การเสริมฤทธิ์ของสารฟลาโวนอยด์และยาปฏิชีวนะต่อเชื้อแบคทีเรียดื้อยาในกลุ่มบีตาแลคแทม

นางสาวสุพัชรี ศิริวงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์
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SYNERGISTIC EFFECTS OF FLAVONOIDS AND ANTIBIOTICS ON BETA-LACTAM ANTIBIOTIC RESISTANT BACTERIA

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

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SYNERGISTIC EFFECTS OF FLAVONOIDS AND ANTIBIOTICS ON BETA-LACTAM ANTIBIOTIC 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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สุพัชรี ศิริวงศ์ : การเสริมฤทธิ์ของสารฟลาโวนอยด์และยาปฏิชีวนะต่อเชื้อแบคทีเรียดื้อยาในกลุ่มบีตาแลคแทม (SYNERGISTIC EFFECTS OF FLAVONOIDS AND ANTIBIOTICS ON BETA-LACTAM ANTIBIOTIC RESISTANT BACTERIA) อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ผู้ช่วยศาสตราจารย์ เภสัชกร ดร.เกรียงศักดิ์ เอื้อมเก็บ, 128 หน้า.

การใช้ยาปฏิชีวนะในกลุ่มบีตาแลคแทมอย่างแพร่หลายในการรักษาการติดเชื้อแบคทีเรียนำไปสู่การดื้อยาของเชื้อเพิ่มขึ้น ซึ่งทำให้การรักษายากขึ้นและนำไปสู่การเสียชีวิตของผู้ป่วย เชื้อสแตปฟีโลคอคคัส อีพิเดอมิดิสและสเตรปโตคอคคัส พายโอจีเนส เป็นเชื้อที่ก่อโรคในมนุษย์ โดยเชื้อเหล่านี้สามารถพัฒนาให้ดื้อต่อยาปฏิชีวนะได้หลายชนิดรวมถึงยาปฏิชีวนะในกลุ่มบีตาแลคแทม รายงานวิจัยหลายฉบับกล่าวถึงประสิทธิภาพในการยับยั้งเชื้อแบคทีเรียก่อโรคของสารฟลาโวนอยด์ที่สกัดได้จากพืช อย่างไรก็ตาม การวิจัยเกี่ยวกับการเสริมฤทธิ์กันและกลไกการออกฤทธิ์ของสาร ฟลาโวนอยด์ต่อแบคทีเรียดื้อยายังมีค่อนข้างน้อย วัตถุประสงค์ของการวิจัยนี้เพื่อทดสอบความสามารถของสารฟลาโวนอยด์และการเสริมฤทธิ์ของสารฟลาโวนอยด์กับยาปฏิชีวนะในกลุ่มบีตาแลคแทมในการยับยั้งเชื้อแบคทีเรียสแตปฟีโลคอคคัส อีพิเดอมิดิส ดีเอ็มเอสที 15505 ดื้อยาอะม็อกซีซิลลิน (เออาร์เอสอี) และเชื้อสเตรปโตคอคคัส พายโอจีเนส ดีเอ็มเอสที 30653 (เอสพี) วิธีบรอทมาโครไดลูชันและเชคเกอร์บอร์ดถูกใช้ในการทดสอบความไวของสารฟลาโวนอยด์และยาปฏิชีวนะต่อแบคทีเรีย ได้ค่าเอ็มไอซีของยาอะม็อกซีซิลลินและสารฟลาโวนอยด์ ลูทีโอลิน ไบคาลีนและเคอเซติน เมื่อใช้เดี่ยวๆ ในการยับยั้งเชื้อเออาร์เอสอี เท่ากับ 16 32 32 และ 256 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ และค่าดรรชนีเอฟไอซีเท่ากับ >1 1 และ 0.25 ตามลำดับ และได้ค่าเอ็มไอซี ของยาเซพตาซิดีมและสารฟลาโวนอยด์ ลูทีโอลิน ไบคาลีนและเคอเซติน เมื่อใช้เดี่ยวๆ ในการยับยั้งเชื้อเอสพีเท่ากับ 0.25 128 >256 และ 128 ไมโครกรัมต่อมิลลิลิตรตามลำดับ และค่าดรรชนีเอฟไอซีเท่ากับ 0.625 <0.625 และ 0.531 ตามลำดับ แสดงให้เห็นว่าเมื่อใช้ยาปฏิชีวนะร่วมกับสารฟลาโวนอยด์เกิดการเสริมฤทธิ์ในการยับยั้งเชื้อเออาร์เอสอีและเอสพีได้ ผลการทดสอบการดูดกลืนแสงที่ค่าความยาวคลื่น 260 นาโนเมตร แสดงให้เห็นว่าภายหลังที่เชื้อเออาร์เอสอีและเอสพีถูกเลี้ยงกับสารผสมของฟลาโวนอยด์และยาปฏิชีวนะ การดูดกลืนแสงที่ค่าความยาวคลื่นนี้ เพิ่มขึ้นจาก 0-0.02 เป็น 0.3-0.8 เมื่อเทียบกับกลุ่มควบคุม ซึ่งแสดงให้เห็นถึงเยื่อหุ้มเซลล์ชั้นใน (ซีเอม) ถูกทำลาย ผลการทดสอบการยับยั้งการทำงานของเอนไซม์บีตาแลคแทมเมสของสารฟลาโวนอยด์ เปรียบเทียบกับยาปฏิชีวนะ พบว่าสารฟลาโวนอยด์มีความสามารถในการยับยั้งการทำงานของเอนไซม์บีตาแลคแทมเมสมากกว่ายาปฏิชีวนะ ผลจาก

กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านแสดงให้เห็นว่า เซลล์แบคทีเรียภายหลังจากที่ถูกเลี้ยงกับสารผสมของฟลาโวนอยด์และยาปฏิชีวนะพบว่า ทำให้สัณฐานวิทยาของเซลล์ได้รับความเสียหายอย่างเห็นได้ชัด เปปทิโดไกลแคนและเยื่อหุ้มเซลล์ชั้นในได้รับความเสียหายและขนาด (พื้นที่)โดยเฉลี่ยของเซลล์เปลี่ยนแปลงไป ผลจากเอฟทีไออาร์แสดงให้เห็นว่า เมื่อใช้เคอเซตินผสมกับอะม็อกซิซิลลิน และเคอเซตินหรือไบคาลีนผสมกับเซพตาซิดีมต่อเซลล์ของเออาร์เอสอีและเอสพี ทำให้กรดไขมันและกรดนิวคลิอิกลดลงในขณะที่โปรตีนเอไมด์เพิ่มขึ้น เมื่อเทียบกับกลุ่มควบคุม

ดังนั้นการค้นพบนี้เป็นรายงานชิ้นแรกที่พบว่าสารฟลาโวนอยด์เหล่านี้ออกฤทธิ์เสริมกับยาปฏิชีวนะในกลุ่มบีตาแลคแทม ต้านแบคทีเรียเออาร์เอสอีและเอสพีให้กลับไปไวต่อยาปฏิชีวนะเหมือนเดิม โดยผ่านกลไกการออกฤทธิ์สี่แบบ ได้แก่ยับยั้งการสังเคราะห์เปปทิโดไกลแคน ยับยั้งการออกฤทธิ์ของเอนไซม์บีตาแลคแทมเมส เพิ่มการซึมผ่านของเยื่อหุ้มเซลล์ชั้นใน และทำให้สารชีวโมเลกุลภายในเซลล์เปลี่ยนแปลง สารฟลาโวนอยด์เหล่านี้มีศักยภาพเด่นชัดที่จะพัฒนาเป็นสารชนิดใหม่ที่ใช้ผสมกับยาปฏิชีวนะในกลุ่มบีตาแลคแทมในการยับยั้งแบคทีเรียเหล่านี้ งานวิจัยในอนาคต ควรทดสอบประสิทธิภาพและพิษในสัตว์ทดลองและมนุษย์ ในทำนองเดียวกันการเสริมฤทธิ์กันในเลือดและเนื้อเยื่อควรได้รับการทดสอบว่าถึงระดับที่ออกฤทธิ์ได้

สาขาวิชาเภสัชวิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SUPATCHAREE SIRIWONG : SYNERGISTIC EFFECTS OF FLAVONOIDS AND ANTIBIOTICS ON BETA-LACTAM ANTIBIOTIC RESISTANT BACTERIA. THESIS ADVISOR : ASST. PROF. GRIANGSAK EUMKEB, Ph.D. 128 PP.

FLAVONOIDS/QUERCETIN/BETA-LACTAM/MECHANISM OF ACTION/RESISTANT BACTERIA/FT-IR MICROSPECTROSCOPY

The wide use of β-lactam antibiotic in the treatment of bacterial infections has led to the emergence and spread of resistant strains. This evidence has increased risk of worse clinical outcomes and death. *Staphylococcus epidermidis* and *Streptococcus pyogenes* are bacterial pathogens of human and have developed resistance to most antibiotics and β-lactam antibiotics. Many researchers reported that some flavonoids isolated from plants could efficiently inhibited some pathogenic bacteria. However, little works have investigated the synergistic effect and modes of action of flavonoids against drug-resistant bacteria. The objectives of this study were to test the inhibitory effect of flavonoids and synergistic effect of flavonoids plus β-lactam antibiotic against amoxicillin resistant *S. epidermidis* DMST 15505 (ArSE) and *S. pyogenes* DMST 30653 (SP). The broth macrodilution and checkerboard assay were used for antibacterial susceptibility testing. The minimum inhibitory concentrations (MICs) of amoxicillin, luteolin, baicalein and quercetin alone against ArSE were 16, 32, 32 and 256 µg/ml, respectively, and the fraction inhibitory concentration (FIC) indices were >1, 1 and 0.25, respectively. The MICs of ceftazidime, luteolin, baicalein and quercetin alone against SP were 0.25, 128, >256 and 128 µg/ml, respectively, and the

FIC indices were 0.625, <0.625 and 0.531, respectively. These results proposed that β-lactam antibiotic in combination with flavonoids showed a synergistic effect against both ArSE and SP. The results from OD_{260} UV-VIS spectroscopy showed that after treatment, the OD_{260} of both ArSE and SP cells treated with a combination of flavonoids plus β-lactam antibiotic were increased from 0-0.02 to 0.3-0.8. These results suggest that cytoplasmic membrane (CM) was damaged. The effect of these flavonoids on β-lactamase enzyme activity compared to that of β-lactam antibiotic showed that these flavonoids had higher inhibitory effect than β-lactam antibiotic. The transmission electron microscopy (TEM) results exhibited the morphological, peptidoglycan and CM damage and average cell areas were changed of the cells treated with the combination. Fourier transform infrared (FT-IR) microspectroscopy revealed that the combination of quercetin plus amoxicillin and quercetin or baicalein plus ceftazidime on ArSE and SP cells cause the decrease in fatty acid and nucleic acid whereas protein amide was shown to increase compared to controls. These findings are the first report that these flavonoids have the synergistic effect with β-lactam antibiotic against ArSE and SP to its original susceptibility *via* four modes of actions, inhibit peptidoglycan synthesis, restrain β-lactamases activity, increase CM permeability and cause biomolecules change in these cells. These flavonoids have the dominant potentiality to be a new agent for synergistic use with β-lactam antibiotic to treat these bacteria. Future work should focus on the efficacy and toxicity in animals and in humans, as well as, the synergistic effect on blood and tissue.

School of Pharmacology Student's Signature_____

Academic Year 2014 Advisor's Signature _____

Co-advisor's Signature _____

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Supatcharee Siriwong

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LIST OF ABBREVIATIONS

°C	=	Degree Celsius
mg	=	Milligram per liter
gm	=	Gram
µg	=	Microgram
µm	=	Micrometer
nm	=	Nanometer
ml	=	Milliliter
AR	=	Analytical reagent
r.p.m.	=	Revolution per minute
MIC	=	Minimum inhibitory concentration
ATCC	=	American Type Culture Collection
DMST	=	Department of Medical Sciences, Thailand
FIC	=	Fraction inhibitory concentration
DMSO	=	Dimethylsulfoxide
CM	=	Cytoplasmic membrane
TEM	=	Transmission electron microscopy
cfu	=	Colony forming unit
min	=	Minute
h	=	Hour
OD	=	Optical density

LIST OF ABBREVIATIONS (Continued)

UV	=	Ultra violet
VIS	=	Visible
PBS	=	Phosphate buffer saline
CLSI	=	Clinical and laboratory standards institute
W/V	=	Weight by volume
kV	=	Kilo volt
FT-IR	=	Fourier transform infrared
PC	=	Principal component
PCA	=	Principle component analysis

CHAPTER I

INTRODUCTION

1.1 Introduction

Most of the coagulase-negative *Staphylococci* associated with clinical disease are common inhabitants of the skin and mucous membranes. *S. epidermidis* is the most prevalent species, accounting for approximately 60-70% of all coagulase-negative *Staphylococci* on the skin. Coagulase-negative staphylococci are frequently associated with nosocomial infections and bacteremia. Moreover, they are also one of the leading causes of bloodstream infections (Otto, 2004; 2009; Vuong and Otto, 2002).

Streptococcus pyogenes is also known as Group A *Streptococci* (GAS). They are important human pathogen. They can cause of pharyngitis, cellulitis, skin infections and lead to toxic shock syndrome (TSS). GAS is well known as a highly adhesive cellular organism, the virulence of which is related to the production of exotoxins (Wong and Yuen, 2012).

