การตรวจสอบฤทธิ์ของสารในกลุ่มฟลาโวนอยด์ที่คัดเลือกต่อแบคทีเรียที่ดื้อต่อยาปฏิชีวนะในกลุ่มเบตาแลคแทม

นายสมนึก ฉู่กระโทก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรดุษฎีบัณฑิต
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INVESTIGATION OF THE EFFECT OF SELECTED FLAVONOIDS ON β-LACTAM ANTIBIOTIC RESISTANT BACTERIA

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

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INVESTIGATION OF THE EFFECT OF SELECTED FLAVONOIDS ON β-LACTAM ANTIBIOTICS 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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สมนึก ฉู่กระโทก : การตรวจสอบฤทธิ์ของสารในกลุ่มฟลาโวนอยด์ที่คัดเลือกต่อแบคทีเรียที่ดื้อต่อยาปฏิชีวนะในกลุ่มเบตาแลคแทม (INVESTIGATION OF THE EFFECT OF SELECTED FLAVONOIDS ON β-LACTAM ANTIBIOTIC RESISTANT BACTERIA) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ เภสัชกร ดร. เกรียงศักดิ์ เอื้อมเก็บ, 156 หน้า.

ทุกวันนี้แบคทีเรียชนิด *S. aureus* ที่ดื้อต่อยาปฏิชีวนะเมทิซิลินและแบคทีเรียชนิด *E. cloacae* ที่ดื้อต่อยาเซฟตาซิดีมมีการดื้อต่อยาปฏิชีวนะหลายชนิดมากขึ้นเรื่อยๆ ทำให้เพิ่มความเสี่ยงต่อชีวิตของผู้ป่วยและผู้ให้การดูแลในโรงพยาบาลที่จะถูกคุกคาม งานวิจัยที่จะหาสารต้านแบคทีเรียใหม่ๆที่ทำให้ยาต้านเชื้อแบคทีเรียชนิดเบตาแลคแทมเหล่านั้นนำกลับมาใช้ได้เหมือนเดิมจึงเป็นเป้าหมายที่สำคัญและความต้องการอย่างเร่งด่วน งานวิจัยนี้ได้ทำการตรวจสอบฤทธิ์ในการต้านเชื้อแบคทีเรียของฟลาโวนอยด์ที่มีอยู่ตามธรรมชาติ โดยเมื่อใช้ยาปฏิชีวนะแอมพิซิลิน คลอกซาซิลิน และเซฟตาซิดีมร่วมกับไบคาลีนที่ปริมาณ 5 ไมโครกรัมต่อมิลลิลิตร ความเข้มข้นต่ำสุดของยาปฏิชีวนะทั้งสามข้างต้นในการต้านเชื้อแบคทีเรียที่ได้จากการคัดแยกทางคลินิกที่ดื้อต่อยาเมทิซิลินชนิด *S. aureus* ลดลงจาก 100 มากกว่า 1,000 และ 50 ไมโครกรัมต่อมิลลิลิตร เป็น 5 ไมโครกรัมต่อมิลลิลิตรในยาทั้งสามชนิดข้างต้น และยิ่งไปกว่านั้นแบคทีเรียที่ดื้อต่อยาปฏิชีวนะเซฟตาซิดีมชนิด *E. cloacae* ที่ได้จากการคัดแยกทางคลินิกที่มีค่าการยับยั้งของยาเซฟตาซิดีมต่ำสุดมากกว่า 1,000 ไมโครกรัมต่อมิลลิลิตร กลับมาไวต่อยาปฏิชีวนะดังกล่าว เมื่อใช้ร่วมกับเอพิจีนินหรือลูทิโอลิน 5 ไมโครกรัมต่อมิลลิลิตร โดยค่าการยับยั้งของยาเซฟตาซิดีมต่ำสุดลดลงเหลือ 5 ไมโครกรัมต่อมิลลิลิตร จากการตรวจนับจำนวนเซลล์ที่มีชีวิตอยู่ของเชื้อแบคทีเรียชนิด MRSA เมื่อใช้ยาปฏิชีวนะแอมพิซิลินหรือคลอกซาซิลินในปริมาณ 10 ไมโครกรัมต่อมิลลิลิตรร่วมกับไบคาลีน 10 ไมโครกรัมต่อมิลลิลิตรพบว่ามีฤทธิ์เสริมกัน เซฟตาซิดีม 10 ไมโครกรัมต่อมิลลิลิตรผสมกับไบคาลีนหรือเคอเซติน 10 ไมโครกรัมต่อมิลลิลิตร สามารถลดจำนวนเซลล์ของแบคทีเรียที่ดื้อต่อยาเมทิซิลินชนิด *S. aureus* ให้อยู่ในระดับต่ำสุดที่สามารถนับได้ (10^3 CFU/ml) ในช่วงเวลาตั้งแต่ 6 ชั่วโมงและมากกว่า ส่วนแบคทีเรียที่ดื้อต่อยาปฏิชีวนะเซฟตาซิดีมชนิด *E. cloacae* เหลืออยู่ในระดับต่ำสุดในช่วงเวลา 6 ถึง 24 ชั่วโมง จากการใช้ยาเซฟตาซิดีม 10 ไมโครกรัมต่อมิลลิลิตรร่วมกับลูทิโอลินหรือเอพิจีนิน 10 ไมโครกรัมต่อมิลลิลิตร จากการตรวจสอบด้วยกล้องจุลทรรศน์อิเลคตรอน เห็นอย่างชัดเจนว่าเมื่อใช้ไบคาลีน 10 ไมโครกรัมต่อมิลลิลิตรร่วมกับ 10 ไมโครกรัมต่อมิลลิลิตรของแอมพิซิลินหรือคลอกซาซิลิน และเซฟตาซิดีม 10 ไมโครกรัมต่อมิลลิลิตรผสมกับ 10ไมโครกรัมต่อมิลลิลิตรของไบคาลีน

หรือกาแลนจินหรือเคอเซติน ทำให้เกิดการทำลายโครงสร้างของแบคทีเรียที่ดื้อต่อยาเมทิลินชนิด *S. aureus* ให้เกิดการสูญเสีย เมื่อใช้เซฟตาซิดีม 10 ไมโครกรัมต่อมิลลิลิตรร่วมกับ 10 ไมโครกรัมต่อมิลลิลิตรของเอพิจีนินหรือลูทิโอลิน สามารถทำลายรูปร่างและโครงสร้างของแบคทีเรียที่ดื้อต่อยาปฏิชีวนะเซฟตาซิดีมชนิด *E. cloacae* จนสูญเสียโครงร่างอย่างเห็นได้ชัด โดยผลดังกล่าวสามารถยืนยันโดยแคพิลลารี อิเลคโทรโฟเรซิส การวิเคราะห์ด้วยวิธีเอ็นไซม์ชี้ให้เห็นว่าไบคาลีน เคอเซตินหรือกาแลนจินเมื่อผสมกับเซฟตาซิดีมมีผลยับยั้งต่อเอ็นไซม์เบตาแลคแทมเมส I ของแบคทีเรียชนิด *B. cereus* เอพิจีนินและเคอเซตินเมื่อผสมกับเซฟตาซิดีมสามารถยับยั้งเอ็นไซม์เพนิซิลิเนส IV ของแบคทีเรียชนิด *E. cloacae* ได้อย่างมีประสิทธิภาพ

จากการศึกษานี้สรุปได้ว่าไบคาลีน กาแลนจินหรือเคอเซตินมีประสิทธิภาพในการทำให้แบคทีเรียชนิด *S. aureus* ที่ดื้อต่อยาปฏิชีวนะในกลุ่มเบตาแลคแทมกลับมาไวต่อยาดังกล่าวได้ดังเดิม ลูทิโอลินหรือเอพิจีนินเมื่อใช้ร่วมกับเซฟตาซิดีมมีประสิทธิภาพในการเสริมฤทธิ์กันเพื่อทำลายแบคทีเรียชนิด *E. cloacae* ที่ดื้อต่อยาปฏิชีวนะเซฟตาซิดีม เมื่อมองในแง่ความเป็นพิษที่ต่ำของสารฟลาโวนอยด์เหล่านี้แล้ว สารที่มีคุณค่าเหล่านี้ควรถูกนำมาพัฒนาเป็นสารที่นำมาใช้ร่วมกับยากลุ่มเบตาแลคแทม เพื่อเป็นยารักษาโรคที่เกิดจากแบคทีเรียที่ดื้อต่อยาปฏิชีวนะเหล่านี้ที่ไม่สามารถรักษาได้ในปัจจุบัน

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษา_____

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SOMNUK CHUKRATHOK : INVESTIGATION OF THE EFFECT OF SELECTED FLAVONOIDS ON β-LACTAM ANTIBIOTIC RESISTANT BACTERIA. THESIS ADVISOR : ASST. PROF. GRIANGSAK EUMKEB, Ph.D. 156 PP.

ANTIBACTERIAL ACTIVITY/ β-LACTAM ANTIBIOTICS/MINIMUM INHIBITORY CONCENTRATION (MIC)/METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)/CEFTAZIDIME-RESISTANT *ENTEROBACTER CLOACAE* (CREnC)

Today, strains of methicillin-resistant *S. aureus* (MRSA) and ceftazidime-resistant *Enterobacter cloacae* (CREnC) are usually multiply resistant to many antibiotics and pose life-threatening risks to the hospitalised patients and their care givers. The search for new antibacterial agents and compounds that can reverse the resistance to β-lactam antibiotics are research objectives of far reaching importance and urgently needed. In this study, we have examined the antibacterial action of naturally occurring flavonoids. When combined ampicillin, cloxacillim or ceftazidime with baicalein 5 μg/ml, minimum inhibitory concentrations (MICs) of these drugs against clinical isolates of MRSA were reduced from 100, > 1,000 and 50 μg/ml to 5, 5 and 5 μg/ml respectively. Furthermore, clinical isolates of CREnC with MICs of ceftazidime >1,000 μg/ml had their resistance to these drugs reversed by apigenin 5 μg/ml or luteolin 5 μg/ml to MICs of ceftazidime 5 μg/ml. Viable counts showed that the killing of MRSA cells by 10 μg/ml ampicillin or cloxacillin was potentiated by 10

μg/ml baicalein. Ceftazidime 10 μg/ml in combination with 10 μg/ml of baicalein or quercetin also reduced the CFU/ml of MRSA to low level (1×10^3 CFU/ml) over 6 h. The killing curve of CREnC were also maintained at low level from 6 to 24 h by 10 μg/ml ceftazidime in combination with 10 μg/ml of luteolin or apigenin. Electronmicroscopy clearly showed that the combination of 10 μg/ml baicalein with 10 μg/ml of ampicillin or cloxacillin and 10 μg/ml ceftazidime with 10 μg/ml of baicalein or galangin or quercetin caused damage to the ultrastructure of MRSA. Ceftazidime 10 μg/ml in combination with 10 μg/ml of luteolin or apigenin also caused marked morphological damage for CREnC. These results were also confirmed by capillary electrophoresis. Enzymes assays indicated that baicalein, quercetin or galangin plus ceftazidime had inhibitory activity against β-lactamase I from *B. cereus.* Moreover, the combination of ceftazidime plus apigenin and quercetin showed efficiently inhibitory activity against penicillinase type IV from *Enterobacter cloacae.*

From the study, it was concluded that baicalein, galangin or quercetin have the potential to reverse bacterial resistance to β-lactam antibiotics against MRSA. Luteolin or apigenin have synergistic effect with ceftazidime against CREnC. In view of their limited toxicity, these tested flavonoids offer for the development of a valuable adjunct to β-lactam treatments against otherwise resistant strains of currently almost untreatable microorganisms.

School of Biology Student' Signature_____

Academic Year 2006 Advisor' Signature_____

Co-advisor's Signature_____

Co-advisor's Signature_____

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Somnuk Chukrathok

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LIST OF ABBREVIATIONS

ASEnC	Ampicillin-Sensitive *Enterobacter cloacae*
AICD	Activation-Induced Cell Death
BDH	Standard Protein
CAMHB	Cation- Adjusted Hueller-Hinton
CE	Capillary Electrophoresis
CFU	Colony Forming Unit
CM	Cytoplasmic Membrane
CREnC	Ceftazidime-Resistant *Staphylococcus aureus*
Da	Dalto
KDa	Kilo Dalton
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DNP	Dinitropyrene
EDTA	Ethylenediamine Tetraacetic Acid
EMCV	Enceplalomycarditis
FIC	Fractional Inhibitory Concentration
g	Gram
h	Hour
HEPES	N-2-Hydroxyethyl piperazine-N'-ethanesulphonic acid
HSVI	Herpes Virus Type I

LIST OF ABBREVIATIONS (Continued)

IFN	Interferon
KV	Kilo Volts
IV	Intravenous
LPS	Lipopolysaccharide
MIC	Minimum Inhibitory Concentration
ml	Millilitre
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MW	Molecular Weight
NaCl	Sodium Chloride
NAT	N-Acetyltransferase
NO	Nitrocoxide
NT	Nitropyrene
ODC	Ornithine decarboxylase
OM-PG	Outer Membrane and Peptidoglycan
OsO_4	Osmium Tetroxide
PMF	Protein Motive Force
PMSF	Phenyl Methyl Sulphonyl Fluoride
POX	Peroxidase
RNA	Ribonucleic Acid
s	Second

LIST OF ABBREVIATIONS (Continued)

Sarkosyl	Sodium-N-Lauryl sarcosinate
	Electrophoreis
TEM	Transmission Electron Microscopy
TNF	Tumor Necrosis Factor
Tris-HCL	Trizma Hydrochloride
TSS	Toxic Shock Syndrom
VSV	Vesicular Stomatitis Virus

CHAPTER I

INTRODUCTION

For thousands of years human have used natural substances, especially plants, to relieve pain, heal wound and maintain health. Throughout the world today, especially in the developing countries, people recognize the value of medicinal plants in treating and preventing common diseases. Herbal medicine is one of the most important resources which can be mobilized for the attendance of WHO's goal of health for all by the year 2000. It has contributed significantly to man's struggle against diseases and has been an important component of health care system for thousands of years.

Now, a serious world wide problem in the hospital is drug-resistant bacteria including Thailand. Due to indiscriminate use of antimicrobial drugs, microorganisms have developed resistance to many antibiotics and that have created immense clinical problems in the treatment of infectious diseases (Davis, 1994). The bacterial resistant antibiotics have several groups including β-lactam antibiotic drugs which are very expensive. In the past, antibiotics are used for effective treatment of many diseases, but now they can not cure those diseases because the bacteria can resist those drugs. Strains of ceftazidime-resistant *Escherichia coli* (CREsC), ceftazidime-resistant *Enterobacter cloacae* (CREnC) including β-lactam resistant *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) have a posing serious problem to hospitalized patients and their care providers (Maharat Nakhon Ratchasima hospital,

2000; Lui, Durham, and Richards, 2000).

MRSA are the most common organisms causing infections of the urinary tract, surgical wounds, skin respiratory and gastrointestinal tract including *Escherichia coli* and *Enterobacter spp*. (Biedenbach et al., 2004; D'A gata, 2004; Farr, 2002; Hull and Beck, 2004; Schaberg et al., 1190). In addition, antibiotics are sometimes associated with adverse effects on host which include depletion of beneficial gut and mucosal microorganisms, immunosuppression, hypersensitivity and allergic reaction. The drugs-resistant bacteria have further complicated the treatment of infectious diseases in immonocompromised, AIDS and cancer patients, especially in the case of nosocomial infections (McGaw, Jager, and Staden, 2000). There is not only the lost of an effective antibiotic against multi-drugs resistant bacteria, but also a global problem for the lost of budget for treatment of infectious diseases.

In the present, scenario of emergence of drug resistant in human pathogenic organisms, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases. One approach is to screen new, cheap and effective drugs from other sources, including plants, for possible antimicrobial properties that will be able to act for longer periods before resistance set in. In recent years, antimicrobial properties of medicinal plants have been reported from different parts of the world including Thailand, in which indigenous culture possess a rich heritage of healing with medicinal plants (Roengsumran et al., 1997; Ahmad and Beg, 2001; Brantner and Grein, 1994; Mehmood and Mohamad, 1998; Samy et al., 1999; Sokmen et al., 1999). Previous study, Tor, Klara, Sigmund, Chun, Victoria, and Olaf (2003) found that marine organisms were a rich source for discovering antimicrobial and other bioactive compound such as flounder found that marine organisms were a rich source

for discovering antimicrobial and other bioactive compound such as flounder (*Pleuronectes platessa*), saithe skin (*Pollachius virens*) had antibacterial activity against *Listonella anguillarum*. The 6 novel AMPs (antimicrobial peptides) isolated from the coelomocytes was shown to have a MIC of 1.2-5.0 μM against *E. coli* and *S. aureus*. Ara-A (acyclovir) and AZT (zidovudine) isolated from sponge were shown antiviral activity.

Thailand, there are many medicinal plants which have antiseptic properties and are used for antimicrobial activity including Zingiberaceae family such as *Alpinia galanga (A. galanga), Alpinia nigra (A. nigra) and Alpinia officinarum (A. officinarum)* which have some chemicals of flavonoids such as galangin. Some flavonoids such as baicalin can affect synergy with β-lactam antibiotics for anti-MRSA and penicillin-resistant *Staphylococcus aureus* (Lui et al., 2000). Previously antibacterial studies found that galangin has an effect on anti-MRSA when combined with β-lactam antibiotic drugs (Griangsak Eumkeb and Richards, 2003).

Maharat Nakhon Ratchasima hospital is the largest hospital in Nakhon Ratchasima province, northeast of Thailand. The problem of drug-resistant bacteria in many sections of the hospital has been previously reported such as MRSA, CREsC and CREnC (Maharat Nakhon Ratchasima hospital, 2001).

However, there are no researchers investigate about the used of galangin, baicalein, apigenin, luteolin and quercetin alone and in combination with β-lactam antibiotics to treat MRSA and CREnC. These bacteria have had drug-resistance in high level in many sections of Maharat Nakhon Ratchasima hospital and other hospitals in Thailand and world wide (Jungthirpanich, Tungsathapornpong and

Chaumrattanakul, 2000; Kusum and Dejsirilert, 2003; Nakhon Ratchasima hospital, 2001; National Nosocomial Infection Surveillance, 2000).

1.1 Objectives of Study

1.1.1 To test the effectiveness of selected flavonoids (apigenin, baicalein, galangin, luteolin or quercetin) whether they take effect on β-lactam antibiotic resistant bacteria.

1.1.2 To test the effectiveness of selected flavonoids (apigenin, baicalein, galangin, luteolin or quercetin) when combined with β-lactam antibiotics whether they take affect on β-lactam antibiotic resistant bacteria.

1.1.3 To investigate the mechanism of action of selected flavonoids on β-lactam antibiotics resistant bacteria when use singly and in combination with β-lactam antibiotics by examining morphology with transmission electronmicroscopy (TEM), capillary electrophoresis and enzyme assay.

1.2 Research hypothesis

1.2.1 Apigenin, baicalein, galangin, luteolin or quercetin could show antibacterial activities against clinical isolates of methicillin-resistant *S. aureus* (MRSA), ceftazidime-resistant *E. cloacae* (CREnC) and ampicillin-sensitive *E. cloacae* (ASEnC).

1.2.2 Apigenin, baicalein, galangin, luteolin or quercetin in combination with β-lactam antibiotics could show antibacterial activities against clinical isolates of MRSA, CREnC, and ASEnC.

1.3 Scope and limitation of the study

1.3.1 Bacterial suspension standard curves for clinical isolates of MRSA, CREnC and ASEnC were done.

1.3.2 Minimal inhibitory concentrations (MICs) of cloxacillin, ampicillin, ceftazidime and the flavonoids were done for clinical isolates of MRSA, CREnC, and ASEnC.

1.3.3 Checkerboard assay of these combinations that show the lowest MIC were selected to do further investigations such as viable counts, TEM and electrophoresis.

1.4 Expected results

1.4.1 Some selected flavonoids could show antibacterial activity against β-lactam antibiotic resistant bacteria.

1.4.2 Some selected flavonoids could show synergism activity against β-lactam antibiotic resistant bacteria when combine with β-lactam antibiotics.

1.4.3 The preliminary informations of the mechanism of action of some flavonoids on β-lactam antibiotics resistant bacteria when they are used singly and in combination with β-lactam antibiotics by morphology examining with transmission electronmicroscopy (TEM), capillary electrophoresis and enzyme assay will be elucidated.

1.4.4 Providing additional scientific data on synergism antimicrobial activity between the combination of some flavonoids and β-lactam antibiotics.

CHAPTER II

LITERATURE REVIEWS

The drug-resistant bacteria posses a serious world-wide problem that need immediately solution because it is harmful to human. Drug resistant microbial is an unavoid consequence of the utilization of antimicrobial agents in a given environment. Nowhere is the importance of resistance more evident than among agents of the β-lactam antibiotic family (Sanders and Sanders, 1992).

β-lactam antibiotics include penicillin, cephalosporins and related compounds, are active against many gram-positive, gram-negative and anaerobic organisms. Information based on "expert opinion" and antimicrobial susceptibility testing support certain antibiotic choices for the treatment of common infections, but less evidence-based literature is available to guide treatment decision. Evidence in the literature supports the selection of amoxicillin as first-line antibiotic therapy for acute otitis media. Alternative drugs, such as amoxicillin-clavulanate, trimethoprim-sulfamethoxazole and cefuroxime axetil, can be used to treat resistant infections. Penicillin V remains the drug of choice for the treatment of pharyngitis caused by group A *Streptococci*. Inexpensive narrow-spectrum drugs such as amoxicillin or trimethoprim-sulfamethoxazole are first-line therapy for sinusitis. Animal and human bites can be treated most effectively with amoxicillin-clavulanate. For most outpatient procedures, amoxicillin is the preferred agent for bacterial endocarditis prophylaxis. β-lactam antibiotics are usually not the first choice for empiric outpatient treatment of

community-acquired pneumonia. Based on the literature, the role of β-lactam antibiotics in the treatment of bronchitis, skin infections and urinary tract infections remains unclear (Keith and Edward, 2000).

β-lactam antibiotics, which are named for the β-lactam ring in their chemical used structure, include the penicillin, cephalosporins and related compounds. These agents are active against many gram-positive, gram-negative and anaerobic organisms. The β-lactam antibiotics exert their effect by interfering with the structural crosslinking of peptidoglycans in bacterial cell walls. Because many of these drugs are well absorbed after oral administration, they are clinically used in the outpatient setting (Keith and Edward, 2000).

MRSA stands for methicillin resistant *Staphylococcus aureus*. It is a type of bacterium commonly found on the skin and/or in the nose of healthy people. Although it is usually harmless at these sites, it may occasionally get into the body (e.g., through breaks in the skin such as abrasions, cuts, wounds, surgical incisions or in dwelling cathesters) and cause infections. These infections may be mild (e.g., pimplea or boils) or serious (e.g., infection of the bloodstream, bones or joints). The treatment of infections due to *Staphylococcus aureus* was revolutionised in the 1940s by the introduction of the antibiotic penicillin. Unfortunately, most strains of *Staphylococcus aureus* are now resistant to penicillin. This is because *Staphylococcus aureus* has 'learnt' to make substance called β-lactamase, that degrades penicillin, destroying its antibacterial activity. It is a bacterium that has developed a resistance to most antibiotics commonly used for *Staphylococcus* infection. These drugs include methicillin, oxacillin, nafcillin, imipenem and other β-lactams. MRSA can affect people in two different ways, colonization or infection. When the person carries the

flora on the skin or in the nose without showing signs or symptoms of infection, the person is said to colonize. If a person has signs of infection that are caused by MRSA (such as abscesses, wound infection, pneumonia, respiratory infections, blood, stool or urinary tract infection), the person is said to be infected. MRSA most often spread from person to person by direct contact, for example, in medical setting. MRSA is most commonly spread from patient to patient by health care worker's hands (Barret et al., 1968; Chamber, 1997; http:// www. netdoctor.co.uk /diseases/ facts/mrsa.htm).

2.1 Microorganisms

2.1.1 Bacterial structure

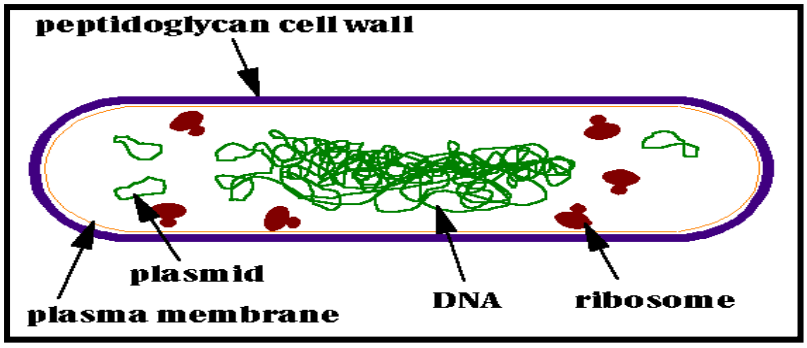


Figure 1 Show structure of bacterial cell

(www. microbeworld.org/img/aboutmicro/bacteria/bactdiag.gif)

The cytoplasmic membranes of gram-positive and gram-negative bacteria are indistinguishable. Each is composed of protien, lipids, phospholipids and a small amount of carbohydrate. It acts as an osmotic barrier, synthesizes the cell wall and provides a site to implant the chromosome. The other important functions are serving

as the site of selective permeability, cytochrome activity, carrier-mediated transport and generation of proton motive force (PMF).

The periplasm is the space between the inner and outer membrane of a gram-negative bacterium, and the cell wall lies within it. The periplasm contains enzymes that hydrolyze large molecules, hydrolyze antibiotics, and binding protein that facilitate transport.

The cell wall is a web-like structure that is sometimes called the murien sacculus. It is composed of peptidoglycan. The cell wall provides the cell with its sharp and osmotic stability. The cell wall constituents are peptidoglycan, teichoic acids and lipoteichoic acids.

Only gram-negative bacteria have an outer membrane. Porins and porin-like protiens in the outer membrane allow the membrane to act as a molecular sieve, restricting the access of some molecules to the cell wall and periplasm. The most clinical significant component of the outer membrane is a phospholipid like molecule called lipopolysaccharide (LPS) (Walker, 1999).

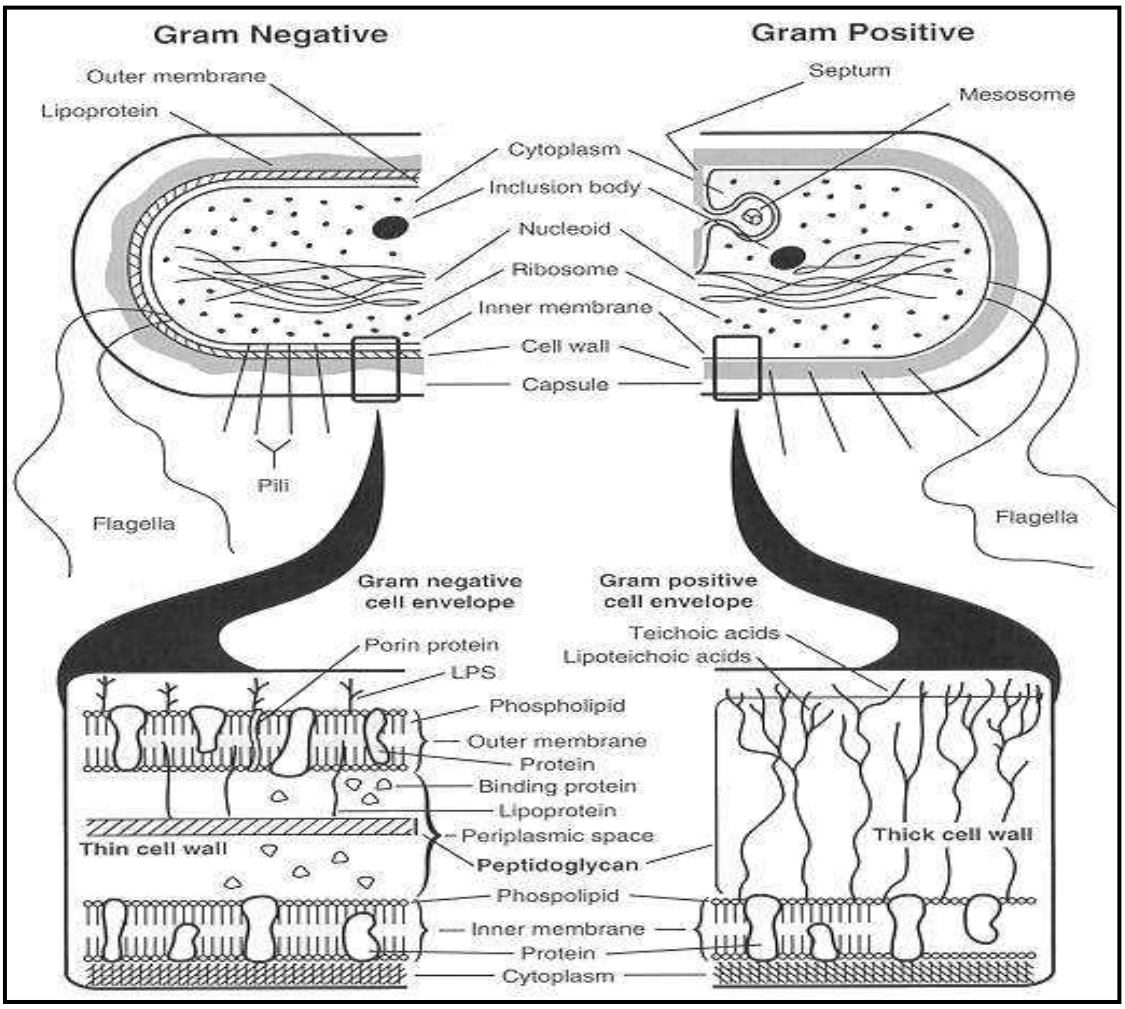


Figure 2 Comparison of the thick cell wall of gram-positive bacteria with the comparatively thin cell wall of gram-negative bacteria. Note the complexity of the gram-negative cell envelope (outer membrane, its hydrophobic lipoprotein anchor; periplasmic space).

(www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=mmed.figgrp.294)

2.1.2 Bacteria types

2.1.2.1 *Staphylococcus aureus (S. aureus*)

Family: Micrococcaceae

General characteristics:

The *S. aureus* is gram-positive cocci. It is a spherical cell (0.5 to 1.5 μm) that appears singly, in pairs, and in irregular clusters. This organism is non-motile, non-spore forming, facultative anaerobe and chemoorganotrope (both respiratory and fermentative metabolism). Colonies appear creamy, white or light gold and sometimes yellow to orange. The optimum temperature for *S. aureus* is 30-37ºC (Holt, Krieg, Sneath, Staley, and Williams, 1974, 1994).

Clinical significance:

S. aureus is responsible for a wide variety of infections and disease due to toxins. Infections caused by *S. aureus* are supparative and pyogenic. Some of the common skin infections are boils, carbuncles, folicullitis and bullous impetigo. These opportunistic infections occur usually as a result of previous skin injuries (Mahon and Manuselis, 2000; Shimeld and Rodgers, 1999).

