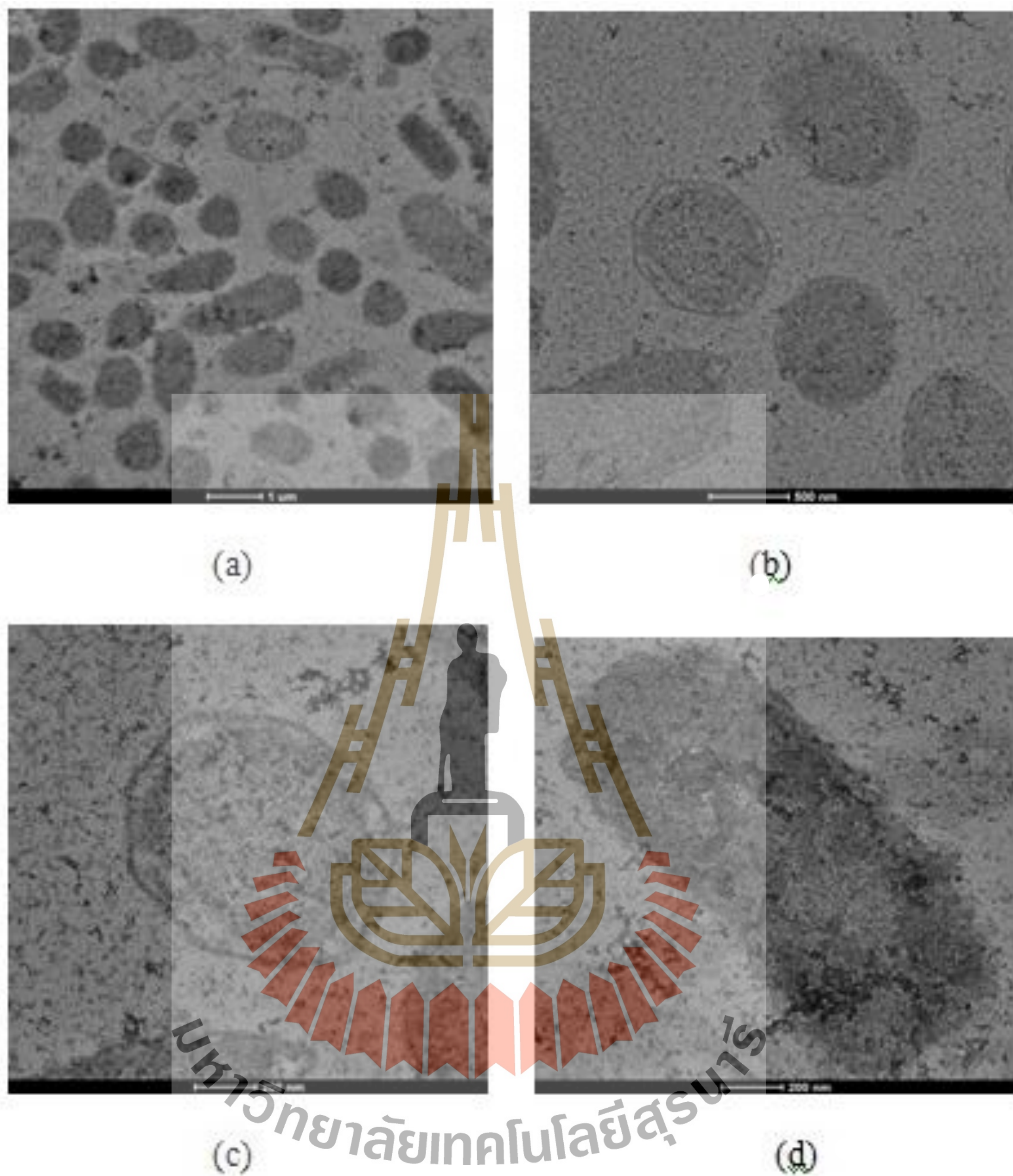

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Figure 4.16 Ultrathin sections of log phase of clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 grown for 4 h in Mueller–Hinton broth: (a), (b), (c) and (d) represent separated fraction P1(512 mg/mL). x5,000, bar = 1 µm (a); x15,000, bar = 500 nm (b); x29,000, bar = 200 nm (c); x29,000, bar = 200 nm (d).

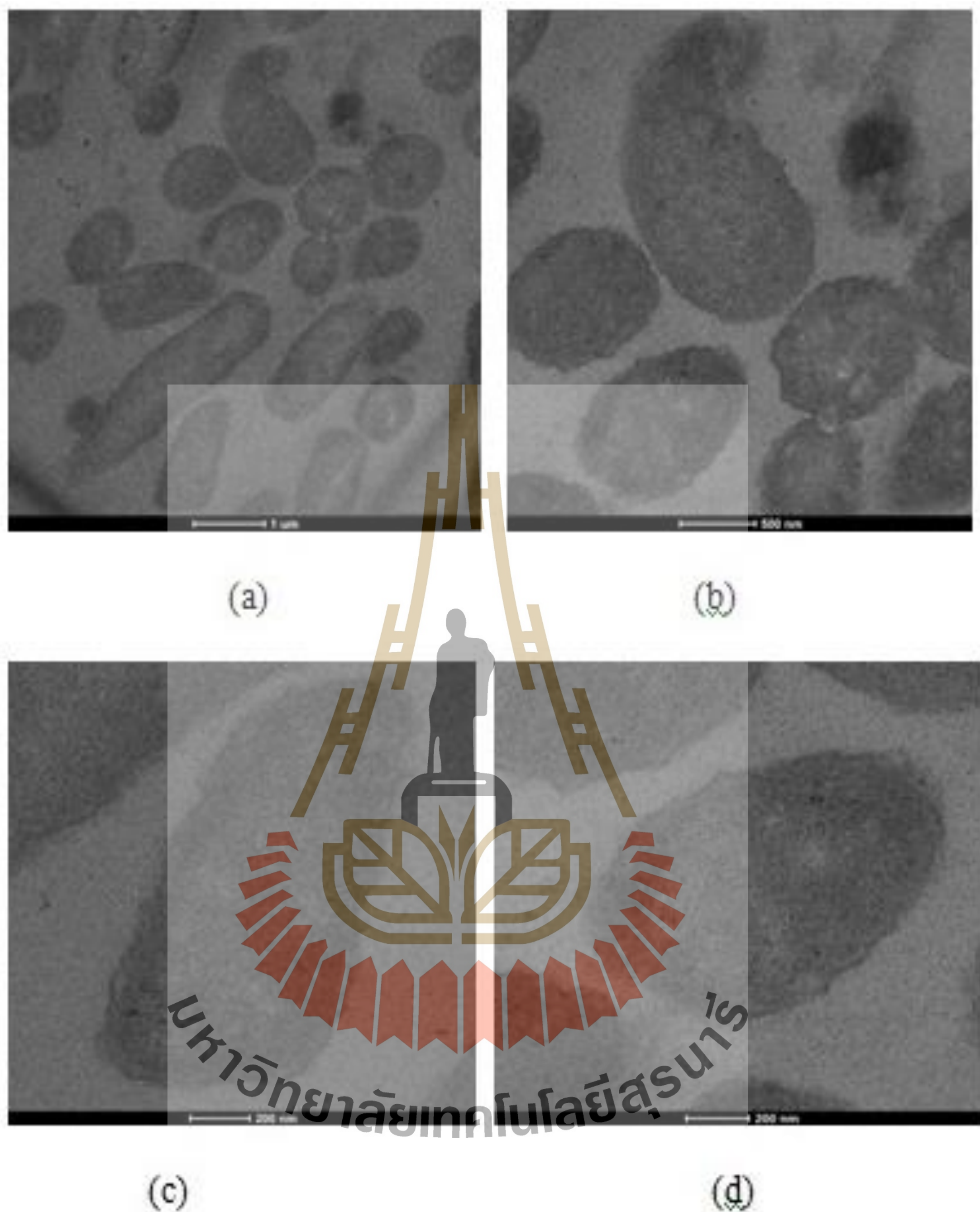

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Figure 4.17 Ultrathin sections of log phase of clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 grown for 4 h in Mueller–Hinton broth: (a), (b), (c) and (d) represent separated fraction P5 (512 mg/mL). x7,000, bar = 1 µm (a); x15,000, bar = 500 nm (b); x29,000, bar = 200 nm (c); x29,000, bar = 200 nm (d).