Normally, β-lactam antibiotics are first choices to treat *Staphylococc*i and *Streptococcus* infections. These antibiotics are highly specific inhibitors of the metabolism of peptidoglycan and their targets are membrane bound D, D-peptidase domain of the penicillin binding proteins (PBPs) (Ghuysen, 1994). These peptidases cross link the bacterial peptidoglycan cell wall which maintains the integrity of the cell. Coagulase-negative *Staphylococci* may rarely be penicillin-susceptible, but the

majority of this strain is resistant based on the production of an inducible enzyme, including β-lactamase or penicillinase mediated through a plasmid-located gene known as *blaZ* or other mechanisms to resist. Staphylococcal penicillinases confer resistance to penicillins, ampicillin, amoxicillin and piperacillin. Some strains of *Staphylococci* may only produce detectable amounts of enzyme only after exposure to an inducing agent, which may be generally a β-lactam (Archer and Scott, 1991; Olsen et al., 2006).

Recommended therapies for *Streptocooci* sp. infections also include penicillin and cephalosporin which are grouped in β-lactam antibiotic. However, number resistant strain of *Streptococcus* sp. to these drugs also increases through many mechanisms of resistance (Passali et al., 2007; Sutcliffe et al., 1996). Some of *Streptococcus* sp. gained resistance to β-lactam antibiotics by mutation of *PBP 2x* gene resulting in low affinity to drug which is an important step in resistance development (Grebe and Hakenbeck, 1996).

Recommended therapies for *S. pyogenes* infections include penicillins and cephalosporin. However, the failure of penicillin to eradicate *Streptococci* from the throat occurs in up to 35% of patients with pharyngeal-tonsillitis (Passali et al., 2007). Van and coworkers investigated that penicillin tolerance could not be ruled out as a cause of penicillin treatment failures due to the lack of evidence. (Van Asselt et al., 1996). Previous research found that 1.9% of *S. pyogenes* was intermediately resistant to ampicillin (Gur et al., 2002). Idrees and Saeed found that *S. pyogenes* was resistive to cloxacillin, oxacillin, and cefoparazone (Idrees and Saeed, 2013). Moreover, only two *S. pyogenes* were β-lactamase producers and *bla*-TEM was detected and amplified on genomic DNA of 28 β-lactam resistant from 42 *S. pyogenes* isolated

from Mansoura university hospitals (Hassan et al., 2010). The coexistence of oropharyngeal β-lactamase-producing bacteria (BLPB) may not have only caused the bacteria to survive penicillin therapy, but could also have protected other penicillin-susceptible bacteria from penicillin. So, the increased failure rate of penicillin in eradication of otitis, sinusitis and pharyngo-tonsillitis infections by these bacteria had been reported (Brook, 2007). Effective antibiotics available for the treatment of *S. pyogenes* and the coexistence of the BLPB infections are frequently associated with the failure of β-lactams and unwanted side effects. Invention of new combination agent to treat these bacteria that can overcome β-lactams failures in the treatment of *S. pyogenes* and the resurgence of the BLPB infections and reduce adverse drug effect is urgently needed.

This situation has created new challenges of drug discovery and much effort has been undertaken in the area of medicinal plants since plant sources are highly relevant for the identification of compounds which can result in the development of novel and safe therapeutic agents. In recent years, many researchers have been testing and developing the activity of new agents to treat resistant strain of *Staphylococcus* sp. and *Streptococcus* sp. Available reports show that secondary metabolites of phenolic nature, including flavonoids are responsible for the variety of pharmacological activities (Curcic et al., 2012; Dryden, 2014; Zimmer et al., 2014). One of their activities is antibacterial activity. Flavonoids are known to be synthesized by plants and response to microbial infection. It also is effective antimicrobial substance against a wide array of microorganisms. Several flavonoids including apigenin, baicalein, galangin, catechins, quercetin and naringenin have been shown to possess potent

antibacterial activity (Fowler et al., 2011; Hemaiswarya et al., 2008; Kumar and Pandey, 2013).

Flavonoids can exert antibacterial activities through multiple mechanisms, such as disruption of cytoplasmic membrane, inhibition of nucleic acid synthesis, inhibition of energy metabolism, inhibition of cell wall synthesis, and inhibition of cell membrane synthesis (Cushnie and Lamb, 2011; Lee et al., 2013). Several reports suggested that flavonoids usually have multiple mechanisms of action (Vikram et al., 2010; Wu et al., 2008). However, the antibacterial targets remain largely unknown for most flavonoids. Then, to overcome multidrug resistance in the antimicrobial therapy combination of drugs has to be used. Some research notes that the β-lactam antibiotic combination with isolated flavonoids is effective as an antibacterial agent. Plant extracted flavonoids, together with antimicrobial drug could be advantageous in the treatment of many strains of drug resistant bacteria (Da Silva et al., 2014; Wojtyczka et al., 2013). However, a few studies have been performed on the synergy and mechanism of action of these flavonoids when used alone or in combination against *S. pyogenes* and amoxicillin-resistant *S. epidermidis*. So, this study was aimed to investigate the synergistic interactions of the secondary metabolites of plants such as flavonoids with β-lactam antibiotics in the treatment of β-lactam antibiotic resistant *Staphylococcus* sp. and *Streptococci* sp. The understanding of the primary mechanisms of synergy of the combination will provide a new strategy for the treatment of these bacterial infections.

1.2 Research objectives

(1) To test the effectiveness of selected flavonoids alone on drug resistant bacteria.

(2) To test the effectiveness of selected flavonoids in combination with antibiotic drugs against drug resistant bacteria.

(3) To investigate the primary mechanism of action of selected flavonoids on drug resistant bacteria when used singly and in combination with antibiotic drugs.

(4) To examine FT-IR spectral feature of control and bacterial treated cells for determination of the variation in composition and distribution of the biochemical components of these cells by using FT-IR microspectroscopy.

CHAPTER II

LITERATURE REVIEWS

2.1 Bacterial cell wall

Fundamental differences in ultrastructure of the cell wall are responsible for the reaction of bacteria towards the Gram's stain. A major characteristic for classifying bacteria is the gram stain reaction which distinguishes two types of bacterial cell walls. Gram stain dye process stains gram positive bacteria dark violet and gram negative bacteria red. In both types of cell, the cytoplasmic membrane is surrounded and supported by a cell wall, which provides strength, rigidity and shape. Schematic cross sections of these structures are given below (Figure 2.1) (Scheffers and Pinho, 2005).

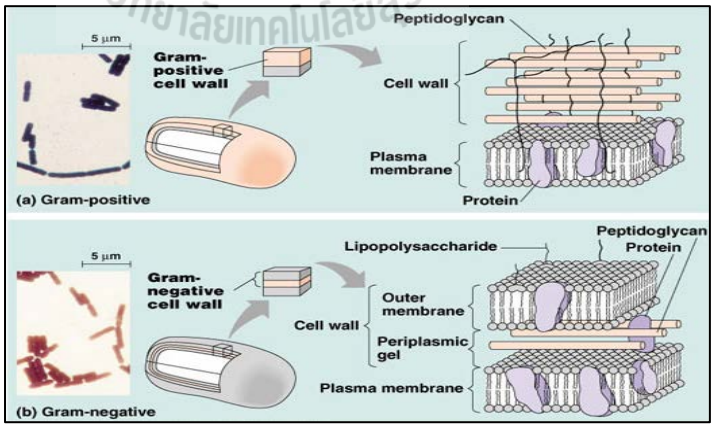

Figure 2.1 Structure of gram positive and gram negative bacteria. Gram negative bacteria have a thin layer of peptidoglycan and outer membrane (a). Gram positve bacteria have a thick peptidoglycan layer (b) (Scheffers and Pinho, 2005).

2.1.1 Gram positive bacterial cell wall

In gram positive bacterial cell wall, the peptidoglycan layer is thick, comprising up to 40-80% of the cell wall. Most gram positive cell walls contain additional substances such as teichoic acid and teichuronic acid. These are water soluble polymers of ribitol or glycerol. There are two types of teichoic acid, wall teichoic acid (linked to peptidoglycan) and lipoteichoic acid (linked to membrane). Some gram positive bacteria may lack wall teichoic acid, but all contain lipoteichoic acid. The teichoic acid constitutes major antigens of cells that possess them. Teichoic acid binds to magnesium ions and plays a role in the supply of this ion in the cell. Teichuronic acids are produced in place of teichoic acid when phosphate is limiting. Gram positive cells stain purple due to retention of the crystal violet dye during the gram stain procedure. If peptidoglycan is digested away from the cell, gram positive cells lose their cell walls and become protoplasts while the gram negative cells become spheroplasts (Navarre and Schneewind, 1999; Silhavy et al., 2010).

2.1.2 Gram negative bacterial cell wall

Gram negative cells consist of a relatively thin layer of peptidoglycan comprising 5-10% of cell wall material. The outer membrane is the major permeability barrier in gram negative bacteria. The space between the inner and outer membranes is known as the periplasmic space, which contains digestive enzymes and other transport proteins. Gram negative cell walls contain three components that locate outside the peptidoglycan layer and lipoprotein, outer membrane and lipopolysaccharide (LPS). Lipoprotein stabilizes the outer membrane by anchoring it to peptidoglycan. The outer membrane is a phospholipid bilayer in which the outer phospholipids are replaced by LPS. The outer membranes contain several important

porins, which specifically allow transport of solutes. LPS consists of a polysaccharide core, a complex lipid called “lipid A” and a terminal series of repeat units. The polysaccharide core is similar in all gram negative bacteria. Each species contains unique terminal repeat units. LPS is toxic in nature and is called endotoxin because it is firmly bound to the cell wall and released only when the cell is lysed. Endotoxin can trigger fever and septic shock in gram negative infections. LPS also protects the cell from phagocytosis and lysozyme (Silhavy et al., 2010).

2.2 Structure of peptidoglycan

Peptidoglycan is the macromolecule that surrounds the bacterial cell and is a principal determinant of bacterial shape. Peptidoglycan consists of a backbone of alternating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). Each NAM molecule carries a short peptide made up of five amino acids, which cross-link (via an interpeptide bridge composed of further amino) to provide the characteristic rigidity (Vollmer et al., 2008).

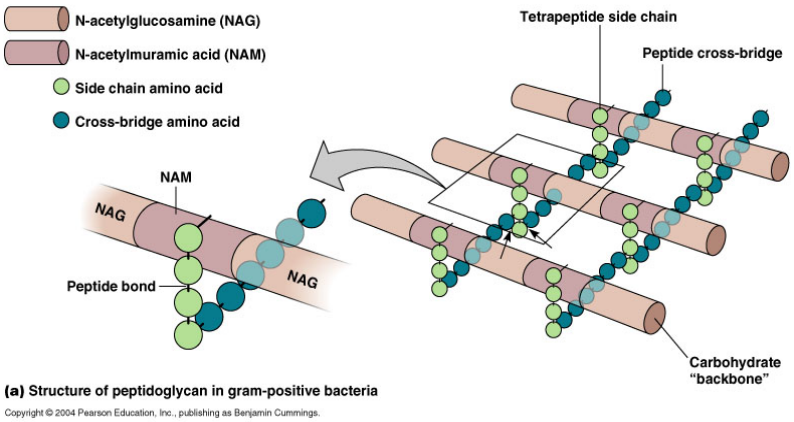

Figure 2.2 General structure of the peptidoglycan component of the cell wall (Vollmer et al., 2008).

2.2.1 Peptidoglycan synthesis

The bacterial peptidoglycan is assembled form units of NAG, initially linked to uridine diphosphate (UDP). UDP-NAM units are manufactured from UDP-NAG by the addition of a lactic acid moiety derived from phosphoenolpyruvate. The NAM then receives, one by one, three amino acids usually L-alanine, D-glutamic acid and either L-lysine (in gram positive) or meso-diaminopimelic acid (in gram negative). Meanwhile, two D-alanine residues, produced from L-alanine by an enzyme called alanine racemase, are joined together by another enzyme, D-alanine synthetase. The linkage unit, D-ala-D-ala, is added to the tripeptide side chain of NAM and the NAM-pentapeptide thus formed is passed to a lipid carrier in the cell membrane. Here a UDP-NAG unit transfers its NAG to the NAM-pentapeptide and any amino acids needed for interpeptide bridges are added to the L-lysine of the pentapeptide side-chain. The lipid carrier transports the whole building block across the cell membrane and the unit is added to the end of the growing peptidoglycan chain of the existing cell wall, where the final cross-linking reaction takes place. The cross-linking reaction is catalyzed by membrane bound transpeptidases (Blumberg and Strominger, 1974; Scheffers and Pinho, 2005). The process is illustrated in outline in Figure 2.3.

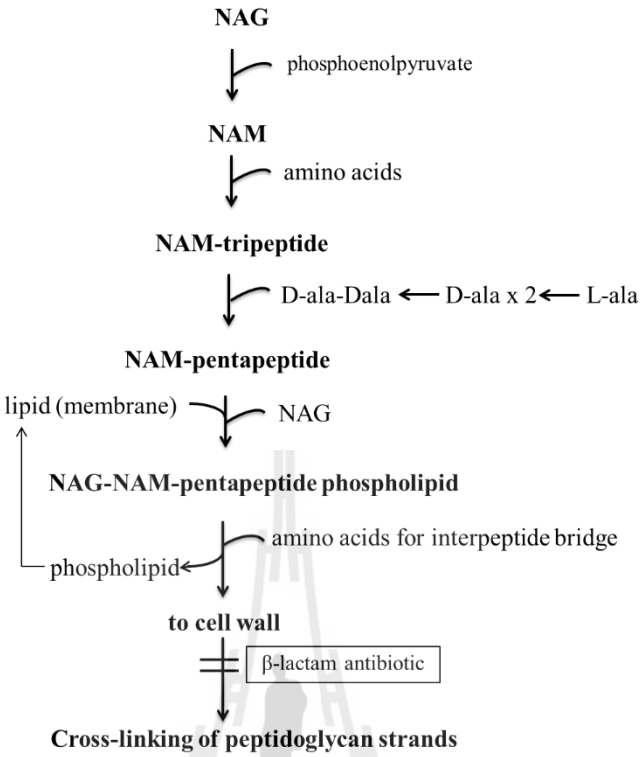

Figure 2.3 Peptidoglycan syntheses (Scheffers and Pinho, 2005).