Antibiotic susceptibility characteristics:

Penicillin became more widely available and used. By the year 1950, isolated strain of *S. aureus* was resistant to penicillin by producing an enzyme that cleaves its β-lactam ring. The penicillinase-resistant penicillins, which were nafcillin, methicillin and oxacillin, were used to treat the more resistant isolates. The 1970s, resistance developed to these compounds. The MRSA have become a costly problem in

hospitals. The rest anti-infective therapy against MRSA is vancomycin (Shimeld and Rodgers, 1999).

Diseases associated with *S. aureus* are systemic infections (bacteremia, septicemia), toxin productions (food poisoning, scaled skin syndrome, toxic shock syndrome (TSS)) (Mahon and Manuselis, 2000; Shimeld and Rodgers, 1999).

2.1.2.2 *Enterobacter cloacae (E. cloacae)*

Family: *Enterobacteriaceae*

General characteristics:

E. cloacae is a gram-negative, straight rod (0.6-1.0 μm wide x1.2-3.0 μm long). It is facultative anaerobe and chemoorganotrop. The optimal temperature for *E. cloacae* is 30-37ºC. It widely distributed in nature and it can be found in the soil, dairy products, water and sewage. It may also be present in the intestinal tract of humans and animals (John et al., 1994; Shimeld and Rodgers, 1999).

Clinical significance

E. cloacae generally does not cause disease in healthy individuals but sigificantly cause the infections in immunocompromised or otherwise debilitated patient. This species is an opportunistic pathogen causing burn, wound and urinary tract infection and occasionally septicemia and meningitis (Mahon and Manuselis, 2000).

Antibiotic susceptibility characteristics

Most isolates of *Enterobacter* are resistant to ampicillin and first-generation cephalosporins. Second- and third-generation cephalosporins may be effective (Shimeld and Rodgers, 1999).

2.2 Antibiotics

Antibiotics are chemical substances produced from various microorganisms (bacterial and fungus) that kill or suppress the growth of other microorganisms. The term is also uses for synthetic antimicrobial agents such as sulfonamides and quinolones (Salerno, 1999).

2.2.1 Mechanisms of action of clinically used antimicrobial drugs

Selective toxicity is a drug that harmful to a parasite without being harmful to the host. The mechanisms of action can be placed under four headings:

a) Inhibition of cell wall synthesis.

b) Inhibition of cell membrane function.

c) Inhibition of protein synthesis (i.e, inhibition of translation and transcription of genetic material).

d) Inhibition of nucleic acid synthesis (Brooks, Butel, Ornston, Jawetz, Melnick, and Aldelberg, 1995).

Antibiotics that inhibit bacterial cell wall synthesis

The two most important classes of antibiotics that inhibit bacterial cell wall synthesis are β-lactam and glycopeptides. In this literature review describe only β-

lactam that is one of antibiotic group associated with study (Page, Curtis, Sutter, Walker, and Hoffman, 1997).

2.2.2 β-lactam

β-lactam antibiotics possess a four-member nitrogen-containing β-lactam ring. It interferes with bacterial cell wall synthesis by inhibiting the cross-linkage of the peptide side chains of the bacterial cell wall. β-lactams are mainly bactericidal and exhibit time-dependent killing (Page et al., 1997).

2.2.2.1 Penicillin They are a byproduct of *Penicillium notatum*. It consists of a β-lactam ring fused to a five-member, sulfur-containing thiazolidine ring. Modification of the side chain at position six of the β-lactam ring results in drugs with different antibacterial and pharmacological properties. There are four classes of penicillins, natural penicillins, antistaphylococcal penicillins, aminopenicillins and antipseudomonal penicillins (Page et al., 1997).

2.2.2.2 Cephalosporins The first cephalosporin was discovered from *Cephalosporium acremonium*. It consists of a β-lactam ring fused to a six-member sulfur-containing dihydrothiazine ring. Individual cephalosporins are created by side-chain substitutions at position seven of the β-lactam ring and position three of the dihydrothiazine ring. Cephalosporins are traditionally classified into first-, second- and third-generation drugs based on their spectrum against aerobic gram-negative bacilli, which increases from first to third generation (Page et al., 1997).

Resistance to β-lactam antibiotics

Bacterial resistance against β-lactam antibiotics is increasing at a significant rate and become a common problem in primary care medicine. There are several mechanisms of antimicrobial resistance to β-lactam antibiotics. One important mechanism is the production of β-lactamase, which is enzyme that cleave the β-lactam ring. β-lactamase activity can occur in gram-positive organisms (*Staphylococcus aureus* and *Staphylococcus epidermidis*); gram-negative organisms (*Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Moraxella* [formerly Branhamella] *catarrhalis, Escherichia coli* and *Proteus, Serratia*, *Pseudomonas* and *Klebsiella* species); anaerobic organisms (*Bacteroides* species). The newer β-lactam antibiotics can be highly effective in combating infections caused by β-lactamase producing organisms. When used alone, β-lactamase inhibitors (clavulanate, sulbactam and tazobactam) have weak intrinsic antibacterial activity, but their effectiveness increase when they are combined with β-lactam antibiotics (e.g., amoxicillin-clavulanate [Augmentin®] (Keith and Edward, 2000).

Spectrum including gram-positive bacteria antibiotics, and some β-lactams have been developed which inhibit a range of gram-negative bacteria. It may be innate resistance to the original β-lactams including the outer membrane of the gram-negative cell wall or may be acquired resistance including β-lactam enzymes which may be acquired by resistance transfer (Katzung, 1998).

Antibiotic that inhibit bacterial deoxyribonucleic acid synthesis

Each year, more than 500 metric tons of chemotherapeutic agents of various types are manufactured and used. In 1994, 37% cephalosporins, 17% penicillins and

14% quinolones are produced and used world wide (Madigan, Martinko, and Parken, 2000).

Ceftazidime is a third generation cephalosporin that active against aerobic gram-negative bacilli particularly Enterobacteriaceae. Usual adult dose for this antibiotic is 200 mg every 12 h or 0.5-2 g every 8-12 h for intravenous (IV) (Salerno, 1999). A cost per vial (1 g) of ceftazidime is about 400 baht.

Methicillin is a penicillinase resistant penicillin, which is the usual treatment for *S. aureus*. Over the past 30 years, most strains of *S. aureus* have become resistant to commonly used antibiotics. This makes infections caused by this organism more difficult and expensive to treat. Usual adult dose for the methicillin is 1 g every 4-6 h for intramuscular (IM) and 1 g every 6 h for IV (Salerno, 1999).

Cloxacillin is a semisynthetic antibiotic of the penicillin group that is used primarily to treat infections caused by *Staphylococci, Streptococci*, or *Pneumococc*i. Cloxacillin is for use against *Staphylococci* that produce beta-lactamase. It is sold under a number of trade names, including Cloxapen ® and Orbenin ®.

2.2.3 Major groups of antimicrobial compounds from plants

Plants are rich in wide variety of secondary metabolites. Useful antimicrobial phytochemicals can be divided into several categories (Cowan, 1999; Kaufman, Cseke, Warben, Duke, and Brielmann, 1999).

Phenolics and polyphenols

2.2.3.1 Simple phenols and phenolic acid

Most of the simple phenols are monomeric components of the polymeric polyphenols and acid which make up plant tissue, including lignin, melanin, flavolin and tannins. The sites and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity. The mechanisms though to be responsible for phenolic toxicity to microorganisms including enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins.

Phenolic compound possessing a C_3 side chain at a lower level of oxidation, containing no oxygen are classified as oils and often cited as antimicrobial as well (Cowan, 1999; Kaufman et al., 1999).

2.2.3.2 Flavones, flavonoids and flavonols

Flavones are phenolic structures containing one carbonyl group. The flavonoids have two benzene rings separated by a propane unit and are derived from flavone. Flavonoids are group of naturally occuring compounds which are widely distributed in nature and are ubiquitous in vegetables, berries and fruits.

Flavonoids are polyphenolic compounds that are present in plants. Flavonoids comprise the most common group of plant polyphenols and provide much of the flavor and color to fruits and vegetable. They have been shown to posses a variety of biological activity at non-toxic concentrations in organism (Heo et al., 2001).

Heijnen and co-workers (2000) have reported that flavonoids effeciently protect against peroxynitrite toxicity. Two pharmacophores have been identified in

flavonoids, namely the catechol group in ring B and the hydroxyl (OH) group at the 3-positoin. Cowan (1999) and Kaufman et al. (1999) examined the structure-activity relationship. It was found that catechol (1, 2-dihydroxybenzene) is a potent peroxynitrite scavenger, whereas phenol (hydroxybenzene) is not (Cowan, 1999; Kaufman et al., 1999).

2.3 Resistance to antibacterial agents (antibiotics)

2.3.1 Spectrum of activity of an antibiotic

The spectrum of activity is the range of microbial species and strains which the antibiotic is effective against some species. Some strains naturally resist (innate resistance) due to structure and properties of the microbe which normally sensitive become resistant (acquired resistance).

2.3.2 Microbial resistance to antibiotics

Origins of resistance in a popular of microbes is mutation in chromosome which transfers resistant genes from one microbe to another via conjugation (involving plasmids) or transduction (involving bacteriophages). Multiplication and spread of resistant microbes are selectively enhanced by the use of antibiotics in patients.

Significances of resistance are limitation on the use of antibiotics and cost of developing new antibiotics.

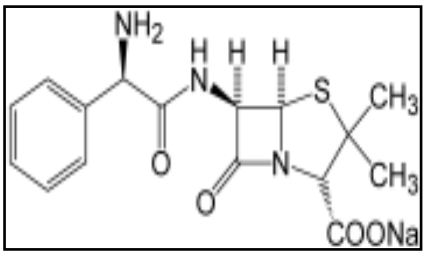
A resistant organism is an organism that will not be inhibited or killed by antibacterial agents at concentrations of the drug achievable in the body after normal dosage (Mims, Playfair, Roitt, Wakelin, and Rosamund, 1998).

2.4 The using β -lactam antibiotics

2.4.1 Ampicillin; Ampicillin sodium;

6-[D-α-aminophenylacetamido] penicillanic acid;

D (-) -α-aminobenzyllpenicillin



Molecular formular: $C_{16}H_{18}N_3O_4S.Na$

Molecular weight: 371.4

Appearance: white or almost white powder

Solubility: soluble in water

Constituted solution:

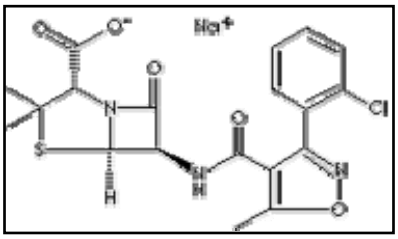
Completenness of solution: the solid dissolves completely

Clarity of solution: clear

Paticulate matter: essentially free from particles

(www.sandoz.com/site/en/business/ant_infectives/product_list/products/471122AmpiNasteril.sh)

2.4.2 Cloxacillin; Cloxacillin sodium



Molecular formular: $C_{19}H_{17}N_3O_5S.Na.H_2O$

Molecular weight: 475.9

Appearance: white or almost white, crystalline powder

Solubility: soluble in water

Appearance of solution:

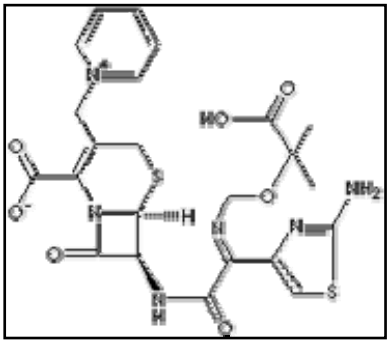
Completenness of solution: the solid dissolves completely

Clarity of solution: clear

Paticulate matter**:** essentially free from particles

(www.sandoz.com/site/en/business/ant_infectives/product_list/products/471122CloxaNasteril.sh)

2.4.3 Ceftazidime; 1-[[(6R, 7R)-7-[2-amino-4-thiazoly) glyoxylamido] -2-carboxy-8-oxo-5-thai-1-azabicyclo-[4.2.0] oct2-en-3-yl] methyl] pyridinium hydroxide inner salt



Molecular formular: $C_{22}H_{22}N_6O_7S_2.5H_2O$

Molecular weight: 636.6

Appearance: white to almost white powder

Solubility: soluble in water

Appearance of solution:

Completenness of solution: the solid dissolves completely

Clarity of solution: clear

Paticulate matter**:** essentially free from particles

(www.sandoz.com/site/en/business/ant_infectives/product_list/products/471122CeftaNasteril.sh)

2.5 Medicinal plants used

Extracts of various plant parts of *Aerva persica* has been tested for their antimicrobial activity against human pathogenic bacterial strains of *Staphylococcus aureus* and *Salmonella typhi* and plant pathogenic fungal species *Macrophomina phaseolina.* Aqueous and alcoholic extracts were tested against all the microorganisms. All the plant parts showed antibacterial and/or antifungal activity (Gehlot and Bohra, 1998).

The resistance inhibitory activities of fifty-four mixtures (essential oil) from forty-one Korean aromatic herbs were tested against multi-drugs resistant *Staphylococcus aureus* SA2, which has resistances to ten usual antibiotics including chloramphenicol. As results, combinations of twenty-eight kinds of samples from twenty-one herbs and chloramphenicol have resistance inhibitory activities in dose dependent manner (Lee, Kim, Moon, and Shin, 1998).

With the therapeutic concept of using the defensive ability of plants against microbial infections, phytoalexin, an antimicrobial phytochemical was studied for its ability to inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA). Extracts from *Sophola exigua* (Leguminaceae) were fractionated by serial chromatography and the anti-MRSA activity of each fraction was determined by the agar-plate method. Among the activity isolates, 5, 7 ,2', 6'-tetrahydroxy-6-isoprenyl-8-lavandulyl-4'-methoxyflavone (exiguaflavonone D) completely inhibited the growth of all the MRSA strains examined at the concentration of 1.56-6.25 μg/ml and 5, 2', 6'-trihydroxy-8-lavandulyl-7-methoxyflavone (exiguaflavone B) inhibited at a concentration of 50 μg/ml. This former compound is accepted to be a phytotherapeutic agent for MRSA infection as an alternative to conventional antibiotics with unwanted side-effects or the appearance of antibiotic-resistant bacteria (Linuma et al., 1994).

Differently substituted flavanones were isolated from Leguminosae and their antibacterial activity was comparatively studied against methicillin-resistant *Staphylococcus aureus* (MRSA). The minimum inhibitory concentrations (MICs) of phytochemical flavones to clinical isolates of MRSA were determined by a serial agar dilution method. The structure-activity relationship has indicated that 2', 4'- or 2', 6'-

dihydroxylation of the B ring and 5, 7-dihydroxylation of the A ring in the flavone structure were important for significant anti-MRSA activity and that substitution with a certain aliphatic group at the 6- or 8-position also enhanced the activity. Among the thirteen flavanones tested, tetrahydroxyflavanones with these structural characteristics isolated from *Sophora exigua* and *Echinosophora koreansis* showed intensive activity to inhibit the growth of all MRSA strains at 3.13-6.25 μg/ml. The present hydroxyflavanones would be useful in the phytotherapeutic strategy against MRSA infections (Tsuchiya et al., 1996).

Potential uses of flavonoids

Most flavonoids have anti-inflammatory, anti-microbial activity, anti-carcinogenicity properties, and are powerful antioxidants. Extracts from onion and various flavonoids induced the cellular antioxidant system. Onion extract and quercetin were able to increase the intracellular concentration of glutathione by approximately 50% (www.nal.usda.gov/fnic/foodcomp/Data/flav.html.).

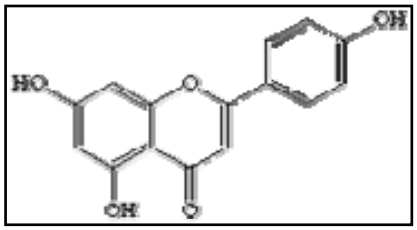
Flavonoids are generally found in plants as their glycosides. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More phynolic flavonoids may also disrupt microbial membrane (www.nal.usda.gov/fnic/foodcomp/Data/Flov/flav.html).

Rhizomes of *A. galanga*, *A. officinarum* and, *A. nigra* contain volatile oil (0.5-1.0%), resin, galangol, kaempferide, galangin, alpinin and starch which roots and rhizomes are used for stomachace upset, indigestion, stomache and flatulence. They have not the effect on mutation and all both acute and chronic toxicity. So it is not danger to human (Smitinand, 2001; Temsirikul et al., 2001; Heo et al., 2001). Baicalin

is a flavonoid which was extracted from edigible medicinal plant from China. It was called Xi-nan Huangqin (*Scutellaria amoena* C.H.Wright) that had effect combined with β-lactam antibiotics such as ampicillin, amoxycillin, benzylpenicillin, methicillin and cefotaxime for inhibiting methicillin-resistant *S. aureus* (MRSA) and penicillin - resistant *S. aureus* (Liu, Durham, and Richards, 2000).

2.6 The using flavonoids

2.6.1 Apigenin; Versulin; 4′, 5-, 7-trihydroxyflavone; 5, 7- dihydroxy-2-(4 hydroxyphenyl)-4H-1-benzopyran-4-one; 2-(ρ-hydroxyphenyl)-5, 7-dihydroxychromone



Molecular formula: C_{15} H_{10} O_5

Solubility: Soluble in hot alcohol and dimethylsulfoxide (DMSO).

Insoluble in water.

Melting Point: 345-350°C

Appearance: Yellow needle (Susan, 1996)

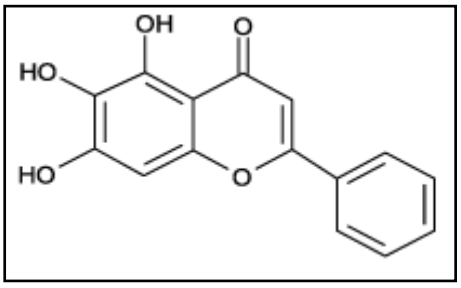
Apigenin is a flavonoid found in parsley, artichoke, basil, celery and other plants. Apigenin, a widely distributed plant flavonoid, was previously found to inhibit

chemically induce ornithine decarboxylase (ODC) activity and skin tumor promotion and may be developed as a promising chemopreventive and/or chemotherapeutic agent against prostate cancer (Birt, Mitchell, Gold, Pour, and Pinch, 2002; Gupta, Afaq, and Mukhtar, 2001).

Apigenin strongly inhibited the bacterial mutagenesis induced by nitropyrenese. Apigenin protected against the cytotoxicity induced by 1-nitropyrene (1-NP) and 1, 6-dinitropyrene (1, 6-DNP) and gave a significant reduction of the frequency of sister chromatid exchange (Kuo, Lee, and Lin, 1992).

Oksus et al. (1984) found that the MIC for apigenin against *Proteus vulgaris, Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pnemoniae* ranged from 54 to 219 µg/ml and that apigenin also proved to be active against the spore-former *Bacillus subtilis*.

2.6.2. Baicalein ; noroxylin; 5,6,7-trihydroxyflavone; 5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one



Molecular formular: $C_{15}H_{10}O_5$

Solubility: Soluble in alcohol, methanol, ether, acetone, ethyl acetate,

hot gacial acetic acid and DMSO

Melting Point: 264-265°C

Appearance: Yellow needle and light yellow powder (Susan, 1996)

The dried roots of *Scutellaria baicalensis* (*S. baicalensis*) Georgi (common name: Huangqin in China) have been widely employed for many centuries in traditional Chinese herbal medicine as popular antibacterial and antivirus agents. It had effective against *Staphylococci*, cholera, dysentery, *pneumococci* and influenza virus. Baicalein, one of the major flavonoids contained in the dried roots, possesses a multitude of pharmacological activities (Huang, Tsang, Yao, and Chen, 2005).

Fujita et al. (2005) found that the effect of baicalein on the efflux of tetra cycline, using *Escherichai coli* KAM32/pTZ1252 carrying the tetK. Baicalein showed strong inhibition of transport of tetracycline with membrane vesicles prepared from *Escherichai coli* KAM32/pTZ1252.

Baicalein inhibited thrombin-induced product of plasminogen activator inhibition-1, and interluekin-1β-and tumor necrosis factor-α-induced expression in cultured human umbilical vein endothelial cells. The pharmacological findings had highlighted the therapeutic potentials of using plant-derived baicalein and its analogs for the treatment of arteriosclerosis and hypertension (Huang et al., 2005).

Baicalein halted enzymatic activity, adminished endothelial cell migration and differentation, and reduced anticancer response in several assays (Fung, 2003).

Kyoungho, Heasuk, Sang, Gyeong, and Wan (2003) found that baicalein selectively inhibited the nitric-oxide (NO) dependent apoptotic pathway of activated microglia by supreesing cytotoxic NO production. Also, the activation-induced cell

death (AICD) inhibiting effects of baicalein were specific for inflammatory stimulus activated microglia.

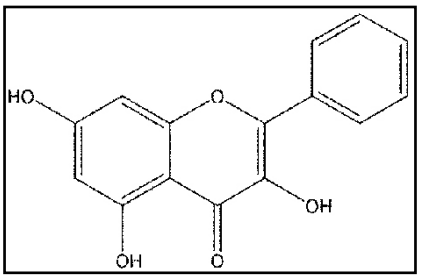
Baicalein is a potent scavenger, attenuates oxidant stress during hypoxia, ischemia, reperfusion and mitochondrial ETC inhibiting and protects against cell death in an ischemia and reperfusion model (Zuo et al., 2002).

The glycoside of baicalein is a potent anti-flammatory and anti-tumor agent. In the previous study, it was investigated the in vitro effects on the growth, viability and induction of apoptosis in several human prostate cancer cell lines (Chan et al., 2000).

Schinazi et al. (1997) showed that baicalein inhibited certain viruses in vitro, including the Rauscher murine luekemia virus and the HIV virus, as well as cellular DNA polymerases, and that the inhibition of reverse transcriptase by the flavone baicalein was highly specific. These facts suggested that the flavone baicalein may be less toxic than the flavonols to the DNA and RNA polymerases in the host cell infected with retroviruses.

Platelet 12-lipoxygenase was inhibited by baicalein with an ID_{50} value of 0.12 μg /mL, with minimal inhibition of platelet cyclooxygenase-1 (IC_{50} = 0.83mM) and baicalein inhibited lipid peroxydation, as assessed by production of TBARS, with an IC_{50} value of 5 μM. In addition to these effects, baicalein may play a role in apoptosis, as the compound inhibited cell growth of three human hepatocellular carcinoma celllines with IC_{50} values ranging from 17-70 μg /ml (Gao et al., 1996; Matsuzaki et al., 1996; Sekiya, and Okuda, 1996).

2.6.3 Galangin; norizalpinin; 3, 5, 7-trihydroxyflavone; 3, 5, 7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one



Molecular formular: C_{15} H_{10} O_5

Solubility: Soluble in chloroform, benzene, ethanol, ether, and DMSO

Melting point: 214-215°C

Appearance: Yellowish needle (Susan, 1996)

Galangin, a number of the flavonol class of flavonoid, is presented high concentrations in medicinal plants (e.g., *Alpinia officinarum*) and propolis, a natural beehive product (Heo et al., 2001). Galangin is a flavonol that does not have any hydroxyl group in the B ring and has been suggested to be a substrate of cytochromes P450 which, through the hydroxyl of the B ring, could metabolise it to more genotoxic products. Galangin was more often acknowledged for its potential in cancer chemoprevention. And relatively nontoxic antioxidant and free radical scavenger that was capable of enzyme modulation demonstrates proapoptosis in leukemic cell lines and suppresses chemical genotoxicity (Duarte et al., 1997).

Tim and Andrew (2005) clearly demonstrates that galangin causes a significant increase in potassium loss from *S. aureus* cells, which may be attributed to

either direct damage to the cytoplasmic membrane or indirect damage effected through autolysis/weakening of the cell way and consequent osmoticlysis. The MIC of galangin against multiple-resistant *Staphylococcus aureus, Enterococcus* spp. and *Pseudomonas aeruginosa* strains was significantly lower 0.16 to 0.44 mg/ml (Pepeljnjak and Kosalec, 2004).

The MIC of galangin against *Pseudomonas aeruginosa* strains was present at 0.17 ± 0.05 mg/ml. (Stjepan et al., 2004). Galangin was shown to have a minimum inhibitory concentration (MIC) of 25 to 50 μg/ml against all six strains of *Staphylococcus aureus (S. aureus)* that it caused a 100,000-fold decreasing in the viability of growing population of *S. aureus* NCTC 6571 within the frist two hours of treatment. Decreases in viability of *S. aureus* NCTC 11561 and NCIMB 9968 populations were also observed (Gushnie et al., 2003).

A combination of galangin or 3, 5, 7-trihydroxyflavone with vancomycin may be used to sensitize resistant strains of *Enterococcus faecalis* and *Enterococcus faecium* to the level of vancomycin-sensitive strains. Minimum inhibitory concentrations (MICs) of vancomycin against 67% of resistant clinical isolates and a type strain of *Enterococci* were lowered from > 250 μg/ml to 4 μg/ml in the presence of galangin (12.5 μg/ml) or 3, 5, 7-trihydroxyflavone (6.25 μg/ml). This combination action in reversing vancomycin resistance of *Enterococci* highlights novel drug targets has importance in the design of new therapeutic regimes against resistant pathogens (Lui et al., 2001).

Evaluation of the antibacterial activity of the galangin against ten randomly selected bacteria indicated sinificant activity against all the gram-positive bacteria tested with the minimum inhibitory concentration (MIC) ranging from 0.1 to 0.5

mg/ml, and against *Enterobacter cloacae* which was significantly inhibited at an MIC of 0.1 mg/ml. Galangin indicated considerable activity against the fungi tested appeared to be particularly susceptible at a concentration of 0.01 mg/ml (Afolayan and Meyer, 1997).

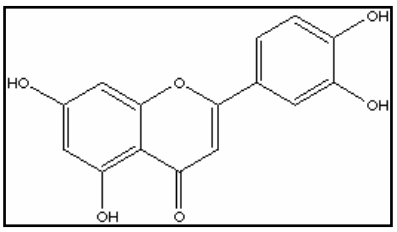
The major antimicrobial compound isolated from the shoots of Helichrysum aureonitens plant in South Africa was found that it was able to inhibit some gram-positive bacteria such as *Bacillus cereus, Bacillus, pumilus* and *Staphylococcus aureus* in the minimal inhibitory concentration (MIC) at 0.1-0.5 mg/ml but was not able to inhibit gram-negative bacteria except *Enterobacter cloacae* in the MIC at 0.1 mg/ml. It had affected antifungal activity at 0.01mg/ml concentration (Afolayan, and Meyer,1997). Furthermore, galangin could affect to herpes simplex virus type I (HSVI), Coxsackie B virus type I (Cox.B1) at 12.47 μg/ml but least effected to retrovirus and no effect to adenovirus type 31 (Ad.31) (Meyer et al., 1997).

Galangin may be used for cancer chemoprevention because it could be synergetic with enzyme and suppressing genotoxicity of chemical (Heo et al., 2001). Cipak and other researchers (2003) found that galangin combined with cisplatin could eradicate cell toxicity in L 1210 murine leukemia cells. This may treat cancer in cooperation with cisplatin. Researchers found that galangin (with 3, 5, 7 OH-group position) could reduce toxicity of peroxynitrite because it has catechol at ring B or OH-group at 3, 5, 7 position (Heijnen et al., 2001).

Twelve clinical isolates of resistance *Staphylococcus aureus (S. aureus)* and four isolates of methicillin-resistance *S. aureus* were inhibited when treated with amoxicillin plus galangin 12.5 μg/ml. Furthermore, six clinical isolates of

ceftazidime-resistant *S. aureus* had theirs resitance to ceftazidime by galangin at MICs 25 μg/ml to < 0.25 μg/ml (Graingsak Eumkeb and Richards, 2003).

2.6.4 Luteolin; digitoflavone; 3′, 4′, 5, 7-tetrahydroxyflavone; 2-(3, 4-dihydroxyphenyl)-5, 7-dihydroxy-4H-1-benzopyran-4-one



Molecular formular: C_{15} H_{10} O_6

Melting point: 328-330°C

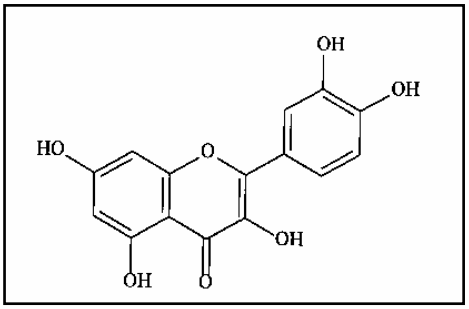
Solubility: Soluble in ethyl acetate, formic acid, water, alkaline, and DMSO

Appearance: Yellow needle (Susan, 1996)

Luteolin, a naturally occurring flavonoid, is abundant in our daily dietary intake. It exhibits a wide spectrum of pharmacological properties, but little is known about its biochemical targets other than the fact that it induces topoisomerase II-mediated apoptosis. One study, showed that luteolin completely inhibits the catalytic activity of eukaryotic DNA topoisomerase I at a concentration of 40 μM, with an IC50 of 5 μM and further study supported its therapeutic potential as a lead anti-cancer compound (Chowdhury et al., 2002).

Luteolin could inhibit *Neisseria gonorrhoeae* and *Helicobacter pylori* N-acetyltransferase (NAT) activity and their growth (Chung et al., 2001; Tsou et al., 2001).

2.6.5 Quercetin; Quercitin; Quercetrin; meletin; sophoretin; 3, 3', 4', 5-7- pentahydroxyflavone; 2-(3,4-dihydroxyphe-nyl)-3, 5, 7- trihydroxy-4H-1-benzopyran-4-one



Molecular formular: $C_{15}H_{10}O$

Melting point: 314°C

Solubility: Soluble in glacial acetic acid, aqueous alkaline, alcohol, water and DMSO

Appearance: Yellow needle (Susan, 1996)

Quercetin belongs to a class of water-soluble plant pigments called flavonoid. Quercetin can be found in onions, apples, green tea and black tea. Smaller amounts

are found in leafy green vegetables and beans. Quercetin is chemically related to a class of flavonoids called (pro) anthocyanins.

Quercetin acts as an antihistamine, antiinflammatory, antiallergic, antiviral properties. As antioxidant, it protects LDL cholesterol (the "bad" cholesterol) from becoming damage. A variety of evidence indicated that quercetin possesses potent antioxidant properties. Cardiologists believe that damage to LDL cholesterol is an underlying cause of heart disease. Quercetin blocks an enzyme that leads to accumulation of sorbital, which has been linked to nerve, eye and kidney damage in those with diabetes. Quercetin is active against Bacillus cereus when present in the amount of 2.5 μg/ml (Wang et al., 1989).

Schinazi, Hughes, and Chen (1997) showed that quercetin inhibited certain viruses in vitro, including the Rauscher leukemia virus and HIV virus at nontoxic concentrations and quercetin was strong inhibitor of DNA polymerase-β.

Quercetin has been shown to cause chromosomal mutations in certain bacteria in test tube studied (Stoewsand, Anderson, Boyd, and Hrazdina, 1984).