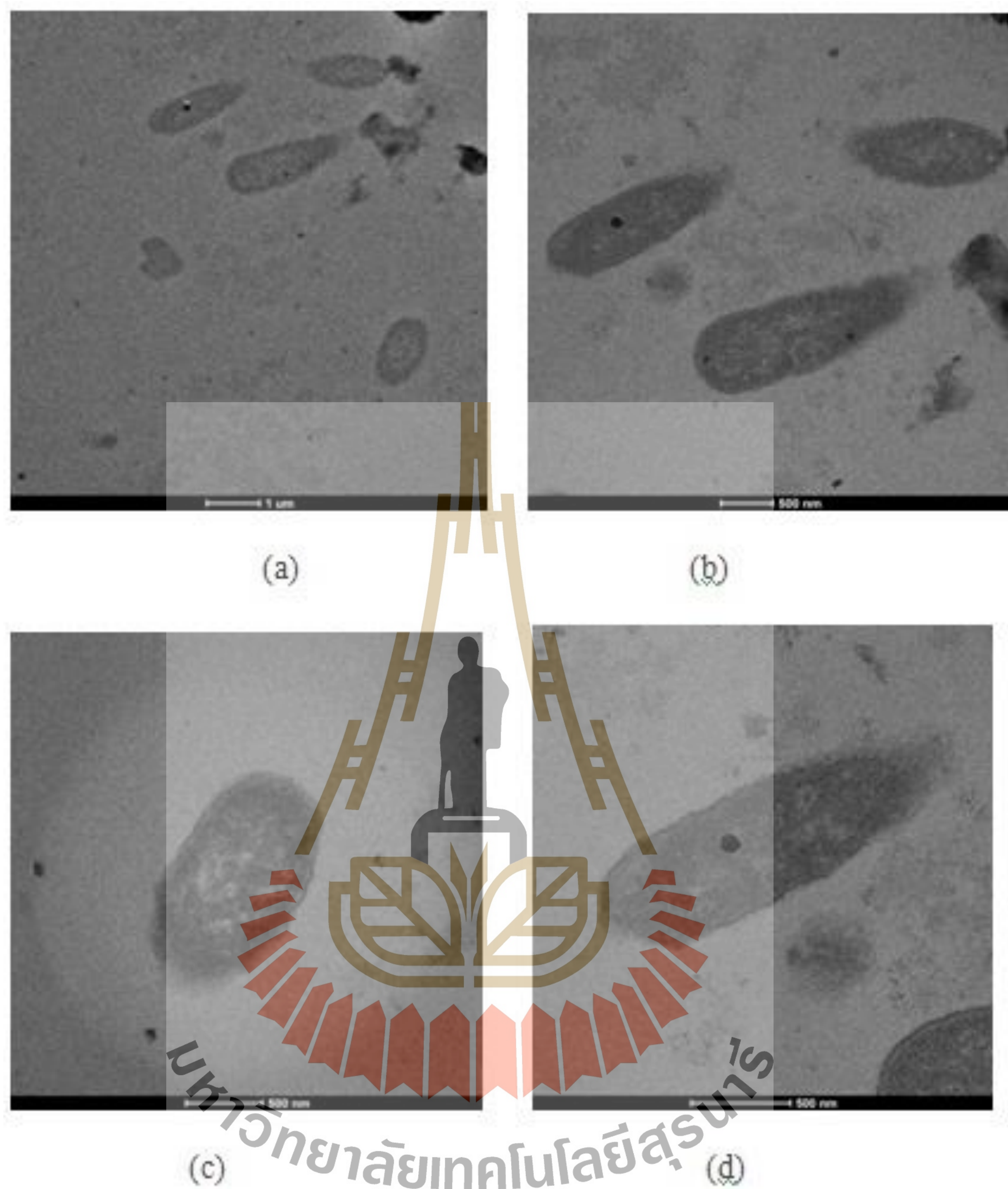

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Figure 4.18 Ultrathin sections of log phase of clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 grown for 4 h in Mueller–Hinton broth: (a), (b), (c) and (d) represent Ceftazidime (16 µg/mL). x5,000, bar = 1 µm (a); x9,900, bar = 500 nm (b); x15,000, bar = 500 nm (c); x19,500, bar = 500 nm (d).

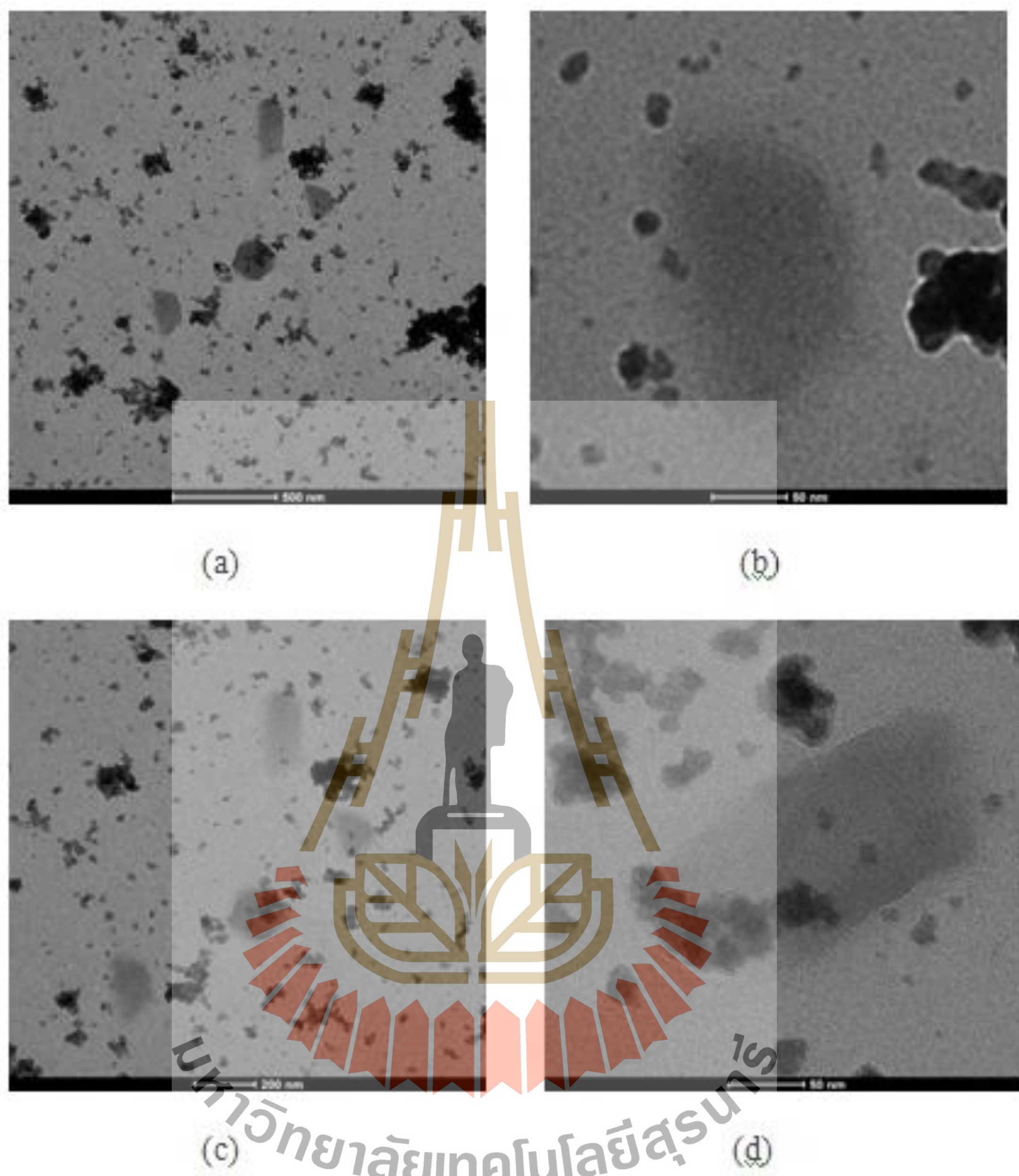

Figure 4.19 Ultrathin sections of log phase of clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 grown for 4 h in Mueller–Hinton broth: (a), (b), (c) and (d) represent Ceftazidime (16 μg/mL) plus separated fraction P1 (32 mg/mL). x5,000, bar = 1 μm (a); x14,500, bar = 50 nm (b); x29,000, bar = 200 nm (c); x14,500, bar = 50 nm (d).