2.3 β-lactam antibiotics

β-lactam antibiotics are a group of antibiotics characterized by possession of a β-lactam ring and they include penicillins, amoxicillin, cephalosporins, carbapenems, oxapenams, and cephamycins, which share the structural feature of a β-lactam ring. In the penicillin, β-lactam ring is fused to a five membered thiazolidine ring (Figure 2.4). The β-lactam ring is the weakness of this group of antibiotics because many bacteria possess enzymes (β-lactamase) that are capable of breaking open the ring and rendering the molecule antibacterial inactive (Figure 2.5) (Periti and Nicoletti, 1999).

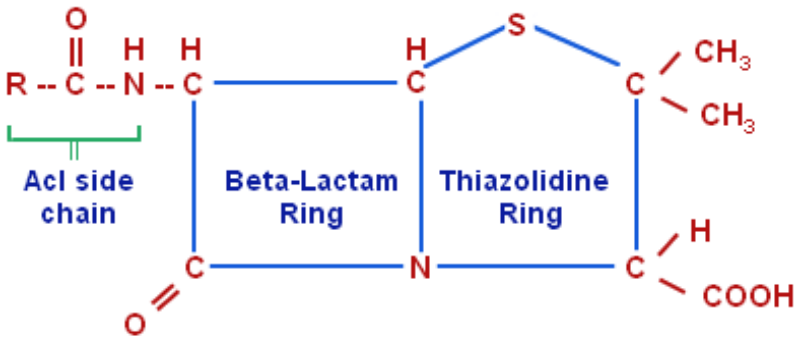

Figure 2.4 General structure of penicillin (Bassetti et al., 2008)**.**

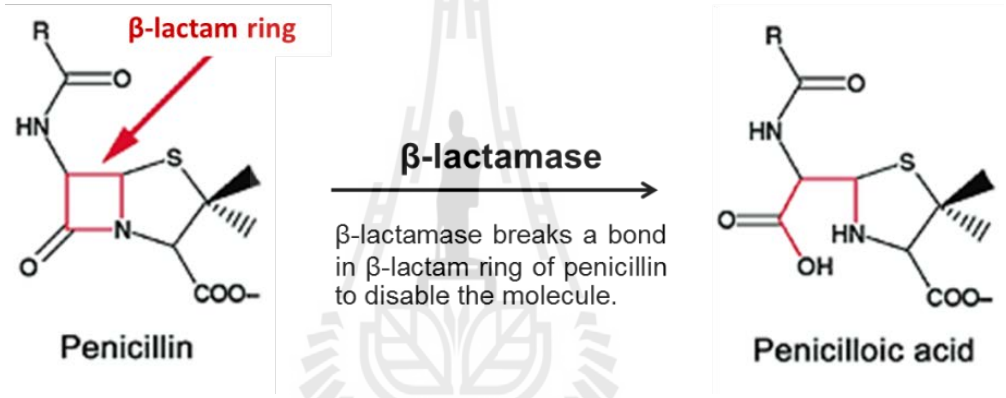

Figure 2.5 β-lactamase breaks the β-lactam ring (Samaha-Kfoury and Araj, 2003).

2.3.1 Mechanism of action of clinically used β-lactam antibiotics.

The major structural component of most bacterial cell walls is the peptidoglycan layer. The basic structure is consisting of NAG and NAM. The building of the chains and cross-links is catalyzed by specific enzymes that are members of a large family of serine proteases. These regulatory enzymes are called penicillin-binding protein (PBPs), which are the target of β-lactam antibiotics. When growing bacteria are exposed to these antibiotics, the antibiotic binds to specific PBPs in the bacterial cell wall and inhibits assembly of the peptidoglycan chains. This in turn activates autolysins that degrade the cell wall, resulting in bacterial cell death (Leski and Tomasz, 2005).

β-lactam antibiotics interfere with the final transpeptidation reaction that gives the cell wall its strength (Figure 2.6). However, several forms of transpeptidase, some of which also have transglycosylase activity, are needed to maintain the molecular architecture of the cell and these are differentially inhibited by various β-lactam agents. These target enzymes belong to a group of proteins to which penicillin and other β-lactam antibiotics bind (penicillin-binding proteins; PBPs) (Blumberg and Strominger, 1974).

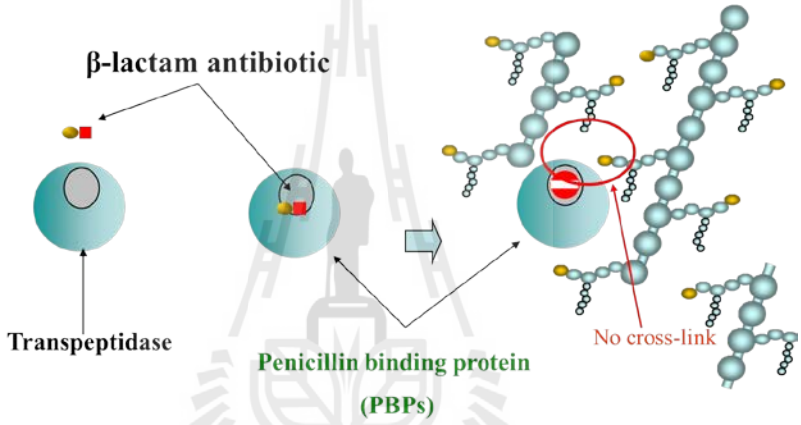

Figure 2.6 β-lactam antibiotic inhibit peptidoglycan synthesis (Kong et al., 2010).

2.4 β-lactam antibiotic resistance bacteria

Resistance genes can reside on the bacterial chromosome or on small rings of DNA called plasmids. Some of the genes are inherited and emerge through random mutations in bacterial DNA and some are imported from other bacteria. Resistance can spread either vertically by dissemination of resistant clones or horizontally by inter- and intra- species specific gene transfer such as transduction, conjugation and transformation. Bacteria can become resistant to β-lactam antibiotics by three general mechanisms (Figure 2.7) (Livermore, 1995).

2.4.1 Decreased penetration to the target site

The outer membrane of gram negative bacilli provides an efficient barrier to the penetration of β-lactam antibiotics to their target PBPs in the bacterial plasma membrane. β-lactam usually must pass through the hydrophilic porin protein channels in the outer membrane of gram negative bacilli to reach the periplasmic space and the plasma membrane. In some bacteria, resistance can result from changes to the size or function of the porins, so that passage of the antibiotic is prevented. In a few instances, non-specific changes in cell permeability to β-lactam antibiotics are encoded by genes carried on plasmids. The permeability barrier of the outer membrane is a major factor in the resistance of *Pseudomonas aeruginosa* to many β-lactam antibiotics (Tenover, 2006).

2.4.2 Alteration of the target site

The target sites for the β-lactams are the PBPs in the cytoplasmic membrane. Alterations in PBPs may influence their binding affinity for β-lactam antibiotics and therefore the sensitivity of the altered bacterial cell to inhibition by these antibiotics. Such a mechanism is responsible for penicillin resistance in *Pneumococci* spp. and methicillin (oxacillin) resistance in *Staphylococcus* spp. and for bacteria with increasing intrinsic resistance to β-lactams, such as *Enterococci* spp. and *Haemophilus influenza* (Mulligan et al., 1993; Tenover, 2006; Tomasz, 1997).

2.4.3 Inactivation by a bacterial enzyme

Some bacteria appear to contain enzymes capable of hydrolyzing β-lactam ring to form an inactive product. Indeed, it has been suggested that the normal function and evolutionary origin of β-lactamase is to break a β-lactam structure that is a transitory intermediate in cell wall synthesis. These inherent enzymes are encoded

by the chromosome, and are normally bound closely to the cell membrane. *Enterobacter* spp., *Acinetobacter* spp., *Citrobacter* spp. and *P. aeruginosa* and *Staphylococcus* spp. production of these chromosomal enzymes has been associated with treatment failures (Drawz and Bonomo, 2010; Tenover, 2006).

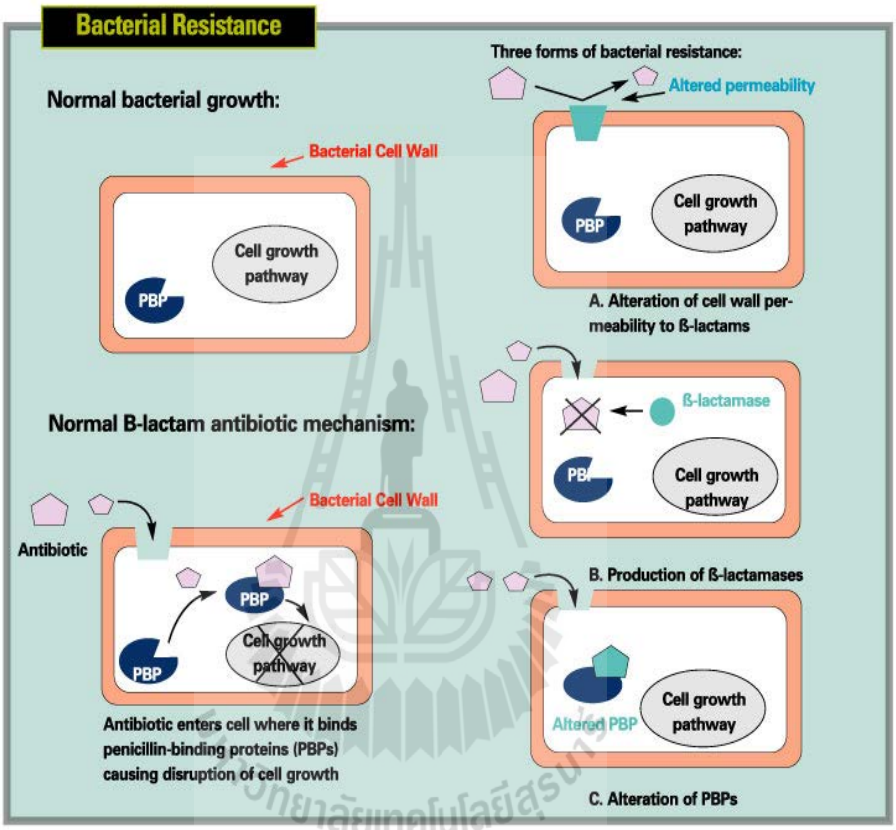

Figure 2.7 Mechanisms of bacterial resistance to antibiotic (Livermore, 1995).

2.5 General characteristics of *Staphylococcus epidermidis*

Staphylococci are spherical gram-positive bacteria, which are immobile and often stick together in grape-like clusters. They belong to the family *Micrococcacea*. With a few exceptions, all species are catalase-positive and they are all facultative anaerobe. The genus can be separated into two groups based on the ability to produce coagulase, an enzyme that causes clotting of blood plasma, including the coagulase-

positive *Staphylococci* and the coagulase-negative *Staphylococci* (Casey et al., 2007; Vuong and Otto, 2002).

2.5.1 *S. epidermidis* clinical significance

Ubiquitous colonization of *S. epidermidis* on the human skin and mucus membranes gives them the opportunity to cause infections under special circumstances. However, in general *S. epidermidis* are low virulent bacteria with few virulence factors (Otto, 2004; 2009). *S. epidermidis* has emerged as an important opportunistic human pathogen, reflecting the increased use of indwelling medical devices and an increasing number of patients with impaired immune systems and is now considered one the most frequent causes of nosocomial infections (Vuong and Otto, 2002).

2.5.2 Virulence factors

S. epidermidis does not produce many toxins as *S. aureus*. There are a very small number of them and their role as specific virulence factors has yet to be determined in detail. Generally, the success of *S. epidermidis* as a pathogen has to be attributed to its ability to adhere to surfaces and to remain there, under the cover of a protecting extracellular material, in relative silence (Vuong and Otto, 2002).

2.5.2.1 Biofilm

Biofilm formation is the most important virulence factor of *S. epidermidis*. The adaptation to environmental factors and the metabolic shift contributes to *S. epidermidis* success in colonization of host tissue and medical devices, protects the bacteria against the host immune system and attempts of antibiotic treatments. It is now generally accepted that *S. epidermidis* infections are dependent on the species ability to adhere to artificial surfaces and to assemble

biofilm consortia (Otto, 2008). Biofilm formation is commonly described as two different stages with;

(1) The primary attachment of the bacteria to the material.

(2) The formation of multi-layered cell clusters with cell-cell adherence depending on the production of a “slimy” extracellular substance.

A final detachment phase after steady-state has been acquired then follows. The detachment phase involves the detachment of single cells or cell cluster by various mechanisms and is believed to be crucial for the dissemination of the bacteria (Donlan and Costerton, 2002; Irie and Parsek, 2008; Vuong and Otto, 2002).

2.5.2.2 Toxins

Staphylococcal toxins are the phenol soluble modulins (PSM). PSM is small peptide toxins with a common amphipathic alpha helical region, which is thought to enable the disruption of the cell membrane leading to cell death. The expression of the gene encoding the PSMs is under the control of the accessory gene regulator (agr) system. At the high cell density, the bacteria secrete of a variety of toxins such as α-toxin, γ-toxin and toxic shock syndrome toxin and produce enzyme such as proteases, lipases, hyaluronidase and nuclease. These toxins are involved in tissue destruction (Chamberlain and Brueggemann, 1997; Rosenstein and Gotz, 2000; Vuong and Otto, 2002).

2.5.2.3 Protective exopolymers

Pathogen survival in the human body requires evasion of host defenses. The subset of host defense mechanisms such as antimicrobial peptides (AMPs) is present in the human skin. Multiple species of coagulase-negative *Staphylococci*, including *S. epidermidis* can produce exopolymer name Poly-γ-DL-

glutamic acid (PGA) that protect from important mechanisms of host defense and facilitate colonization of human skin. This polymer is synthesized by the product of *cap* gene and is important for *S. epidermidis*, which resistance to neutrophil phagocytosis and AMPs (Otto, 2009).