Quercetin significantly reduced superoxide dismutase activity and increase the malonaldehyde content in SV40-transformed cell line (BNL SV A.8) cells. These are thought to be closely related to quercetin-mediated apoptosis. Young et al. (2004) suggested that quercetin was a dietary flavonoid that is capable of inducing selective growth inhibition and apoptosis in hepatic tumor cells. Flavone, quercetin is effective inhibiting the growth of the organisms. The most active extracts were white birch (*Betula pubescens* Ehrh.), pine (*Pinus sylvestris* L.) and potato (*Solanum tuberosum* L.) against gram-positive *Staphylococcus aureus* (Rauha et al., 2000). The antibacterial activitiy of *Rubus ulmifolius* extracts and some isolated constituents,

quercetin-3-O -beta-D- glucuronide showed a high antimicrobial activity (Panizzi et al., 2002). Quercetin, up to 100 μg/mL, had any significant activity on selected gram-positive strains, gram-negative strains, and yeasts. It effected on anti-HIV activity (80% inhibition at 40 μM), which might depend on the free hydroxyl in the C-3 position, as suggested by the lack of activity of the 3-O-acylquercetines (Gatto et al., 2002).

Oral treatment with quercetin protected ABD2F1/Jena mice significantly against intraperitoneal encephalomyocarditis, Col, Sk, MM, Men M, L and Mengo M virus infections. Tumor necrosis factor (TNF) produced a dose-dependent inhibition of vesicular stomatitis virus (VSV), encephalomyocarditis (EMCV) and herpes simplex virus type I (HSV-I) replication in WISH cells. The antiviral activity of TNF against VSV and EMVC was greatly enhanced by combination with quercetin. Induction of 2, 5-oligo-adenylate (2-5A) synthetase by TNF was also enhanced by quercetin. Addition of polyclonal antibodies to human interferon (IFN)-beta completely blocked both enhancement of antiviral activity and 2-5A synthetase induction (Ohnishi and Bannai, 1993). An antifungal agent 3, 4-dihydroxybenzoic acid was formed by peroxidase (POX)-dependent oxidation of quercetin on browning of onion scales (Takahama and Hirota, 2000).

Apigenin and quercetin were effective in reducing DNA oxidative damage and inhibited to poisomerase-catalyzed DNA irregularities. Furthermore, galangin and quercetin also displayed antiviral including anti-HIV activity. It was found that the flavonols were more active than flavones against herpes simplex virus type 1 (Panizzi et al., 2002). The aqueous organic solvent extracts of the leaves of Begonia malbarica Lam (Begoniaceae) were also tested against human pathonic bacteria and fungal

strains by the agar-well diffusion method. The aqueous extracts showed activity against the gram-negative bacteria. The chloroform and methanol extracts showed activity against all the tested bacteria. The study supported the claim of the usefulness of the plant in respiratory tract infections and also suggested its use in diarrhoea and skin diseases caused by pathogenic bacteria (Ramesh, Viswanathan, Sarawathy, Balakrishna, Brindha, and Lakshmanaperumalsamy, 2002).

In Thailand, there are many medicinal plants which have antiseptic properties, antimicrobial activity including Zingiberaceae family such as *A. galanga, A. nigra* and *A. officinarum*. These medicinal plants have some chemicals of flavonoids such as galangin and baicalin could affect synergy with β-lactam antibiotics agianst MRSA and penicillin-resistant *Staphylococcus aureus* (Lui et al., 2000). Previously antibacterial studies found that galangin had inhibitory effect against MRSA strain when combined with β-lactam antibiotic drugs (Griangsak Eumkeb and Richards, 2003).

2.7 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).

2.7.1 Susceptibility test method

Dilution Testing:

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).

2.7.2 Mechanism

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) (American Society for Microbiology, 2004; John, 2004; Odds, 2003).

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 synerg; a deviation away from the axes is often taken to indicate antagonism, although in difference may also produce this result (Sawan and Manivannan, 2000).

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Test organisms

Bacterial strains

Staphylococcus aureus (methicillin-resistant) and *Enterobacter cloacae* (ceftazidime-resistant) were obtained from Maharat Nakhon Ratchasima hospital, Nakhon Ratchasima province, Thailand.

3.1.1.1 Identification of isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and ceftazidime-resistant *Enterobacter cloacae* (CREnC)

Identification of MRSA

MRSA was identified by biochemical characteristic tests following Holt et al. (1974, 1994). The results obtained were a gram-positive spherical. Colonies on nutrient agar were opaque, creamy, mucoid and smooth with entire margin. Catalase coagulase and VP tests were positive. Oxidase test was negative. The bacteria could hydrolyse starch. Acids were produced from carbohydrates, namely mannose and lactose. Nitrate was reduced to nitrite (Table 1). In this results, the characteristics of

MRSA were similar to biochemical characteristic tests of *S. aureus* by Holt et al. (1974, 1994).

Identification of CREnC

CREnC was identified by biochemical characteristic tests following Holt et al. (1974, 1994). The results obtained were a gram-negative straight rod. Colonies on nutrient agar were yellow and mucoid. Catalase and VP tests were positive. Idole production, oxidase and MR tests were negative. The bacteria could hydrolyse gelatin and starch. Acids were produced from many carbohydrates, namely arabinose, cellobiose, lactose, maltose, melibiose, mannose, rhamnose, raffinose, sucrose, trehalose and xylose (Table 1). In this results, the characteristics of CREnC were similar to biochemical characteristic tests of *E. cloacae* by Holt et al. (1974, 1994).

Table 1 Characteristic of isolates of methicillin-resistant *S. aureus* and ceftazidime-resistant *E. cloacae* from Maharat Nakhon Ratchasima hospital

Biochemical Test	Clinical isolates of MRSA	Clinical isolates of CREnC
Gram reaction	+	-
Coagulase test	+	ND
Catalase test	+	+
Oxidase test	-	-
Starch hydrolysis	+	+
Indole production	ND	-
Gelatin liquefaction	ND	-
MR test	ND	-
VP test	+	+
Mannose	+	+
Maltose	-	+
Sucrose	-	+
Lactose	+	+

Note: + Positive reaction

- Negative reaction

ND = Not Determined

3.1.1.2 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 4°C. Fresh slope cultures were prepared every 3-4 weeks (Griangsak Eumkeb, 1999).

3.1.2 β-lactam antibiotics

Cloxacillin, ceftazidime and ampicillin were obtained from Sigma, Glaxo, Welcome (distributor in Thailand). β-lactamases of *S. aureus* and *E. cloacae* were obtained from Sigma (Poole, England).

3.1.3 Culture media

Iso-sensitest broth, agar and Mueller-Hinton nutrient broth, agar were obtained from Oxiod. Apigenin, baicalein, galangin, luteolin and quercetin were obtained from Indofine chemical (The Flavonoid Company, USA).

Approximate formula per liter of each medium was as following:

3.1.3.1 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	

3.1.3.2 Nutrient broth

Difco nutrient broth was used as the basic liquid culture medium for growing the overnight cultures.

The formula was:

	g/litre
Beef extract	3.0
Peptone	5.0
pH 6.8±0.2	

3.1.3.3 Iso-sensitest broth

Oxoid Iso-sensitest broth was developed specifically for antimicrobial susceptibility testing. It was used in the investigations to determine the permeability of the cytoplasmic membrane (CM) and outer membrane (OM) and the effect of flavonoids on the cell structure of MRSA, CREnC and ASEnC.

The formula was:

	g/litre
Hydrolysed casein	11.0
Peptones	3.0
Sodium chloride	2.0
Soluble starch	1.0

	g/litre
Disodium hydrogen phosphate	2.0
Sodium acetate	1.0
Magnesium glycerophosphate	0.2
Calcium gluconate	0.1
Cobaltous sulphate	0.001
Cupric sulphate	0.001
Zinc sulphate	0.001
Ferrous sulphate	0.001
Manganous chloride	0.002
Menadione	0.001
Cyanocobalamin	0.001
L-cysteine hydrochloride	0.02
L-trypophan	0.02
Pyridoxine	0.003
Pantothenate	0.003
Nicotinamide	0.003
Biotin	0.0003
Thiamine	0.00004
Adenine	0.01
Gaunine	0.01
Xanthine	0.01
Uracil	0.01
pH 7.4 ±0.2	

3.1.3.4 Iso-sensitest agar

Oxiod Iso-sensitest agar was the medium used for determining the activity of the antibacterial agent by the able count method and for preparing the bacterial suspension standard curve by determining the absorbance and viable count for a series of suspensions.

The formula was:

	g/litre
Hydrolysed casein	11.0
Peptones	3.0
Glucose	2.0
Sodium chloride	3.0
Soluble starch	1.0
Disodium hydrogen phosphate	2.0
Sodium acetate	1.0
Magnesium glycerophosphate	0.2
Calcium gluconate	0.1
Cobaltous sulphate	0.001
Cupric sulphate	0.001
Zinc sulphate	0.001
Ferrous sulphate	0.001
Manganous chloride	0.002
Menadione	0.001

	g/litre
Cyanocobalamin	0.001
L-cysteine hydrochloride	0.02
L-trypophan	0.02
Pyridoxine	0.003
Pantothenate	0.003
Nicotinamide	0.003
Biotin	0.0003
Thiamine	0.00004
Adenine	0.01
Gaunine	0.01
Xanthine	0.01
Uracil	0.01
Agar	8.0
pH 7.4±0.2	

3.1.3.5 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
Casien hydrolysate	17.5
Soluble starch	1.5

pH 7.4 ± 0.2 at 37ºC

Mueller-Hinton had been cation-adjusted that has had the correct concentrations of the divalent cations of Ca^{++} 20 mg/l and Mg^{++} 10 mg/l (MBH). All culture media were dissolved by water.

3.1.3.6 Mueller-Hinton agar

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
Casien acid hydrolysate	17.5
Solube starch	1.5
pH 7.3±0.2 at 25ºC	

3.1.4 Chemicals

All chemicals used were laboratory grade otherwise specified.

Dimethylsulfoxide (DMSO)	AR grade
Disodium tetraborate	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
Aradite	Lab grade
Agarose	Lab grade
Uranyl acetate	Lab grade
Lead acetate	Lab grade
HEPES buffer	Lab grade
Phosphate	Lab grade
PMSF	Lab grade
Triton X-100	Lab grade
Cloxacillin	AR grade
Ceftazidime	AR grade
Ampicillin	AR grade
Apigenin	AR grade
Baicalein	AR grade
Galangin	AR grade
Luteolin	AR grade

Quercetin	AR grade
β-lactamases	AR grade

3.1.5 Equipment

3.1.5.1 Apparatus

Whlimixer	Labinco BV
Spectronic 21	Milton Roy
Labofuge 400R	Heraeus
Autoclave	Yamato
Laminar air flow	Woerden
Hot air oven	Shellab
Shaking incubator	Heto
Hot plate	VELP scientifica
Refrigerated Incubator	VELP scientifica
Ultramicrotome	JEM
Micropipettors (2-20 µl)	Witeg
Micropipettors (100-1000 µl)	Witeg
Cenrtifuge tubes	Pyrex
Spectraphysics	Agilent

3.1.5.2 Glassware

Beakers (50, 100, 250, 500, 1000 ml)	Pyrex
Pipettes (1, 5, 10 µl)	Brand
Measuring cylinder (10, 20 µl)	Brand

Petri dishes	Pyrex
Test tubes	Pyrex

3.2 Methods

3.2.1 Preparation of test solution and inoculum

Antibiotic test solutions were prepared by dissolving cloxacillin, ceftazidime and ampicillin (1 mg/ml) in sterile water. Baicalein and other four flavonoids (apigenin, galangin, luteolin and quercetin (500 μg/ml)) were dissolved in DMSO solution and were diluted with sterile water with adjustment to give the required test concentrations. Test organisms were incubated in 20 ml Cation-adjusted Muller-Hinton broth (CAMHB) for MRSA and Iso-sensitest broth for CREnC for 18 h at 37º C. The cell cultures were centrifuged at 4,000 r.p.m/min. The cell pelletes was washed with saline, recentrifuged and resuspended in saline. The cell concentrations were adjusted with saline to give 10^{12} CFU /ml using a predetermined calibration curve of absorbance at 500 nm against viable count (Richards and Xing, 1993). This cell suspension was diluted with double strength broth to 10^{6} CFU/ml. Overdried CAMH agar plates for MRSA and Iso-sensitest agar plates for CREnC were used for determining CFU (Iain et al., 1999).

3.2.2 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

nutrient 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 5 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 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 overdried agar plate counting method. (Griangsak Eumkeb, 1999; Richards, Gregory, and Xing, 1993).

3.2.3 MICs determination

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 broth medium and a standard inoculum of the test strain (commonly 100,000 bacteria) was introduced into each tube. The test was incubated at 37ºC 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 serves to indicated that the organism is viable in case the end-point was missed (Greenwood, 2000).

MICs was determined using a broth macrodilution method.The sterile wire loop test organism from a slope culture was inoculated into nutrient 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 $5x10^7$ CFU/ml by using the absorption of bacterial suspension viable count standard curve. The inoculum of 0.1 ml of standard of suspension (18 h culture) of each strain of the test bacteria was added to triplicate tubes containing 9.90 ml CAMHB for MRSA and Iso-sensitest broth for CREnC plus serial dilutions of the antibacterials, to give approximately $5x10^5$ CFU/ml. Tubes of broth without antibacterials were used as the control for each of the test bacteria. Incubation was at 37ºC for 24 h. The MIC was defined as the lowest concentration of antibiotic at which there is no visible growth in the triplicate tubes (Griangsak Eumkeb, 1999).

3.2.4 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 (Griangsak Eumkeb, 1999; Lorian, 1991).

Checkerboard determinations in antimicrobial combinations were performed following the method of Sabath (1967) with slight modification (Griangsak Eumkeb, 1999). Antibacterial agent “A” and antibacterial agent “B” were diluted to1/10 of their MICs along the ordinate and abcissa respectively.

An 18 h culture of each of the testbacteria was prepare. The test bacterial

suspensions were adjusted to 5 X 10^7 CFU/ml using the absorption of bacterial suspension from the previously determine standard curve. 0.1 ml of the bacterial suspension was added to a series of 9.9 ml CAMHB for MRSA and Iso-sensitest broth for CREnC, plus 10% serial dilutions of the antibacterial combinations, to give $1x10^5$ CFU/ml. Tubes of broth without antibacterials were used as the controls for each of the test bacteria. The culture was incubated for 24 h at 37ºC. The test was carried out in triplicate. MICs were determined for each antibacterial combination and the isobolograms were plotted. The calculation of the FIC (Minimum Inhibitory 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) $\leq$ 0.5	Synergy
FIC (A+B) $>$ 0.5-4.0	No interaction
FIC (A+B) $>$ 4.0	Antagonism

(American Society for Microbiology, 2004; Johnson, 2004; Odds, 2003)

3.2.5 Killing curve determinations

Viable counts for the determination of killing curve was performed as previously described (Richards and Xing, 1993) using a culture medium volume of 100 ml. Inocula of 5 X 10^5 CFU/ml was exposed to the antibacterials either singly or in combination with galangin or other four flavonoids (apigenin, baicalein, luteolin

and quercetin) at an incubation temperature of 37°C. After contact time of 0, 0.5, 1, 2, 4, 6 and 24 h, a 1 ml sample of each incubated mixture was inactivated by addition of nutrient broth (9 ml) containing 0.125% lecithin and 3% (v/v) Tween 80. Subsequent dilution plating on overdried MH agar plates for MRSA and Iso-sensitest agar plates for CREnC in quadruplicate and incubation at 37°C for 18 h was allowed counting of growing colonies. The lowest detectable limit for counting was 10^3 CFU/ml. Positive controls were used containing similar cell and solvent concentrations (Iain et al., 2000).

3.2.6 Transmission electronmicroscopy (TEM) method

Preparation of cultures

To examine the effect of apigenin, baicalein, galangin, luteolin and quercetin on the cell structure of MRSA or CREnC the following mehtods were used.

MRSA or CREnC or ASEnC were incubated separately in 10 ml nutrient broth for 18 h at 37°C. A 0.2 ml volume of 18 h culture was inoculated into a 250 ml conical flask containing 98 ml CAMHB for MRSA and Iso-sensitest broth for CREnC 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 5 min in 0.9% NaCl. Volume of 10 ml of 5 X 10^6 of CFU/ml in 0.9% NaCl, the 4 h log phase culture was inoculated into 250 ml conical flasks each containing 90 ml CAMHB for MRSA and Iso-sensitest broth plus galangin and/or other four flavonoids plus selected antibacterial to give approximately 5 X 10^5 CUF/ml final concentration. A flask containing 90 ml CAMHB for MRSA and Iso-sensitest broth for CREnC without any antibiotics was used as the control. The log phase cultures together with

the appropriate flavonoid plus antibacterial and log phase control cultures was incubated for 4 h shaking at 100 oscillations/min in a water bath at 37°C (Richards et al., 1993; Xing, 1994).

The *E. cloacae* 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 in 4%v/v 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 resuspension in distilled water. The final pellet 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 Aradite (Agar Scientific Ltd, Stansted, Essex) thin sections will be cut with a diamond knife on a RMC ultramicrotome model MTX, stained with uranyl acetate and lead citrate, and examined in a JEOL, JEM 2010 electron microscope at 80-100 kV.

Log phase of *S. aureus* (MRSA) cultures in cubated for 4 h in the presence of the appropriate concentrations of flavonoid plus selected antibacterial was centrifuged (4,000 g, 15 min, 4°C) and the supernatants were removed. The cell pellets of MRSA were fixed for 12 h respectively at 4°C with 2% w/v paraformaldehyde plus 2.5% w/v glutaraldehyde in 0.1 M phosphate buffer pH 7.2 containing 2.5 mM $MgCl_2$. After washing with buffer the fixed cells were suspended in 1% w/v OsO_4 for 6 h for MRSA at 20°C. The specimen embeding sectioning, staining and examining were as described previously (Richards et al., 1993).

3.2.7 Capillary electrophoresis

To examine the effect of some flavonoids as previously described above on the outer membrane (OM) and peptidoglycan associated proteins of MRSA or CREnC the following methods were used.

MRSA or CREnC was incubated separately in 100 ml quantities nutrient 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 Iso-sensitest broth which was place in a water bath shaking at 100 oscillations/min for 4 h at 37°C. Volumes of 100 ml of the 4 h log phase culture were inoculated into 250 ml conical flasks each containing 100 ml Iso-sensitest broth plus galangin and/or other four flavonoids plus selected antibacterial. A flask containing 100 ml Iso-sensitest broth without any antibacterials was used as the control. The log phase cultures together with the appropriate flavonoid plus antibacterial and log phase control culture was incubated for 4 h shaking at 100 oscillations/min in a water bath at 37°C. The bacterial concentrations used at 1/2 MIC were as followed. Selected flavonoids and drugs were used singly or combination for clinical isolates of MRSA or CREnC (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 (3 X 60s 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. A 1 ml 20% (w/v) sodium-N-lauroyl sarcosinate (Sarkosyl) was mixed with supernatant and incubated for 30 min at room temperature. A visible clearing of the envelope suspension was indicated

solubilization of the cytoplasmic membrane (CM). The OM 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 and peptidoglycan protein extract of each sample from MRSA or CREnC was resuspended in 0.5 ml of (distilled water + 2ml/ml PMSF). Therefore, the same quatity of OM and peptidoglycan extract of each sample (50 mg/ml) from MRSA or CREnC could be investigated. These extract proteins were then stored frozen at -70°C (Markwell, Hass, Bieber, and Tolbert, 1978; Richards and Xing, 1996; Williams and Gledhill, 1991).

Peptidoglycan-associated protein (PG) extract (0.25 ml) was mixed with 0.5M sodium borate solution (pH 9, 0.2 ml) and internal standard albumin 50 μl (20 μg/ml). Samples were then separated by capillary electrophoresis (CE). The column was given a 1-min flush with 0.1M sodium hydroxide (100 μl), a 1-min flush with methanol (100 μl), then a 1-min flush with running buffer (100 μl) by syringe and followed by 5 min running with buffer under high voltags to equilibrium. The sample was loaded over 10s and the electrophoresis was at 20 kilo voltages (kV). The column eluent was monitored for ultraviolet (UV) absorbance at a wavelength of 205 nm.

A volume of protein extract was mixed with a volume of sample buffer and incubated at 20°C for 10 min. Each protein extrac suspension was injected to identify the relative protein by CE. The protein peak having the same migration time in CE was regarded to be that same protein.

Quantification was performed by comparison of the peak areas of the individual compound relative to those of the appropriate internal standard. The

reproducibility of the assay was determined by carring out five independent replicate determinations.

The capillary electrophoresis (CE) system used for the experiments was the electropherograph from Agilent (Germany). The fused-silica capillary was 50 μm I.D. and had a total length of 64.5 cm and a separation length of 40 cm. The sample loading data was collected with Spectraphysics Agilent Technologies Model 4270 integrator (Xing and Richards, 1996).

3.2.8 Enzyme assay

β-lactamases of *Bacillus cereus (B. cereus)* and *E. cloacae* were obtained from Sigma (Poole, England). Enzyme activities were adjusted to concentration sufficient to hydrolyse 50-60% substrate in 5 minute. Flavonoids 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, cloxacillin and ampicillin 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 500 µg/ml of ceftazidime, cloxacillin and ampicillin within 5 min. 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 (100 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).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Bacterial suspensions viable count absorption standard curve

The results of the bacterial suspensions viable count standard curve for methicillin-resistant *S. aureus* (MRSA), ceftazidime-resistant *E. cloacae* (CREnC) and ampicillin-sensitive *E. cloacae* (ASEnC) are shown in Figures 3 to 5.

Figures 3, 4, and 5 indicate that approximately $5X10^{12}$ CFU/ml of clinical isolates of methicillin-resistant *S. aureus*, ceftazidime-resistant *E. cloacae* and ampicillin-sensitive *E. cloacae* have absorption at 500 nm of 0.14, 0.15, and 0.13 respectively.

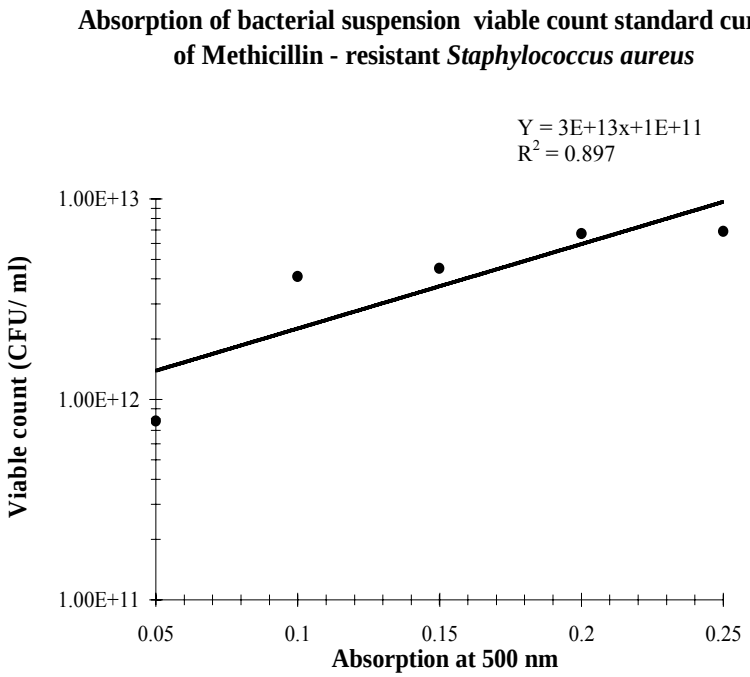


Figure 3 Standard curves for suspension of methicillin-resistant *Staphylococcus aureus* (MRSA)

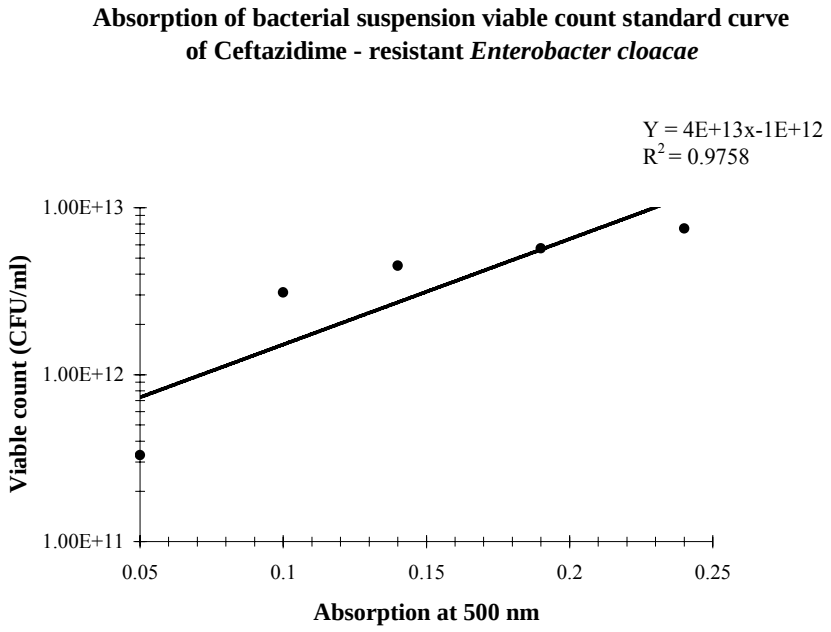


Figure 4 Standard curves for suspensions of ceftazidime-resistant *Enterobacter cloacae* (CREnC)

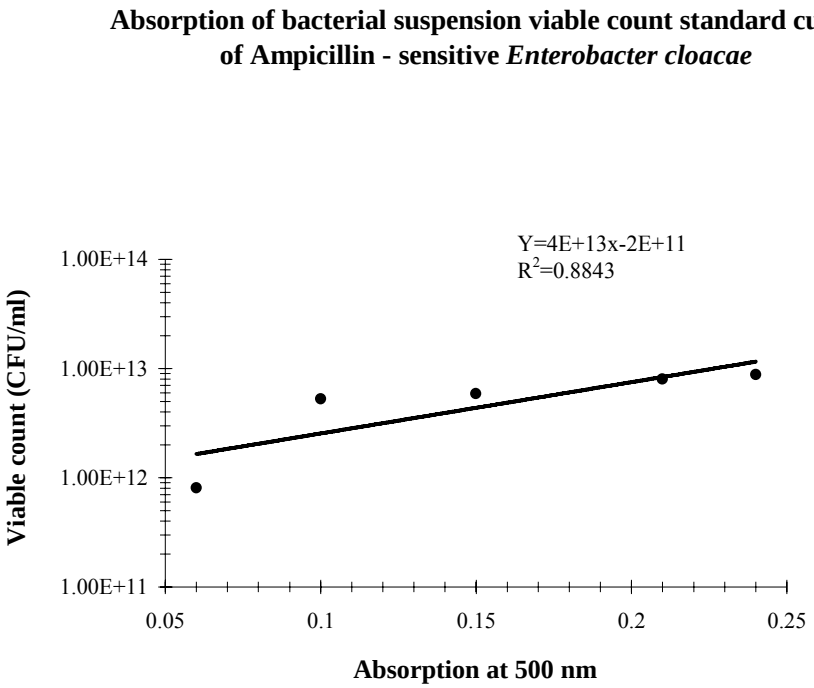


Figure 5 Standard curves for suspensions of ampicillin-sensitive *Enterobacter cloacae* (ASEnC)

4.2 MIC determination

In present study, several flavonoids such as galangin, apigenin, baicalein, quercetin, and luteolin and β-lactams such as methicillin, cloxacillin, and ceftazidime were tested against clinical isolates of methicillin-resistant *S. aureus*, ceftazidime-resistant *E. cloacae* and ampicillin-sensitive *E. cloacae.* The MIC values of the flavonoids and β-lactams quantitatively assessed by macrobroth dilution method as described previously (Lui, Durham, and Richards, 2000). The MICs of five flavonoids and three β-lactams in the present study are summarized in Table 2. The results showed that some antibacterial activities against drug-resistant isolates of bacteria tested, both gram-positive and gram-negative bacteria. The MICs of ceftazidime, ampicillin and cloxacillin against clinical isolates of methicillin-resistant *S. aureus* were 50, 100, and > 1,000 μg/ml respectively. The MICs of these drugs against clinical isolates of ceftazidime-resistant *E. cloacae* were > 1,000 μg/ml. The MICs of ampicillin, ceftazidime and cloxacillin against clinical isolates of ampicillin-sensitive *E. cloacae* were 5, 10, 100 μg/ml respectively. While all of these bacteria showed resistance to all five flavonoids at MICs > 400 μg/ml.

Disscussion

From these results showed MIC values of the flavonoids and β-lactam antibiotics against clinical isolates of MRSA, CREnC and ASEnC. Ampicillin and ceftazidime alone showed that some antibacterial activities against clinical isolates of MRSA at MICs 100 and 50 μg/ml respectively. But CREnC showed MICs of ampicillin,

ceftazidime and cloxacillin alone at > 1000 μg/ml for all of three drugs. Ampicillin, ceftazidime and cloxacillin alone showed MICs at 5, 10, and 100 μg/ml respectively for ASEnC. All tested flavonoids alone showed MICs at > 400 μg/ml for MRSA, CREnC and ASEnC. These results showed that MRSA and CREnC were strong resistant to all tested antibacterial drugs and flavonoids.

The previously researcher reported the antibacterial activities of β-lactam antibiotics and flavonoids alone. Variation between the results reported by previous workers and this study could be due to differences in concentration of β-lactam antibiotics, flavonoids and strains of test microorganism. The β-lactam antibiotics did not inhibit gram-negative bacteria, CREnC but showed activities against gram-positive, MRSA and ampicillin-sensitive *E. cloacae* (ASEnC) bacteria (Youichi et al., 2003; Griangsak Eumkeb and Richards, 2004). From this study, gram-negative drug-resistant bacteria were greater resistant to β-lactam antibiotics. These were likely to be the result of the difference in cell wall structure between gram-positive and gram-negative bacteria. The gram-negative has a multi-layered and complex structure. The outer membrane can act as a barrier to many environmental substances including antibiotics (Essawi and Srour, 2000).

Table 2 Minimum inhibitory concentration (μg/ml) of the following β-lactams and flavonoids againsclinical isolates of MRSA, CREnC, and ASEnC

Organisms	Ampicillin	Ceftazidime	Cloxacillin	Baicalein	Apigenin	Luteolin	Quercetin
MRSA	100	50	> 1000	> 400	> 400	> 400	> 400
CREnC	> 1000	> 1000	> 1000	> 400	> 400	> 400	> 400
ASEnC	5	10	100	> 400	> 400	> 400	> 400

*MIC presented as Geomean of 3 observations

4.3 Checkerboard determination

Some flavonoids showed diverse combinative effects on sensitive of MRSA and CREnC to β-lactam antibiotics. MIC determination are shown in Tables 3-4. The synergistic activity for all combination of baicalein and all tested β-lactams against clinical isolates of MRSA (FIC ≤ 0.5). The MICs of ampicillin, cloxacillin and ceftazidime were reduced from 100, > 1000, and 50 μg/ml to 5, 5, and 5 μg/ml respectively when combined with baicalein 5 μg/ml against this strain. Luteolin 5 μg/ml can also reduce MICs of ampicillin, cloxacillin and ceftazidime against clinical isolates of MRSA to 50, 50, and 30 μg/ml respectively.