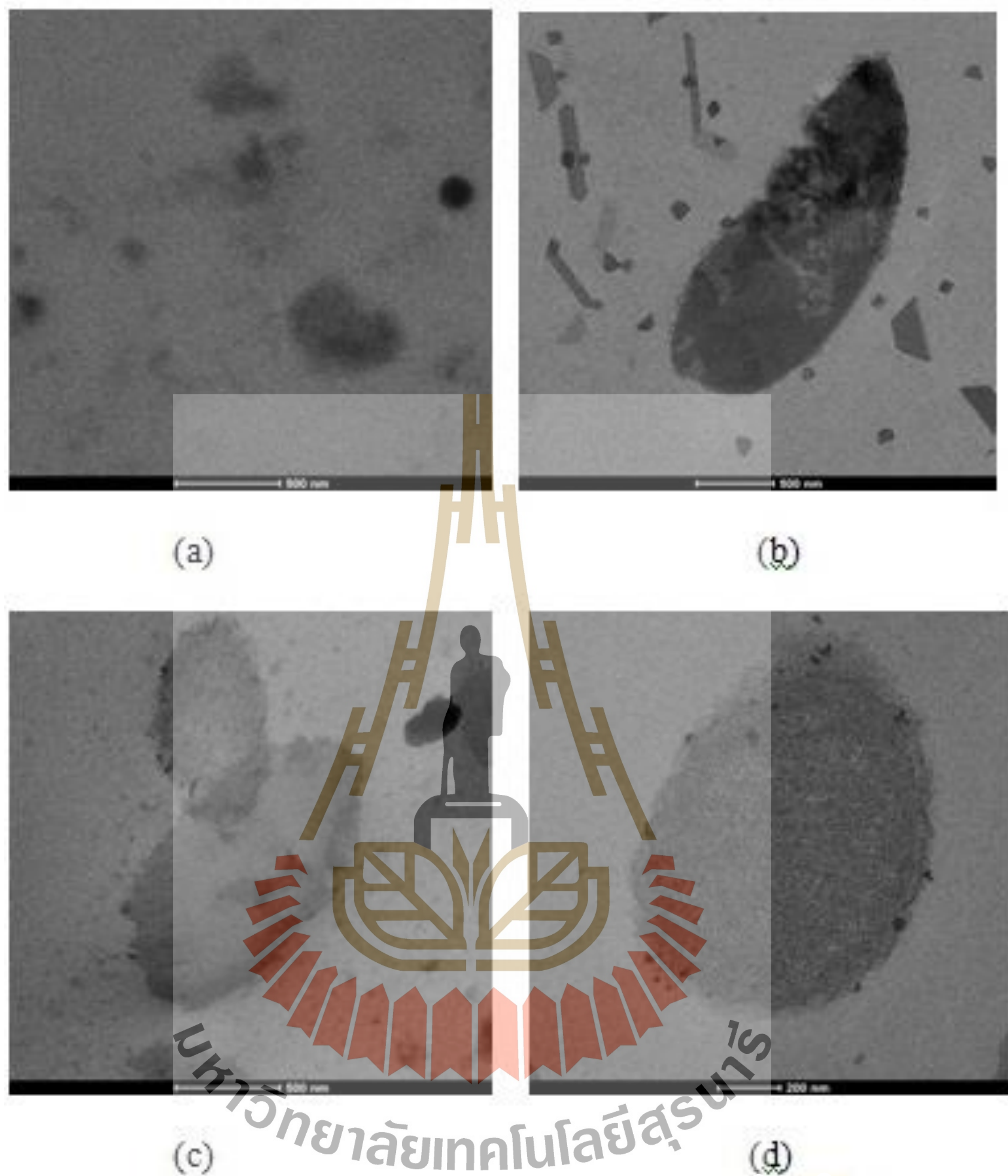

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Figure 4.20 Ultrathin sections of log phase of clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 grown for 4 h in Mueller–Hinton broth: (a), (b), (c) and (d) represent Ceftazidime (16 µg/mL) plus separated fraction 5 (32 ug/mL). x19,500, bar = 200 nm (a); x15,000, bar = 500 nm (b); x19,500, bar = 500 nm (c); x29,000, bar = 200 nm (d).

The morphology of normal log phase cells of clinical isolate of CREnC 21394 strain are presented in Figure 4.15. The cell wall and cytoplasmic membrane can be undoubtedly distinguished. The eletronmicroscopic study for the effect of 512 mg/mL separated fraction P1 on clinical isolate of CREnC 21394 exhibited slightly damage to cell envelope. The periplasmic space of about 40-50% of these cells treated with separated fraction P1 alone seemed to have the of periplasmic space cells broader than control (Figure 4.16). The effects of 512 mg/mL separated fraction P5 against CREnC 21394 are shown in Figure 4.17. The cell shape of about 80-90% of these treated cells appeared slight distortion with the roughed and irregular cell surface. The cell wall or outer membrane seemed slightly damaged in about 60-70% of these cells. Similarly, the effect of 16 μg/mL cetazidime against CREnC 21394 revealed scarce distortion in around 10-20% of the cells. The cell wall or outer membrane damage were also observed (Figure 4.18). The micrographs of log phase cells of clinical isolates of CREnC 21394 strain after treatment with separated fraction P1 at 32 mg/mL plus ceftazidime 16 μg/mL are illustrated in Figure 4.19. Around 70-80% of these treated cells exhibited quite enormous cell shape distortions and markedly damaged to cell envelope. Also, the inner membrane and outer membrane of about 50-60% of these treated cells could not be distinguished. Most of these cells were destroyed and damaged. The similar results with separated fraction P5 at 32 mg/mL plus ceftazidime 16 μg/mL treated cells were also observed, but the effect of this combination seemed lesser effect than cells treated with separated fraction P1 plus ceftazidime (Figure 4.20). Furthermore, the cell size of these cells, n ≥ 10, from micrographs were calculated by measuring cell width multiplied by cell length (μm^2) in order to confirm

the effects of these agents on cell size when used either single or in combination. The results of cell size among groups were compared and shown in Figure 4.21.

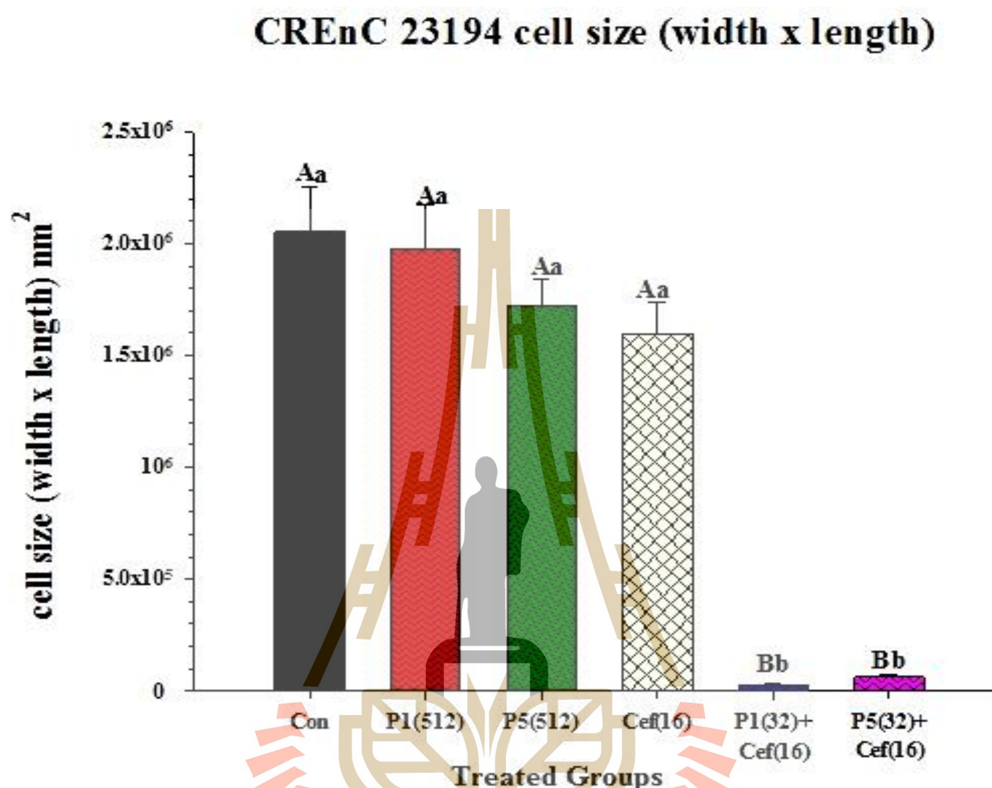

Figure 4.21 The comparison of cell size for cell grown in the presence of either separated fraction P1 (512 mg/mL), P5 (512 mg/mL), ceftazidime (16 µg/mL) alone or in combination. The mean ± SEM for ten or greater, n ≥ 10, treated cells in each group are presented. The graph shows area of cell determined by cell width x cell length (nm^2). The different superscript alphabet are significantly different from each other. Each treated group was compared using one-way ANOVA and Tukey's HSD Post-hoc test, $p<0.05$ (a, small alphabet) and $p<0.01$ (A, capital alphabet) are presented. Con = control, P1(512) = P1 at 512 mg/mL, P5(512) = P5 at 512 mg/mL, Cef(16) = ceftazidime at 16 µg/mL, P1(32)+Cef (16) = P1 at 32 mg/mL + ceftazidime at 16 µg/mL, P5(32)+Cef(16) = P5 at 32 mg/mL + ceftazidime at 16 µg/mL.

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The cell size of clinical isolate CREnC 21394 treated with absence of any antibacterial agents (Control; 2054421.77±197082 nm^2) seemed higher than cells treated with separated fraction P1 (1972789.116±201178 nm^2), P5 (1724561.40±120169 nm^2) and ceftazidime (1597959.18±139628 nm^2) alone, but not significant difference were observed (p>0.05). However, the combination of ceftazidime plus either P1 (27182.24±5841.26 nm^2) or P5 (63045.07±10754.59 nm^2) showed significant dramatically smaller than those of control, separated fraction P1, P5 and ceftazidime alone (p<0.01).