2.5.3 *S. epidermidis* antibiotics resistant

Staphylococci causing hospital-acquired infections are becoming increasingly resistant to multiple antimicrobial agents. This multiresistant phenotype invariably includes penicillin resistance mediated by β-lactamase, intrinsic resistance to β-lactam antibiotics (Allen et al., 1981; Archer and Climo, 1994).

An increasing prevalence of antibiotic resistance in *S. epidermidis* is partly due to the increasing use of broad-spectrum antibiotics, which encourage selection of multiresistant strains. Hospital-acquired *S. epidermidis* often display resistance against many antimicrobials in use today, such as methicillin and aminoglycosides (Biswas et al., 2013; Klingenberg et al., 2004; Raad et al., 1998). Methicillin-resistant *S. epidermidis* is cross-resistant to all β-lactam antibiotics. This resistance was plasmid-mediated. The methicillin-resistance in *Staphylococci* is mediated by the *mecA*-gene encoding a PBP2a with reduced affinity for all β-lactam antibiotics. The *mecA* gene is integrated in the *SCCmec* element (Casey et al., 2007; Fuda et al., 2004; Katayama et al., 2000; Tsubakishita et al., 2010). Quinolones were initially shown to have activity against methicillin-resistant *S. epidermidis*. However, similar to methicillin-resistant *S. aureus* and *S. epidermidis* strains emerged that was resistant to the quinolones (Linde et al., 2001; Yamada et al., 2008). Glycopeptide resistant *Staphylococci* have rarely been reported and vancomycin is the drug of choice for methicillin-resistant *S. epidermidis* (Biavasco et al., 2000). But,

vancomycin-resistant *S. epidermidis* is also found and mechanism of vancomycin-resistant *S. epidermidis* is still unknown. Sanyal and colleague reported that low level resistance to glycopeptide antibiotics in *S. epidermidis* is constitutive rather than inducible (Sanyal and Greenwood, 1993). Resistance was attributed to the increased capacity of the cell wall of resistant strains to bind and sequester glycopeptides possibly at sites other than the D-alanyl-D-alanine target site to which they normally bind (Wu et al., 2008).

2.6 General characteristics of *Streptococcus pyogenes*

S. pyogenes is a gram positive spherical bacteria that grows in long chains. They belong to family Streptococcaceae. *S. pyogenes* is classified as a group A streptococcus (GAS) because of its display antigen on its cell wall. *S. pyogenes* regularly produces zones of β-hemolysis when cultured on blood agar plates and are therefore also called Group A (β-hemolysis). They can cause cutaneous and skin infections and are able to destroy red blood cells and other cells using toxins known as streptolycins cause severe infections (Wong and Yuen, 2012).

2.6.1 *S. pyogenes* clinically significant

S. pyogenes causes a variety of infections and symptoms associated with GAS diseases. Infections range from minor to severe and invasive. Infections typically originate within the throat or the skin such as pharyngitis and impetigo. Symptoms of these infections vary depending on which area is affected such as sore throat, pus-filled blister. Symptoms of more serious infection may include fever, rapidly increasing swelling, severe pain, redness at a wound site, dizziness and influenza like symptoms. When the bacterium becomes invasive, conditions like necrotizing

fasciitis, which destroys fat, skin and muscle tissue and streptococcal toxic shock syndrome (STSS) that causes a decrease in blood pressure and organ failure (Sutcliffe et al., 1996; Wong and Yuen, 2012).

2.6.2 Virulence factor

S. pyogenes possession a wide variety of virulence factor can cause severe and invasive infections and large number of diseases. Although some factors are expressed by all clinical isolates, others are variably present among *S. pyogenes* strains. This variation is due to the transfer of virulence genes among strains by bacteriophages.

2.6.2.1 Capsule

Some strains of *S. pyogenes* have a capsules compose of hyaluronic acid resulting in large mucoid colonies on blood agar. The strains producing hyaluronic acid capsule were resistant to phagocytosis and were virulent in mice, but the hyaluronic acid deficient strains were killed by phagocytes (Wessels et al., 1991).

2.6.2.2 M protein

M protein is an important surface-located virulence factor of *S. pyogenes*. The expression of M protein is primarily controlled by *Mga* transcriptional activator protein. More than 80 different M-protein types of GAS are currently described. The M-protein was encoded by *emm* gene. These proteins can bind various plasma proteins of the host, including fibrinogen, plasminogen, albumin and immunoglobulin. As well as masking the bacterial surface with host proteins, these affinities are responsible for the ability of M-proteins to against phagocytosis by leukocytes (Fischetti, 1991; Fischetti et al., 1995).

2.6.2.3 Hyaluronidase

GAS are capable of producing two types of hyaluronidase, a bacteriophage associated enzyme and an extracellular hyaluronidase that is secreted from the cell**.** This extracellular enzyme hydrolyzes hyaluronic acid, the ground substance of host connective tissue and may facilitate the spread of infection along fascial planes. Anti-hyaluronidase titers rise following *S. pyogenes* infections, especially those infections involving the skin (Wessels et al., 1991).

2.6.2.4 Exotoxin

Most strains of *S. pyogenes* can produce an exotoxin that is called “pyrogenic” toxin can induce lymphocyte, induce fever and suppress antibody synthesis. Some of these exotoxins can cause the rash of scarlet fever and systemic toxic shock syndrome. The genes for exotoxins are transmitted by a bacteriophage (Barsumian et al., 1978; Nida and Ferretti, 1982).

2.6.3 *S. pyogenes* antibiotic resistant

GAS exhibits a high degree of clinically relevant phenotypic diversity. Strains vary widely in terms of antibiotic resistance. Recommended therapies for GAS infections include penicillin, erythromycin, and clindamycin. Interestingly, in vitro testing of these strains show *S. pyogenes* to be very sensitive to penicillin. However, some of the more aggressive infections with GAS have not responded well to penicillin therapy. Moreover, the prevalence of antibiotic resistant GAS has emerged rapidly. Several reports erythromycin, tetracycline and quinolone resistant among GAS have been published. This strain encodes a ribosomal modification gene (*erm*) that confers decreased susceptibility to macrolides. And the second resistance mechanism against macrolides is associated with the M phenotype, which encodes an

efflux system (*mef*) for macrolide antimicrobials (Lloyd et al., 2007; Yazgi et al., 2002). Previous research found that *S. pyogenes* 1.9% was intermediately resistant to ampicillin (Gur et al., 2002). Khan and co-worker found that *S. pyogenes* was resistive to cloxacillin, oxacillin, and cefoparazone (Idrees and Saeed, 2013). Moreover, only two *S. pyogenes* were β-lactamase producers and *bla*-TEM was detected and amplified on genomic DNA of 28 β-lactam resistant from 42 *S. pyogenes* isolated from Mansoura university hospitals (Hassan et al., 2010).

2.7 Bioflavonoids classification

Flavonoids in the broad sense of the term are virtually universal plant pigments. Almost always water-soluble, they are responsible for the color of flowers, fruits and sometimes leave. Examples are yellow flavonoids (chalcones, aurones and yellow flavonols) and red, blue or purple anthocyanins. When they are not directly visible, they contribute to the color by acting as co-pigments; for example, colorless flavone and flavonol copigments protect anthocyanins. In some cases, the molecule absorbs near-UV radiation; this “color” is only perceived by insects, which are thus efficiently attracted and guided to the nectar and therefore compelled to ensure pollen transport, a necessary condition for the survival of the plant species. Flavonoids are also ubiquitous in the leaf cuticle and epidermal cells where they ensure tissue protection against the damaging effects of UV radiation. All flavonoids have the same basic structural element, namely the 3-phenylchromane skeleton (Figure 2.8). Flavonoids can be divided into 6 major classes base on the basis of their molecular structures, including flavonol, flavone, flavanone, flavanol, isoflavone and anthocyanidine (Figure 2.9) (Cushnie and Lamb, 2005).

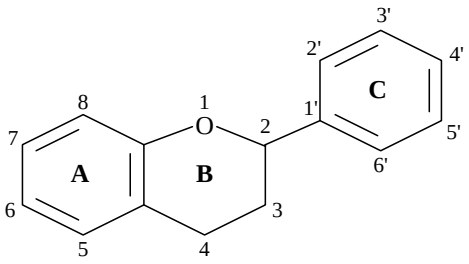

Figure 2.8 The 3-phenylchromane skeleton (Cushnie et al., 2003).

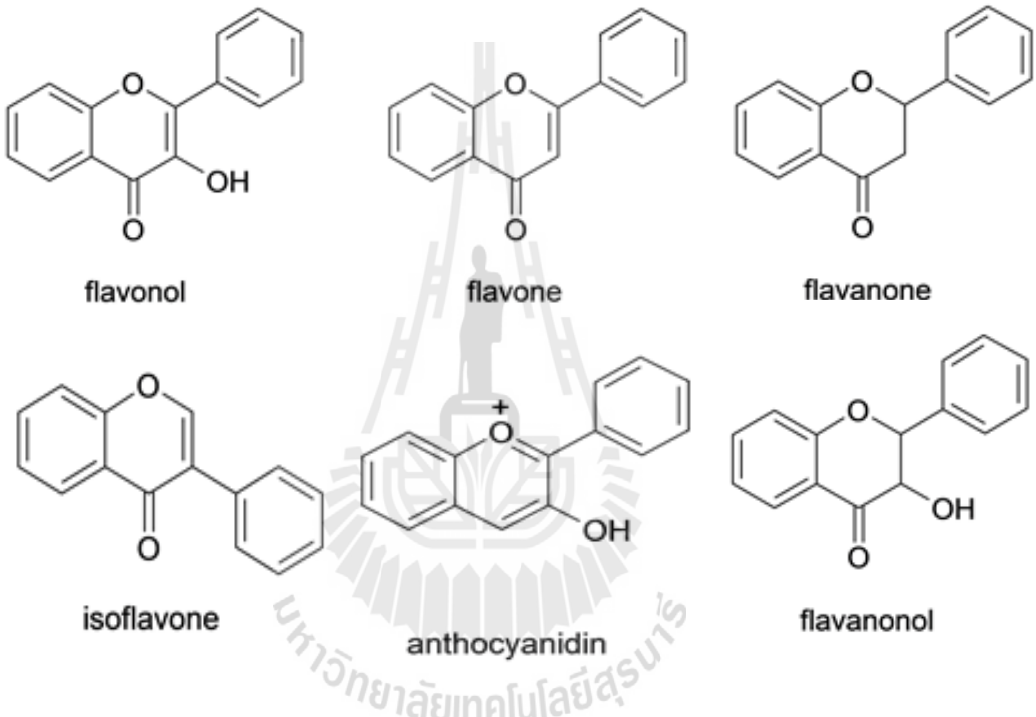

Figure 2.9 Major classes of flavonoids (Cushnie and Lamb, 2005).

2.7.1 Antimicrobial activity of flavonoids

The use of antibiotics is often accompanied by side effects and often the development of resistant strains. At present the search for compounds active against antibiotic-resistant strains of bacteria are continuing among the flavonoids, compounds which are non-toxic or have low toxicity (Narayana et al., 2001).

One of the undisputed functions of flavonoids and related polyphenols is their roles in protecting plants against microbial invasion. This not only involves their presence in plants as constitutive agents, but also their accumulation as phytoalexins

in response to microbial attack (Grayer et al., 1994).The crude extract and isolated flavonoids from *Ficus Chlamydorcapa* and *Ficus Cordata* could inhibit gram positive, gram negative bacteria and fungi (Kuete et al., 2008). Six flavonoids isolated from *Galium fissurense*, *Viscum album* ssp. and *Cirsium hypoleucum* showed *in vitro* antibacterial activity on *Klebsiella pneumonia*. The activity of these flavonoids against extended-spectrum β-lactamase (ESBL)-producing *K. pneumoniae* and natural flavonoids showed a significant synergy effect in combination with antibiotics against ESBL-producing *K. pneumonia*e (Lin et al., 2005; Qzcelik et al., 2008).

Several mechanisms of action have been proposed to explain the actions of flavonoids. It has been suggested this act on the integrity of the cell wall and on septum formation prior to cell division, inhibition of the activity of certain penicillinase enzyme and cause cytoplasmic membrane damage that results in potassium loss (Eumkeb et al., 2010). Some researcher explained that flavonoids could reduce D-alanylation of cell wall teichoic acid or intercalating into the cytoplasmic membrane and reducing structural changes (Cushnie and Lamb, 2011).

2.7.1.1 Apigenin

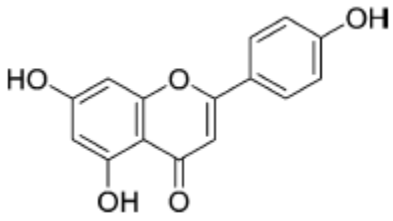

Figure 2.10 Chemical structure of apigenin (Wu et al., 2008).

Apigenin (4′,5,7-trihydroxyflavone) is a common dietary flavonoid abundantly present in fruits and vegetables. Common structure was showed in Figure 2.10. Apigenin effectively disrupts the formation and accumulation of

Streptococcus mutants biofilm and its active constituents act against methicillin-resistant *Staphylococcus aureus* (MRSA) (Sato et al., 2000). Apigenin extracted from *Citrus* spp. significantly inhibited the growth rate and demonstrated poor antagonistic activity against of *Vibrio harveyi* (Lewis, 2001; Vikram et al., 2010). Moreover, apigenin isolated from *Plagiomnium affine* inhibits the growth of *Salmonella typhi, Proteus mirabilis* and *P. aeruginosa*. However, the glycosilation of apigenin causes a reduction in antibacterial power due to the reduction in lipophilia and the consequent diminished ability to penetrate the bacterial membrane (Basile et al., 1999). Apigenin prevent human immunodeficiency virus type 1 (HIV-1) activation via a novel mechanism that probably involves inhibition of viral transcription (Critchfield et al., 1996).