Figure 6 shows effectiveness of the combination between ampicillin plus baicalein at 5:5 μg/ml against MRSA, but ampicillin plus galangin at 50:5 μg/ml, ampicillin plus apigenin at 50:10 μg/ml and ampicillin plus luteolin at 50:5 μg/ml shows

lower antibacterial activities against MRSA.

Figure 7 shows effectiveness of the combination between cloxacillin plus baicalein at 5:5 μg/ml and cloxacillin plus luteolin at 50:50 μg/ml. Figure 8 shows antibacterial activities of the combination of ceftazidime plus galangin, ceftazidime plus baicalein, ceftazidime plus quercetin at 5:5 μg/ml. Whereas ceftazidime plus luteolin at 30:5 μg/ml shows lower antibacterial activity.

The combination between apigenin and tested β-lactams against clinical isolates of CREnC are shown in Figures 9-11. Figures 9 shows antibacterial activities of ampicillin 30 μg/ml plus apigenin or luteolin 5 μg /ml. Figure 10 shows antibacterial activities of cloxacillin plus apigenin at 30:5 μg/ml by reducing the MICs of cloxacillin > 1000 to 30 μg/ml against this strain. Figure 11 indicates the MICs of ceftazidime was reduced from > 1000 to 5 μg/ml with apigenin or luteolin at 5 μg/ml.

FICs Index

The FICs index of five flavonoids plus three β-lactams against clinical isolates of MRSA and CREnC are shown in Tables 3–4. Baicalein at a concentration 5 μg/ml when combined with ampicillin at 5 μg/ml against clinical isolates of MRSA had significantly reduced their MIC values and exhibited FICs index of 0.0625. Baicalein at a concentration 5 μg/ml when combined with cloxacillin at 5 μg/ml against clinical isolates of MRSA had significantly reduced their MIC values and exhibited FICs index of 0.0175. Baicalein or galangin or quercetin at a concentration 5 μg/ml when combined with ceftazidime at 5 μg/ml against clinical isolates of MRSA had significantly reduced their

MIC values and exhibited FICs index of 0.1125. These results showed synergy effect (Table 3).

The FICs index of apigenin or luteolin at a concentration 5 μg/ml when combined with ceftazidime at 5 μg/ml against clinical isolates of CREnC had significantly reduced their MIC values and exhibited FICs index of 0.0175. These results showed synergy effect (Table 4).

Discussion

The lowest fractional inhibitory concentration (FIC) for ampicillin plus baicalein, cloxacillin plus baicalein, ceftazidime plus galangin, ceftazidime plus baicalein, and ceftazidime plus quercetin combinations were 0.0625, 0.0175, 0.1125, 0.1125, and 0.1125 respectively for MRSA. The lowest FICs for ceftazidime plus apigenin or quercetin were 0.0175 for CREnC.

These results indicate a high level synergistic activities since values below 0.5 are widely accepted as representing synergism between two antibacterials (American Society for Microbiology, 2004; Johnson, 2004; Odds, 2003;).

The clinical isolates strains of MRSA and CREnC also had theirs resistance to ampicillin, cloxacillin and ceftazidime. These MICs were reversed when any of these β-lactams was combined with 5 μg/ml of baicalein, galangin or quercetin for MRSA and apigenin or luteolin for CREnC.

Table 3 Minimum inhibitory concentration (μg/ml)* of β-lactams used alone and in combination with different concentrations (μg/ml) of the following flavonoids against methicillin-resistant *S. aureus* (MRSA)

Combination of agents	MIC of MRSA	Approximate FIC index	FIC index value
Ampicillin/Galangin	50:5	0.5125	No interaction
Ampicillin/Baicalein	5:5	0.0625	Synergy
Ampicillin/Apigenin	50:10	0.525	No interaction
Ampicillin/Luteolin	50:5	0.5125	No interaction
Ampicillin/Quercetin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Cloxacillin/Galangin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Cloxacillin/Baicalein	5:5	0.0175	Synergy
Cloxacillin/Apigenin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Cloxacillin/Luteolin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Cloxacillin/Quercetin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Ceftazidime/Apigenin	> 50:50	> 1.125	Antagonism, Synergy, No interaction
Ceftazidime/Luteolin	30:5	0.6125	No interaction
Ceftazidime/Galangin	5:5	0.1125	Synergy
Ceftazidime/Baicalein	5:5	0.1125	Synergy
Ceftazidime/Quercetin	5:5	0.1125	Synergy

*MIC presented as Geomean of 3 observation

Table 4 Minimum inhibitory concentration (μg/ml)* of β-lactams used alone and in combination with different concentrations (μg/ml) of the following flavonoids against ceftazidime-resistant *E. cloacae* (CREnC)

Combination of agents	MIC of MRSA	Approximate FIC index	FIC index value
Ampicillin/Galangin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Ampicillin/Baicalein	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Ampicillin/Quercetin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Ampicillin/Luteolin	30:5	0.0425	Synergy
Ampicillin/Apigenin	30:5	0.0425	Synergy
Cloxacillin/Galangin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Cloxacillin/Baicalein	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Cloxacillin/Luteolin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Cloxacillin/Quercetin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Cloxacillin/Apigenin	5:30	0.08	Synergy
Ceftazidime/Galangin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Ceftazidime/Baicalein	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Ceftazidime/Quercetin	> 50:50	> 0.175	Antagonism, Synergy, No interaction
Ceftazidime/Apigenin	5:5	0.0175	Synergy
Ceftazidime/Luteolin	5:5	0.0175	Synergy

*MIC presented as Geomean of 3 observation

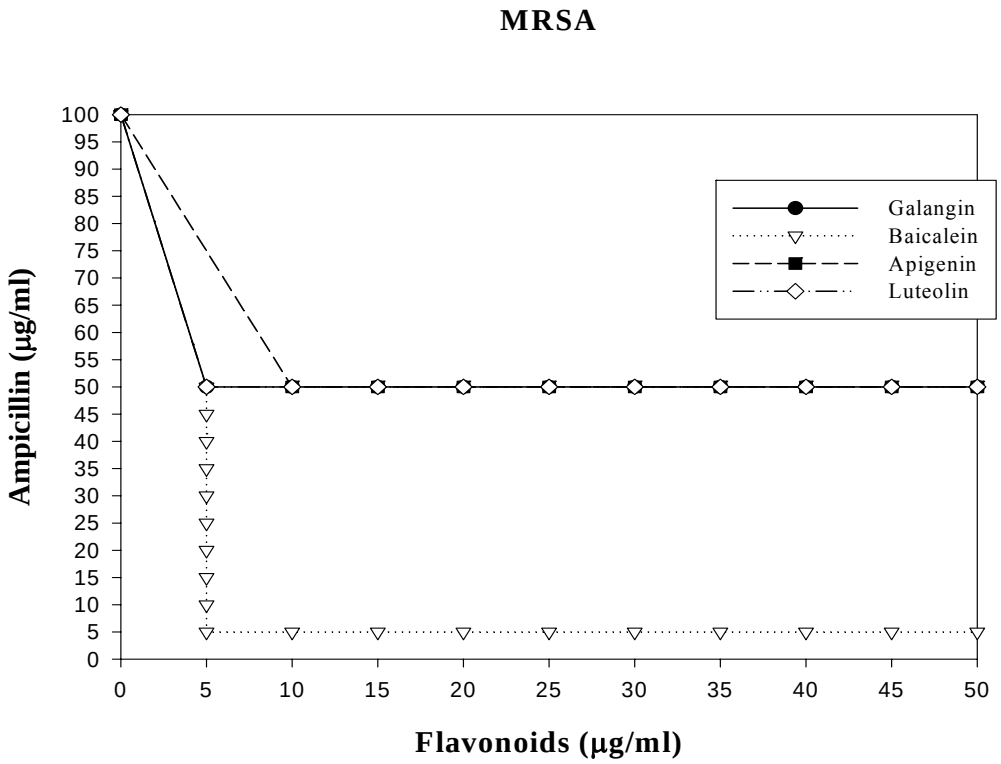


Figure 6 Isobologram constructed from checkerboard MIC data showing antibacterial combination of ampicillin plus tested flavonoids against clinical isolates of MRSA

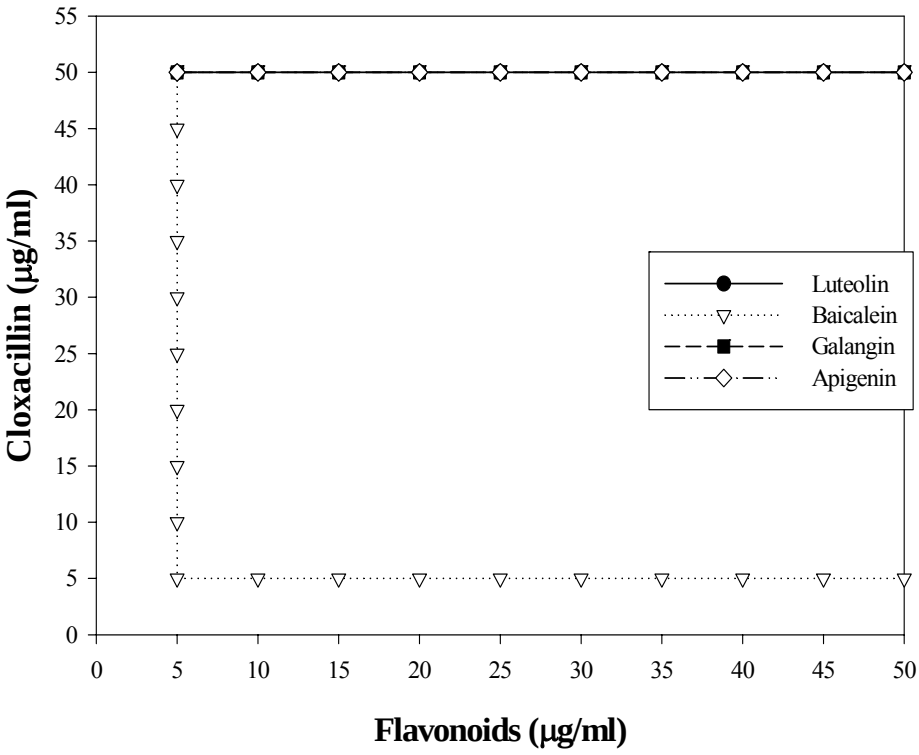


Figure 7 Isobologram constructed from checkerboard MIC data showing antibacterial combination of cloxacillin plus tested flavonoids against clinical isolates of MRSA

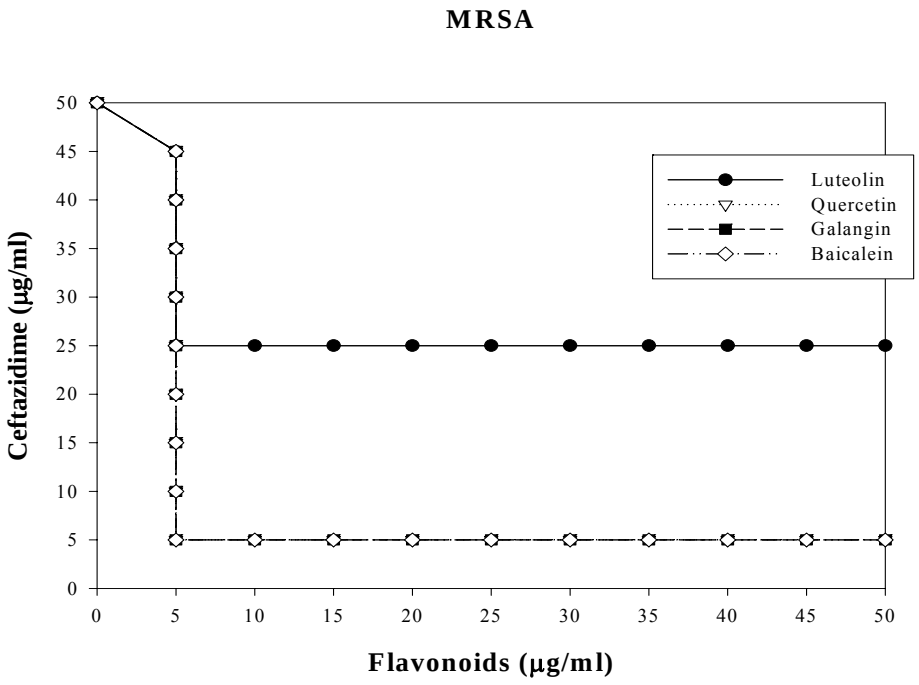


Figure 8 Isobologram constructed from checkerboard MIC data showing antibacterial combination of ceftazidime plus tested flavonoids against clinical isolates of MRSA

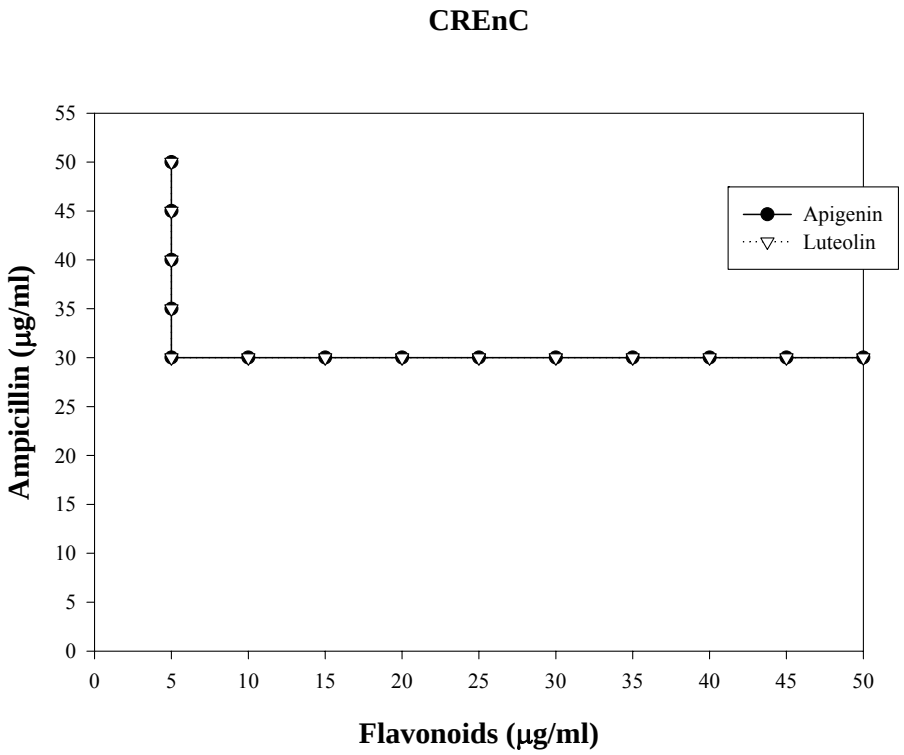


Figure 9 Isobologram constructed from checkerboard MIC data showing antibacterial combination of ampicillin plus tested flavonoids against clinical isolates of CREnC

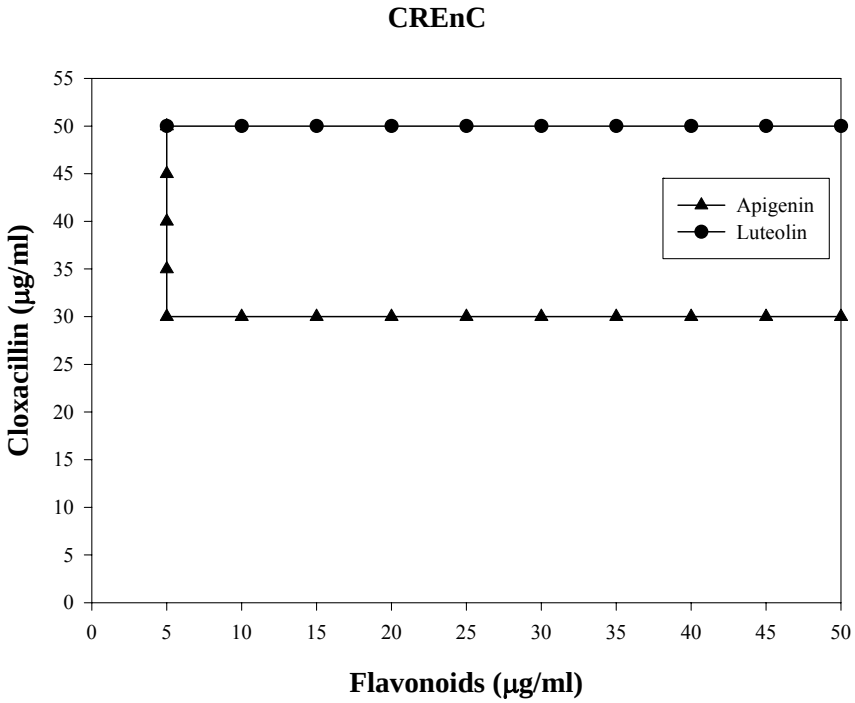


Figure 10 Isobologram constructed from checkerboard MIC data showing antibacterial combination of cloxacillin plus apigenin against clinical isolates of CREnC

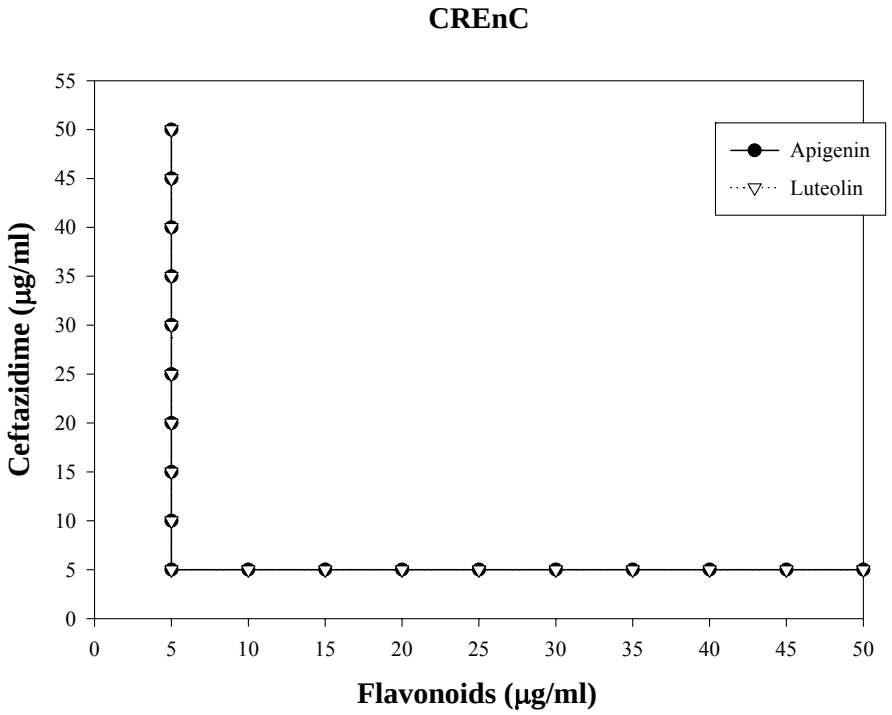


Figure 11 Isobologram constructed from checkerboard MIC data showing antibacterial combination of ceftazidime plus tested flavonoids against clinical isolates of CREnC

From this study, both MRSA and CREnC were found to high resistant to selected antibacterial drugs or flavonoids when use singly. Some flavonoids in combination with β-lactam antibiotics showed synergistic effects on clinical isolates MRSA or CREnC. The results showed synergistic activity for all combination of biacalein and all tested β-lactams against clinical isolates of MRSA, but there were no synergism effect of baicalein and these drugs on clinical isolates of CREnC. These results were supported by ealier studies that *S. aureus* was the most easily inhibited of all the bacteria exposed to flavonoids (Grosvenor, Supriono and Gray, 1995). The greater resistance of gram-negative bacteria to flavonoids had been reported previously (Essawi and Srour, 2000). These observations are likely to be the results of the differences in cell wall structure between gram-positive and gram-negative. The gram-negative has a multi-layered and complex structure, the outer membrane can act as a barrier to many environmental substances including antibiotics (Essawi and Srour, 2000). The results indicated that flavonoids not only have an activity of their own against β-lactam resistant *Staphylococci*, but also have the ability to reverse the resistance of *Enterobacter* bacteria.

4.4 Viable counts

Sampling killing curves resulting from five flavonoids alone and in combination with three β-lactams against clinical isolates of MRSA and CREnC are presented in Figures 12, 13, and 14. The control showed no reduction in the counts of CFU from control inoculum. Figure 12 shows that the combination of baicalein (10 μg/ml) with either cloxacillin or ampicillin (10 μg/ml) caused a reducing of 5 X 10^5 CFU/ml for clinical isolates of MRSA to 10^3 CFU/ml within 6 h. Figure 13 shows that galangin, baicalein and quercetin (10 μg/ml) combined with ceftazidime (10 μg/ml) reduced value count for clinical isolates of MRSA from 5x10^5 to 10^3 CFU/ml within 6 h. Figure 14 shows similar decreases of clinical isolates of CREnC when used ceftazidime (10 μg/ml) in combination with either apigenin (10 μg/ml) or luteolin (10 μg/ml). The viable count of clinical isolates of CREnC was reduced from 5x10^5 to 10^3 CFU/ml within 6 h and throughout 24 h.

Discussion

Results of viable count determinations were consistent with those of the checkerboard tests. Figures 12-13, the results suggested that baicalein in combination with β-lactams (ampicillin, cloxacillin or ceftazidime) had synergistic activity against MRSA by enhancing β-lactam activity in the combination. These results are in substantial agreement with the results from checkerboard that galangin, baicalein or quercetin in combination with ceftazidime had synergistic activity against MRSA by reducing ceftazidime concentration from 50 μg/ml to 5 μg/ml in the combination.

In addition, the results in Figure 14 indicated that apigenin or luteolin in combination with ceftazidime had synergistic activity against CREnC by enhancing

ceftazidime activity in the combination. The concentration of below 17 μg/ml has been regarded by Livemore (1993) as the breakpoint concentration of clinical susceptibility.

These results indicated that the synergistic effects of β-lactam/flavonoid combinations against *S. aureus* may arise from contributions of three distinct types of activities. The first of these due to a weak/moderate direct antibacterial action of flavonoid on cell growth. The second mechanism arises from the ability of flavonoid to inhibit the β-lactamase hydrolysis of susceptible β-lactam antibiotics and hence restore cell sensitivity to the β-lactam antibiotics. The third mechanism involves an action against MRSA or CREnC, which is not dependent upon β-lactamase inhibition, but may be associated with the inhibitory interactions between β-lactams and penicillin binding protein (Iain et al., 2000).

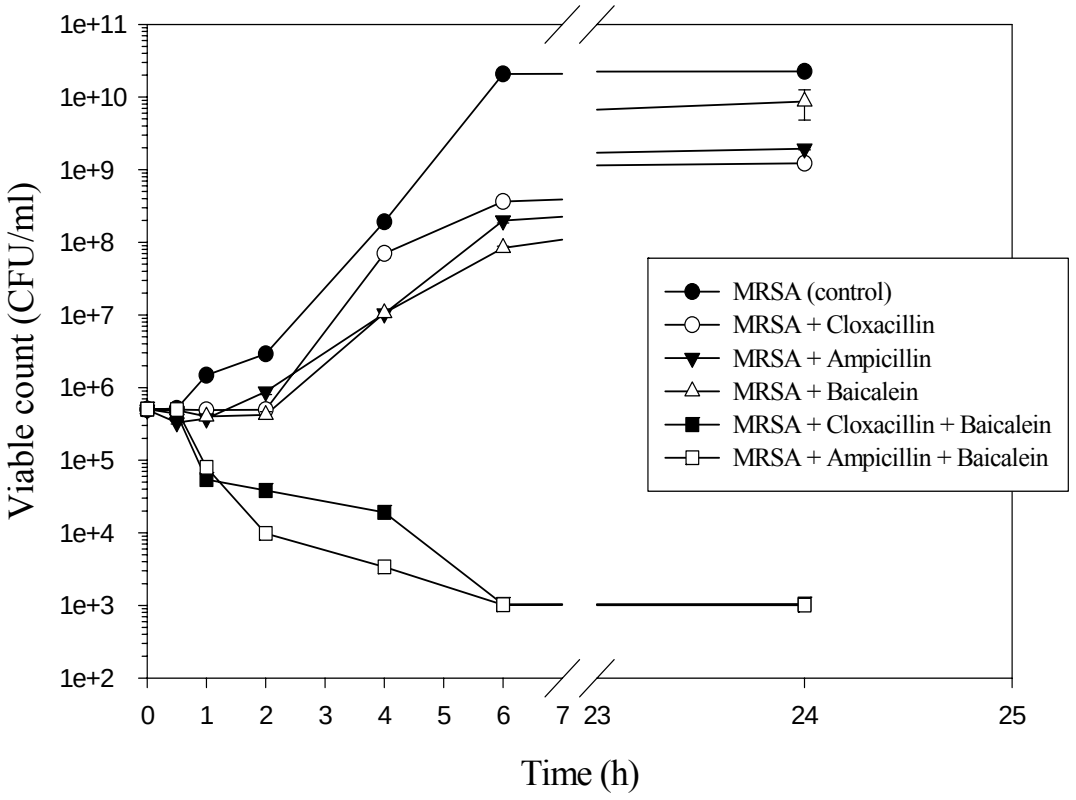


Figure 12 The effect of cloxacillin or ampicillin combined with baicalein on the viable counts of MRSA; ●, control (bacterial culture with corresponding solvent); ○, cloxacillin 50 μg/ml; ▼, ampicillin 50 μg/ml; Δ, baicalein 50 μg/ml; ■ , cloxacillin 10 μg/ml plus baicalein 10 μg/ml; □, ampicillin 10 μg/ml plus baicalein 10 μg/ml; the values plotted are the means of 4 observations and the vertical bars indicate the standard errors of the means.

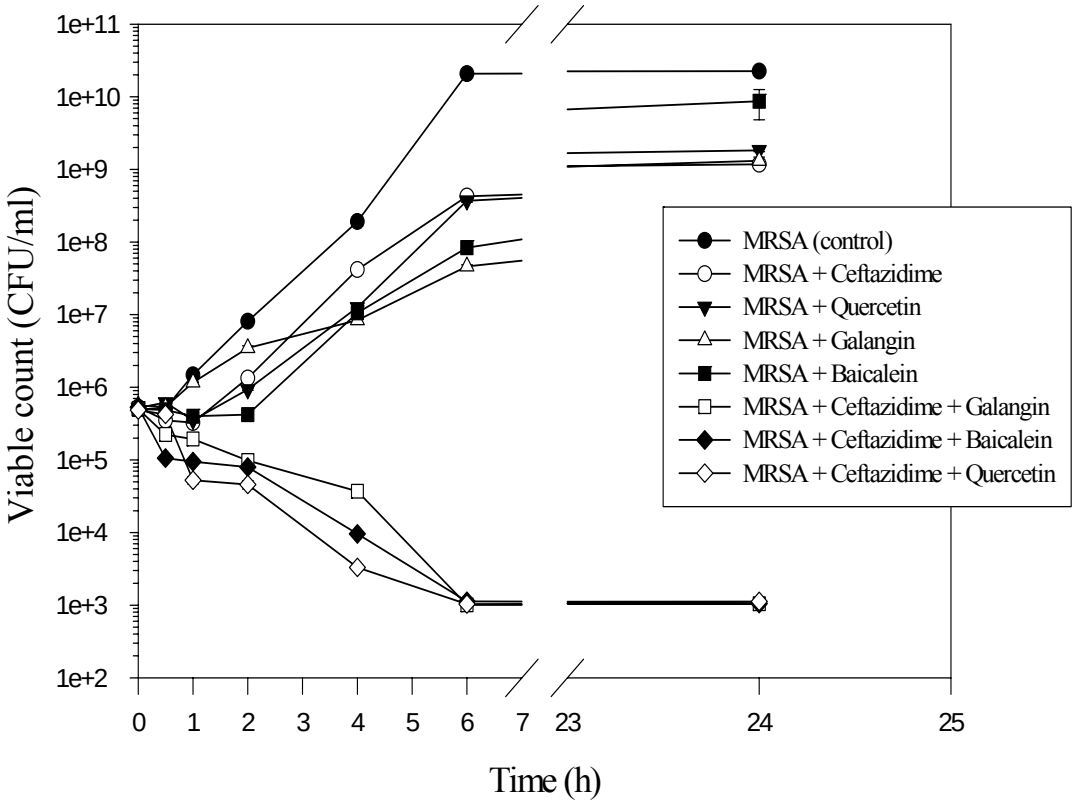


Figure 13 The effect of ceftazidime combined with selected flavonoids on the viable counts of MRSA. ●, control (bacterial culture with corresponding solvent) ;○, ceftazidime 30 μg/ml; ▼, quercetin 50 μg/ml; Δ, galangin 50 μg/ml; ■, baicalein 50 μg/ml; □, ceftazidime 10 μg/ml plus galangin 10 μg/ml; ♦, ceftazidime 10 μg/ml plus baicalein 10 μg/ml; ◊, ceftazidime 10 μg/ml plus quercetin 10 μg/ml; the values plotted are the means of 4 observations and the vertical bars indicate the standard errors of the means.

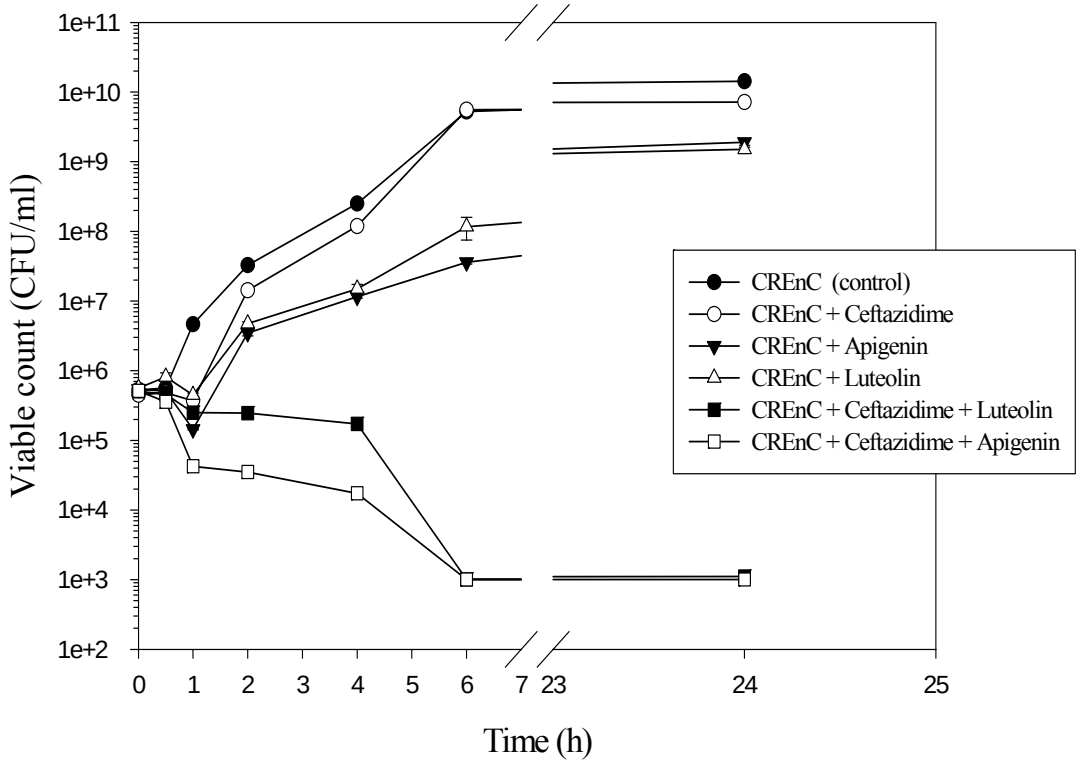


Figure 14 The effect of ceftazidime combined with selected flavonoids on the viable counts of CREnC. ●, control (bacterial culture with corresponding solvent); ○, ceftazidime 30 μg/ml; ▼, apigenin 50 μg/ml; ∆, luteolin 50 μg/ml;■, ceftazidime 10 μg/ml plus luteolin 10 μg/ml; □, ceftazidime 10 μg/ml plus apigenin 10 μg/ml; the values plotted are the means of 4 observations and the vertical bars indicate the standard errors of the means.