The result of micrographs and cell size from electronmicroscopic study suggest that ceftazidime alone exhibit no serious damage to cell membrane and not significant different in cell size compared to control cells. These results provide evidence that clinical isolate of CREnC 21394 has a high resistant to ceftazidime. Furthermore, previous research has also been reported that ceftazidme alone had no significant effect on ultrastructure of these treated cells, whereas the combination of apigenin and ceftazidime against ceftazidime-resistant *E. cloacae* caused seriously damage to the ultrastructure of these treated cells (Eumkeb and Chukrathok, 2013). Apart from this , there were a wealth of evidence supported the effect of crude plasma and fraction from Siamese crocodile (*Crocolylus siamenis*) plasma on cell membrane of *Salmonella thyphi* and *S. aureus* determined by scanning electronmicroscopy, suggested that crude plasma and fraction caused roughen to cell membrane and cell membrane blebbing formations (Preecharram et al., 2010). Similar results have been reported by Kommanee et al. (2012) that the crude plasma from Siamese crocodile (*C. siamenis*) exhibited ability of inducing breakage and roughness of both reference and clinical isolates of *S. aureus*, *S. typhi*, *E. coli*, *Vibrio cholerae*, *Pseudomonas*

aeruginosa and *Staphylococcus epidermidis*. Therefore, crude plasma from crocodile had antibacterial activity against both gram positive and negative bacteria by membrane disruption. These previous results seem consistent with our present finding that separated fractions from crocodile plasma exhibit weak antibacterial activity and show strong synergistic antibacterial activity with ceftazidime against clinical isolates of CREnC 21394. Interestingly, separated fractions from crocodile plasma have ability to exert synergy effect with ceftazidime by reversing the resistant to be susceptible strain to its primary antibiotic.

4.7 Outer membrane (OM) permeability

The separated fraction P1 and P5 induced OM permeabilization of clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 (CREnC 21394) was carried out by NCF assay. The results of this investigation are presented in Figure 4.22. The OM permeabilization of clinical isolates of CREnC 21394 was done with ceftazidime, separated fraction P1 and P5 at concentrations of 32 µg/mL, 512 mg/mL and 512 mg/mL, respectively when used alone, whereas when used in combination at concentration of ceftazidime 32 µg/mL plus separated fraction P1 32 mg/mL, and ceftazidime 32 µg/mL plus separated fraction P5 32 mg/mL. Polymixin B (PMX) 7 µg/mL was used as permeabilizing probe and nitrocefin 20 µg/mL was used as a substrate for β-lactamase, which localized within periplasm. The results for OM permeabilization with nitrocefin assay revealed that separated fraction P1 and P5, and ceftazidime alone caused slight alteration of the OM permeability of clinical isolates of CREnC 21394 compared to control (p<0.01).

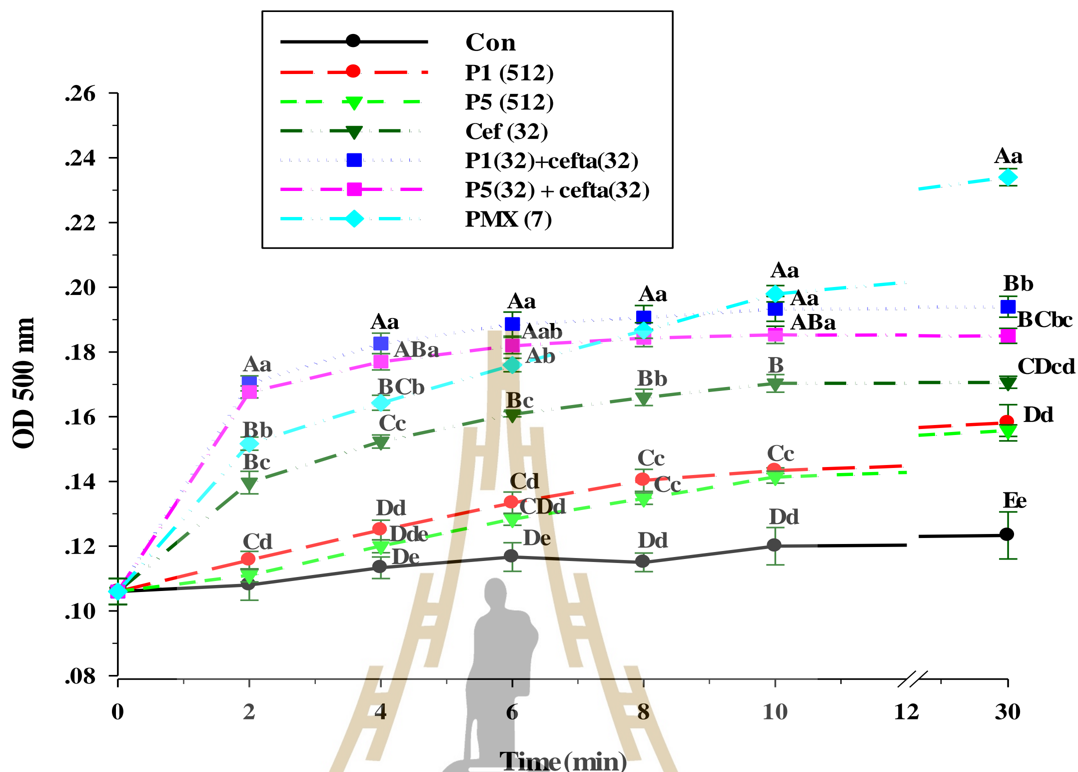


Figure 4.22 Outer membrane permeabilization of clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 by either separated fraction P1 (512 mg/mL), P5 (512 mg/mL) and ceftazdime (32 µg/mL) alone or in combination and lysis caused by subsequent treatment with 20 µg/mL of NCF and 7µg/mL of PMX. The bars represent the standard deviations of three replicates. All data are expressed as mean ± SEM. The different superscript alphabet are significantly different from each other. Each treated group was compared using one-way ANOVA and Tukey's HSD Post-hoc test, $p<0.05$ (a, small alphabet) and $p<0.01$ (A, capital alphabet) are presented. Con = control, P1(512) = P1 at 512 mg/mL, P5(512) = P5 at 512 mg/mL, Cef(32)= ceftazidime at 32 µg/mL, P1(32)+Cef(32) = P1 at 32 mg/mL + ceftazidime at 32 µg/mL, P5(32)+Cef(32) = P5 at 32 mg/mL + ceftazidime at 32 µg/mL. The bars represent the standard deviations of 3 replicates.

The results of either P1 (512 mg/mL), P5 (512 mg/ml), ceftazidime (32 µg/mL) alone or in combination exhibited significantly increased OM permeabilization of CREnC 21394 compared to control (p<0.01). Moreover, the combination of P1 (32 mg/mL) plus ceftazifime (32 µg/mL) exhibited greater significantly increased in OM permeabilization of CREnC 21394 than those of P1, P5 and ceftazidime alone (p<0.01), but did not reveal significant difference compared to P5 (32 mg/mL) in combination with ceftazidime (32 µg/mL) (p>0.01). The OM permeabilization of all treated groups were significantly lower than PMX (p<0.01) (Figure 4.22). These results seem correspondence with previous findings that the outer membrane of ceftazidime-resistant *E. cloacae* was significantly altered by ceftazidime plus apigenin combination (Eumkeb and Chukrathok, 2013) as well as peptide–peptide nucleic acid conjugate (Eriksson et al., 2002). The results of separated fractions in increasing OM permeabilization of CREnC 21394 in the present study may be assumed that cationic peptide in separated fractions may involve either hydrophilic interactions with polysaccharide core of lipopolysaccharide (LPS) or electrostatic interactions causing disturbance of polar core region and interference of saccharide-saccharide interaction (Junkes et al., 2008; 2011). The mechanisms of PMX and other cationic antimicrobial peptides are believed that these act as a competitor for magnesium ion binding sites within the LPS layer as part of their membrane-permeabilizing activities (Hancock, 1997). Similarly, cyclic cationic antibacterial peptides containing lipophilic and hydrophilic groups that binds to lipid A can interfere LPS functions (Cardoso et al., 2007). In addition, the novel antibacterial peptide Leucrocin I and Leucrosin II isolated from white blood cells of *C. siamensis* have been reported that these peptides showed ability to combat *S.*

epidermidis, *S. typhi* and *V. clolerae* by increasing outer membrane permeability of these tested strains (Pata et al., 2011). In particular, separated protein fractions from crocodile plasma used in this study were obtained by ion exchange and gel infiltration, thus cationic peptides were obtained and may play an important role in increase OM permeability. Therefore, the present study is in substantial agreement with those of previous studied. In addition, these findings indicate that increase in OM permeability in CREnC 21394 caused by either separated fraction alone or in combination with ceftazidime may exert its effects through one of several important mechanism of actions and leads to cell lysis.

4.8 Cytoplasmic membrane (CM) permeability

The separated fraction P1 and P5 induced CM permeabilization of clinical isolates of CREnC 21394 was evaluated by measuring the access of Ortho-Nitrophenyl-*β*-Galactoside (ONPG) to cytoplasm as well described in Chapter 3. The results of this assay are illustrated in Figure 4.23. The CM permeabilization of clinical isolates of CREnC 21394 was performed with ceftazidime, separated fraction P1 and P5 at concentrations of 32 µg/mL, 512 mg/mL and 512 mg/mL, respectively when used alone. Besides, the combination of ceftazidime 32 µg/mL plus separated fraction P1 32 mg/mL, and ceftazidime 32 µg/mL plus separated fraction P5 32 mg/mL were used. Polymixin B (PMX) at concentration of 7 µg/mL was used as permeabilizing probe and ONPG 100 µg/mL was used as a substrate for *β*-galactosidase, which localized within cytoplasm. In normal cell, ONPG cannot enter through inner membrane, if cell damage the ONPG can be cleaved by *β*-galactosidase. The results of CM permeabilization showed that separated fraction P1 or ceftazidime alone

caused alteration of the CM permeability of clinical isolates of CREnC 21394, whereas not significant difference were observed in separated P5 compared to control ($p>0.01$, $p>0.05$) .