2.7.1.2 Baicalein

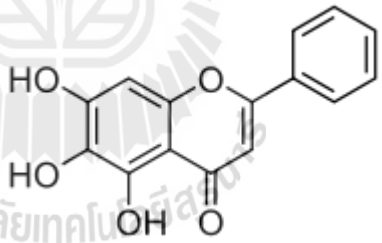

Figure 2.11 Chemical structure of baicalein (Psotova et al., 2004).

Baicalein (5,6,7-trihydroxyflavone) is a typical flavonoid compound and is the main component of a traditional Chinese herbal medicine *Scutellaria baicalensis*. Common structure was showed in Figure 2.11. Baicalein has multiple biological activities including antiallergic, anticarcinogenic and anti-HIV properties (Zhu et al., 2004). Baicalein did not inhibit the growth of gram negative and showed a weak effect against the gram positive, spore-forming *Bacillus subtilis* and the gram positive coccus *S. aureus* (Ali et al., 1998). *S. aureus* was treated with the

combination of baicalein and penicillin, which act as a bacteriocide, while the combination of baicalein and notopterygium ethanol extracts (NEE) was bacteriostatic. These results showed that baicalein can synergist with both penicillin and NEE but has different modes of action (Meng et al., 2006). Moreover baicalein and ampicillin showed synergistic activity against *P. aeruginosa*. Meng and coworker suggested that baicalein can interfere with quorum sensing system of *P. aeruginosa* and might be developed as an antibacterial agent with a novel mechanism (Meng et al., 2006).

2.7.1.3 Luteolin

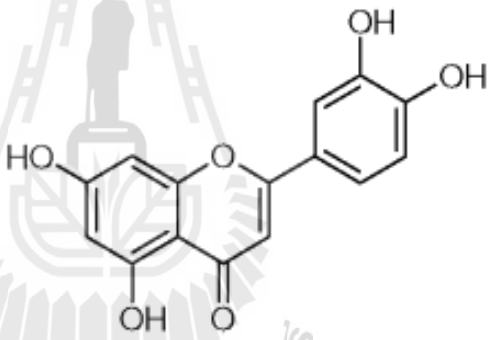

Figure 2.12 Chemical structure of luteolin (Psotova et al., 2004).

Luteolin (3′,4′,5,7-tetrahydroxyflavone) is naturally occurring flavonoids found in edible plants such as broccoli, celery, green chili and carrots at high concentration. Common structure was showed in Figure 2.12. Luteolin has been shown previously to have antibacterial activity against a number of bacteria. Luteolin isolated from the seeds of *Senna petersiana* showed antibacterial activity against gram positive bacteria. It partially inhibited the growth of *Bacillus cereus* and *S. aureus* (Chiruvella et al., 2007). Synergism has been demonstrated between various combinations of flavones and flavonols. For example, kaempferol and luteolin showed

synergy against herpes simplex virus (HSV) (Amoros et al., 1992). Luteolin exhibited antibacterial activity against some gram positive coccus including *S. aureus* and *S. epidermidis, but* luteolin is characterized as selectively inhibiting the growth of drug resistant *S. aureus, including* MRSA (Sato et al., 2000).

2.7.1.4 Quercetin

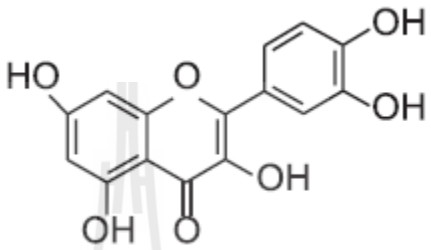

Figure 2.13 Chemical structure of quercetin (Materska, 2008).

Quercetin (3,3',4',5,7-Pentahydroxyflavone) is one of the most prominent dietary antioxidants. Common structure was showed in Figure 2.13. It is ubiquitously present in foods, including vegetables, fruit, tea, onions and wine as well as countless food supplements and is claimed to exert various beneficial health effects (Boots et al., 2008). Quercetin and oxacillin in combination also displayed the lowest fraction inhibitory concentration (FIC) index, which suggested flavonoids-base combination regimens may be a useful candidate for treatment options of vancomycin intermediate *S. aureus* infection (Basri et al., 2008). Quercetin potentiates the effects of 5-ethyl-2'-dioxyuridine and acyclovir against HSV and pseudorabies infection (Mucsi et al., 1992).

2.7.1.5 Kaempferol

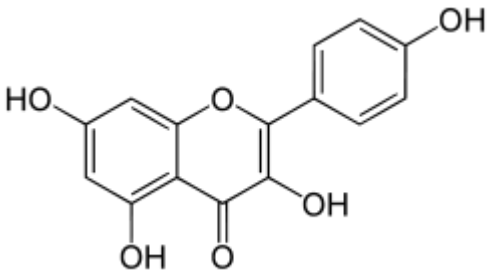

Figure 2.14 Chemical structure of kaempferol (Psotova et al., 2004).

Kaempferol (3,4′,5,7-tetrahydroxyflavone) is a strong antioxidant and help to prevent oxidative damage of the cells. Common structure was showed in Figure 2.14. *In vitro, both* tryptanthrin and kaempferol significantly decreased the numbers of *Helicobecter pylori* colonies in a dose dependent manner. An additive effect on colony formation was observed with the combined use. For *in vivo* experiment, oral administration of tryptathrin and kaempferol significantly decreased the numbers of colonies in the gerbils' stomachs. Thus kaempferol would be safe *in vivo* (Kataoka et al., 2001). The combination of clindamycin or erythromycin with kaempferol showed synergic inhibition of *Propionibacterium acnes* (Lim et al., 2007).

2.8 Infrared (IR) spectroscopy

Infrared (IR) spectroscopy is the measurement of the wavelength and intensity of the absorption of infrared light by a sample. Infrared light is energetic enough to excite molecular vibrations to higher energy levels. This technique is based on a change in the dipole moment as a bond vibrates. Infrared spectroscopy has been widely used for a routine analytical technique. By combining spectroscopy with microscopy, the applicability of IR microspectroscopy can be achieved to obtain

molecular information at high spatial resolution (Dumas and Miller, 2003; Parker, 1983). The vibration levels and hence, infrared spectra are generated by the characteristic twisting, bending, rotating and vibrational motions of atoms in a molecule (Figure 2.15). All of the motions can be described in terms of two types of molecular vibrations. One type of vibration, a stretch, produces a change of bond length. A stretch is a rhythmic movement along the line between the atoms so that the interatomic distance is either increasing or decreasing. The second type of vibration, a bend, results in a change in bond angle. These are also called scissoring, rocking or wigwag motions. Each of these two main types of vibration can have variations. A stretch can be symmetric or asymmetric. Bending can occur in the plane of the molecule or out of the plane; it can be scissored, like blades of a pair of scissors, or rocking, where two atoms move in the same directions (Garip, 2005).

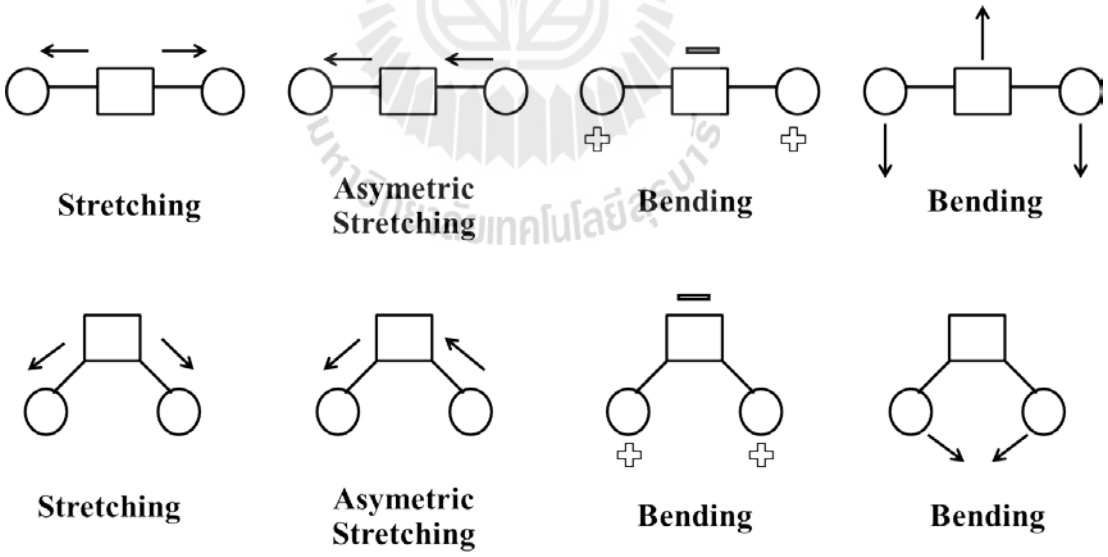

Figure 2.15 Types of normal vibrations in a linear and non-linear triatomic molecule (Garip, 2005).

The IR bands of intact microorganisms still have to be assigned unambiguously. Preliminary conclusions can already show in Table 2.1.

Table 2.1 Tentative assignments of some bands frequently found in biological FT-IR spectra (Yu and Irudayaraj, 2005).

Frequency (cm^{-1})	Assignment
~3200	N–H str (amide A) of proteins
2959	C–H str (asym) of $–CH_3$
2934	C–H str (asym) of $>CH_2$
2921	C–H str (asym) of $>CH_2$ in fatty acids
2898	C–H str of →C–H methane
2872	C–H str (sym) of $–CH_3$
2852	C–H str (sym) of $>CH_2$ in fatty acids
1741–1715	>C=O str of esters, >C=O str of carbonic acids, nucleic acids
~1700-1600	Amide I band components resulting from antiparallel pleated sheets and β-turns of proteins
~1655	Amide I of α-helical structures
~1637	Amide I of β-pleated sheet structures
1548	Amide II
1515	Tyrosine band
1468	C–H def of $>CH_2$
~1400	C=O str (sym) of COO^-
1310–1240	Amide III band components of proteins
1250–1220	P=O str (asym) of $>PO_2^-$ phospodiesters
1200–900	C–O–C, C–O dominated by ring vibrations of carbohydrates C–O–P, P–O–P

def = deformation, str = stretching, sym = symmetric, asym = asymmetric

FT-IR microspectroscopy technique can be used as an analytical tool in various fields (clinical, environmental, food microbiology) for the very rapid classification, differentiation of diverse microbial species and strains. Spectroscopic techniques are characterized by a minimum of sample handling: no extractions, amplifications, labeling, or staining steps of any kind are required. FT-IR spectra reflect the overall molecular composition of a sample. Since different organisms differ in overall molecular composition, their FT-IR spectra will also be different. The spectra can serve as spectroscopic fingerprints that enable highly accurate identification of microorganisms (Astrid et al., 2006; Becker et al., 2006; Fischer et al., 2006; Mariey et al., 2001; Stuart and Ando, 1997).

2.8.1 Infrared spectroscopy of microbial cells (band assignments)

Absorption bands observed in the mid-IR region of the electromagnetic spectrum between 4000 and 400 cm^{-1} can usually be associated with the fundamental vibrational modes of a particular functional group or bonds in a molecule. The mid-IR spectra of many molecules of biological importance are now available from spectral reference databases and from the literature, which in turn can now be used to identify unknown pure compounds routinely (Ojeda and Dittrich, 2012). It is a matter of fact that different species and strains of bacteria differ qualitatively and quantitatively in the chemical composition and chemical structures present. Thus, it can be expected that each species or strain will produce a unique IR spectrum. (Al-Qadiri et al., 2008; Huleihel et al., 2009; Ojeda and Dittrich, 2012).

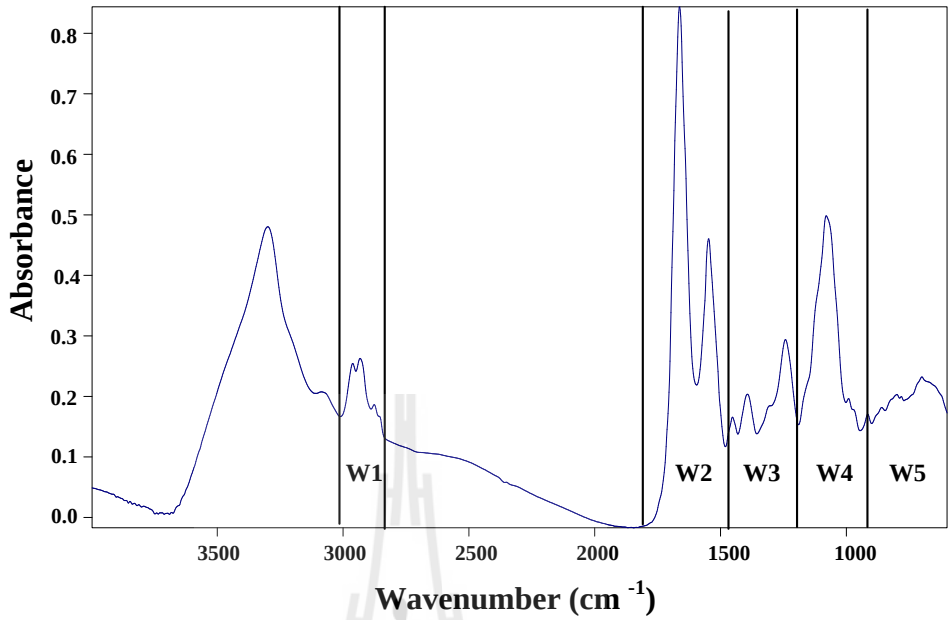

Figure 2.16 FT-IR spectrum from *S. pyogenes* (Goodwin, 2006).