4.5 Electronmicroscopy

Electronmicroscope investigations clearly showed the combination of β-lactams with selected flavonoids caused damage to ultrastructures of MRSA and CREnC cells (Figures 15-32).

Figure 15 shows the apperance of normal log phase cells of clinical isolates of MRSA. The cell wall and cytoplasmic membrane can be distingquished. The electron dense ribosomes can be scanned in the great number in cytoplasm. Figures 16-18 show the effect of cloxacillin, ampicillin and ceftazidime respectively on clinical isolates of MRSA strain. All tested antibiotics showed no activity against the clinical isolates of MRSA when used alone at 20 μg/ml.

Figure 16 shows the micrographs of log phase cells of clinical isolates of MRSA after exposure to cloxacillin 20 μg/ml. Some of the bacteria had a little larger than those of the control cells in Figure 15.

Figure 17 shows the micrographs of log phase cells of clinical isolates of MRSA after treatment with ampicillin 20 μg/ml. The micrographs showed that some of the bacteria had slightly larger gap between the cytoplasmic membrane and cell wall. Figure 18 shows the micrographs of log phase cells of clinical isolates of MRSA after expossure to ceftazidime at 20 μg/ml. Several cells of bacteria had rather larger gap between the cytoplasmic membrane and cell wall.

Figures 19-21 show the effect of selected flavonoids use singly on the MRSA cells which had been grown in Cation-adjusted Mueller-Hinton broth. Figure 19 shows the micrographs of log phase cells of clinical isolates of MRSA after treatment with baicalein at 20 μg/ml. The baicalein treated cells were slightly bigger than the normal MRSA cells. Several cells of bacteria had ratherlarger gap between the

cytoplasmic membrane and cell wall. Figure 20 shows thw micrographs of log phase cells of clinical isolates of MRSA after treatment to galangin at 20 μg/ml. The bacterial cells showed no observable morphological cell damage but a few cells had slightly larger shape than normal cells. Figure 21 shows the MRSA cells after treatment with 20 μg/ml quercetin. The cells were approximately the same as control cells.

Figure 22-26 show the effect of selected flavonoids plus β-lactams antibiotics on clinical isolates of MRSA. Figure 22 shows the effect of the combination of ampicillin 10 μg/ml plus baicalein 10 μg/ml on clinical isolates of MRSA. Most of these bacteria exhibited cell wall and cell shape morphological damage. Many cells lost ribosome, consequently had big hole in cytoplasm. Figure 23 shows the micrographs of log phase cell of clinical isolates of MRSA after exposure to cloxacillin plus baicalein at 10:10 μg/ml. A majority of these bacteria exhibited electron-transparent areas devoid of ribosomes (holes) clearly visible within the cytoplasm. A lot of cells lost some organells from cytoplasm such as chromosomes etc. Some of these cells showed considerably longer than normal cells. Figure 24 shows the effect of the combination of ceftazidime plus baicalein at 10:10 μg/ml on clinical isolates of MRSA. These bacteria precisely exhibited morphological damage of cell wall, cytoplasmic membrane and lost of some organells from cytoplasm. Figure 25 indicate the micrographs of log phase cells of clinical isolates of MRSA after treatment with ceftazidime 10 μg/ml plus galangin 10 μg/ml. Some of these bacteria exhibited cell wall damage. Also electron-transparent area devoid of ribosomes and lost of some organells from cytoplasm. Figure 26 shows the

micrographs of log phase cells of clinical isolates of MRSA after treatment with 10 μg/ml ceftazidime plus 10 μg/ml quercetin. Most of these bacterial cells showed very larger gap between cytoplasmic membrane and cell wall. Several bacterial cells clearly exhibitedelectron-transparent area in cytoplasm due to lost most of organells.

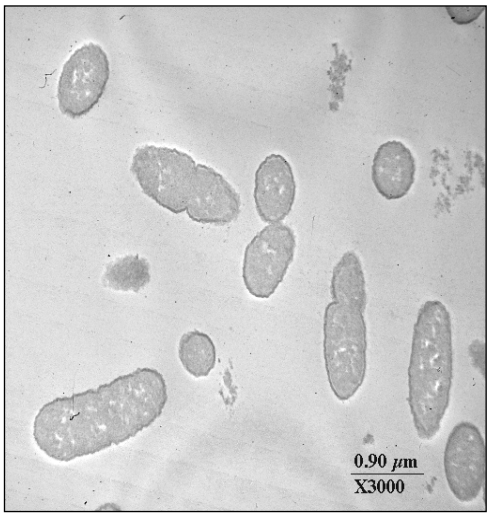
Figures 27-32 show the effects of ceftazidime and/or selected flavonoids on log phase cells of clinical isolates of CREnC. Figure 27 shows the micrographs of normal log phase cells of clinical isolates of CREnC. The outer membrane and cytoplasmic membrane can be distinquised. The electron dense ribosomes can be observed in numerous numbers in cytoplasm. Figure 28 indicates the micrographs of log phase cells of clinical isolates of CREnC after treatment with ceftazidime at 20 μg/ml. Some of these bacterial cells exhibited larger gap between outer membrane and cytoplasmic membrane in a few area of the cells.

Figures 29 and 30 show the micrographs of log phase cells of clinical isolates of CREnC after exposure to either luteolin or apigenin at 20 μg/ml respectively. These micrographs showed no observable morphological damage and were precisely the same as control cells. Figure 31 indicates the effect of the combination of ceftazidime plus luteolin at 10:10 μg/ml on clinical isolates of CREnC. Most of these bacterial cells obviously exhibited morphological cell wall and cell shape damage. A lot of bacterial cells clearly exhibited electron-transparent areas in cytoplasm due to losing most of organells. Some bacterial cells showed distortion of cell wall and broken cells. Figure 32 shows the micrographs of log phase cells of clinical isolates of CREnC after treatment with 10 μg/ml ceftazidime plus 10 μg/ml apigenin. Most of bacteria showed obvious detachment of cell wall and cytoplamic membrane. Also,

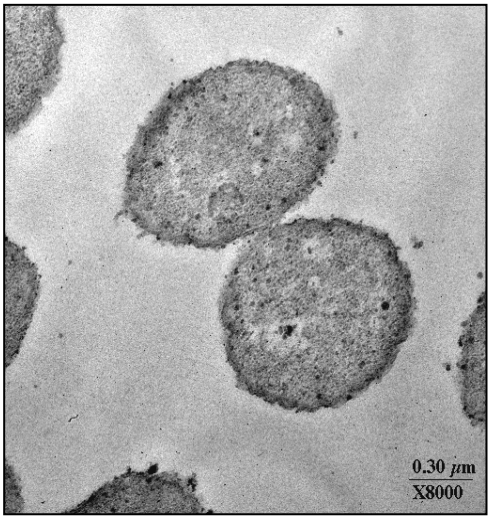
electron-transparent areas were appeared in cytoplasm due to losing of ribosomes and other organells.

Discussion

From these results, it can conclude that both selected antibacterial drugs or flavonoids had little activity against MRSA or CREnC when use singly. However the combination of these drugs and flavonoids showed obvious great synergism activity against both strains, result in morphological damage. Many previous researchers reported the antibacterial activities of β-lactam plus flavonoid. These caused marked morphological damage to cells. The damage including loosening or detachment of outer membrane which may have resulted from damage to the peptidoglycan layer internal to the OM. Some of the bacteria exhibited electron-transparent areas devoid of ribosomes in the cytoplasm. Most of the treated bacteria were considerably larger and longer than the control bacteria. This results are in substantial agreement with those obtained with different bacterial species by Richards et al. (1993C) and Richards et al. (1996). The found that selected β-lactam plus selected flavonoids had an effect on the production of peptidoglycan in *E. cloacae.* These results are also in substantial agreement with those of Zhu (1995) who reported that detachment of the outer membrane from the cytoplasmic membrane of *E. coli* 326 cells after treatment with either SD and/or TMP.

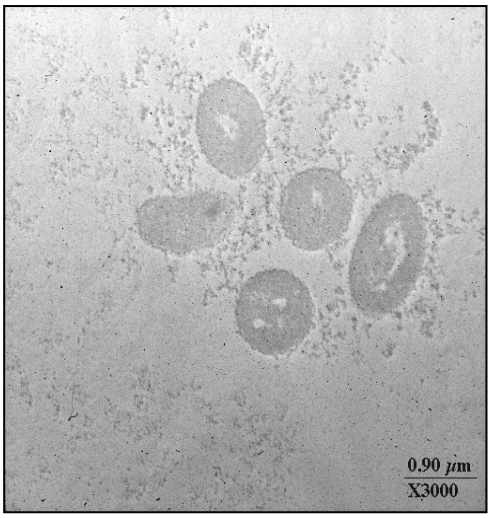


(a)



(b)

Figure 15 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth: (a), (b), control (no antibacterial agent). Bar = 0.9 μm (a); 0.3 μm (b)



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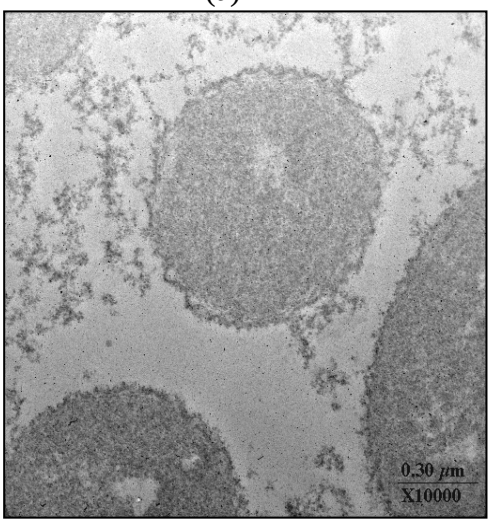
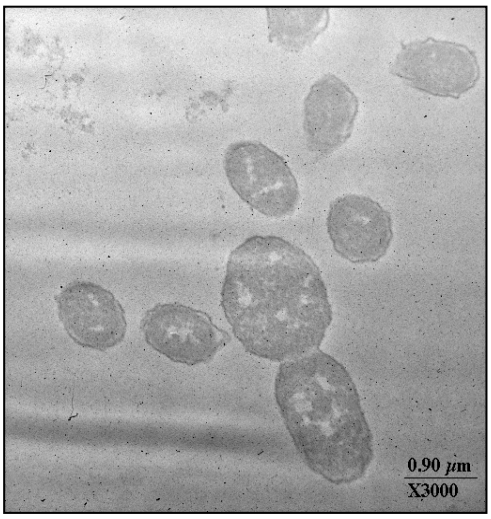


(b)

Figure 16 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), cloxacillin (20 μg/ml). Bar = 0.9 μm (a); 0.3 μm (b)



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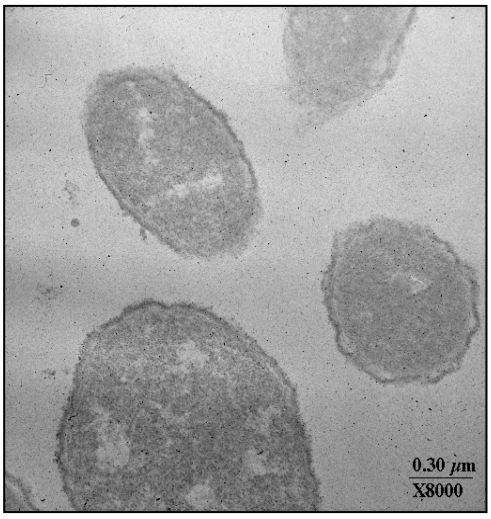
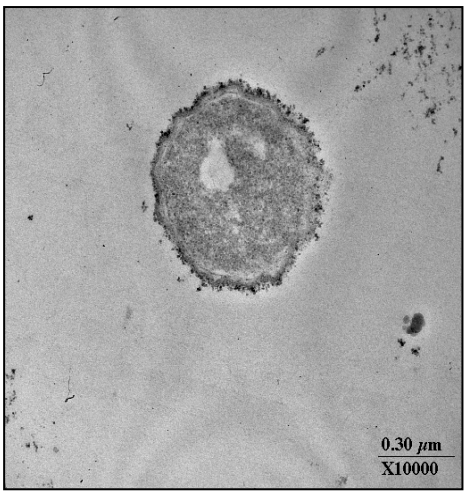


(

(b)

Figure 17 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), ampicillin (20 μg/ml). Bar = 0.9 μm (a); 0.3 μm (b)



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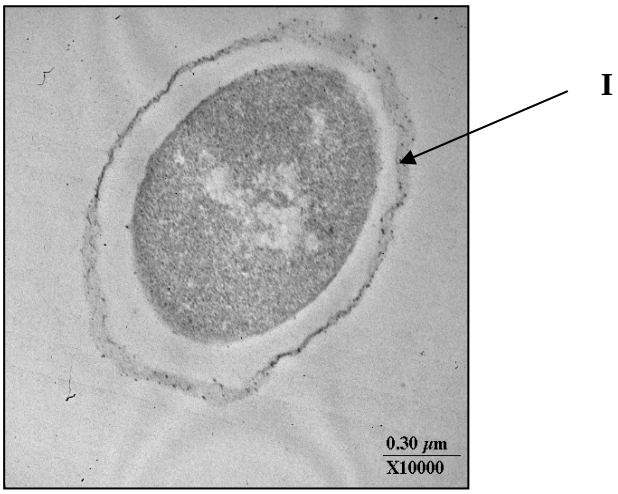


(b)

Figure 18 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), ceftazidime (20 μg/ml): I = Larger gap between cell wall and cell membrane. Bar = 0.3 μm (a), (b)

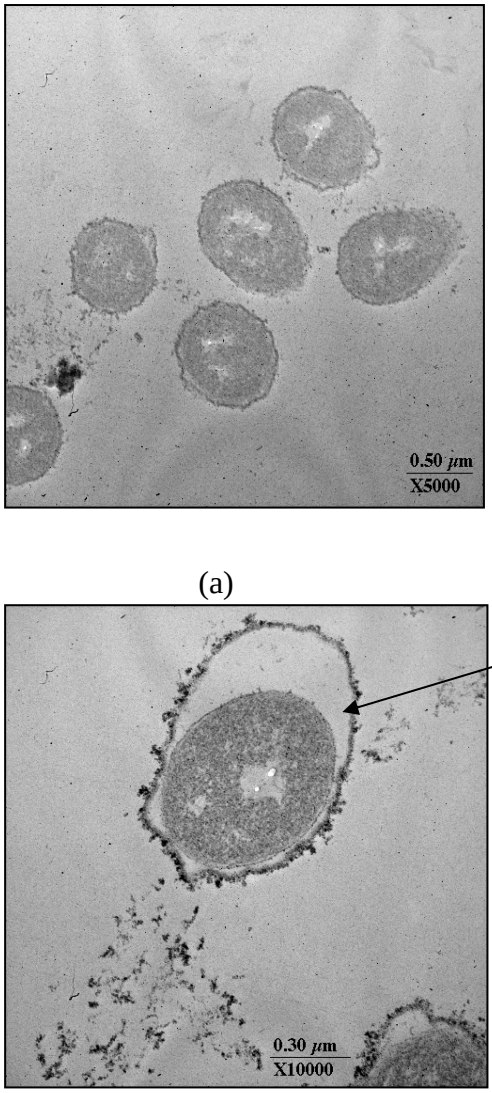
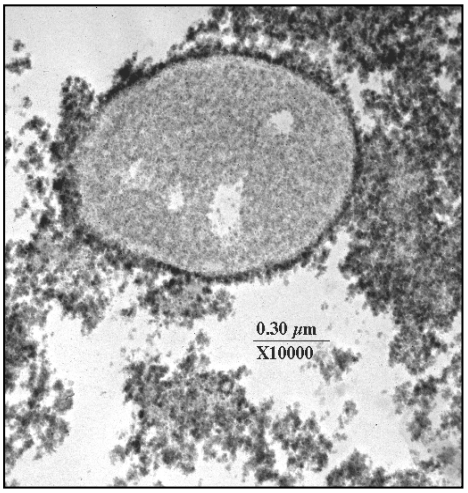


Figure 19 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), baicalein (20 μg/ml): I=Larger gap between cell wall and cell membrane. Bar = 0.5 μm (a); 0.3 μm (b)



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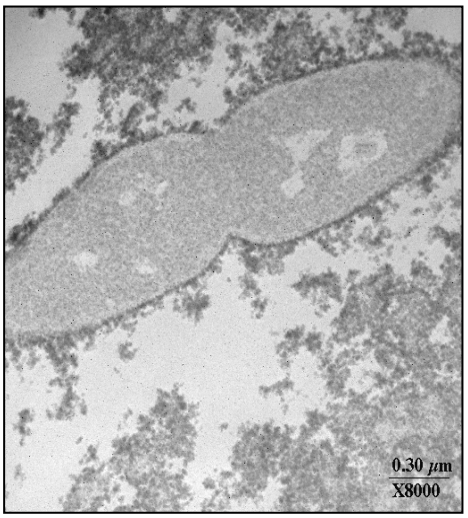
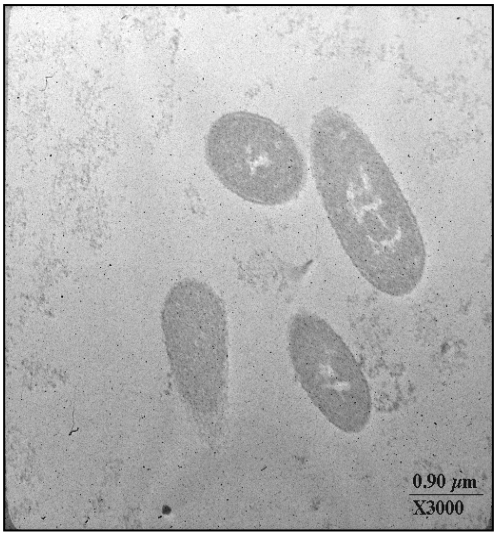


(b)

Figure 20 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), galangin (20 μg/ml). Bar = 0.3 μm (a), (b)



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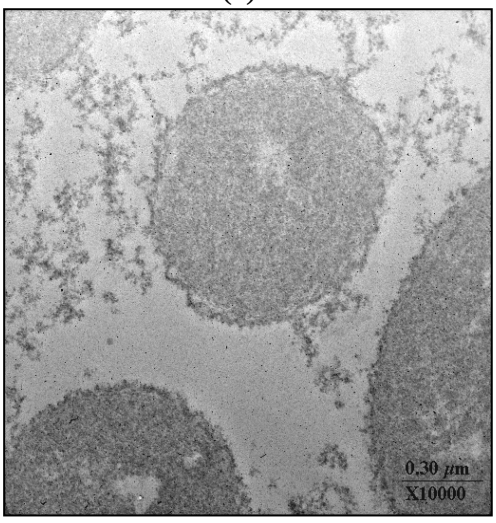
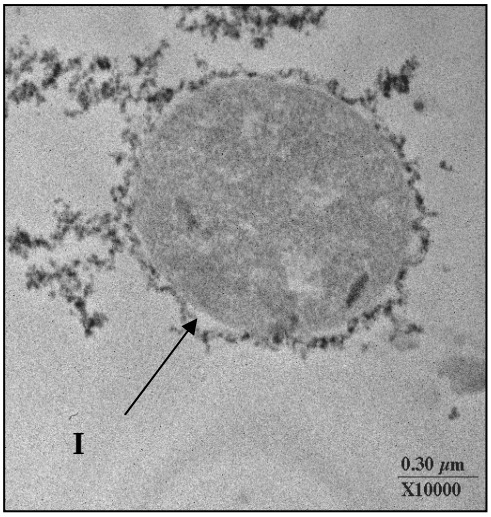


(b)

Figure 21 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), quercetin (20 μg/ml). Bar = 0.9 μm (a); 0.3 μm (b)



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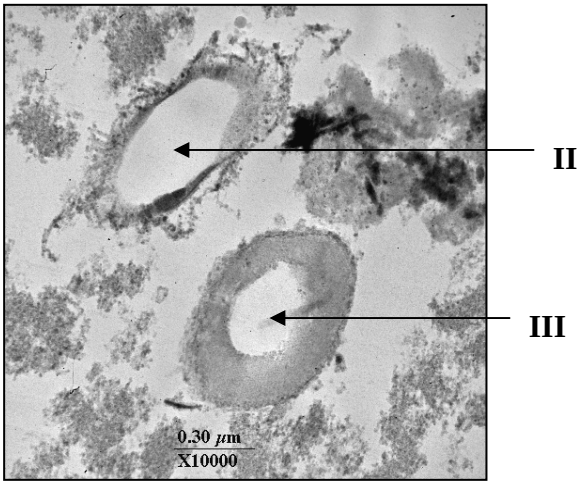


(b)

F**igure 22** Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), ampicillin (10 μg/ml) plus baicalein (10 μg/ml): I = Gap between cell wall and cell membrane: II, III = Electron-transparent area devoid of ribosomes. Bar = 0.3 μm (a), (b)

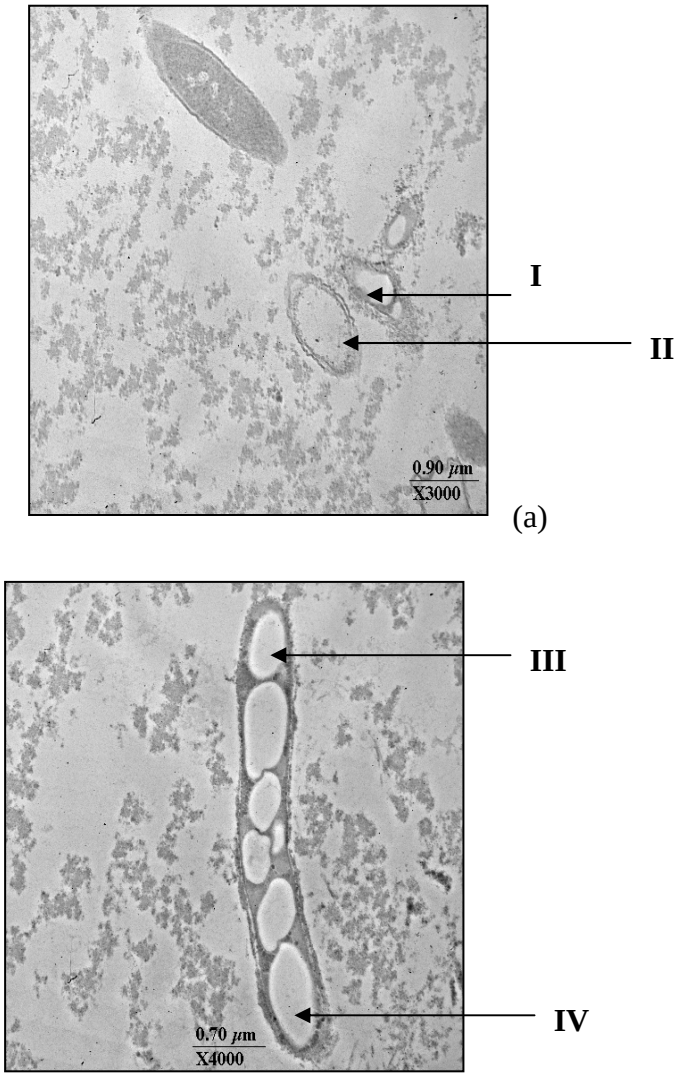
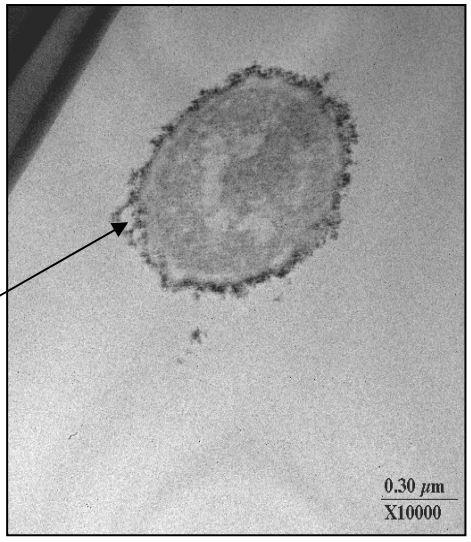


Figure 23 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), cloxacillin (10 μg/ml) plus baicalein (10 μg/ml): I, II, III, IV = Electron-transparent area devoid of ribosomes. Bar = 0.3 μm (a); 0.2 μm (b)



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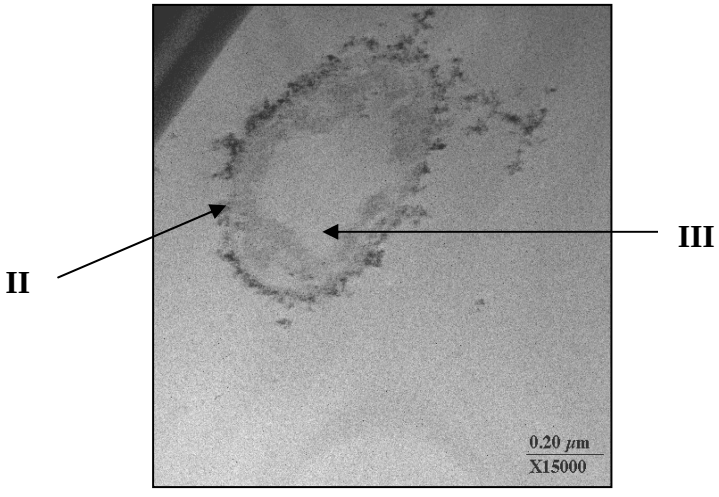


(b)

Figure 24 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), ceftazidime (10 μg/ml) plus baicalein (10 μg/ml); I, II: Morphological damage of cell wall and cytoplasmic membrane; III: Lost of some organells from cytoplasm. Bar = 0.3 μm (a); 0.2 μm (b)

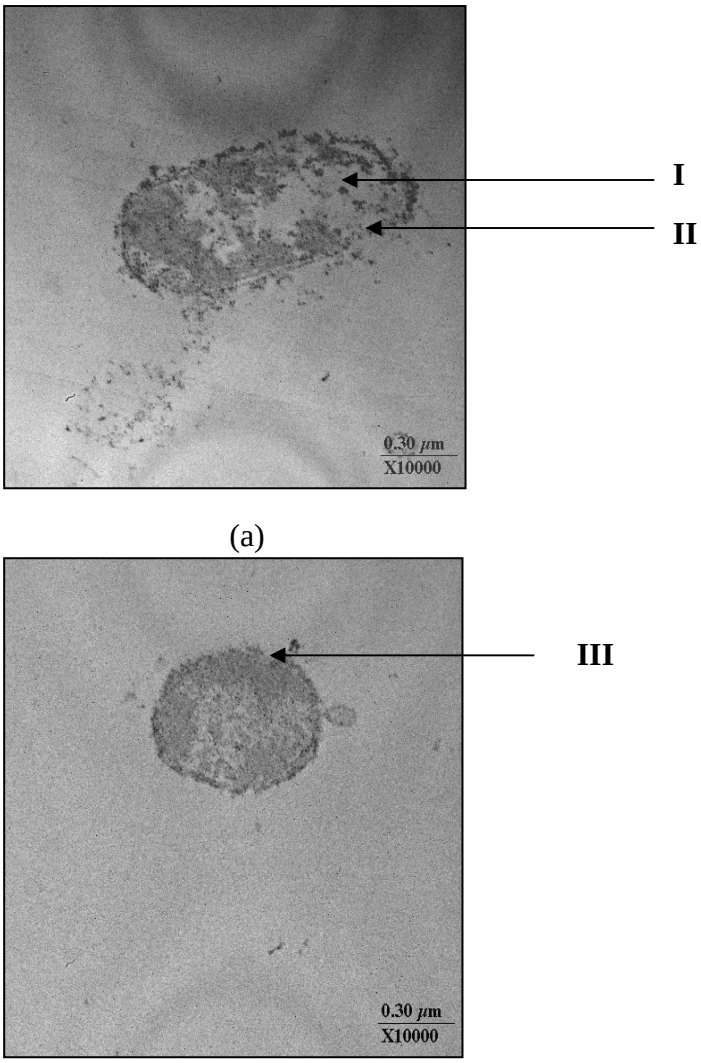
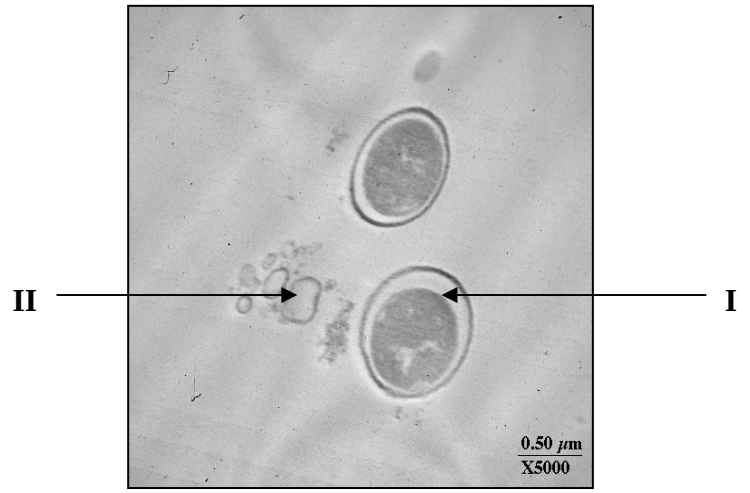
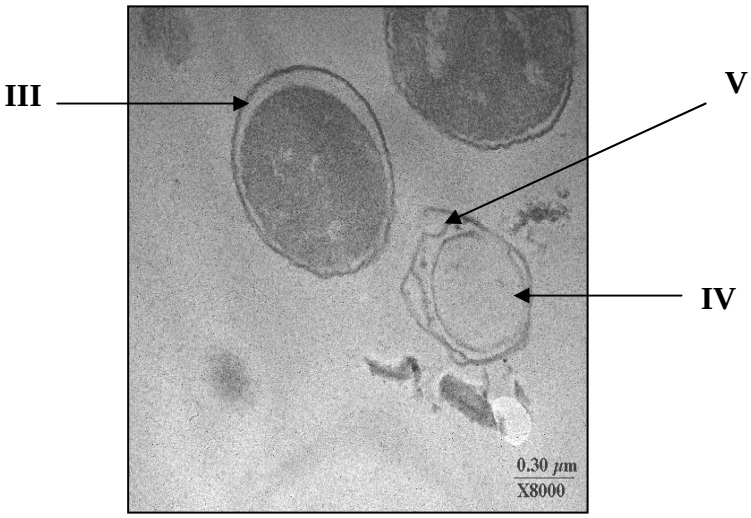