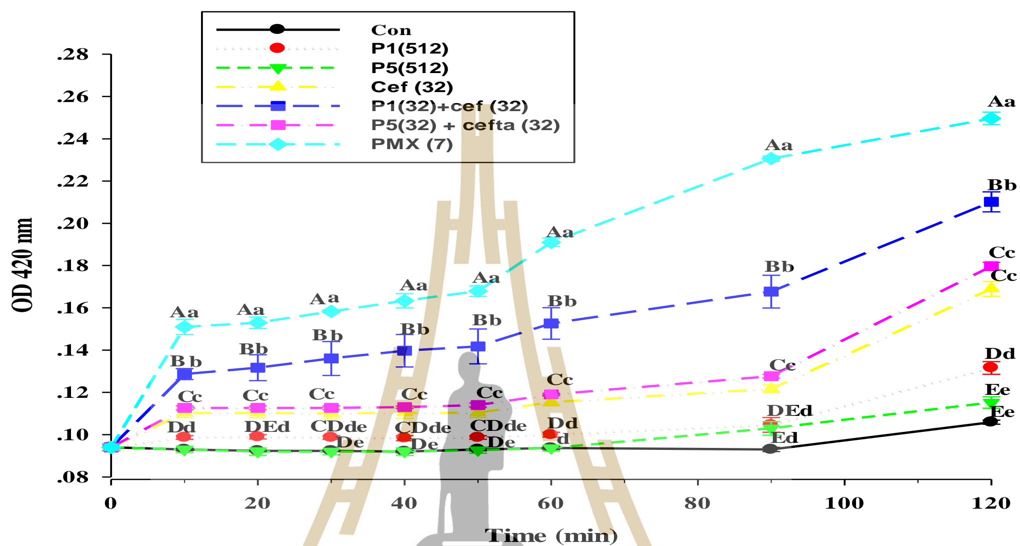


Figure 4.23 Cytoplasmic membrane permeabilization of clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 either separated fraction P1 (512 mg/mL), P5 (512 mg/mL), ceftazidime (32 µg/mL) alone or in combination and lysis caused by subsequent treatment with 100 µg/mL of ONPG and 7µg/mL of PMX. The bars represent the standard deviations of three replicates. All data are expressed as mean ± SEM. The different superscript alphabet are significantly different from each other. Each treated group was compared using one-way ANOVA and Tukey's HSD Post-hoc test, $p<0.05$ (a, small alphabet) and $p<0.01$ (A, capital alphabet) are presented. Con = control, P1(512) = P1 at 512 mg/mL, P5(512) = P5 at 512 mg/mL, Cef(32) = ceftazidime at 32 µg/mL, P1(32)+Cef(32) = P1 at 32 mg/mL + ceftazidime at 32 µg/mL, P5(32)+Cef(32) = P5 at 32 mg/mL + ceftazidime at 32 µg/mL. The bars represent the standard deviations of 3 replicates.

Interestingly, the results of the ceftazidime plus either separated fraction P1 or P5 combination exhibited markedly significant difference of CM permeabilization alteration of CREnC 21394 compared to control. In addition, ceftazidime or P1 alone exhibited gradually significant higher than control (p<0.01). However, the P5 alone did not reveal significant difference from control. The CM permeabilization of PMX was significantly higher than control and those of all treated groups (p<0.01) (Figure 4.23). This hyper-permeability of IM caused by separated fractions may due to leakage of ions and extensive loss of other cellular contents, including the intracellular proteins resulting in cell death. These results are consistent with crystal violet assay that eugenol increased the permeability of *S. typhi* membrane results in the deformation of macromolecules in the membrane (Devi et al. 2010). The results of present study are also consistent with previously research that the inner membrane of ceftazidime-resistant *E. cloacae* was significantly altered by ceftazidime plus apigenin combination (Eumkeb and Chukrathok, 2013) and peptide–peptide nucleic acid conjugate (Eriksson et al., 2002). The Mechanisms of PMX and other cationic antimicrobial peptides are believed that these agents act as a competitor for magnesium ion binding sites within the LPS layer as part of their membrane-permeabilizing activities (Hancock, 1997). In the same way, previous study reported the novel antibacterial peptides Leucrocin I and Leucrosin II isolated from white blood cells of *C. siamensis* to possess potential antibacterial activity against *S. epidermidis*, *S. typhi* and *V. clolerae* by targeting membrane sites and causing increase of inner membrane permeability of these tested strains (Pata et al., 2011). In this case, separated protein fraction from crocodile plasma used in this study obtained by ion exchange and gel infiltration, thus cationic peptides were occurred. These

cationic peptides may play an important action in increase CM permeability similar to previous outcomes. So, these findings lead us to believe that either separated fraction alone or in combination with ceftazidime increase CM permeability of CREnC 21394 because these separated fractions may exert its effects through one of several important mechanism of actions and leads to cell lysis.

4.9 Electrophoresis

SDS-PAGE was carried out in order to examine the effect of antibacterial characteristic from separated fraction P1 and P5 when used alone and in combination with ceftazidime on outer membrane and peptidoglycan associated protein (OM-PG). The result of SDS-PAGE is shown in Figure 4.24.

The OM-PG associated protein bands of clinical isolates of ceftazidime-resistant *E. cloacae* after treatment with separated fractions from crocodile plasma, ceftazidime alone and drug free (control) presented the main proteins band at MW 25 kDa of lane S1-S6. Although, either ceftazidime alone or in combination with P1 bands appeared slightly paler than others. In addition, the 35 and 45 kDa protein bands of ceftazidime plus either P1 or P5 were also minimally paler than control. The results form SDS-PAGE of the present study suggest that OM-PG associated protein synthesis of this strain may be interfered by the combination of ceftazidime plus either P1 or P5.

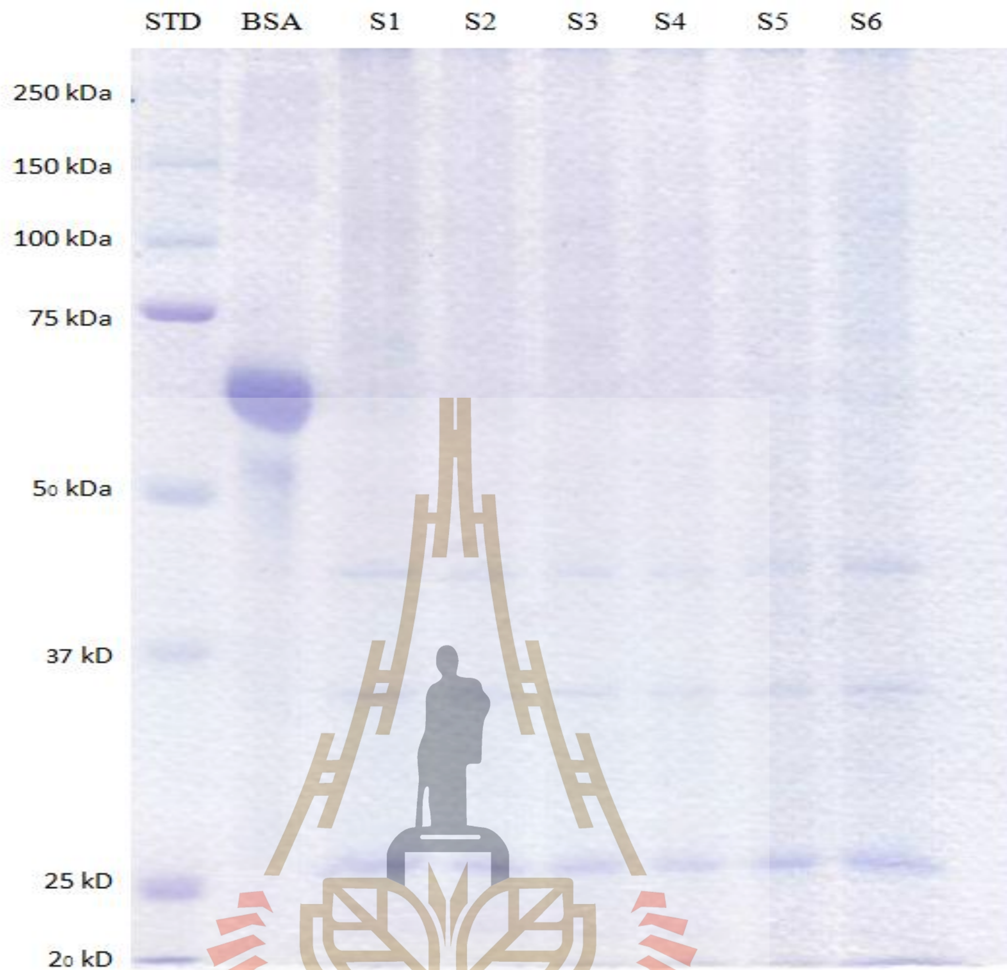


Figure 4.24 SDS-PAGE showing the outer membrane and peptidoglycan associated protein (OM-PG) of clinical isolates of ceftazidime-resistant *E. cloacae* DMST 21394 grown in the absence of drug (control; lane S1, 1.103 mg/mL), separated fraction P1 512 mg/mL (lane S2, 0.816 mg/mL), separated fraction P5 512 mg/mL (lane S3, 0.017 mg/mL), Ceftazidime 16 µg/mL (lane S4, 0.366 mg/mL), ceftazidime 16 µg/mL plus separated fraction P1 32 mg/mL (lane S5, 0.654 mg/mL), ceftazidime 16 µg/mL plus separated fraction P5 32 mg/mL (lane S6, 0.632 mg/mL), BSA; standard bovine serum albumin and std; molecular weight marker proteins (kDa).