Figure 2.16 showed the type of spectrum feature of *S. pyogenes*. The regions between 3000 and 2800 cm^{-1} (W1) induced by functional groups of membrane fatty acids and by some amino acid side-chain vibrations. The window between 1800 and 1500 cm^{-1} (W2) influenced by amide I and amide II belongs to proteins and peptides. The window between 1500 and 1200 cm^{-1} affected by mixed regions of protein, fatty acid and phosphate carrying compounds. The regions between 2900 and 900 cm^{-1} (W4) due to the stretching vibration of PO_2 group found in nucleic acids and stretching of CO in carbohydrate and polysaccharide in the cell wall. The regions between 900 and 700 cm^{-1} (W5), which is called fingerprint region, hold very specific weak spectral patterns from aromatic ring vibration of aromatic amino acids (tyrosine, tryptophan, phenylalanine) (Goodwin, 2006).

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2.8.2 Macromolecules of the prokaryotic cell

Aside from water and macromolecules, small molecules and inorganic ions are the constituents of cells. Essentially the large molecules or macromolecules are polymers made from small monomeric molecules. There are 4 major types of macromolecules which are present in microbial cells. The macromolecules are listed in the table below with the substituent groups, where these macromolecules are found and the approximate percentage composition range (Goodwin, 2006; Vendeville et al., 2011).

Table 2.2 The macromolecules of the prokaryotic cell, location and percentage composition (Goodwin, 2006).

Macromolecule	Primary subunits	Location in cell	% Composition range
Proteins	Amino acids	Cell wall, Cytoplasmic membrane, ribosome, cytoplasm	40-60
Polysaccharides	Sugar or other carbohydrate molecules	Cell wall, capsule	10-20
Phospholipids	Fatty acids	Membrane	10-15
Nucleic acids	Nucleotides	Nucleus, plasmid, ribosome, cytoplasm	5-25

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Bacterial strains

Amoxicillin-resistant *S. epidermidis* DMST 15505 (ArSE), *S. pyogenes* DMST 30653 (SP) and drugs-sensitive *S. aureus* ATCC 29213 were obtained from the Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand.

3.1.2 Preparation and maintenance of stock cultures

The clinical isolates of bacteria were inoculated on Mueller-Hinton agar (MHA) or Nutrient agar (NA) slant for ArSE and Brain Hearth Infusion agar (BHIA) slant for SP and were incubated overnight at 37 °C. These cultures were stored in a refrigerator at 4 °C. Fresh agar slant cultures were prepared every 3-4 weeks. Before use, the culture was pre-incubated in Mueller-Hinton broth (MHB) for ArSE and Brain Hearth Infusion broth (BHIB) for SP at 37 °C for 18-24 h (Matthew et al., 2013; Eumkeb, 1999).

3.1.3 β-lactam antibiotics

Amoxicillin, penicillin and ceftazidime were obtained from Sigma Aldrich (UK). Antibiotic test solutions were prepared by dissolving amoxicillin, penicillin and ceftazidime in sterile water.

3.1.4 Flavonoids

Luteolin, baicalein and quercetin were obtained from the Indofine Chemical Company, USA. Solutions of flavonoids were dissolved with 5% dimethylsulfoxide (DMSO).

3.2 Methods

3.2.1 Preparation of test solution and inoculums

Antibiotic drug test solutions were prepared by dissolving in sterile water. Solutions of flavonoids were prepared with DMSO with adjustment to give the required test concentrations.

The culture was incubated in 100 ml cation-adjusted Mueller-Hinton broth (CAMHB) for ArSE and BHIB for SP with incubation time for 18-24 h at 37 °C. The cell culture was centrifuged at 4,000 r.p.m. for 10 min to harvest cell pellets. The cell pellets were washed with 0.9% NaCl to remove media, re-centrifuged and re-suspended in 0.9% NaCl. The cell concentrations were adjusted with 0.9% NaCl to give 5 x 10^6 cfu/ml using a predetermine calibration curve of absorbance at 500 nm against viable count (Matthew et al., 2013; Liu et al., 2000). The MICs of flavonoids or antibiotics alone and flavonoids in combination with antibiotic will be examined.

3.2.2 Bacterial suspension standard curves

Standard curve method was used to select bacterial suspension with a known viable count. Loopful from culture slant was inoculated in 100 ml of CAMHB for ArSE and BHIB for SP. The cultures were incubated at 37 °C for 18-24 h. The bacterial cells were pelleted by centrifugation at 4,000 r.p.m. for 10 min and were

washed twice by re-suspending and centrifuging at 4,000 r.p.m. for 5 min in 10 ml of 0.9% NaCl. The cells were re-suspended in 10 ml of sterile 0.9% NaCl. The cell suspensions were diluted so that 5-6 spectrophotometer readings could be 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 agar plate counting method (Matthew et al., 2013; Eumkeb et al., 2010; Liu et al., 2000). Over dried agar plates were used for determining colony forming unit (cfu).

3.2.3 MICs determination

The Minimum Inhibitory Concentrations (MICs) are defined as the lowest concentration of antibacterial agents that able to inhibit growth of bacteria. MICs can be determined by agar or broth dilution methods following a guideline of reference of CLSI. The MICs are used for determining the susceptibility of organisms to antimicrobials and are used in diagnostic laboratories to confirm unusual resistance. MICs were measured by broth macrodilution method. The sterile wire loop test organism from a slope culture was inoculated into CAMHB for ArSE and BHIB for SP with incubation time for 18 h at 37 °C. Then, the preparation of a bacterial suspension, the density of the bacterial suspension in 0.9% NaCl were adjusted to approximately 5 x 10^6 cfu/ml by using the absorption of bacterial suspension viable count standard curve. The inoculum of 0.25 ml of standard suspension (18-24 h) of each strain of the test bacteria was added to triplicate tubes containing 2.25 ml CAMHB for ArSE and BHIB for SP plus an antibiotic drugs or flavonoids, to give approximately 5 x 10^5 cfu/ml. Tubes of the broth without antibacterial were used as the control for each of the test bacteria. Incubation is at 37 ºC for 20 h. The MICs

were defined as the lowest concentration of antibiotic at which there was no visible growth in the triplicate tubes (Bassetti et al., 2008; Matthew et al., 2013).

3.2.4 Checkerboard determination

Broth macrodilution checkerboard was used to evaluate combinations of antimicrobial agent against resistance bacteria. This method is based on CLSI broth dilution susceptibility methods for evaluating the inhibitory or bactericidal activity of specific concentrations in combination at a fixed time. In vitro interactions are calculated and interpreted as synergism, no interaction or antagonism.

Checkerboard method was used to determine the bactericidal interaction of the flavonoids plus antibiotic against resistant bacteria. Checkerboards titrations are relatively simple to perform and allow the assessment synergy at 24 h. The 18 h cultures of each of the test bacteria were prepared on CAMHB for ArSE and BHIB for SP. The test bacterial suspensions were adjusted to give 5 x 10^6 cfu/ml using the absorption of bacterial suspension from the previously determined standard curve. The 0.25 ml of the bacterial suspension was added to a series of 2.25 ml CAMHB for ArSE and BHIB for SP, plus 10% serial dilutions of the antibacterial combinations, to give 5 x 10^5 cfu/ml. Tubes of the broth without antibacterial cell were used as the controls. The cultures were incubated for 20 h at 37 ºC. The tests were carried out in triplicate. MICs were determined for each antibacterial combination and the isobolograms were plotted (Bonapace et al., 2002; Matthew et al,. 2013; Fowler et al., 2011). The calculation of the Fraction Inhibitory Concentration (FIC) index for each antibacterial combination was undertaken as follows (Wagner and Ulrich-Merzenich, 2009):

$$I = \Sigma i(xi/Xi) = \begin{cases} < 1 \text{ synergism} \\ 1 \text{ no interaction} \\ > 1 \text{ antagonism} \end{cases}$$

I = interaction index

xi = dose of the individual component in the combination

Xi = dose of the individual component which generate the same effect as the combination

i = the i^{th} individual component in the combination

3.2.5 Killing curve determination

The killing curve technique measures the bactericidal or the inhibitory activity of the combination. Killing curve provided a dynamic picture of antimicrobial action and interaction over time based on serial colony counts.

Killing curve is a result of the interaction between flavonoids and antibacterial agent. The culture was prepared on CAMHB for ArSE and BHIB for SP with incubation time for 18-24 h at 37 °C. The 2 ml of culture was added to 250 ml conical flasks containing 98 ml CAMHB for ArSE and BHIB for SP in shaking water bath at 37 °C and shaking at 100 r.p.m. for 4 h to give log phase. Cultures of bacteria in 0.9% NaCl were adjusted to 5 x 10^6 cfu/ml using the absorption of bacterial suspension from the previously determine standard curve. The 1 ml of log phase cultures was added to 250 ml conical flask containing 99 ml CAMHB for ArSE and BHIB for SP plus antibiotic drugs or flavonoids alone or flavonoids in combination with antibiotic drugs at concentrations ½ or ¾ of their MICs against bacteria. The control flasks containing 99 ml CAMHB for ArSE and BHIB for SP without antibacterial were used as the controls. Bacterial suspensions were exposed to the antibiotic drugs either singly or in combination with flavonoids at an incubation

temperature of 37 °C. Viable count were determined after a contact time of 0, 0.5, 1, 2, 4, 6 and 24 h. Subsequent dilution plating on agar plates in triplicate and incubation at 37 °C for 20 h was allowed the counting of growing colonies (Bassetti et al., 2008; Matthew et al., 2013; Eumkeb, 1999).

3.2.6 Cytoplasmic membrane (CM) permeability

To detect the cytoplasmic membrane damage, the first method detects on only one time. Overnight bacterial cultures in CAMHB for ArSE were adjusted to OD_{600} of 2.0. Cells were harvested by centrifugation at 4,000 r.p.m. for 15 min, the pellet was washed twice and then re-suspended in phosphate buffer saline (PBS) (pH 7.4). Antibiotic drug alone or in combination with flavonoids were added to the cell suspension. Tubes of cell suspension without antibacterial agents were used as the control. The experiment was done in triplicates. All the samples were incubated at 37 °C for 60 min. after treatment, the cell suspension was centrifuged at 13,400 g for 15 min and OD_{260} value of the supernatant was taken as the extracellular UV-absorbing materials released by cells. All the measurements were done in triplicates in Varian Cary 1E UV/ VIS spectrophotometer (Zhou et al., 2008).

The second method, overnight bacterial cultures in BHIB for SP were harvested by centrifugation at 4,000 r.p.m. for 15 min, the pellet was washed twice and then re-suspended in PBS. The log phase of bacterial suspension of SP was adjusted to 5 x 10^5 cfu/ml in 0.9% NaCl plus ceftazidime alone or combined with flavonoids. Tubes of cell suspension without antibacterial cell were used as negative control and tubes with 4 µg/ml nisin (½ MIC) was used as positive control. The experiment was done in triplicates. All the samples were exposed to antibacterial agent at 37 °C and incubated for 0, 0.5, 1, 2, 3 and 4 h. After treatment, the cell

suspension was taken every contact time and filtered through 0.22 µm sterile nitrate cellulose membrane. OD_{260} value of the supernatant was taken as the extracellular UV-absorbing materials released by cells. All the measurements were done in triplicates in Varian Cary 1E UV/ VIS spectrophotometer (Devi et al., 2010).

3.2.7 Enzyme assay

The β-lactamase type IV of *Enterobacter cloacae* was obtained from Sigma (Poole, England). Enzymatic method determines the inhibition of β-lactamase by flavonoids or antibiotic drugs. The β-lactamase was used at a concentration sufficient to hydrolyze 500 µg/ml of antibiotic within 5 min. Flavonoids or antibiotic drugs were pre-incubated with β-lactamase enzyme at 37 °C for 5 min prior to substrate (benzyl penicillin) addition. Assays with pre-incubation were carried out by reacting enzyme with antibiotic drugs or flavonoids for a set time period and then adding the substrate to measure the residual substrate and enzyme activity. High performance liquid chromatography (HPLC) provides a further technique which was used to measure the stability of substrate to β-lactamase in the presence of an enzyme inhibitor. The analyses of the remaining substrate were determined by the reverse-phase HPLC using acetronitrile/ammonium acetate as a mobile phase. Reaction samples (100 µl) were injected at various times on to Waters Bio-Sil C18 HL 90-5s reverse phase column eluted at 1.5 ml/min. Time-course assays were carried out using methanol/acetic acid (100:1) as stopping reagent (Reading and Farmer, 1983).

3.2.8 Transmission electron microscopy (TEM)

Cellular damage of bacteria was examined using TEM. Bacterial cells treated with antibiotic or flavonoids alone and in combination were harvested after log phase of incubation and fixed in 2.5% glutaraldehyde in 0.1 M PBS, for 2 h. The cells

were washed three times with 0.05 M PBS (pH 7.2) and post fixed for 2 h with 1% osmium tetroxide in 0.1 M PBS (pH 7.2) at room temperature. After washed twice in PBS, the cells were dehydrated for 15 min through serial graded concentrations of ethanol (35, 70, 95 and 100%, respectively) 2 times for each, then infiltrated and embedded in Spurr's resin. Ultrathin sections were cut with a diamond knife using an ultra-microtome and then mounted on bare copper grids. Finally, specimens were counterstained with 2% (w/v) uranium for 15 min and then with 0.25% (w/v) lead citrate solution for 15 min and examined with Tecnai G2 electron microscope (FEI, USA) operated at 120 kV (Eumkeb et al., 2010; Kamonwannasit et al., 2013).