Figure 25 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), ceftazidime (10 μg/ml) plus galangin (10 μg/ml); I: Electron-transparent areas devoid of ribosome and lost of some organells from cytoplasm; II, III: Cell wall damage. Bar = 0.3 μm (a), (b)

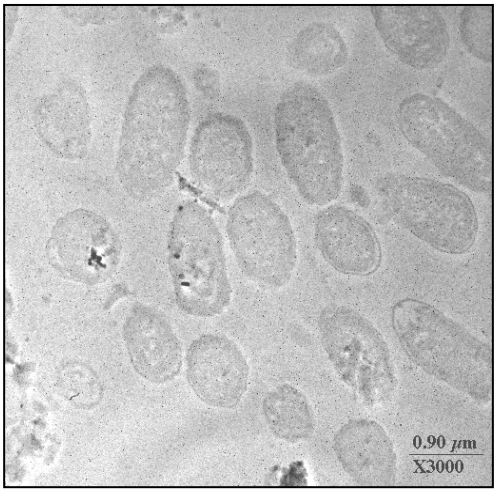


(a)



(b)

Figure 26 Utrathin sections of log phase of clinical isolates of MRSA grown for 4 h in Mueller–Hinton broth containing: (a), (b), ceftazidime (10 μg/ml) plus quercetin (10 μg/ml); I, III: Gap between cell wall and cytoplasmic membrane; II, IV: Electron-transparent areas devoid of ribosomes and lost of some organells from cytoplasm; V: Morphological damage of cell wall and cytoplasmic membrane. Bar = 0.5 μm (a), 0.3 μm (b)



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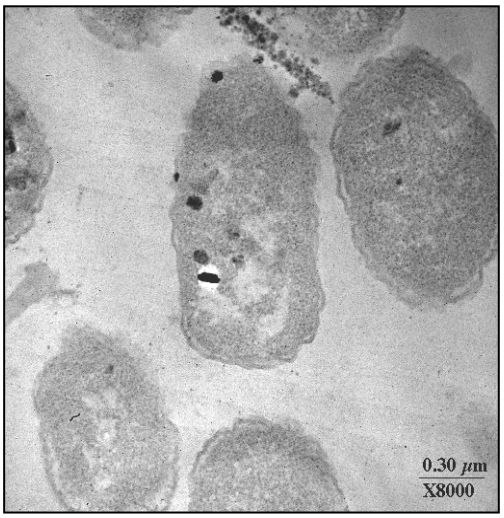
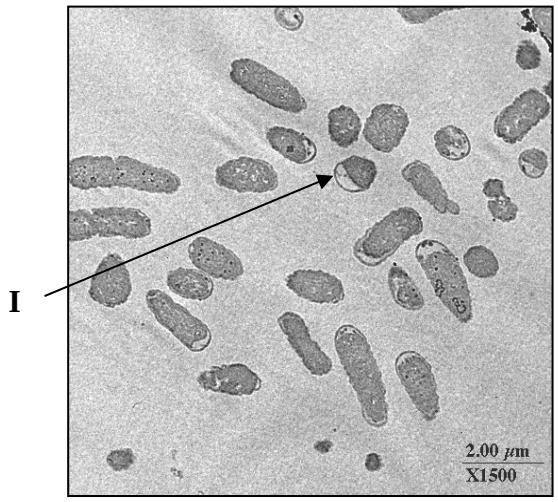


(b)

Figure 27 Utrathin sections of log phase of clinical isolates of CREnC grown for 4 h in Iso-sensitest broth. (a), (b), control (no antibacterial agent). Bar = 0.9 μm (a); 0.3 μm (b)



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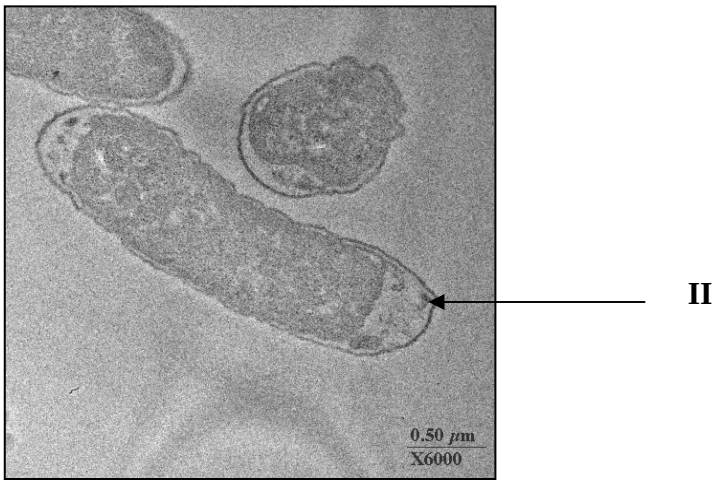
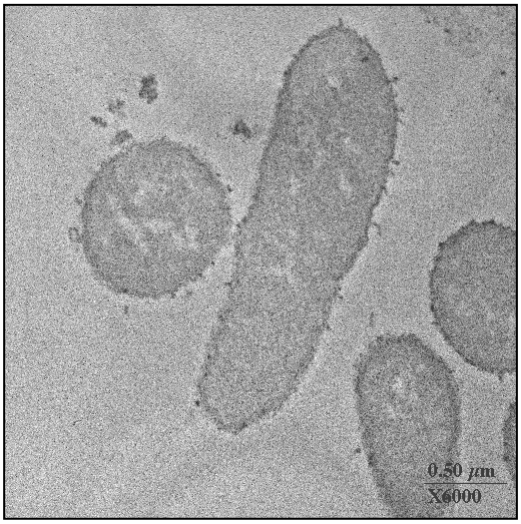


(b)

Figure 28 Utrathin sections of log phase of clinical isolates of CREnC grown for 4 h in Iso-sensitest broth. (a), (b), containing: (a), (b) ceftazidime (20 μg/ml); I, II: Gap between outer membrane and cytoplasmic membrane. Bar = 2 μm (a); 0.5 μm (b)



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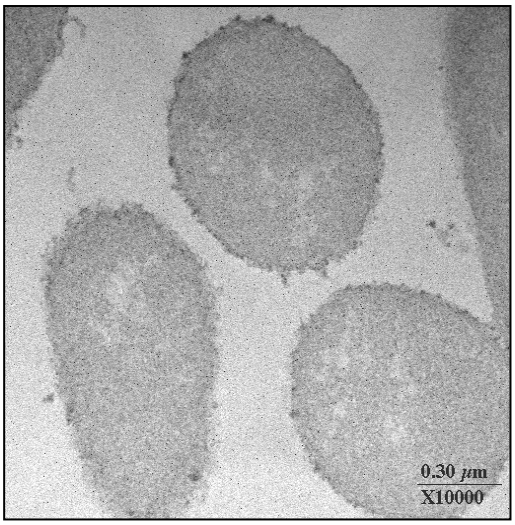
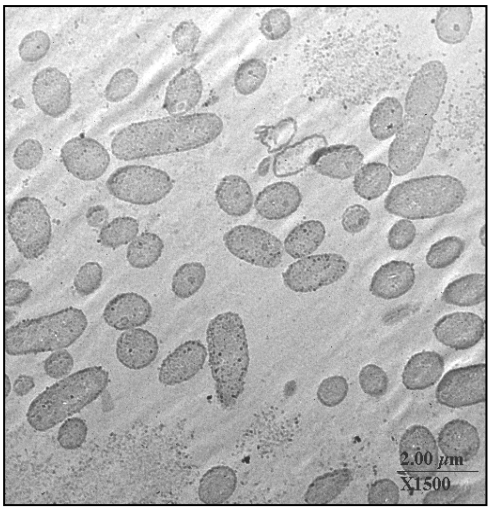
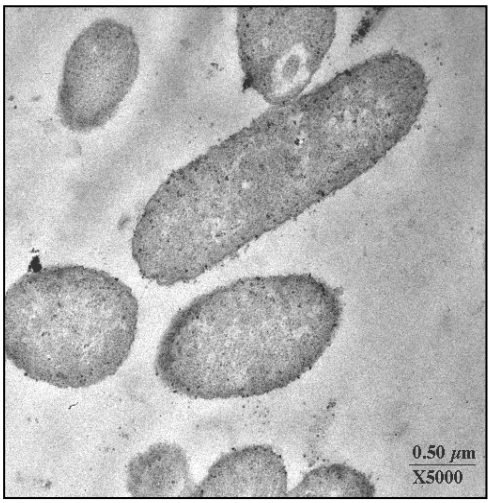


(b)

Figure 29 Utrathin sections of log phase of clinical isolates of CREnC grown for 4 h in Iso-sensitest broth. (a), (b), containing: (a), (b) luteolin (20 μg/ml). Bar = 0.5 μm (a); 0.3 μm (b)



(a)



(b)

Figure 30 Utrathin sections of log phase of clinical isolates of CREnC grown for 4 h in Iso-sensitest broth. (a), (b), containing: (a), (b) apigenin (20 μg/ml). Bar = 2 μm (a) ; 0.5 μm (b)

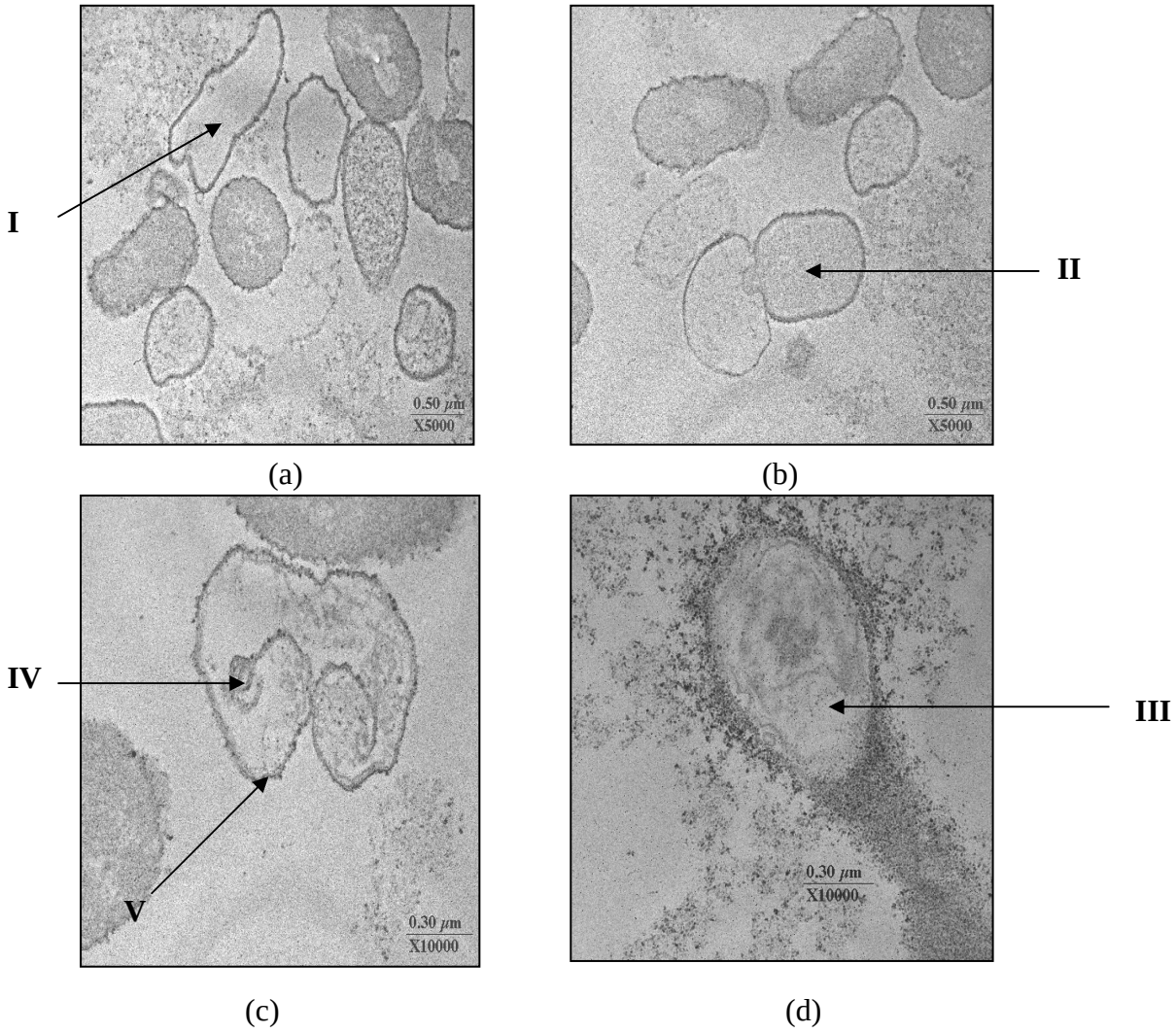


Figure 31 Utrathin sections of log phase of clinical isolates of CREnC grown for 4 h in Iso-sensitest broth. (a), (b), containing: (a), (b), (c), (d) ceftazidime 10 µg/ml plus luteolin 10µg/ml; I, II, III: Electron-transparent areas in cytoplasm and losing most of organells; IV, V: Distortion of cell wall and broken cells. Bar = 0.5 µm (a), (b); 0.3 µm (c), (d)

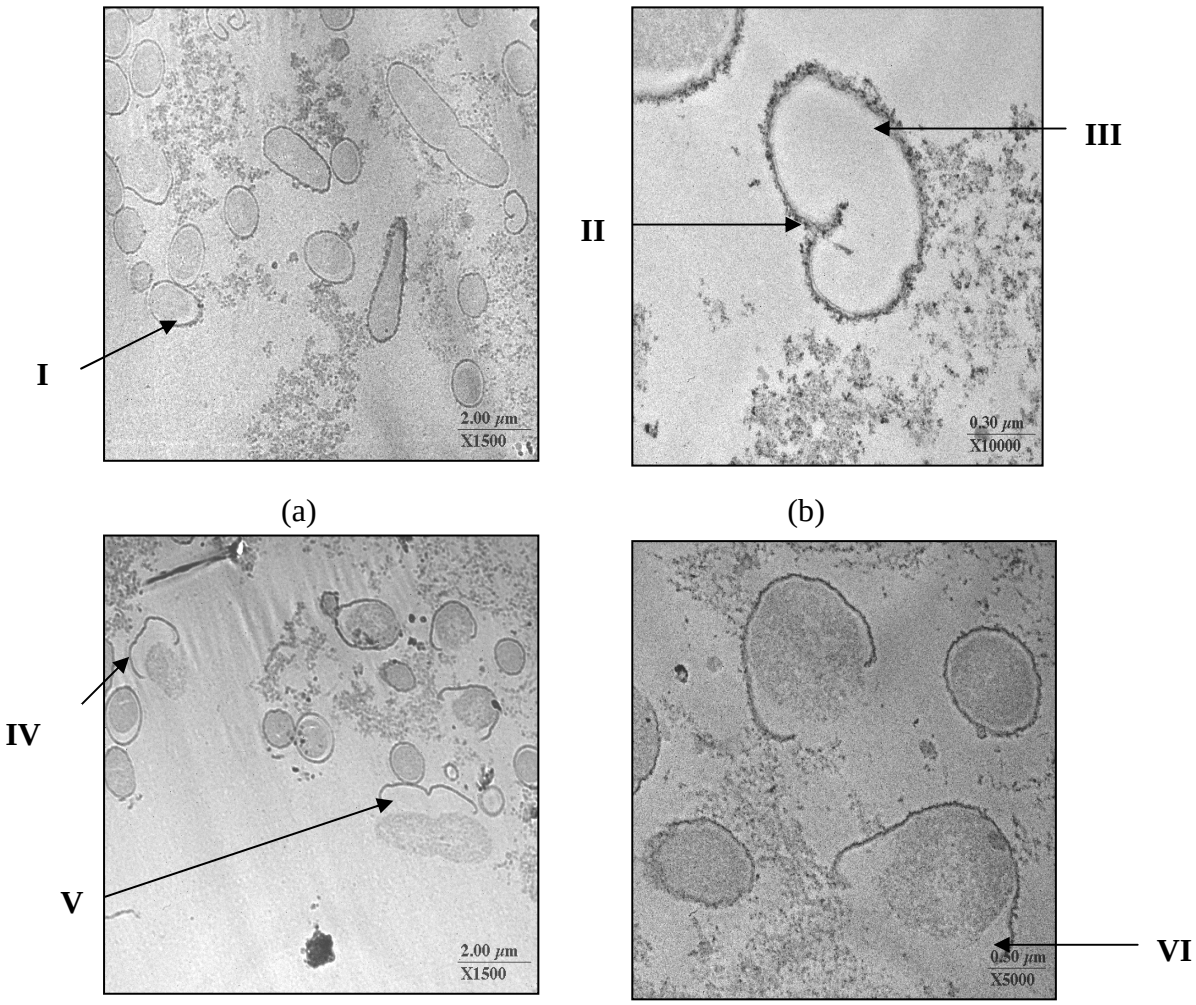


Figure 32 Utrathin sections of log phase of clinical isolates of CREnC grown for 4 h in Iso-sensitest broth. (a), (b), containing: (a), (b), (c), (d) ceftazidime 10 μg/ml plus apigenin 10 μg/ml; I: Gap between outer membrane and cytoplasmic membrane; II: Distortion of cell wall; III: Electron-transparent areas in cytoplasm and losing most of organells; IV, V, VI: Detachment of cell wall and cytoplasmic membrane. Bar = 2 μm (a), (c); 0.3 μm (b), 0.5 μm (d)

4.6 Capillary electrophoresis

Quantification of MRSA and CREnc were performed by comparision of the peak areas of the individual compounds relative to those of the appropriate internal standard is shown in Figure 33. The migration time at 19.014 min. The peak area was 49.8 mAU*s.

The separation electrophoregram by capillary electrophoresis (CE) of peptidoglycan-associated proteins from MRSA control cells plus albumin as internal standard is shown in Figure 34. A good separation of the analyte and the internal standard was achieved by the selected electrophoresis conditions within 20 min. The six major peptidoglycan-associated protein peaks were found at the migration times (the ratio of peak areas) 17.836 (0.14), 18.145 (0.02), 18.344 (0.16), 18.718 (0.17), 19.091 (1), and 20.977 (0.03) respectively. The internal standard albumin was found at 19.091 min. The ratio of peak area in parenthesis means the OM-PG protein of each peak area divided by the IS peak area (OM-PG peak of each area/IS peak area).

The peptidoglycan-associated proteins of MRSA grown in ampicillin at 20 µg/ml is demonstrated in Figure 35. The five protein peaks were found at migration times (ratio of peak areas) 17.748 (2.02), 18.358 (4.28), 18.844 (0.44), 19.042 (1), and 20.976 (1.95) respectively. The internal standard albumin was found at 19.042 min. The protein peak at 18.145 (0.02) of the control seems to be reduced of the ratio of protein peak area.

The peptidoglycan-associated proteins from MRSA exposured to cloxacillin at 20 µg/ml is shown in Figure 36. The four major protein peaks were found at migration times (the ratio of peak areas) 17.309 (0.16), 17.805 (0.40), 18.545 (1), and

20.334 (33.2) respectively. Also, the second protein peak at 18.145 (0.02) of the control is likely to be reduced of the ratio of protein peak area.

The peptidoglycan-associated proteins from MRSA treatment with baicalein at 20 µg/ml is presented in Figure 37. It was found four major proteins peaks. The four protein peaks were found at migration times (the ratio of peak areas) 17.313 (0.45), 17.770 (0.81), 18.393 (1), and 20.281 (0.45) respectively. The second protein peak at 18.145 (0.02) of the control is likely to be reduced of the ratio of proteins peak area.

The peptidoglycan-associated proteins from MRSA exposured to ampicillin plus baicalein, and cloxacillin plus baicalein at 10:10 µg/ml are showed in Figure 38 and 39 respectively. After treated with ampicillin plus baicalein at 10:10 µg/ml, the appearance of four major protein peaks were found at migration times (the ratio of peak areas) 17.377 (0.18), 17.797 (0.22), 18.573 (1), and 20.295(0.11) respectively. Also, the second protein peak at 18.145 (0.02) of the control seems to be reduced of the ratio of protein peak area.

Figure 39 after treated with cloxacillin plus baicalein at 10:10 µg/ml, the appearance of four major protein peaks were found at migration times (the ratio of peak areas) 17.442 (0.58), 17.829 (0.37), 18.621 (1), and 20.319 (0.33) respectively. The second protein peak at 18.145 (0.02), the fourth protein peak at 18.718 (0.17) and the fifth protein peak at 19.091 (1) of the control seem to be reduced of the ratio of protein peak areas, but sixth protein peak at 20.319 (0.03) of the control seems to be increased of the ratio of protein peak area.

The peptidoglycan-associated protein profiles on CE for MRSA grown in ceftazidime at 20 µg/ml are shown in Figure 40. A separation of the analyte and the

internal standard was achieved by the selected electrophoresis conditions within 20 min. Three appeared major protein peaks were found at migration times (the ratio of peak areas) 17.282 (0.21), 17.829 (0.36), and 18.558 (1) respectively. The second protein peak at 18.145 (0.02), the fourth protein peak at 18.718 (0.17) and the sixth protein peak at 20.977 (0.03) of the control are likely to be disappeared of the ratio of protein peak areas.

The peptidoglycan-associated protein profiles on CE for MRSA grown in galangin at 20 μg/ml are shown in Figure 41. A separation of the analyte and the internal standard was achieved by the selected electrophoresis conditions within 20 min. After treatment with galangin at 20 μg/ml, three major protein peaks were found at migration times (the ratio of peak areas) 17.485 (0.83), 17.864 (0.33), and 18.544 (1) respectively. The third protein peak at 18.344 (0.16), the fourth protein peak at 18.718 (0.17) of control are likely to be reduced of the ratio of protein peak areas, but the sixth protein peak at 20.977 (0.03) of the control seems to be disappeared of the ratio of protein peak area.

The peptidoglycan-associated protein profiles on CE for MRSA grown in galangin at 20 μg/ml are shown in Figure 42. A separation of the analyte and the internal standard was achieved by the selected electrophoresis conditions within 20 min. After exposure to quercetin at 20 μg/ml, five major protein peaks were found at migration times (the ratio of peak areas) 17.414 (0.16), 17.947 (0.39), 18.334 (0.32), 18.671 (1), and 20.428 (0.09) respectively. The second protein peak at 18.145 (0.02) of the control is likely to be reduced of the ratio of protein peak area.

The peptidoglycan-associated proteins of MRSA after treatment with ceftazidime plus baicalein, ceftazidime plus galangin and ceftazidime plus quercetin

at 10:10 µg/ml are shown in Figure 43, 44, and 45 respectively.

Figure 43 shows the peptidoglycan-associated proteins of MRSA after treatment with ceftazidime plus baicalein. Three major protein peaks were showed at migration times (the ratio of peak areas) 17.441 (0.22), 17.922 (0.25), and 18.702 (1) respectively. The second protein peak at 18.145 (0.02), the fourth protein peak at 18.718 (0.17) and the sixth protein peak at 20.977 (0.03) of the control are likely to be reduced of the ratio of protein peak areas.

Figure 44 shows the peptidoglycan-associated proteins of MRSA after treatment with ceftazidime plus galangin at 10:10 µg/ml. Four major protein peaks were appeared at migration times (the ratio of peak areas) 17.450 (0.32), 17.973 (0.57), 18.376 (0.35), and 18.711 (1) respectively. The second protein peak at 18.145 (0.02) of the control seems to be reduced of the ratio of peak area. The sixth protein peak at 20.977 (0.03) of the control seems to be disappeared of the ratio of protein peak area.

Figure 45 shows the peptidoglycan-associated proteins of MRSA after treatment with ceftazidime plus quercetin at 10:10 µg/ml. Four major protein peaks were found at migration times (the ratio of peak areas) 17.466 (0.40), 17.882 (0.40), 18.289 (0.37), and 18.606 (1) respectively. The second protein peak at 18.145 (0.02) of the control seems to be reduced of the ratio of protein peak area. In addition, the sixth protein peak at 20.977 (0.03) of the control is likely to be disappeared of the ratio of protein peak area.

The separation electropherogram by capillary electrophoresis of peptidoglycan-associated proteins from CREnC cells control plus albumin as internal

standard is shown in Figure 46. A separation of the analyte and the internal standard was achieved by the selected electrophoresis conditions within 20 min. The five major protein peaks were showed at migration times (the ratio of peak areas) 16.918 (0.21), 17.421 (1.02), 18.125 (4.38), 18.831 (1), and 20.555 (0.31) respectively.

Figure 47 is presented the peptidoglycan-associated proteins peaks of CREnC after treatment with ceftazidime at 20 µg/ml. Four major protein peaks were showed at migration times (the ratio of peak areas) 16.925 (0.23), 17.402 (0.77), 17.983 (1.08), and 18.760 (1) respectively. The second protein peak at 17.421 (1.02), the third protein peak at 18.125 (4.38) of the control seem to be reduced of the ratio of protein peak areas. Also, the fifth protein peak at 20.555 (0.31) of the control is likely to be disappeared of the ratio of protein peak area.

Figure 48 is showed the peptidoglycan-associated proteins peaks of CREnC after exposured to apigenin at 20 µg/ml. Three major proteins peaks were showed at migration times (the ratio of peak areas) 18.323 (0.80), 18.796 (3.05), and 19.602 (1) respectively. The first protein peak at 16.918 (0.21), the second protein peak at 17.412 (1.02) and the third protein peak at 18.125 (4.38) of the control seem to be reduced of the ratio of protein peak areas. Morever, the fifth protein peak at 20.555 (0.31) of the control is likely to be disappeared of the ratio of protein peak area.

Figure 49 is presented the peptidoglycan-associated proteins peaks of CREnC after treatment with luteolin at 20 µg/ml. There were three major proteins peaks by CE at migration times (the ratio of peak areas) 17.1 (0.15), 17.85 (0.15), 18.452 (0.27), and 19.112 (1) respectively. The second protein peak at 17.412 (1.02) and the third protein peak at 18.125 (4.38) of the control seem to be clearly reduced of

the ratio of protein peak areas. In addition, the fifth protein peak at 20.555 (0.31) of the control is likely to be disappeared of the ratio of protein peak area.

Figure 50 and 51 are showed the peptidoglycan-associated protein from CREnC after treatment with ceftazidime plus luteolin and apigenin at 10:10 µg/ml and 10:10 µg/ml respectively.

Figure 50 is presented the peptidoglycan-associated protein peaks of CREnC after treatment with ceftazidime plus luteolin. The results showed four major protein peaks at migration times (the ratio of peak areas) 16.931 (0.09), 17.666 (0.12), 18.677 (0.54), and 19.009 (1) respectively. The first protein peak at 16.918 (0.21), the second protein peak at 17.421 (1.02) and the third protein peak at 18.125 (4.38) of the control seem to be reduced of the ratio of protein peak areas. The fifth protein peak at 20.555 (0.31) of the control is likely to be disappeared of the ratio of protein peak area. However, the fourth protein peak at 18.831 (1) of the control seem to be increased of the ratio of protein peak area.

Figure 51 is showed the peptidoglycan-associated protein peaks of CREnC after treatment with ceftazidime plus apigenin at 10:10 µg/ml. The result showed that three major protein peaks were appeared at migration times (the ratio of peak areas) 16.978 (0.09), 17.662 (0.16), 18.794 (0.32), and 19.111 (1) respectively. The first protein peak at 16.918 (0.21), the second protein peak at 17.421 (1.02) and the third protein peak at 18.125 (4.38) of the control seem to be reduced of the ratio of protein peak areas. The fifth protein peak at 20.555 (0.31) of the control is likely to be disappeared of the ratio of protein peak area. But the fourth protein peak at 18.831 (1) of the control seems to be increased of the ratio of protein peak area.