These results may imply that the combination of either P1 or P5 plus ceftazidime may interfere OM-PG associated protein synthesis of this strain. These findings may compare to previous research that flavonoids isolated from small galangal in combination with amoxicillin and ceftazidme plus apigenin showed ability to inhibit peptidoglycan synthesis of amoxicillin-resistant *E. coli* and ceftazidime-resistant *E. cloacae,* respectively (Eumkeb et al., 2012; Eumkeb and Chukrathok, 2013).

4.10 Enzyme assay

The ability of separated fraction P1 and P5 from crocodile plasma when used alone and in combination with ceftazidime to inhibit β-lactamase type IV isolated from *E. cloacae* activity was assessed with ceftazidime, separated fraction P1 and P5 alone at concentrations of 32 µg/mL, 512 mg/mL and 512 mg/mL, respectively, whereas the combination of ceftazidime 32 µg/mL plus separated fraction P1 32 mg/mL, and ceftazidime 32 µg/mL plus separated fraction P5 32 mg/mL were performed. Figure 4.25 illustrates that the combinations of separated fraction P1 or P5 with ceftazidime have significant inhibitory activity against β-lactamase type IV compared to control and those of separated fraction P1, P5, and ceftazidime alone ($p<0.01$). The combination of separated fraction P1 plus ceftazidime exhibited significantly highest inhibitory β-lactamase type IV activity compared to others ($p<0.01$). However, there were slightly different among control, separated fraction P1, P5 and ceftazidime when used singly were observed ($p>0.01$).

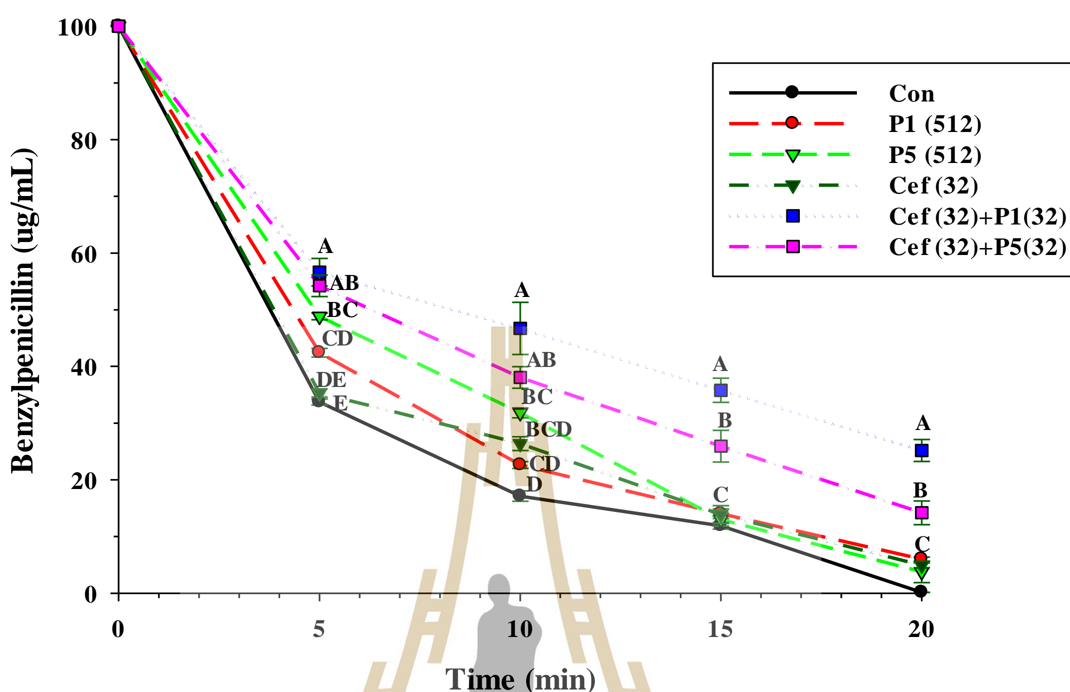
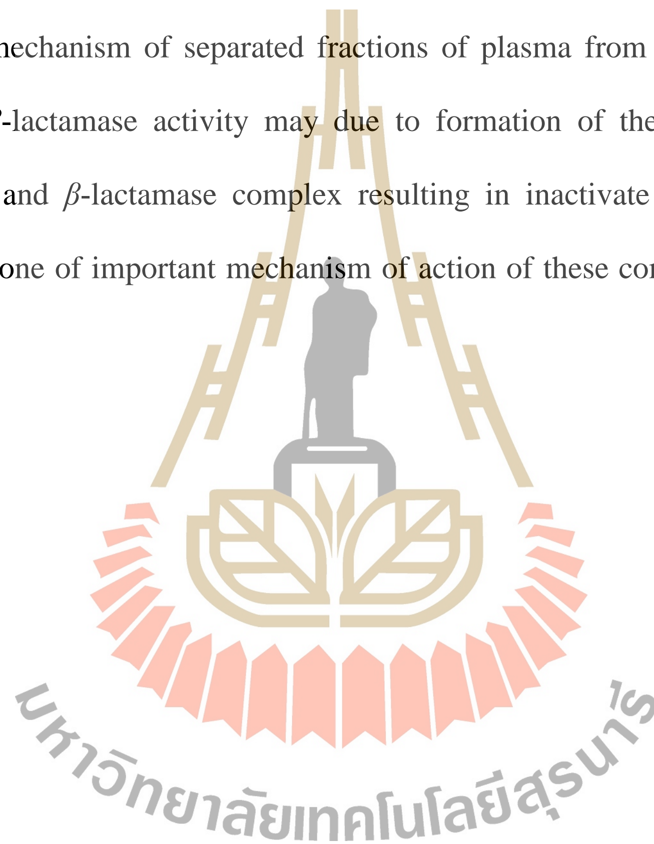


Figure 4.25 Inhibitory activity of separated fraction P1, P5 and ceftazidime when used either alone or in combination in hydrolization of bezylpenicillin by *β*-lactamase type IV obtained from *E. cloacae*. The bars represent the standard deviations of three replicates. All data are expressed as mean ± SEM. The different superscript alphabets are significantly different from each other. Each treated group was compared using one-way ANOVA followed by Tukey's HSD Post-hoc test, $p<0.01$ (A, capital alphabet) are presented. Con = control, P1(512) = P1 at 512 mg/mL, P5(512) = P5 at 512 mg/mL, Cef(32) = ceftazidime at 32 µg/mL, Cef(32)+P1(32) = ceftazidime at 32 µg/mL + P1 at 32 mg/mL, Cef(32)+P5(32) = ceftazidime at 32 µg/mL + P5 at 32 mg/mL. The bars represent the standard deviations of 3 replicates.

β-lactamase has been reported as one of the major resistant mechanism of bacteria to *β*-lactam antibiotics (Tenover, 2006). This study establishes substantial

evidence that the combination of ceftazidime plus either P1 or P5 show inhibitory activity of β-lactamase type IV isolated from *E. cloacae*. Also, these combinations could be offered for the development of novel antibacterial agents. Interestingly, the antibacterial combination approach has been proven by several researches to be an interesting avenue to combat drug resistant bacteria (Eumkeb and Chukrathok, 2013; Eumkeb et al., 2010; Eumkeb et al., 2012; Wagner, 2011; Worthington and Melander, 2013). The mechanism of separated fractions of plasma from Siamese crocodile in inbition of β-lactamase activity may due to formation of these separated fraction combination and β-lactamase complex resulting in inactivate β-lactamase activity. This may be one of important mechanism of action of these combination against this strain.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CHAPTER V