3.2.8.1 Measurement of bacterial cell areas and the average cell division boundary

All cells in each field of each sampling square that can be the most representative of bacterial cells in those groups were collected to measure cell width x cell length using milli-ruler. The average cell areas (mean ± S.E.M) were presented. Moreover, all of the cells that were dividing were chosen to compare cell division boundary. The average cell division boundaries (mean ± S.E.M) were illustrated. Then, these data were compared using one-way analysis of variance (one-way ANOVA) and Tukey HSD post-hoc test was calculated as statistically significant difference at $p<0.05$ or $p<0.01$.

3.2.9 FT-IR microspectroscopy

3.2.9.1 Bacterial cell preparation

Bacterial suspensions were exposed to the antibacterial either singly or in combination with flavonoids at an incubation temperature of 37 °C for 24 h. Bacterial cells were pelleted by centrifugation at 4,000 r.p.m. for 15 min. Each

pellet was washed twice with saline and re-suspended in distill water and then deposited into Mirr IR low e-microscope slides (Kevey slide) used as a substrate for FT-IR microscope analysis. The samples were then desiccated under vacuum for several hours and stored in desiccators to form films suitable before analysis (Naumann et al., 1988).

3.2.9.2 Pre-processing of data

Spectra were collected on a Bruker IR spectrometer (tensor 27) coupled to an IR microscope (Hyperion 2000) with 36x magnification. To achieve high S/N ratios, 64 scans were collected in each measurement in the wavenumber between 4000-400 cm^{-1} and resolution of 6 cm^{-1}. All spectra were processed using opus 6.5 software (Bruker optics) and the Unscrambler 9.7 software (Camo, Norway). Second derivative and vector normalize were manipulated to account for differences in sample thickness, minimize baseline variation and allow visual comparison (Naumann, 2000).

3.2.9.3 Method for cluster analysis

To analyze the effect of variation of the composition and distribution of the biochemical components in bacterial cells during cell cultures, the data were analyzed using principal component analysis (PCA). All data analysis were carried out in the spectral range from 3000-2800 cm^{-1} and 1800-850 cm^{-1}, which cover the mixed region of lipid, protein, polysaccharide and the “true” fingerprint region. The original spectra were extracted and normalized, using the OPUS 6.5 Software (Germany) and generated second derivatives, extended multiplicative signal correction (EMSC) and principal component analysis, using the Unscrambler 9.7 software (Camo, Norway) (Eumkeb et al., 2012; Mariey et al., 2001).

3.2.9.4 Spectrum manipulation

There are a number of techniques available to users of infrared spectrometers that help with both the qualitative and quantitative interpretation of spectra (Mariey et al., 2001).

3.2.9.4.1 Baseline correction

Baseline correction to avoid dissimilarity between spectra due to baseline deviation and to ensure valid comparisons between spectra for absorbance can be made.

3.2.9.4.2 Normalization

The normalization process detects the differences between spectra due to the different amount of sample. The spectra are normalized to the most intense band. This ensures that the thickness differences between samples can be accounted for.

3.2.9.4.3 Second derivative

The second derivative of the spectra is taken to minimize the baseline variability and to better resolution overlaps band especially in amide I.

3.2.9.4.4 Loading plot

The loading plot is a plot of the relationship between original variables and subspace dimensions. It is used for interpreting relationships among variables.

3.2.9.4.5 Principal component analysis (PCA)

Principal component analysis is a multivariate technique for studying one table of observations and variables with the main idea of

transforming the observed variables into a set of new variables, the principal components, which are interrelated and explain the variation in the data. PCA can be used for reducing the dimensionality of the data without decreasing their variance. Each spectrum is then compared to the others in order to make homogeneous groups and scatter plot can then be drawn to visualize the results, each spectrum being represented by a plot.

3.2.9.4.6 Integral area of spectra

A comparative analysis was performed to determine peak integral areas of normalized second derivative spectra using the OPUS software. The correlation between integrated peak area of the biomolecules of bacterial cell after treatment with antibiotic or flavonoids and mode of cell death were assessed using SPSS (Plaimee et al., 2014).

3.2.10 Statistical analysis

All experiment results were expressed as means ± standard error of means (S.E.M) and analyzed by one way ANOVA followed by Tukey's HSD. The results with $p<0.05$ or $p<0.01$ were considered as statistically significant differences.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 MICs and checkerboard determinations

Flavonoids have inhibitory activity against a variety of bacteria. The structure of flavonoids was isolated and identified. Examples of such flavonoids that, possess antibacterial activity include apigenin (Eumkeb and Chukrathok, 2013; Liu et al., 2012), galangin (Denny et al., 2002; Eumkeb et al., 2010; Pepeljnjak and Kosalec, 2004), naringenin (Celiz et al., 2011; Eumkeb and Chukrathok, 2013; Mandalari et al., 2007; Vikram et al., 2010), luteolin (Eumkeb et al., 2012; Li et al., 2009; Wang and Xie, 2010), quercetin, and various quercetin glycosides (Bouchouka et al., 2012; Brown et al., 2011; Chiruvella et al., 2007; Hossion et al., 2010) and kaempferol and its derivatives (Kataoka et al., 2001; Lim et al., 2007).

The MICs of antibacterial agents were determined by broth macrodilution method following the guidelines of Clinical and Laboratory Standards Institute (CLSI) (Matthew et al., 2013). The MICs of amoxicillin and flavonoids against ArSE are shown in Table 4.1. The MICs of amoxicillin, luteolin, baicalein and quercetin alone against ArSE were 16, 32, 32 and 256 µg/ml, respectively. This result showed that flavonoids could have a minor effect to inhibit ArSE. Checkerboard determination was used for investigation the synergistic effect on bacterial from 2 compounds of antibiotic drugs and isolated flavonoids (Wagner and Ulrich-Merzenich, 2009).

The results of checkerboard exhibited that quercetin showed the most synergistic effect with FIC index 0.25 against ArSE compared to others (Table 4.1).

Table 4.1 Minimum inhibitory concentrations (MICs), fraction inhibitory concentrations (FICs) and FIC index determined by checkerboard assay of antibiotic drugs and flavonoids alone and antibiotic drugs in combination with flavonoids against ArSE.

Strains	MIC (µg/ml)	Amo+Fla* (µg/ml)	**FIC Index**
S. epidermidis DMST 15505			
Amoxicillin	16^{R}	-	**-**
Nisin	32		
Flavonoids			
Luteolin	32	>8 + >8	**>1.0**
Baicalein	32	8 + 8	**1**
Quercetin	256	4 + 64	**0.25****
S. aureus ATCC 29213			
Amoxicillin	$<1^{S}$	N/D	**N/D**

Amo = amoxicillin, ^{R} = Resistance, ^{S} = Sensitive, ** = Synergism, Fla* = Flavonoids, N/D = No data

These results are in correspondence with many previous reports that quercetin could inhibit *B. subtilis, C. albicans, E. coli* and *S. epidermidis* (Rattanachaikunsopon and Phumkhachorn, 2010; Silvia Helena et al., 2003). In the same way, earlier data showed that quercetin and quercetin-3-O-arabinoside from *Psidium guajava* leaves exhibited bacteriostatic action against pathogenic bacteria including *B. stearothermophilus, E. coli, L. monocytogenes, S. enteric, S. aureus* and *V. cholera*. Moreover, the quercetin extract from mangosteen and seed were found to be effective against *H. pylori* infected animal resulted in a decline *H. pylori* (Brown et al., 2009; Ruggiero et al., 2007). Likewise, the antibacterial activity of quercetin indicated that the concentration of quercetin 50 µM, significantly inhibited the growth of *S. aureus*

and *S. epidermidis* at 3–9 h of incubation (Hirai et al., 2010). Previous studies reported that the synergism between quercetin and oxacillin display an effect on vancomycin-intermediate *S. aureus* (Basri et al., 2008). In addition, quercetin potentiate the effect of acyclovir against herpes-simplex virus (HSV) and pseudorabies infection (Kaul et al., 1985).

The MICs of ceftazidime and flavonoids against SP are shown in Table 4.2. MICs of ceftazidime, luteolin, baicalein and quercetin alone against SP were 0.25, 128, >256 and 128 µg/ml, respectively. The result of MICs determination explained that the activity of flavonoids could have a little inhibitory effect to SP. This observation is in agreement with the reports of Benso and Mann that flavonoids extracted from *A. Africana* had activity against *B. subtilis*, *S. pyogenes* and *E. coli* (Banso and Mann, 2008). Besides, the extracts of *S. bachiarica* had an effective inhibition of *S. pyogenes* and *S. epidermidis* at the concentration of 2 mg/ml (Heidari-Sureshjani et al., 2014). The combination MICs of ceftazidime plus luteolin, baicalein or quercetin were 0.125+16, 0.125+32 and 0.125+4 µg/ml, respectively, and the FIC indices of these combinations were 0.625, <0.625 and 0.531, respectively. The combination of these flavonoids with ceftazidime showed FIC index less than 1.0. So, these results suggested that flavonoids have a synergistic effect with ceftazidime to inhibit SP (Wagner and Ulrich-Merzenich, 2009). These results are in substantial correspondence with Basri and coworkers that naringenin or quercetin showed the synergistic effect with oxacillin to inhibit vancomycin intermediate *S. aureus* (Basri et al., 2008). In addition, Chang and coworkers also found that baicalein and gentamycin showed a synergy effect against vancomycin resistant strains of *Enterococcus* (Chang et al., 2007).

Table 4.2 Minimum inhibitory concentrations (MICs), fraction inhibitory concentrations (FICs) and FIC index determined by checkerboard assay of antibiotic drugs and flavonoids alone and antibiotic drugs in combination with flavonoids against SP.

Strains	MIC (µg/ml)	Cef+Fla* (µg/ml)	FIC Index
S. pyogenes DMST 30653			
Ceftazidime	0.25[R]	-	-
Nisin	8	-	-
Flavonoids			
Luteolin	128	0.125 + 16	0.625**
Baicalein	>256	0.125 +32	<0.625**
Quercetin	128	0.125 + 4	0.531**
S. aureus ATCC 29213			
Penicillin	≤0.12[S]	N/D	N/D
Amoxicillin	<1[S]	N/D	N/D

Cef = ceftazidime, [R] = Resistance, [S] = Sensitive, ** = Synergism, Fla* = Flavonoids, N/D = No data

These results revealed that these flavonoids displayed synergistic inhibitory effect against ArSE and SP (Wagner and Ulrich-Merzenich, 2009).

4.2 Killing curve determination

Killing curve determination is used for confirmation checkerboard assays that amoxicillin plus quercetin showed a synergistic effect against ArSE. Therefore, the MIC value of combination of amoxicillin plus quercetin that showed a synergistic effect was used. The graph shows viability curve and effect of amoxicillin or quercetin alone and in combination against ArSE.

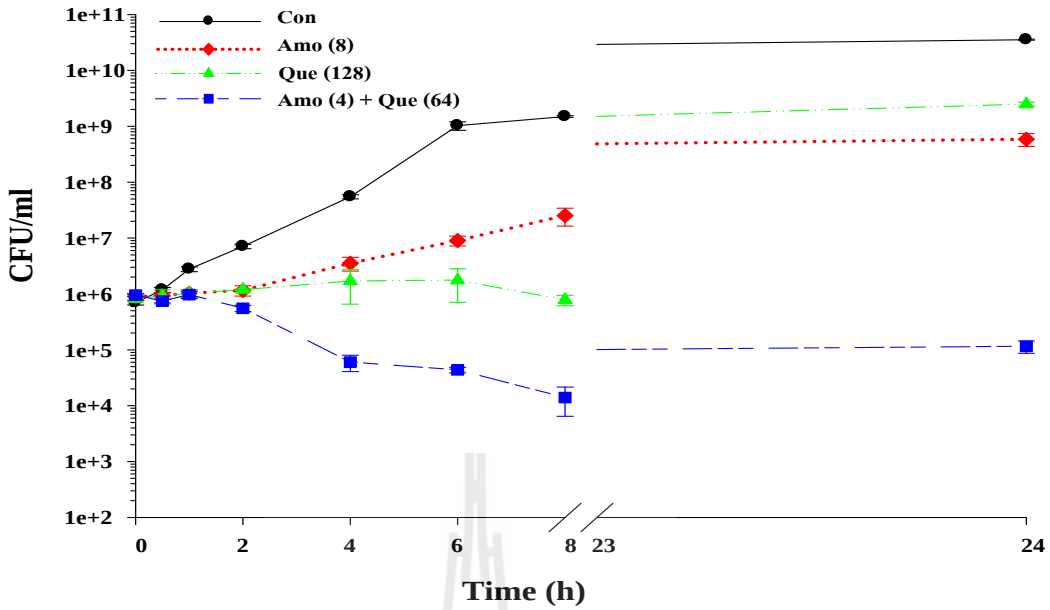

Figure 4.1 The effect of quercetin or amoxicillin alone and in combination on growth of ArSE. Con = Control (drugs free), Amo (8) = 8 µg/ml amoxicillin, Que (128) = 128 µg/ml quercetin, Amo (4) + Que (64) = 4 µg/ml amoxicillin plus 64 µg/ml quercetin. The bars represent the standard error of mean (S.E.M) of three replicates. The values plotted represent the mean of three replicates.

Killing curves in Figure 4.1 shows the bacterial growth in the presence of quercetin or amoxicillin alone. A minimal decrease in viability against ArSE was displayed. Moreover, the amoxicillin alone at 8 µg/ml treated group presented gradual increase within 8 h. Also it was markedly increase to 10^8 cfu/ml at 24 h. On the contrary, the treated group of quercetin alone illustrated a slight decrease in viability of cells over 8 h, however the culture had markedly recovered to 10^9 cfu/ml within 24 h. The combination of amoxicillin 4 µg/ml plus quercetin 64 µg/ml indicated a dramatic decrease in viable cell counts to 10^4 cfu/ml within 8 h. These results had also been confirmed checkerboard determinations that amoxicillin plus quercetin showed synergistic activity due to bacterial cells treated with this combination was decreased

of ≥2 log10 cfu/ml compared to treatment group of amoxicillin alone (Eliopoulos and Moellering, 1996).