Figure 33 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of albumin 100 µg/ml as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate pH 9.1,detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

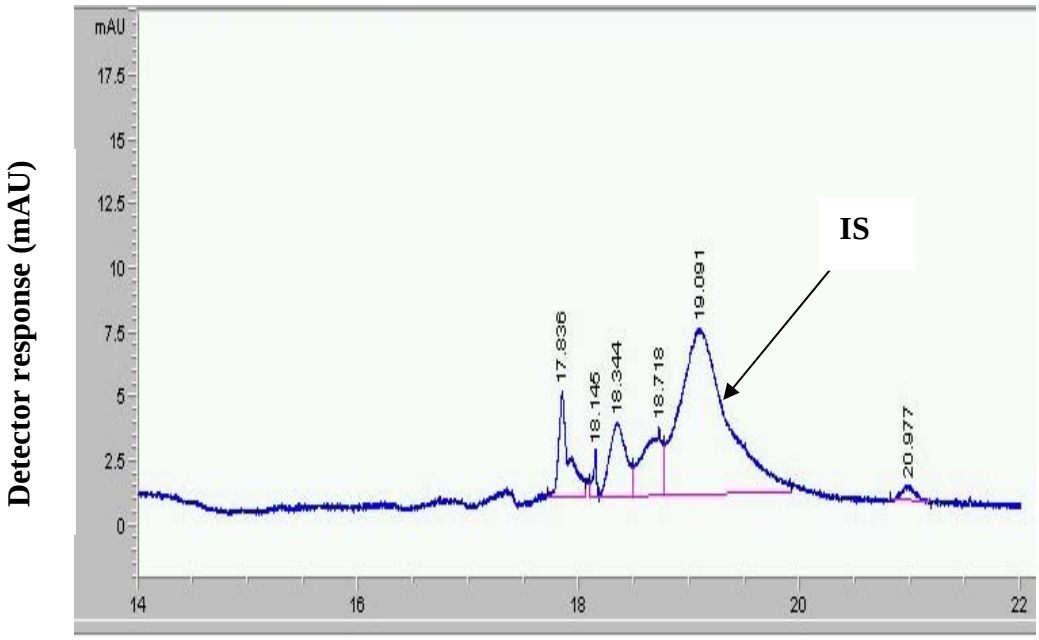


Figure 34 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA control cells plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate pH 9.1; detection at 205 nm; injection, 8 s at 5kV; separation voltage 20 kV; IS = Internal standard

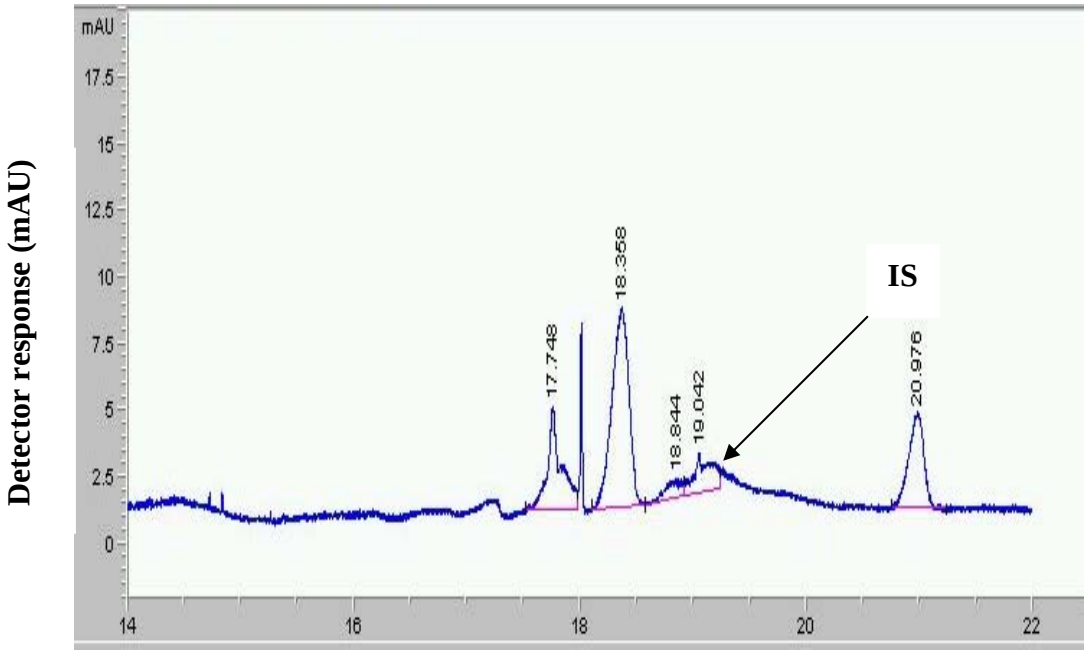


Figure 35 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with ampicillin at 20 µg /ml plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

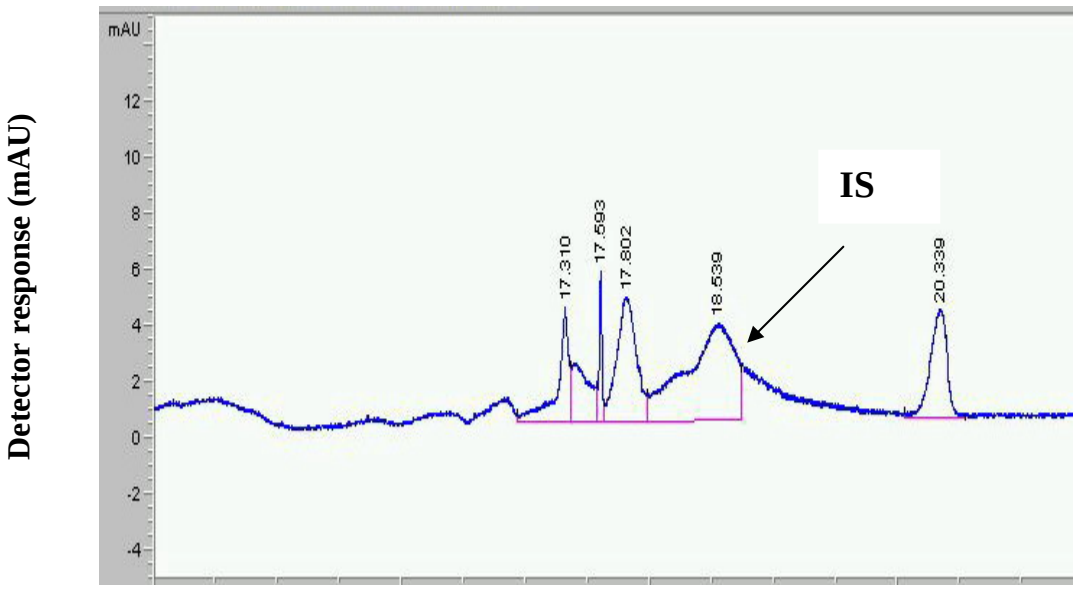


Figure 36 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with cloxacillin at 20 µg/ml plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

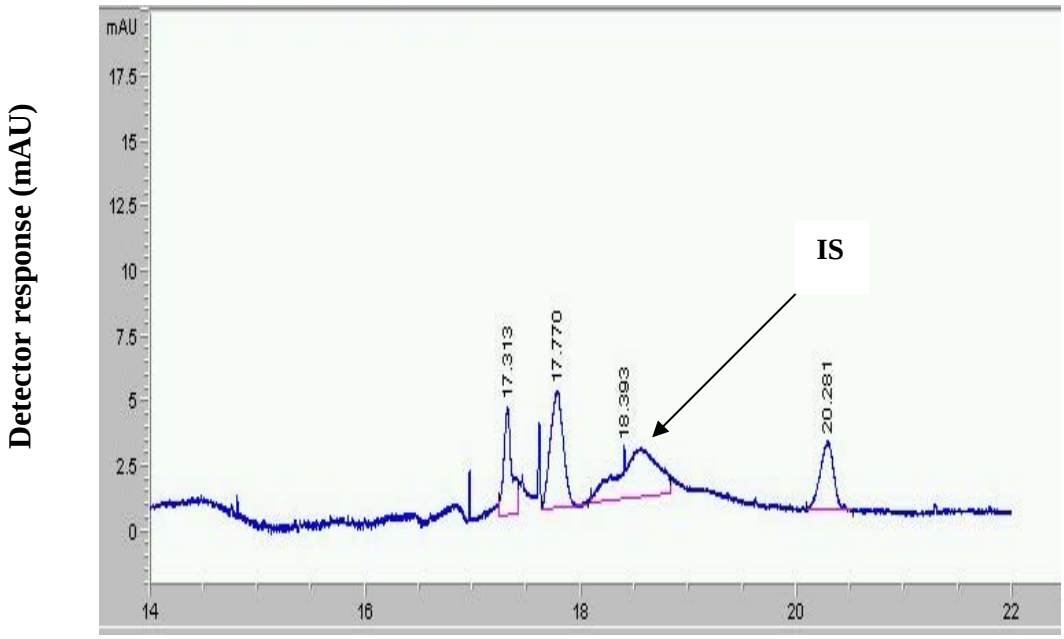


Figure 37 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with baicalein at 20 µg/ml plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

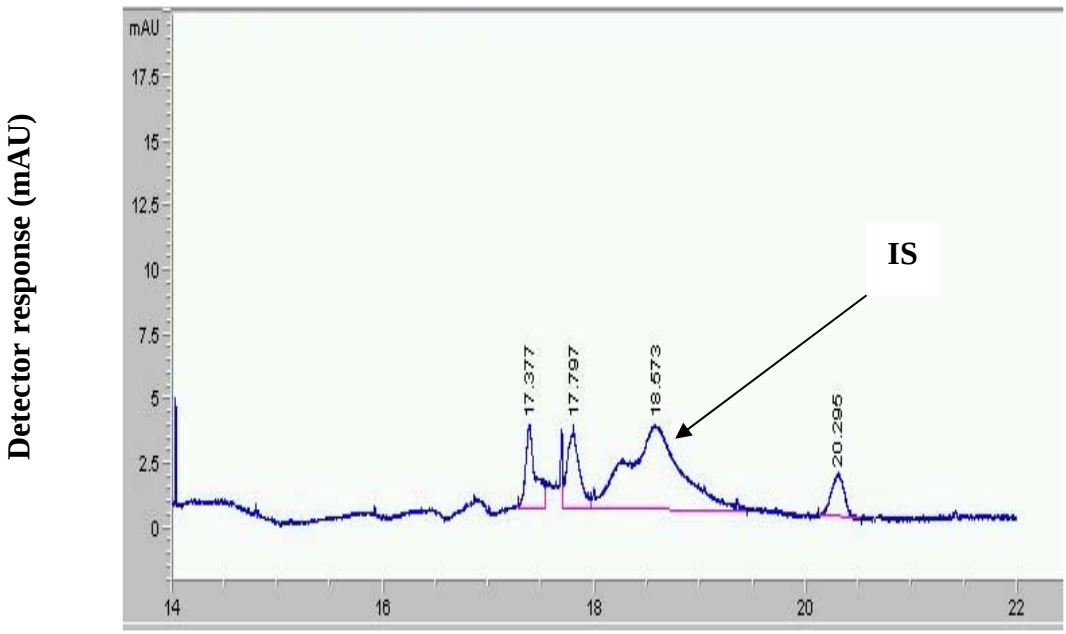


Figure 38 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with ampicillin plus baicalein at 10:10 μg/ml and plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 μm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV;separation voltage 20 kV; IS = Internal standard

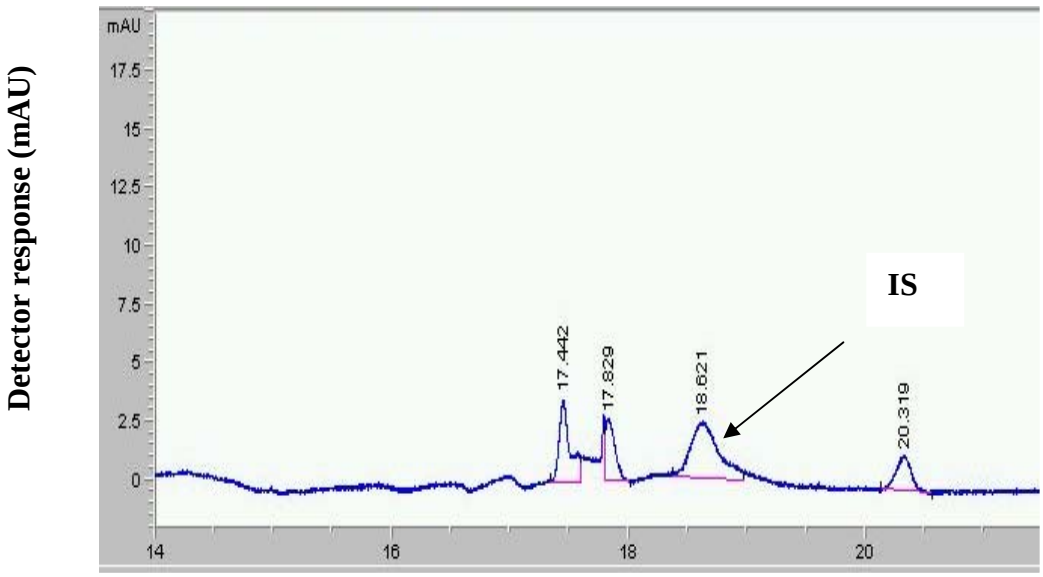


Figure 39 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with cloxacillin plus baicalein at 10:10 µg/ml and plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

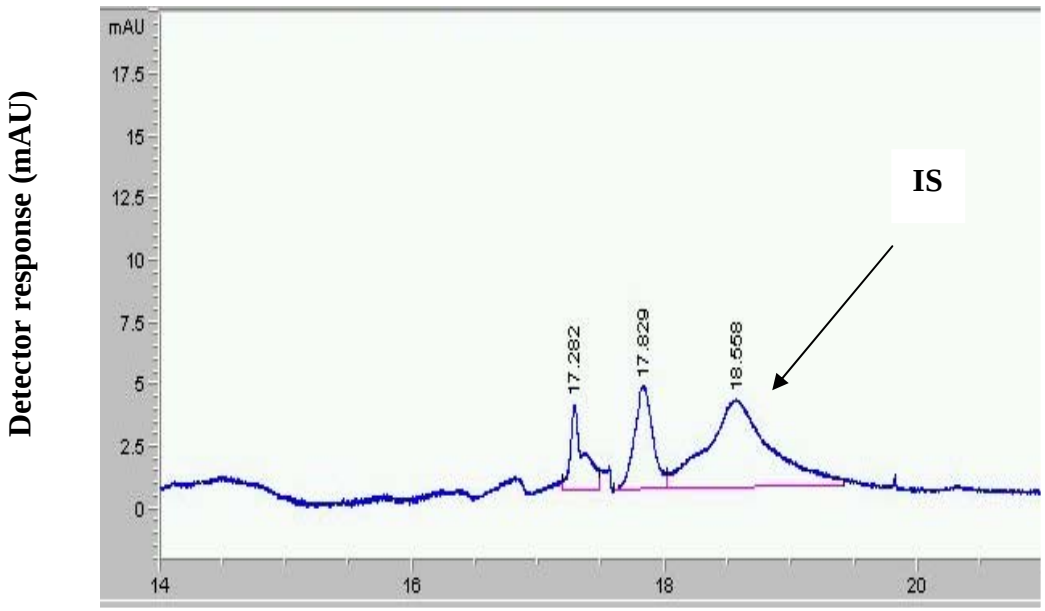


Figure 40 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with ceftazidime at 20 µg/ml plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

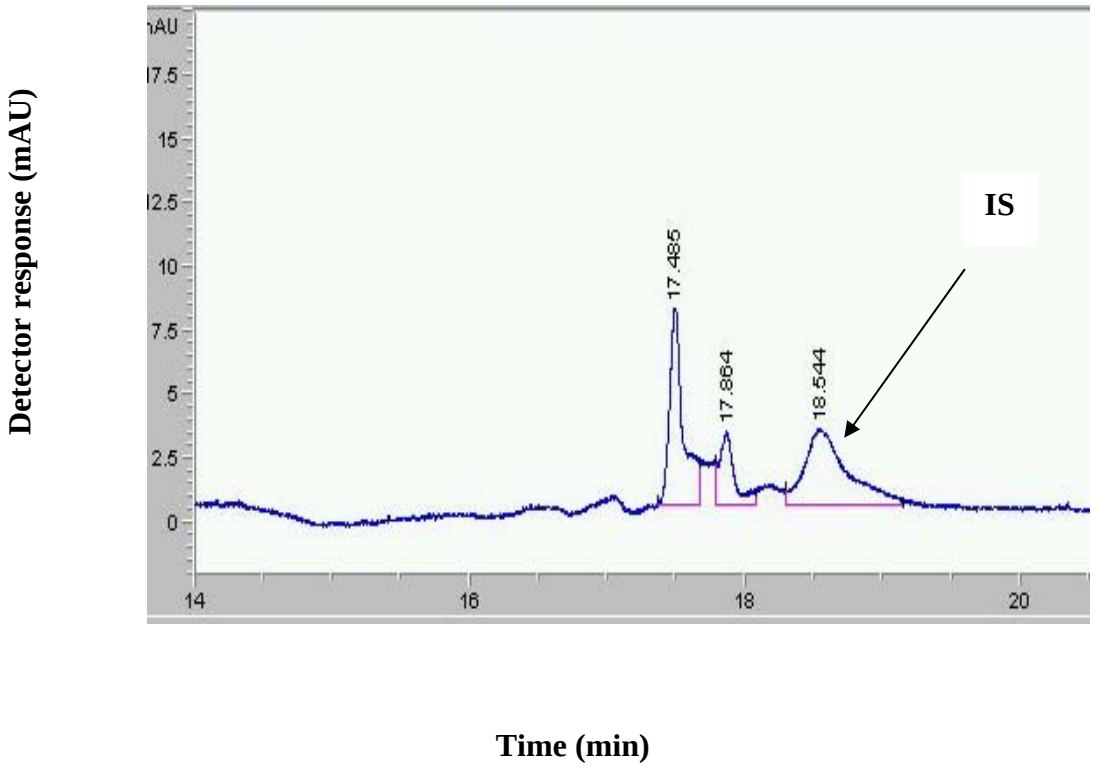


Figure 41 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with galangin at 20 µg/ml plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

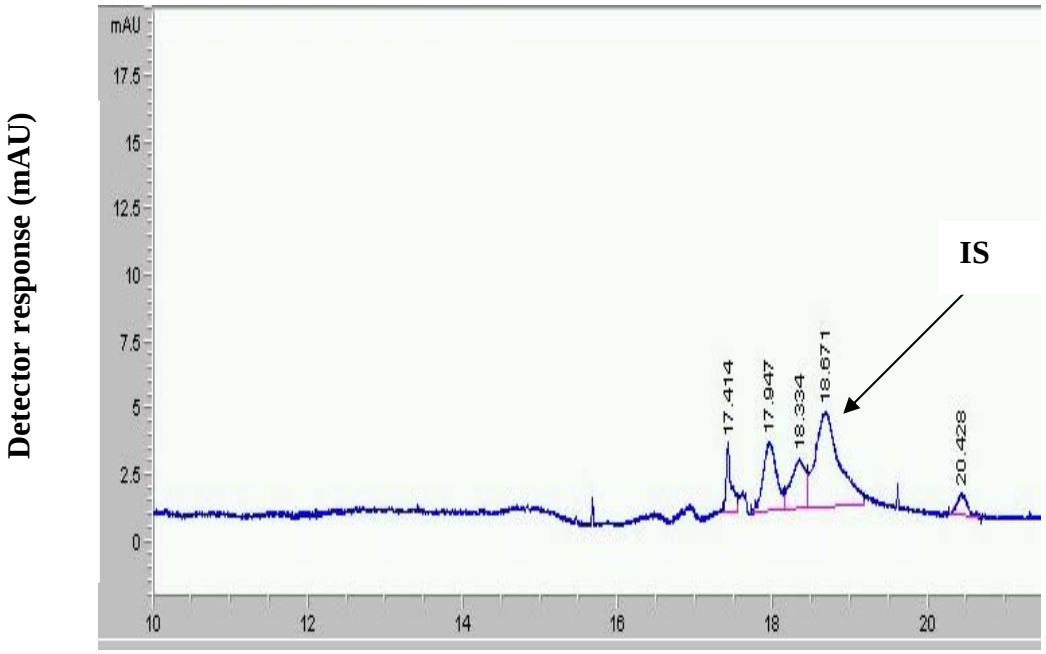


Figure 42 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with quercetin at 20 µg/ml plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

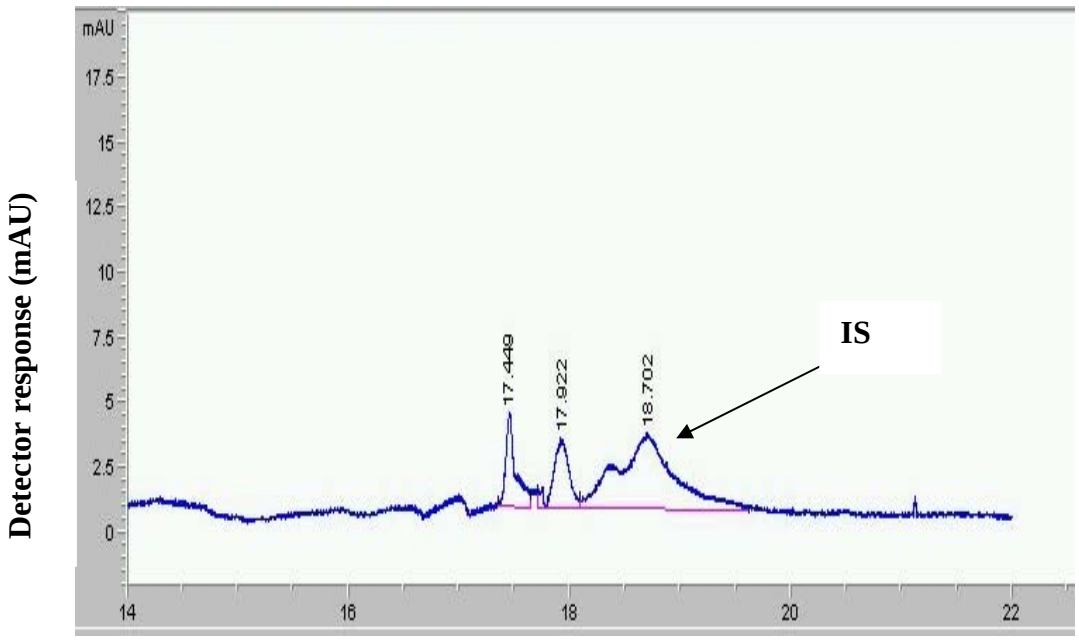


Figure 43 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with ceftazidime plus baicalein at 10:10 µg/ml and plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

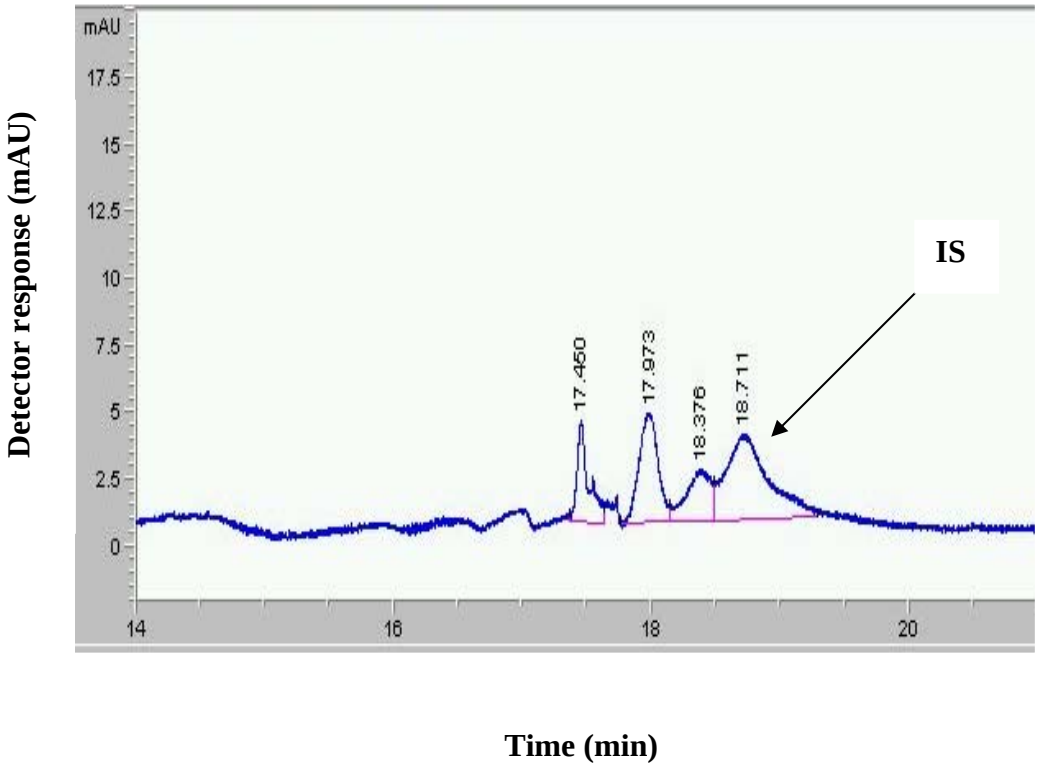


Figure 44 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with ceftazidime plus galangin at 10:10 μg/ml and plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 μm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

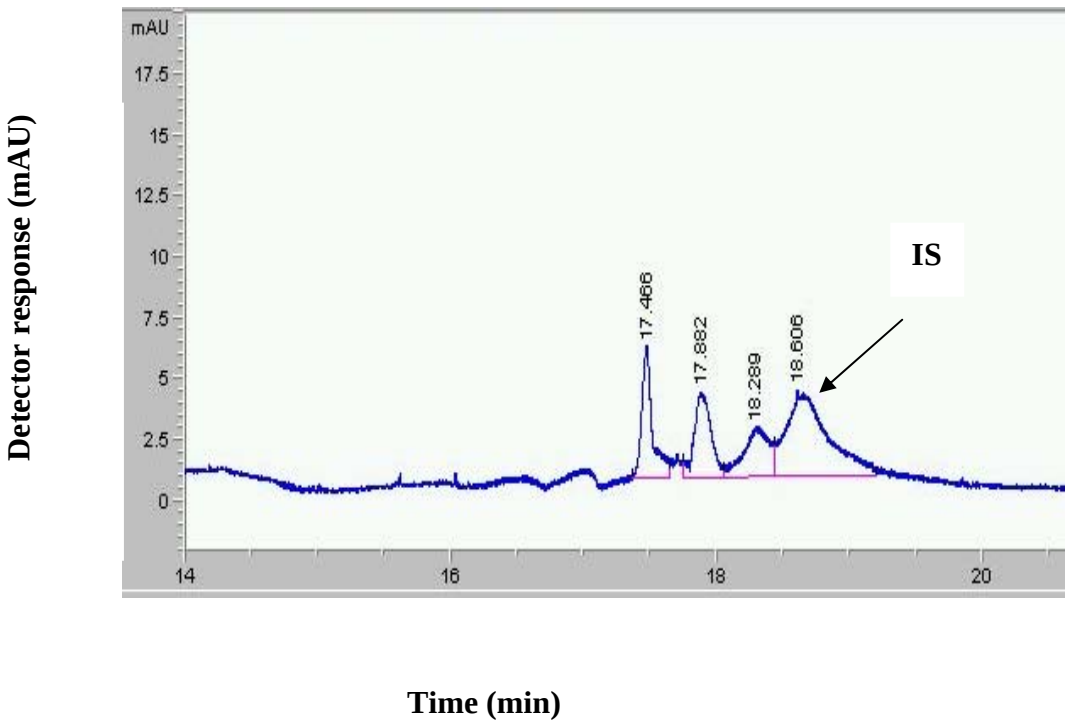


Figure 45 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of MRSA after treament with ceftazidime combined quercetin at 10:10 µg/ml and plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

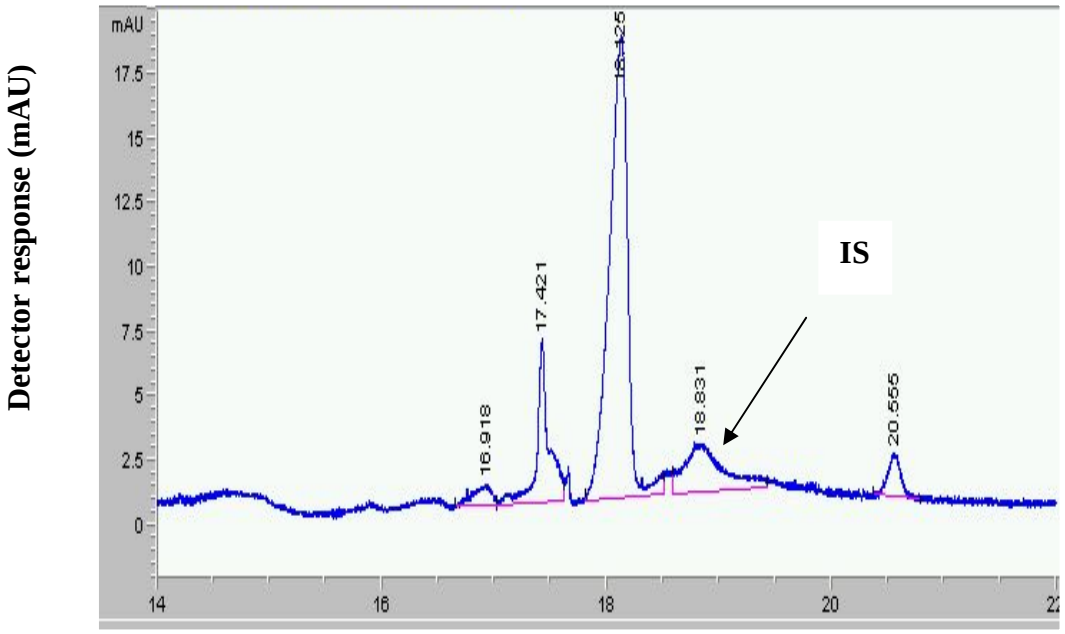


Figure 46 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of CREnC control cells plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total lengt h and 45 cm separation distance; buffer 50 mM disodium tetraborate pH 9.1; detection at 205 nm; injection, 8 s at 5kV; separation voltage 20 kV; IS = Internal standard

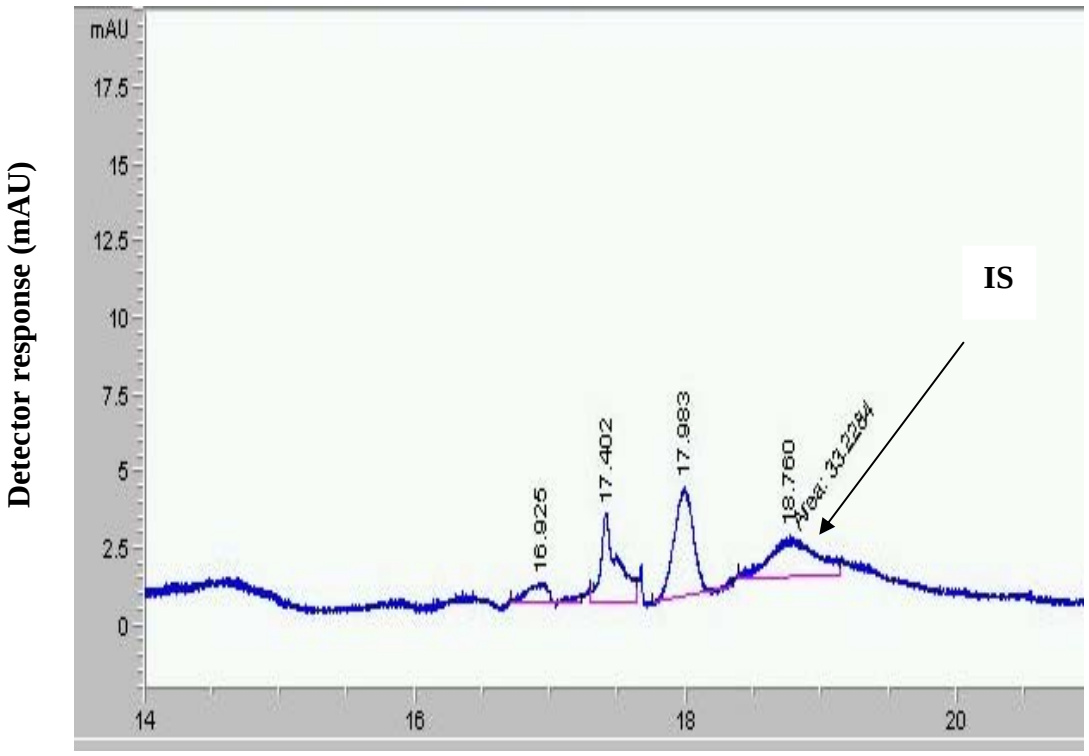


Figure 47 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of CREnC after treament with ceftazidime at 20 µg/ml plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