CONCLUSIONS

Multidrug resistance in microorganisms has surprisingly emerged to be one of the greatest threats to human health worldwide. Therefore, the developments of novel antimicrobial agents and new approaches to combat these drug resistant microorganisms are urgently necessary. The high resistant level of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), or extended *β*-lactamases (ESBL)-producing gram-negative rods have been dramatically reported (Emori and Gaynes, 1993; Leclercq and Courvalin, 1997; Moellering, 1998; Vonberg et al., 2008; Moellering, 2009). In Thailand, the emergence of antimicrobial resistant has also been considerably documented including in many sections of Maharat Nakhon Ratchasima Hospital (Chokejindachai, 2007; Maharat Nakhon Ratchasima hospital, 2012). Furthermore, MRSA, *E. coli*, *S. eidermidis*, *E. cloacae* and *E. faecium* are the most common microorganisms causing infections and resistance to antibiotic of these strains have also been reported (Maharat Nakhon Ratchasima hospital, 2012). Consequently, the practically-prescribed antibiotics to treat these strains are ineffectively used. So, explorations of new alternative antimicrobial drugs for treatment of drug resistant bacteria are immediately required. One of interesting approaches is to find out the novel active ingredients from plants or animals for treatment drug resistant bacteria. Interestingly,

the use of combinations drugs to exert synergy effect has been proven by several studies to overcome drug resistant bacteria. For example, flavonoids isolated from smaller galangal plus amoxicillin combinations against amoxicillin-resistant *Escherichia coli*, galangin plus ceftazidime combinations against β-lactam antibiotic-resistant *S. aureus* and ceftazidime plus apigenin combination against ceftazidime-resistant *Enterobacter cloacae* (Eumkeb et al., 2010; 2012; 2013). Fortunately, the substances from wild crocodiles such as serum from American alligator (*Alligator mississippiensis*) have been reported as much broader spectrum of antibacterial activity against both gram positive and negative bacteria as well as herpes simplex virus type-1, human immunodeficiency virus type-1 and West Nile virus (Merchant et al., 2004). In addition, the peptides from Siamese crocodile (*Crocodile siamenis*) showed antibacterial activity against *Salmonella typhi*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *E. coli*, *Pseudomonas aeroginosa and Vibrio cholerae* (Thammasirirak and Daduang, 2004; Preecharram et al., 2008). However, no work has been done on the effect of Siamese crocodile plasma on drug resistant bacteria such as MRSA, *E. coli* and *E. cloacae*. Therefore, the purpose of this thesis was to examine the antibacterial activity of Siamese crocodile plasma (*C. siamensis*) against these resistant bacterial strains used singly and in combination with β-lactams antibiotic.

The plasma of Siamese crocodiles were separated using ion exchanged chromatography with Q Sepharose and gel filtration chromatography with Sephadex G-50 to give five separated fractions and each fraction was further confirmed its MW using SDS-PAGE. The five plasma protein bands of P1-P5, that appeared MW range between 23-160 kDa, were separated, whereas these results are presumably similar to

previous study that used hold serum occurred the range of serum protein bands at MW between 23-160 kDa (Threenet et al., 2011).

MIC results exhibited that there were strongly resistant to ceftazidime and cloaxacillin, MIC > 1024 µg/mL, in clinical isolated ceftazidime-resistant *E. cloacae* DMST 21394 and Methicillin-resistant *S. aureus* DMST 20651 (MRSA 20651), respectively. Besides, all separated protein fractions revealed very weak activity against these tested strains (MICs 8 - >1024 mg/mL). These results are similar to previous findings that crude plasma from Siamese crocodile (*Crocodylus siamensis*) had antibacterial activities against *S. aureus*, *S. epidermidis*, and *E. coli* with MICs between 10.4-50.0 µg/mL and the diameter of the clear zone about 5 mm (Thammasirirak and Daduang, 2004; Preecharram et al., 2008; 2010; Kommanee et al., 2012). The weak antibacterial activity of these separated fractions of Siamese crocodile plasma may probably because of polypeptide in plasma (Preecharram et al., 2010). To investigate synergistic activity, the checkerboard assay was performed. The results revealed that strong synergistic antibacterial activity were observed in the combination of both P1 and P5 in combination with either ceftazidime or cloxacillin against CREnC 21394 (FIC index 0.062) or MRSA 20651 (FIC index 0.375), respectively. The cationic antibacterial peptides from Siamese crocodile have been previously reported to play a dominant role in combating gram positive and negative bacteria (Thammasirirak and Daduang, 2004; Preecharram et al., 2008) as well as the synergistic interaction between peptides were also reported (Yan and Hancock, 2001). Apart from this, A synergistic inhibitory effect (FICI 0.5) was observed when resistant variants were treated with peptide/antibiotic combinations. The combination of peptide/antibiotic showed the synergistic inhibitory effect (FICI 0.5) on resistant

variants *Pseudomonas fluorescens*. So, the use of antibiotics in medical applications could allow be reduced (Naghmouchi et al., 2012). Our findings lead us to believe that separated protein fractions, comprises of cationic peptides, plays an important role in synergistic activities with these β-lactams against these resistant strains similar to previous report (Naghmouchi et al., 2012).

After all, the killing curve was executed to confirm checkerboard assay. These results revealed that synergistic activity was observed in the combination of separated fraction P1 and P5 plus either ceftazidime or cloxacillin counter CREnC 21394 or MRSA 20651, respectively by dramatic reduction of $5x10^5$ CFU/mL of these strains to 10^3 CFU/mL within 6 h and throughout the remainder of 24 h. Also, synergistic activity was demonstrated with the combination of ceftazidime plus tested flavonoids to conquer MRSA (Eumkeb and Chukrathok, 2013).

To investigate primary mechanism of actions of these combinations, the synergistic activity that revealed the lowest FICI was chosen. The transmission electronmicroscopic study (TEM), OM and CM (IM) permeabilization, OM-PG associated protein alteration using SDS-PAGE and enzyme assay were performed. TEM results demonstrated that about 40-50% of P1 and 80-90% of P5 treated cells alone displayed periplasmic space broader than control and cell wall seemed slightly damaged, respectively. Obviously, about 70-80% of P1 plus ceftazidime combination revealed cell shape distortion, markedly damaged to cell envelope, the inner membrane and outer membrane could not be distinguished. Likewise, the lesser effects than these were manifested after exposure to P5 plus ceftazidime. Furthermore, the synergistic activities of these combinations exhibited significant undoubtedly smaller cell size than control and these agents treated alone (p<0.01).

These results are in substantial correspondence with those of Preecharram et al. (2010) and Kommanee et al. (2012) that crude plasma from Siamese crocodile (*C. siamenis*) revealed to deform and roughen of the cell membranes, causing formation of blebs on the cell surface of *S. aureus*. These findings lend support to the assumption that separated fraction P1 and P5 alone may damage bacterial membranes. In addition, these fractions plus ceftazidime may exert synergistic activity with this drug to inhibit peptidoglycan synthesis and destroy bacterial membrane results in cell shape distortion and cell envelope damage.

The assumption that bacterial membrane may be damaged by either P1 and P5 treated alone or in combination with ceftazidime from TEM results was proved by OM and CM permeabization methods. The OM and CM permeability results were confirmed that OM and CM were increased permeabilization after exposure to P1 and P5 alone compared to control. Obviously, these fractions plus ceftazifime displayed greater significant increase in OM and CM permeabilization than others (p<0.01).

These results seem in agreement with previous research that peptide-peptide nucleic acid (PNAs) increased *E. coli*-permeabilizing nearly as quickly as antimicrobial peptides (Eriksson et al., 2002). So, the results of this study provide evidence that either separated purified fraction alone or in combination with ceftazidime increase OM and CM permeability of this strain and may exert its effects through one of several important primary mechanism of actions and leads to cell lysis.

The OM-PG associated protein band alterations of this strain after exposure to these fractions alone and plus ceftazidime were investigated by SDS-PAGE. These results disclosed that the 35 and 45 kDa protein bands of ceftazidime plus either P1 or

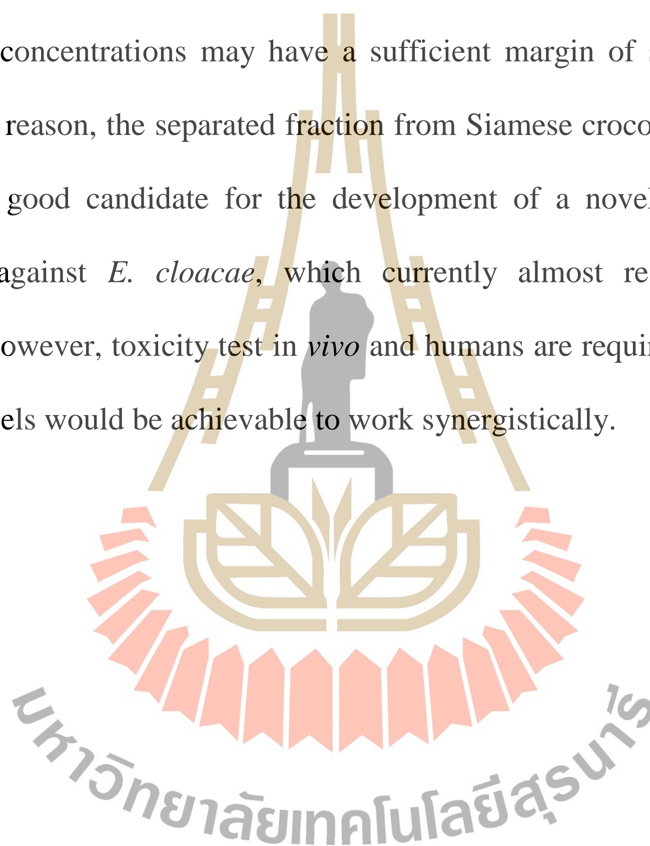
P5 were slightly paler than control. This result imply that OM-PG associated protein synthesis of this strain may be interfered by these combinations.

What is more, the effect of these agents on β-lactamase type IV from *E. cloacae* was studied. The results clearly exhibited that β-lactamase was significantly inhibited by the combinations of P1 or P5 plus ceftazidime compared to control and others (p<0.01). The combination of P1 plus ceftazidime displayed significantly highest β-lactamase inhibitory activity compared to others (p<0.01). This β-lactamase inhibitor activity may due to these combinations should form complex with β-lactamase resulting in inactivate β-lactamase activity. This may be one of important primary mechanism of action of these combination against this strain.

Although there is a limited research evidence regarding on active MW of either crude or fractions plasma from Siamese crocodile in combating bacterial infection, but antibacterial activity of Siamese crocodile serum has been reported at MW less than 1 kDa (Preecharram et al., 2008). Partially purified plasma, crocosin, isolated from Siamese crocodile, has been identified to possess antibacterial activity, but the active MW of it has not been characterized. It has been reported only its amino acid sequences (Preecharram et al., 2008). These results demonstrated that P1 plus ceftazidime showed stronger synergistic antibacterial activity than P5 plus this drug. Therefore, this study provides the first evidence that proteins at MW 67 and/or 80 kDa of P1 may play a dominant role in combating this strain. While the protein bands at MW 80 kDa did not show in P5. So, synergistic activity of P5 might be resulting from protein at MW 23 kDa and/or other bands.

In conclusion, antibacterial and synergistic activities of separated protein fractions in combination with ceftazidime may involve three elementary mechanism

of actions. Firstly, Siamese crocodile plasma shows synergistic effect with ceftazidime and may exert to inhibit cell wall synthesis leads to cell shape distortion and cell envelope damage. Secondly, Increase in OM and CM permeability in this strain. Thirdly, β-lactamase inhibition. Furthermore, the OM-PG associated protein synthesis may be interfered leads to slight paler protein bands at 35 and 45 kDa than control. The separated fractions P1 and P5 from Siamese crocodile plasma at these combination concentrations may have a sufficient margin of safety for therapeutic use. For this reason, the separated fraction from Siamese crocodile plasma would be offered as a good candidate for the development of a novel valuable adjunct to ceftazidime against *E. cloacae*, which currently almost resistant to practically antibiotics. However, toxicity test in *vivo* and humans are required. If possible, blood and tissue levels would be achievable to work synergistically.



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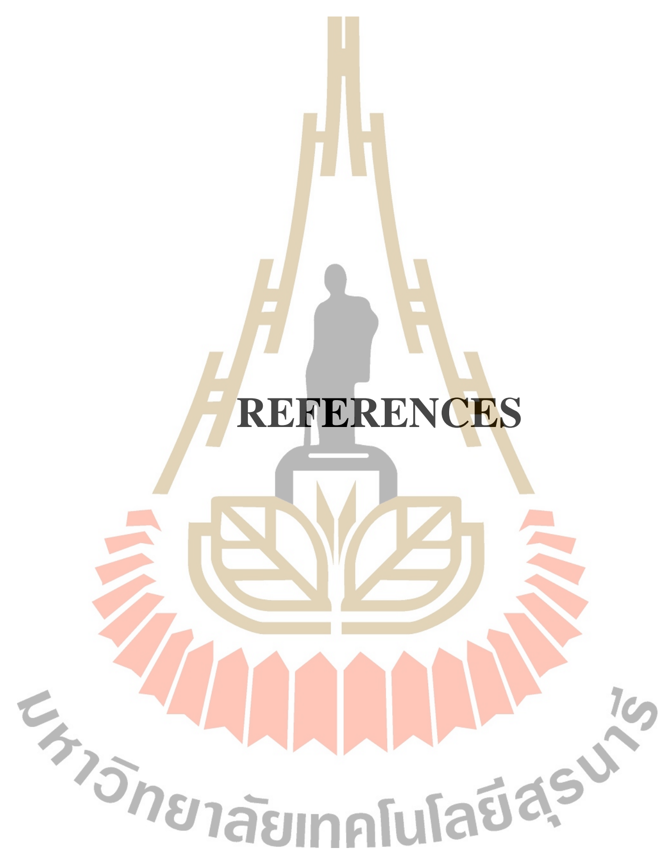

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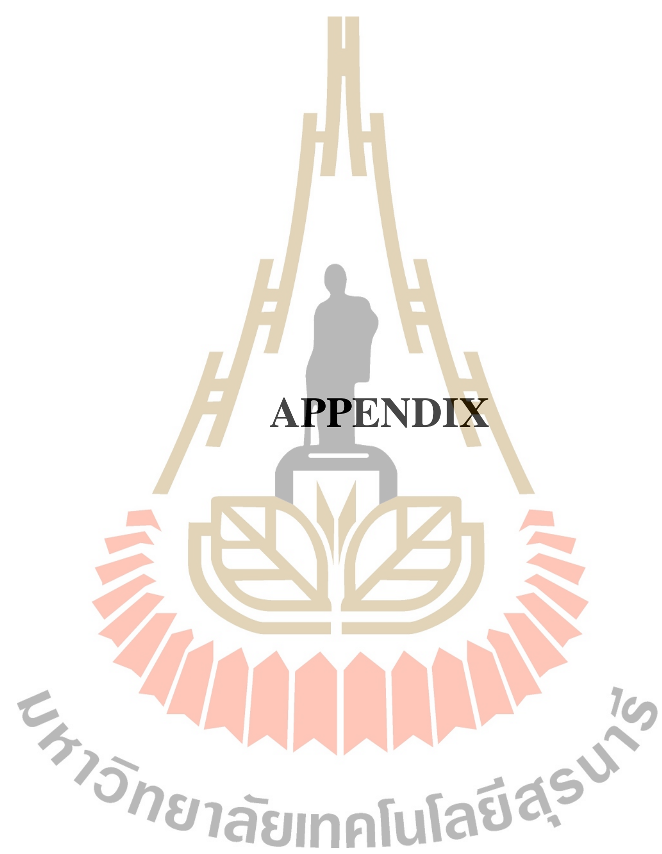
APPENDIX



APPENDIX

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

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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

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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

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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

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Spectraphysics	Agilent
Micro titer plate (96 wells)	Bio-Rad
xMark™ Microplate Absorbance Spectrophotometer	Bio-Rad

3.2 Glassware

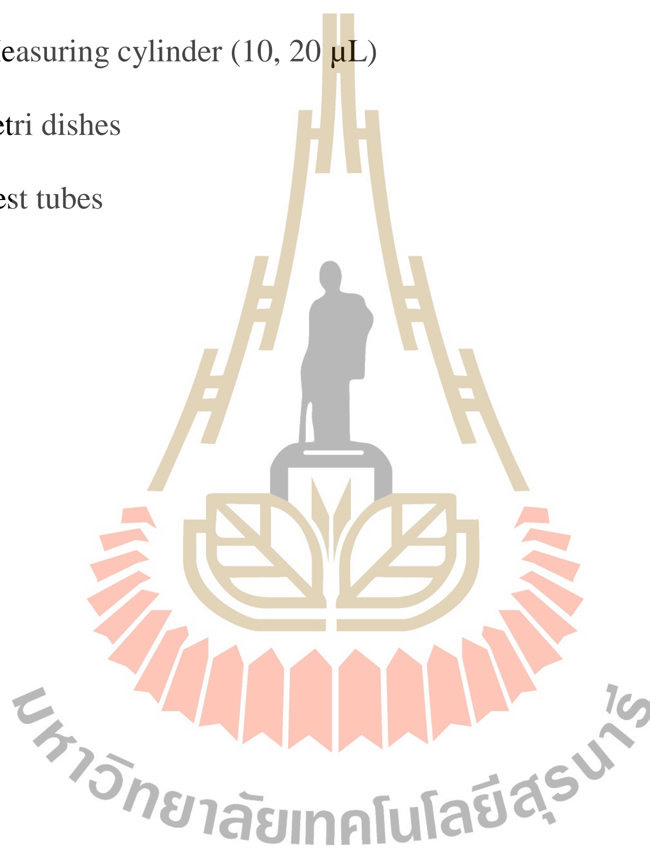
Beakers (50, 100, 250, 500, 1000 mL)

Pipettes (1, 5, 10 μL)

Measuring cylinder (10, 20 μL)

Petri dishes

Test tubes



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Publication

มหาวิทยาลัยเทคโนโลยีสุรนารี

1. Eumkeb, G., Chaisena, N., Rojtinnakorn, N., Siriwong, S., and Aphai Duangkham, A. (2008). Reversing β-Lactam Antibiotic Resistant Bacteria with Flavonoids. **Proceeding of the 36th Congress on Science and Technology of Thailand. Chulalongkorn University, Thailand.**

2. Eumkeb, G., Siriwong, S., Phitaktim, S., Rojtinnakorn, N., and Sakdarat, S. (2011). Synegistic activity and mode of action of flavonoids isolated from smaller galangal and amoxicillin combinations against amoxicillin-resistant *Escherichia coli*. **Journal of Applied Microbiology.** 55-64 : 112.