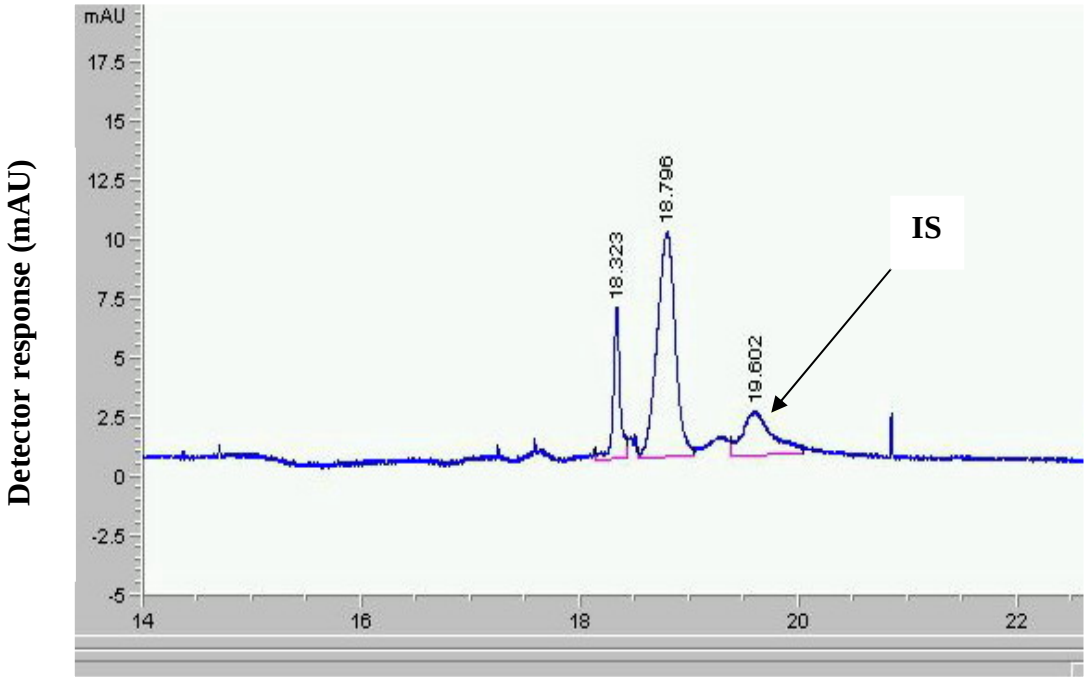


Figure 48 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of CREnC after treament with apigenin at 20 µg/ml plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm; I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate, pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20kV; IS = Internal standard

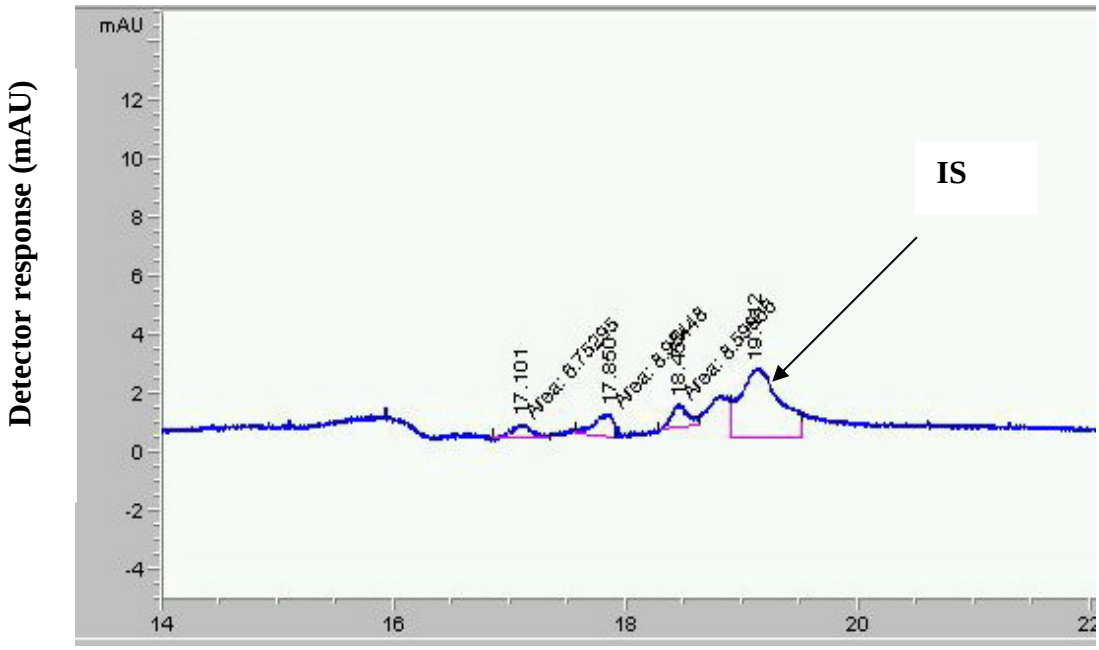


Figure 49 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of CREnC after treament with luteolin at 20 µg/ml plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate; pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

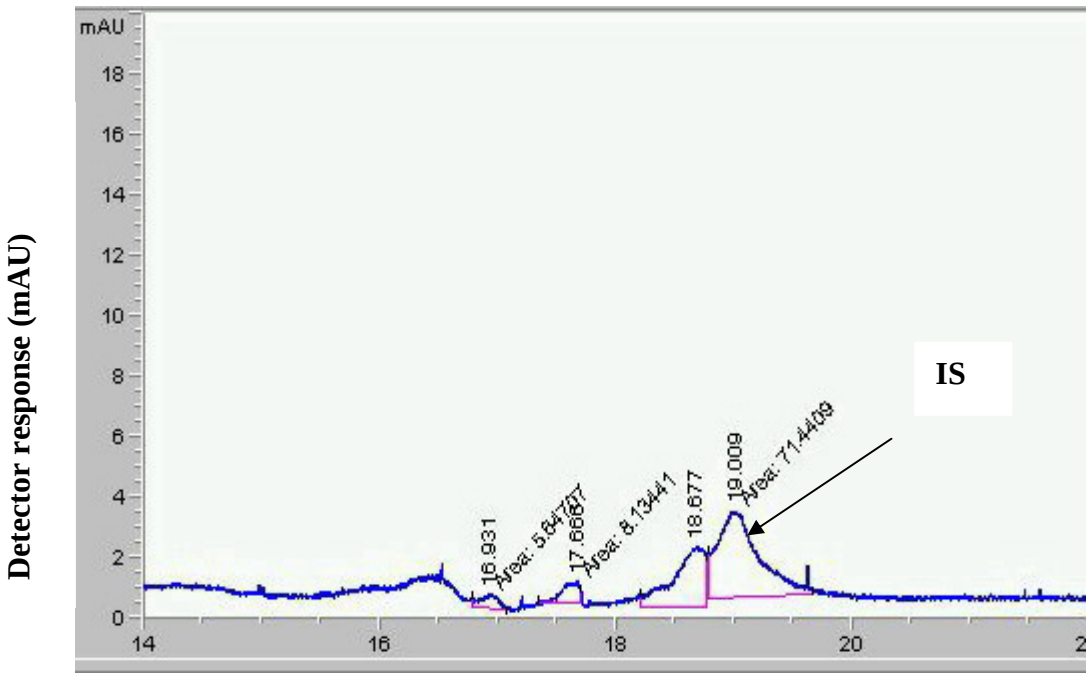


Figure 50 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of CREnC after treament with ceftazidime plus luteolin at 10:10 µg/ml and plus albumin as internal standard.The separation was carried out a fuse silica capillary, 50 µm I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

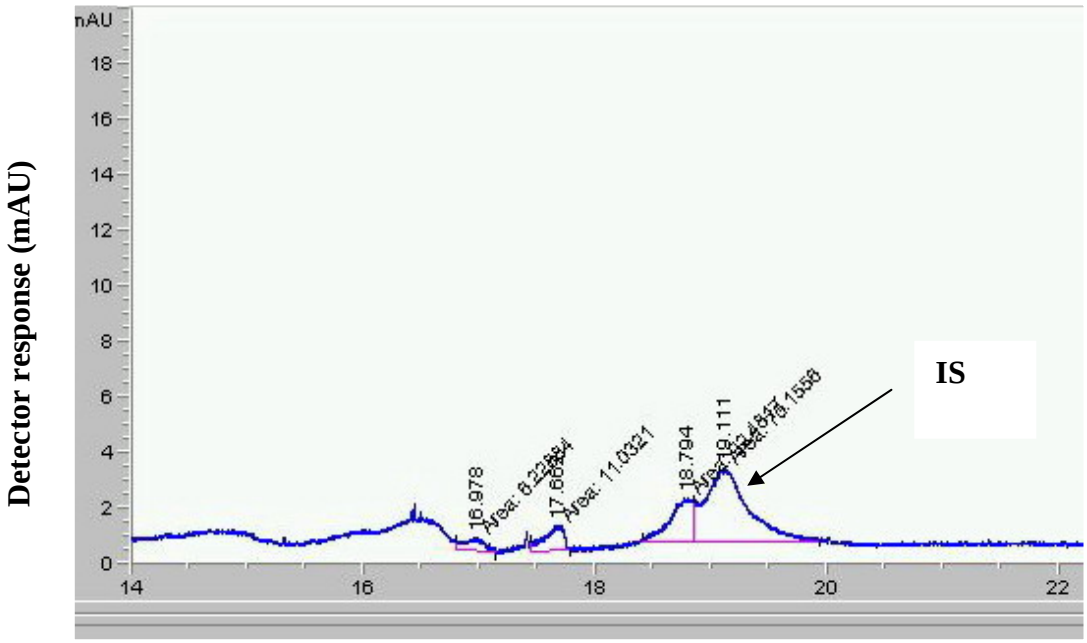


Figure 51 Electrophoregram of the separation by capillary electrophoresis of peptidoglycan-associated protein of CREnC after treament with ceftazidime plus apigenin at 10:10 µg/ml plus albumin as internal standard. The separation was carried out a fuse silica capillary, 50 µm; I.D., 65 cm in total length and 45 cm separation distance; buffer 50 mM disodium tetraborate, pH 9.1; detection at 205 nm; injection, 8 s at 5 kV; separation voltage 20 kV; IS = Internal standard

Discussion

The result in Figure 33 shows that electrophoregram of MRSA control cells albumin as internal standard had six major OM-PG associated protein peak areas at migration time 17.836 (0.14), 18.415 (0.02), 18.344 (0.16), 18.718 (0.17), 19.019 (1), and 20.977 (0.03) respectively. Whereas, Electrophoregram of MRSA when treated with ampicillin:baicalein (10:10 μg/ml) showed migration times at 17.377 (0.18), 17.797 (0.23), 18.573 (1), and 20.295 (0.11) respectively. It was found that the absence and/or reduction of medium MW protein peak areas and increasing in higher and lower MW protein peak areas when compared to control cells. This result was almost the same as MRSA treated cells with cloxacillin:baicalein (10:10 μg/ml) in Figure 39. In addition the extracted OM-PG associated protein peak areas of this MRSA strain treated with ceftazidime:baicalein (10:10 μg/ml) in Figure 43 shows the migration times at 17.441 (0.14), 17.922 (0.25), and 18.702 (1) respectively. The combination of ceftazidime plus baicalein MRSA treated cells showed that the absence and/or reduction of medium and lower MW protein peak areas and increasing in higher MW protein peak areas when compared to control cells. This results were approximately the same as the results of MRSA cells after treatment with ceftazidime :galangin (10:10 μg/ml) or ceftazidime:quercetin (10:10 μg/ml).

The electrophoregram of CREnC control cells in Figure 46 shows the migration times (the ratio of the peak areas) at 16.918 (0.21), 17.412 (1.02), 18.125 (4.38), 18.831 (1), and 20.555 (0.3) respectively. While the results of OM-PG associated protein extracts of this strain when expose to ceftazidime 10 μg/ml plus luteolin or apigenin at 10 μg/ml clearly showed the absence and/or reduction of both higher and lower MW protein peak areas when compared to the CREnC control cells.

The overall results presented here are also in substantial agreement with those obtained by Leying et al. (1986) with *E. coli* D509 grown in 1/4 and 1/6 the MICs of moxalactam. Moxalactam induced a strong reduction of the heavier OM protein bands. The results are also in agreement with those of Suerbaum and co-worker (1987) who concluded that imipenam, cephaloridine and ciprofloxacin had pronounced effects on the quantitative composition of the OM and the capsule information of *E. coli*. These β- lactams and ciprofloxacin seem to induce marked changes of the quantitative composition of the cell enveloped of *E. coli* and the phospholipid/amino acid ratio was reduced in almost all OM preparation from bacteria treated with the antibacterials.

These results may invole two mechanisms of actions. The first is on the integrity to the cell wall and on septum formation prior to cell division, the implies an effect on protein synthesis including an effect on penicillin-binding proteins. The second mechanism of β-lactam activity is via inhibition of the activity of certain β-lactamase enzymes. The first could also include an affect on the production and/or release of β-lactamase enzyme within and from the cell walls (Yam et al., 1998).

4.7 Enzyme assay

The ability of flavonoids plus selected β-lactam antibacterial to inhibit *in vitro* activity of β-lactamases varies considerably. Figure 52 indicated that the flavonoids had an inhibitory activity against β-lactamase I from *B. cereus*. Baicalein had some activity. Baicalein and quercetin in combination at 10 min showed greater activity. Figure 53 shows the ability of baicalein to inhibit *in vitro* activity of β-lactamase to break ampicillin structure. The result showed that baicalein plus ampicillin can inhibit *in vitro* of β-lactamase activity. Figure 54 indicated that baicalein plus cloxacillin had some activity against β-lactamase to break cloxacillin structure. Figure 55 shows activity of apigenin or luteolin against β-lactamase type IV from *E. cloacae*, that can cleave β-lactam bond of ceftazidime. Apigenin showed marked inhibitory activity. Moreover, luteolin showed greater activity.

These results indicated that the combination of ceftazidime plus galangin or quercetin or baicalein, ampicillin plus baicalein and cloxacillin plus baicalein not only had direct effect on cell structure and cell division but also inhibited β-lactamase activity from *B. cereus.* Furthermore ceftazidime plus luteolin or apigenin showed marked inhibitory activity against β-lactamase from *E. cloacae*. The resistance reversing activity of flavonoids against these bacteria might also include inhibition of β-lactamase activity.

Discussion

These results indicated that flavonoids not only have an activity of their own against β-lactam resistant bacteria but also have the ability to reverse the resistance of such bacterial strains to the activity of the primary antibiotics. This may involve two

mechanisms of action by the flavonoids. The first is on the integrity of the cell wall and on septum formation prior to cell division. This implies and effect on protein synthesis including an effect on penicillin-binding proteins.

The second mechanism of β-lactam activity is via inhibition of the activity of certain β-lactamase enzymes. The first action could also include an effect on the production and/or release of β-lactamase enzymes within and from the cell walls (Yam et al., 1998). In the last two decades, β-lactamase inhibitors like clavulanic acid have played an important role in fighting β-lactam resistant bacteria.

These inhibitors work as suicide compound react with enzyme since they share the same key struct with β-lactam antibiotics (Coulton and Francois, 1994). Recent studies demonstrated that clavulanate caused a considerable induction of β-lactamase expression and an increase of clavulanate concentration was followed by an elevation in β-lactamase production (Staplefon et al., 1995; Tzouvelekis et al., 1997).

These results may be explained that the presently available β-lactamase inhibitors can also lose their activity by the same mechanicm as the β-lactam antibiotics. For this research provides an unique example that flavonoids without a β-lactam structure can reverse bacterial resistance to β-lactams via multiple mechanisms. Because of this structural dissimilarity these compounds are unlikely to induce β-lactamase production. It should also be rememberd that conventional β-lactamase inhibitors, unlike flavonoids cannot reverse the resistant of MRSA, which is one of the most dangerous bacterial pathogen (Griangsak Eumkeb and Richards, 2004).

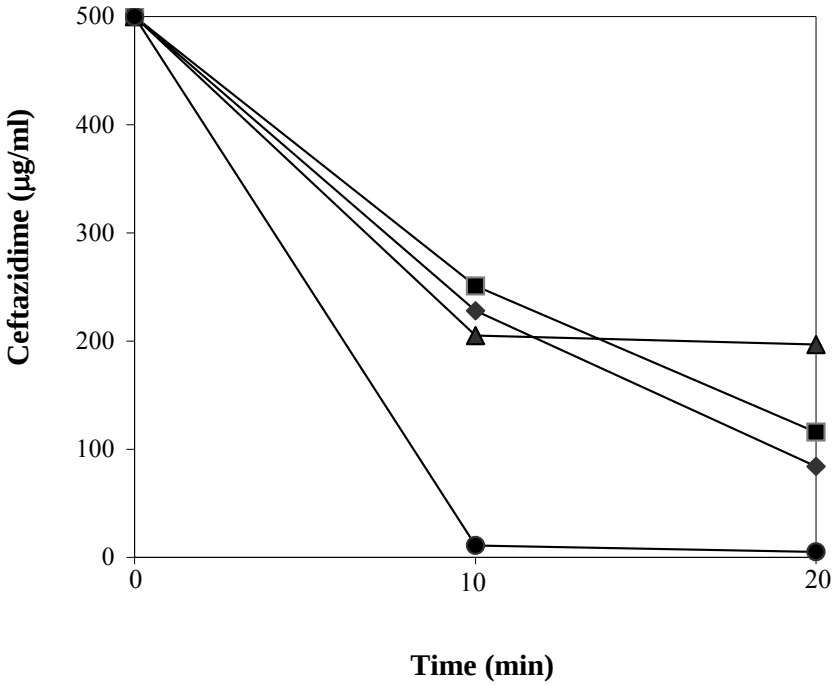


Figure 52 The inhibitory activity of flavonoids against β-lactamase in hydrolyzing ceftazidime. β-lactamase used from *B. cereus*; symbol represents flavonoids (200 µg/ml); ●, control(without flavonoids); ▲ galangin; ■, quercetin; ♦, baicalein

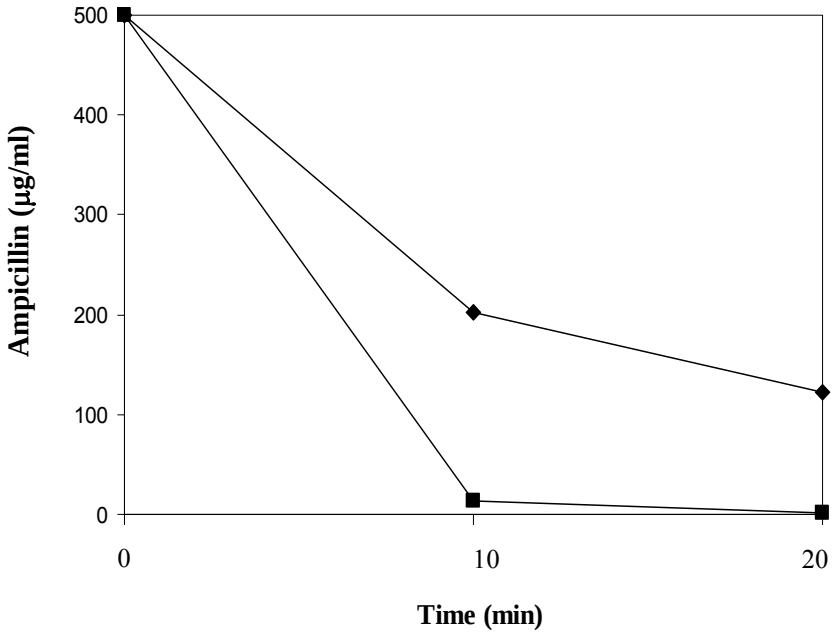


Figure 53 The inhibitory activity of flavonoids against β-lactamase in hydrolyzing ampicillin. β-lactamase used from *B.cereus*; symbol represents flavonoids (200 µg/ml); ■, control (without flavonoids); ♦, baicalein

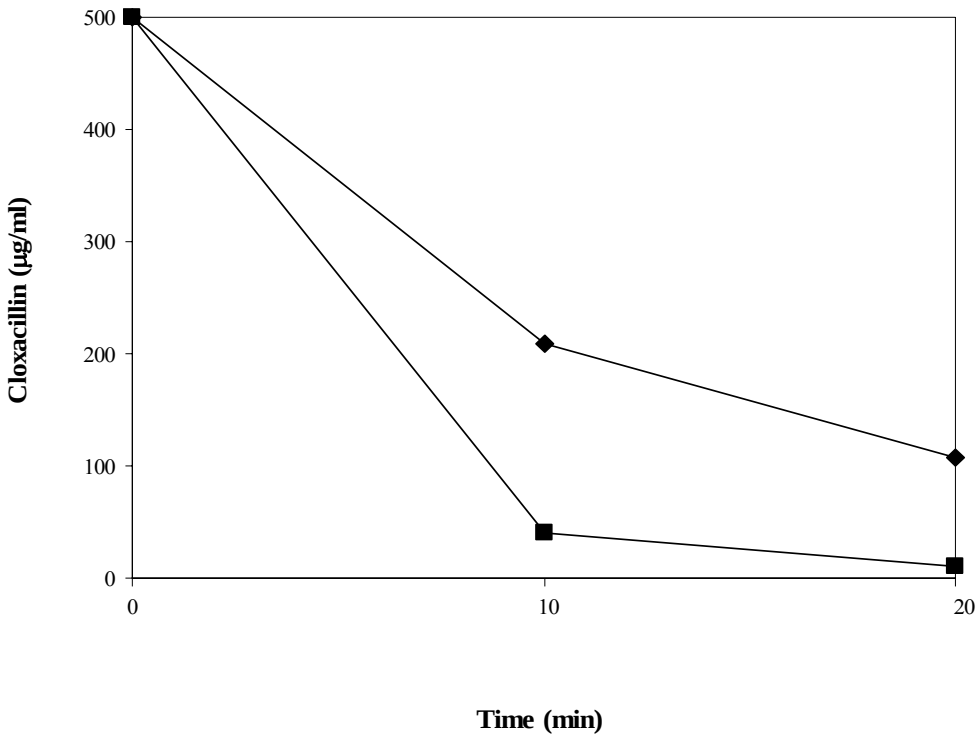


Figure 54 The inhibitory activity of flavonoids against β-lactamase in hydrolyzing cloxacillin. β-lactamase used from *B. cereus*; symbol represents flavonoids (200 µg/ml); ■, control (without flavonoids); ♦, baicalein

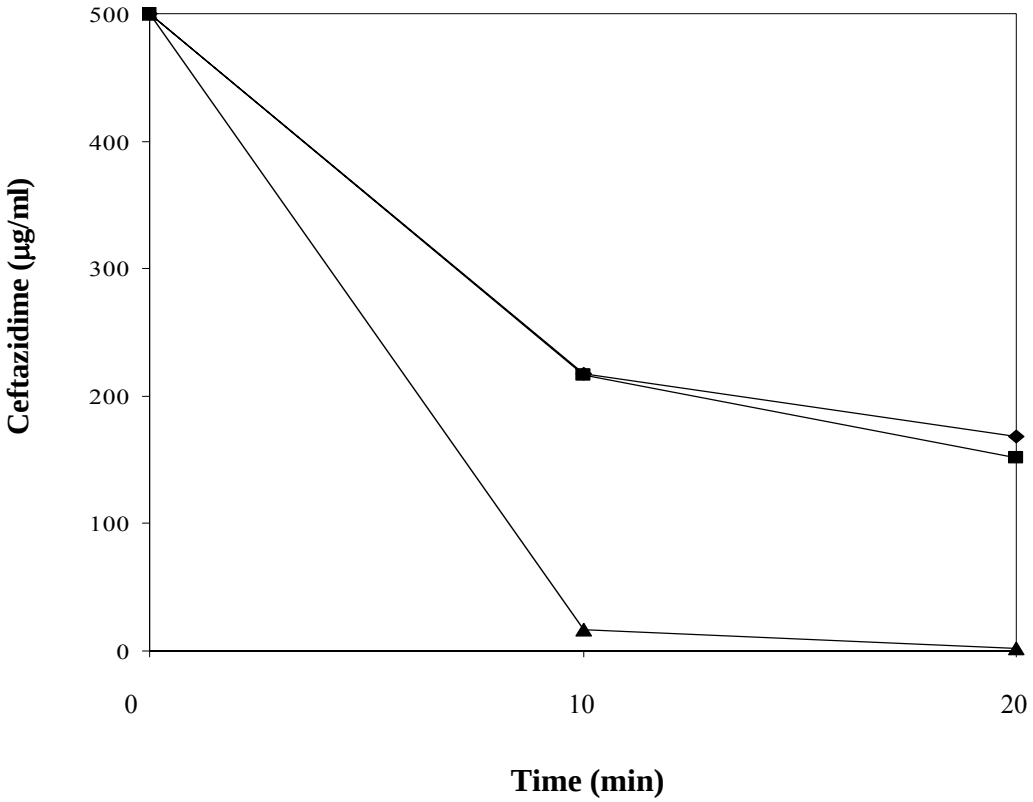


Figure 55 The inhibitory activity of flavonoids against β-lactamase in hydrolyzing ceftazidime. β-lactamase used from *E. cloacae*; symbol reoresents flavonoids (200 μg/ml); ▲, control (without flavonoids); ■, apigenin; ♦, luteolin

CHAPTER V

CONCLUSION

Presently, there is an emergence of multiple drug resistance to human pathogenic organisms. Thus, development of a new antibacterial agents is urgently needed, especially anti-MRSA and CREnC agents that these bacterial caused nosocomial infections increasing. One approach is to search for new inexpensive and effective antibacterial agents from new sources, especially from plants, for possible antimicrobial properties. The present work, flavonoids from plants such as apigenin, baicalein, galangin, luteolin and quercetin plus β-lactam antibiotics were tested against methicillin-resistant *S. aureus* (MRSA) and ceftazidime-resistant *E. cloacae* (CREnC).These clinical isolates of bacteria caused high percentage of drugs-resistance in many sections of the local hospitals and worldwide, as a result, the cost of treating these infection diseases are increasing.

The results from this study showed that two clinical isolated strains (MRSA and CREnC) were resistant to all selected β-lactam antibiotics (ampicillin, cloxacillin and ceftazidime). All tested flavonoids showed no activity against all clinical isolates strains (MRSA, CREnC and ASEnC) when used alone. However, significant synergistic activities against both MRSA and CREnC in combination with β-lactam antibiotics were observed. In checkerboard test, the results showed that when combined ampicillin, cloxacillin and ceftazidime with baicalein 5 μg/ml, minimum

inhibitory concentrations (MICs) of these drugs against clinical isolates of MRSA were reduced from 100, > 1,000, and 50 μg/ml to 5, 5, and 5 μg/ml respectively. Furthermore, clinical isolates of CREnC with MIC of ceftazidime > 1,000 μg/ml had their resistance to these drugs reversed by apigenin 5 μg/ml or luteolin 5 μg/ml to MIC of ceftazidime 5 μg/ml. Viable counts showed that the killing of MRSA cells by 10 μg/ml ampicillin or cloxacillin was potentialed by 10 μg/ml baicalein. Ceftazidime 10 μg/ml in combination with 10 μg/ml of baicalein or galangin or quercetin also reduced the CFU/ml of MRSA to low level ($1X10^3$ CFU/ml) over 6 h. These results are in substantial agreement with those of Iain (2000) that the baicalein had the potential to restore the effectiveness of β-lactam antibiotics against MRSA. The results seem consistent with Sato et al. (2004) that 6, 7 dihydroxyflavone synergistically elevates the susceptibility of MRSA to β-lactam antibiotics from 8 t0 32,000 fold. Morever flavone found to show diverse synergistic effects on the susceptibility of MRSA to β-lactam antibiotics. The killing curve of CREnC cells were also maintained at low level from 6 to 24 h by ceftazidime 10 μg/ml in combination with 10 μg/ml of luteolin or apigenin.

Electronmicroscopy clearly showed that the combination of 10 μg/ml baicalein with 10 μg/ml of ampicillin or cloxacillin caused electron-transparent areas devoid of ribosomes (hole) clearly visible within the cytoplasm. A lot of cells lost some organelles from cytoplasm such as ribosome, chromosomes etc. Some of these showed morphological damages such as cell wall and cell shape distortion. Broken of some cells were also observed. Morever, the combination between 10 μg/ml ceftazidime with 10 μg/ml of baicalein or galangin or quercetin caused damage to the ultrastructure of MRSA such as cell wall damage, electron-transparent areas devoid of

ribosomes and lost some organelles from cytoplasm. The results seem consistent with Eumkeb and Richards (2005) that the combination of β-lactam with galangin caused damage to the ultrastructural of MRSA cells. In addition, the results showed that ceftazidime 10 μg/ml in combination with 10 μg/ml of luteolin or apigenin also caused marked morphological damage for CREnC. A lot of these bacterial cells exhibited morphological damage of cell wall, cell shape and electron-transparent areas in cytoplasm due to losing most of organelles. Several bacterial cells showed broken cell and distortion of cell wall. Some of these cells showed obvious detachment of cell wall and plasma membrane.

In addition, Electrophoregrams show that the extracted OM-PG associated protein peak areas of clinical isolates of MRSA exposed to either ampicillin:baicalein (10:10 μg/ml) or cloxacillin:baicalein (10:10 μg/ml) showed the absence and/or reduction of medium MW protein peak areas and increasing in higher and lower MW protein peak areas. Similarly, the extracted OM-PG associated protein peak areas of this MRSA strain treated with either ceftazidime:baicalein (10:10 μg/ml), ceftazidime:galangin (10:10 μg/ml) or ceftazidime:quercetin (10:10 μg/ml) exhibited the absence and/or reduction of medium and lower MW protein peak areas and increasing in higher MW protein peak areas. Results from electrophoregrams of OM-PG associated protein extracts of CREnC strains when exposed to ceftazidime 10 μg/ml plus luteolin or galangin at 10 μg/ml showed the absence and/or reduction of both higher and lower MW protein peak areas. These absence and/or reduction of medium and higher (or lower) MW OM-PG associated proteins in MRSA and CREnC respectively probably led to the marked morphological damage, detachment of OM and peptidoglycan, the longer and wider cell size in both strains. The results from

electrophoregrams seem consistent with the results from checkerboard, viable counts and TEM.

The results from enzyme assay indicated that β-lactamase activity of *B. cereus* was inhibited by the combinations of ceftazidime plus galangin or quercetin or baicalein and baicalein plus ampicillin or cloxacillin. Morever the β-lactamase activity of *E. cloacae* was marked inhibited by the combinations of ceftazidime plus luteolin or apigenin. The resistance reversing activity of selected flavonoids against these bacteria might also include inhibition of β-lactamase activity. These results are in substantial agreement with earier finding suggesting that flavonoids not only have an activity of their own against β-lactam resistant bacteria but also have the ability to reverse the resistance of these bacterial strains to the activity of the primary antibiotics. This may invole two mechanisms of action by the flavonoids. The first is on the integrity of the cell wall and on septum formation prior to cell division. The second is on the mechanism of β-lactamase enzymes (Yam et al., 1998).

From this study, it was concluded that baicalein, galangin and quercetin have the potential to reverse bacterial resistance to β-lactam antibiotics against MRSA. Luteolin and apigenin have synergistic effect with ceftazidime against CREnC. In view of their limited toxicity, these tested flavonoids offer for the development of a valuable adjunct to β-lactam treatments against otherwise resistant strains of currently almost untreatable microorganisms.

Future Work

1. *In vivo* toxicity test of selected flavonoids and/or plus selected antibacterials.

2. Determination of the sequence of amino acids in the polypeptides in each OM-PG associated protein band in MRSA and CREnC strains by using chemical and the Edman degradation method or an automated device such as a sequentor. Then, comparing the amino acid content of a polypeptide hydrolysate, which can be quantitatively determined by using an automated amino acid analyzer for each OM-PG associated protein band of the control cells and treated cells. By these methods the sequence of amino acid in each protein band of the control cells and treated cells may be compared. The amino acid sequences that combine to from polypeptides (proteins) in each protein band could also be determined. This would elucidate further the mechanism of action of antibacterials.
3. Evaluate the effects of other antimicrobial combination such as other β-lactam antibiotics plus flavonoids.

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