The CM permeability, enzyme assay, TEM and FT-IR microspectroscopy technique were chosen to investigate the elementary mechanism of action of the combination compounds between β-lactam antibiotics and each flavonoid. The concentrations of the combination compounds were performed at ½ or ¾ of their FICs due to the most of injuried cells, not dead, were examined.

4.3 Cytoplasmic membrane permeability

The leakage of CM was analyzed by determining the release of cell materials, including nucleic acid which absorbs light at 260 nm wavelength in the suspensions and demonstrated by UV-VIS absorbing material. The OD_{260} was observed after cells were treated with a combination of amoxicillin and quercetin.

The OD_{260} of materials were released from ArSE cells after treatment with amoxicillin or quercetin alone and in combination for 1 h (Figure 4.2). These results indicated that the combination of amoxicillin and quercetin caused higher cell leakage resulting in losing of nucleic acid, critical molecules and ions (Devi et al., 2010; Zhou et al., 2008). These results suggest that one preliminary mechanism of action of this combination could be cytoplasmic membrane damage of this strain. This result could be cause by the disruption of the formation of pore in the plasma membrane (Zhou et al., 2008). Besides, the release of intracellular proteins is the mark for membrane damage and less membrane integrity (Vaara and Vaara, 1981).

4.3.1 Cytoplasmic membrane permeability of ArSE

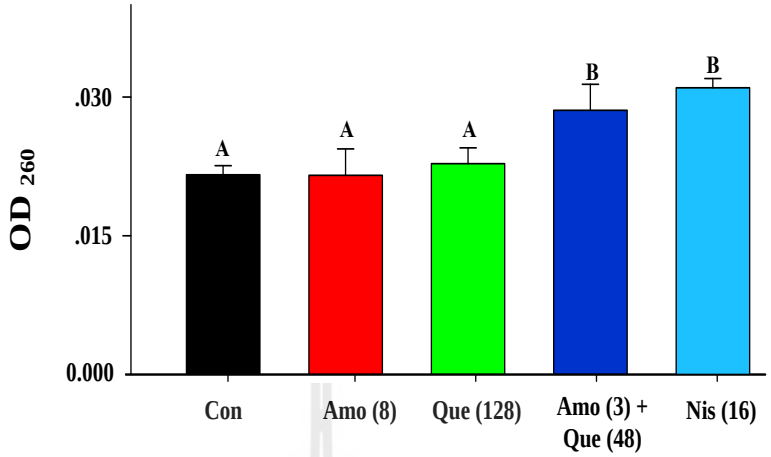

Figure 4.2 Present of OD_{260} nm absorbing material in the supernatants of ArSE treated with quercetin or amoxicillin alone and in combination. The mean ± S.E.M for three replicates are illustrated. Con = Control (drugs free), Amo (8) = 8 µg/ml amoxicillin, Que (128) = 128 µg/ml quercetin, Amo (3) + Que (48) = 3 µg/ml amoxicillin plus 48 µg/ml quercetin, Nis (16) = 16 µg/ml Nisin. Nisin was used as positive control and untreated cells were used as negative control. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, $p<0.01$).

4.3.2 Cytoplasmic membrane permeability of SP

The effect of ceftazidime alone and in combination of each flavonoid on SP cytoplasmic membrane permeability is shown in Figure 4.3 and Table 4.3.

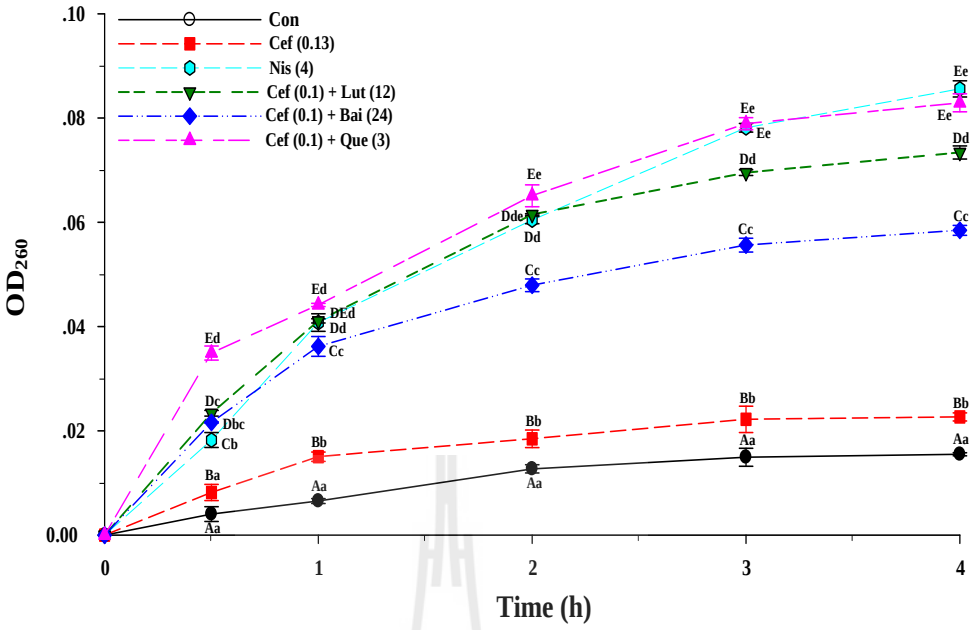

Figure 4.3 Present of OD_{260} nm absorbing material in the supernatants of SP treated with ceftazidime alone and combined with luteolin, baicalein or quercetin. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Nis (4) = 4 µg/ml nisin, Cef (0.1) + Lut (12) = 0.1 µg/ml ceftazidime plus 12 µg/ml luteolin, Cef (0.1) + Bai (24) = 0.1 µg/ml ceftazidime plus 24 µg/ml baicalein, Cef (0.1) + Que (3) = 0.1 µg/ml ceftazidime plus 3 µg/ml quercetin. Nisin was used as positive control and untreated cells were used as negative control. The mean ± S.E.M for three replicates are illustrated. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, A = p<0.05; a = p<0.01).

Table 4.3 OD_{260} absorbing material in the supernatant of SP after treated with ceftazidime alone or in combination with flavonoids. Nisin (4) = Nisin 4 µg/ml, Nisin was used as positive control and untreated cells were used as negative control. The mean ± S.E.M for three replicates are illustrated. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, A = $p<0.05$; a = $p<0.01$).

Groups of treated/OD_{260}	Time (min)				
	0.5	1	2	3	4
Control	0.004^{aA}	0.006^{aA}	0.012^{aA}	0.015^{aA}	0.016^{aA}
Nisin (4)	0.018^{bC}	0.041^{dD}	0.06^{dD}	0.078^{eE}	0.086^{eE}
Ceftazidime (0.13)	0.0082^{aB}	0.0015^{bB}	0.0019^{bB}	0.0022^{bB}	0.0027^{bB}
Ceftazidime (0.1) + Baicalein (24)	0.023^{bcD}	0.041^{cC}	0.062^{cC}	0.07^{cC}	0.073^{cC}
Ceftazidime (0.1) + Luteolin (12)	0.022^{cD}	0.036^{dDE}	0.048^{deD}	0.056^{dD}	0.058^{dD}
Ceftazidime (0.1) + Quercetin (3)	0.035^{dE}	0.044^{dE}	0.065^{eE}	0.079^{eE}	0.083^{eE}

The OD_{260} can detect the extracellular material released by the cells. The results from CM permeability exhibited that ceftazidime alone slightly increased CM permeability of this strain. The ceftazidime treated alone exhibited significantly higher OD_{260} than controls starting from 0.5 h ($p<0.05$). However, nisin showed significantly higher than ceftazidime treated alone ($p<0.01$). In addition, the combination of these flavonoids and ceftazidime significantly dramatic increased CM permeability compared to controls and ceftazidime alone ($p<0.01$). These results are in substantial agreement with previous findings that luteolin either alone or combined with amoxicillin and apigenin alone and in combination with ceftazidime increased CM permeability of amoxicillin-resistant *E. coli* and ceftazidime-resistant *E. cloacae*, respectively (Eumkeb and Chukrathok, 2013; Eumkeb et al., 2012). Besides, these results agree with Zhou that the effect of pantocin on the cytoplasmic membrane of

L. monocytogenes using OD_{260} of intracellular was increased (Devi et al., 2010; Zhou et al., 2008). The release of intracellular material of the cell was indicated the cytoplasmic membrane damaging and loss of membrane integrity (Vaara and Vaara, 1981). So, the increase in CM permeability may be one of the synergistic actions of this combination against *S. pyogenes.* This result could be explained that the formation of pores in the plasma membrane might be disrupted (Zhou et al., 2008).

4.4 Enzyme assay

The enzyme assay used to determine the inhibitory activity of β-lactamase by flavonoids. The remaining of benzylpenicillin from HPLC indicates the inhibitory activity of flavonoids to inhibit β-lactamase. The capability of quercetin to inhibit the activity of β-lactamase enzyme type IV from *E. cloacae* is shown in Figure 4.4 and Table 4.4. The higher benzylpenicillin remainder means the lesser β-lactamase activity. The results demonstrated that amoxicillin alone did not have β-lactamase inhibition activity. Whereas, quercetin displayed β-lactamase inhibition activity in a concentration dependent manner.

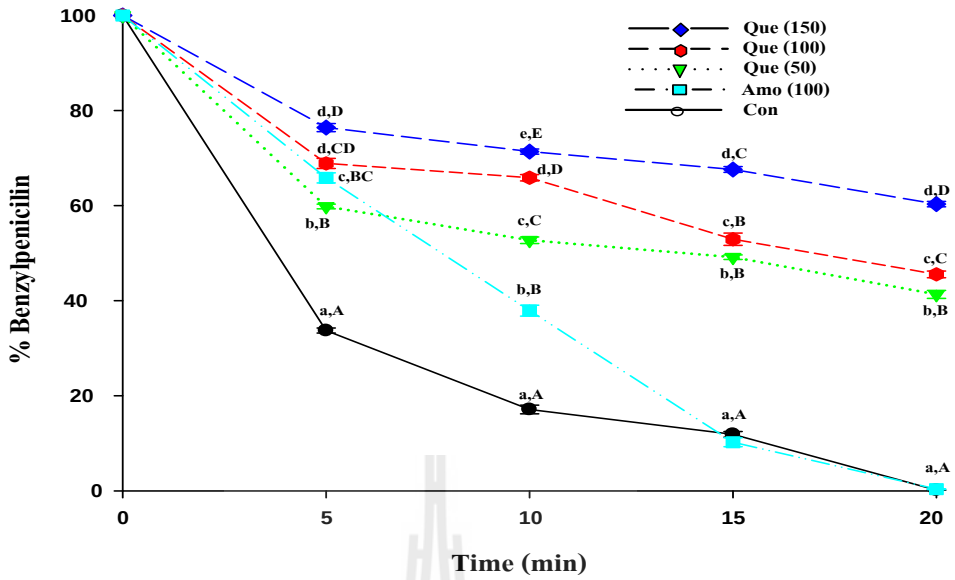

Figure 4.4 The inhibitory effect of quercetin and amoxicillin against β-lactamase in hydrolyzing β-lactamase used from *E. cloacae*. Con = Control (drugs free), Amo (100) = 100 µg/ml amoxicillin, Que (50) = 50 µg/ml quercetin, Que (100) = 100 µg/ml quercetin, Que (150) = 150 µg/ml quercetin. The graph shows the remaining benzylpenicillin at the same time. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, a = $p<0.01$, A = $p<0.05$).

Table 4.4 Benzylpenicillin remaining after treated with combination of β-lactamase enzyme and amoxicillin or quercetin alone. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, a = $p<0.01$, A = $p<0.05$).

Groups of treated/ % Benzylpenicillin	Time (min)				
	0	5	10	15	20
Control	100	33.72^{aA}	17.13^{aA}	11.9^{aA}	0.16^{aA}
Amoxicillin 100 µg/ml	100	65.84^{cBC}	37.92^{bB}	10.24^{aA}	0.37^{aA}
Quercetin 50 µg/ml	100	59.82^{bB}	52.71^{cC}	49.16^{bB}	41.33^{bB}
Quercetin 100 µg/ml	100	68.88^{dCD}	65.87^{dD}	52.92^{cB}	45.54^{cC}
Quercetin 150 µg/ml	100	76.4^{dD}	71.34^{eE}	67.6^{dC}	60.32^{dD}

These results indicate that the activity of quercetin against ArSE might inhibit of β-lactamase activity (Eumkeb et al., 2010). These results are in agreement with those of Eumkeb and coworkers that flavonoids galangin, kaempferide and kaempferide-3-O-β-D-glucoside displayed inhibitory effect on β-lactamase activity. Furthermore, Eumkeb and colleagues suggested that these inhibitions caused reverse the resistance of bacterial strains to the activity of the primary antibiotics (Eumkeb et al., 2012). Our findings imply that quercetin could inhibit β-lactamase activity.

A major mechanism of acquired resistance to the β-lactam antibiotic is production of β-lactamases enzymes to hydrolyze the β-lactam antibiotic. Moreover, some *Streptococci* have developed a different mechanisms of resistance by altering PBPs cause bacterial cell wall synthesis is not disrupted (Grebe and Hakenbeck, 1996). Although penicillin has been the antibiotic of choice for treatment *S. pyogenes* infection, many reports of treatment with penicillin have failures and increased in recent years. Some studies have suggested *Streptocooci* can produce β-lactamases enzymes (Goldstein, 1999). Figure 4.5 and Table 4.5 shows the competency of flavonoids, including baicalein, luteolin and quercetin to inhibit the activity of β-lactamase enzyme type IV from *E. cloacae*.

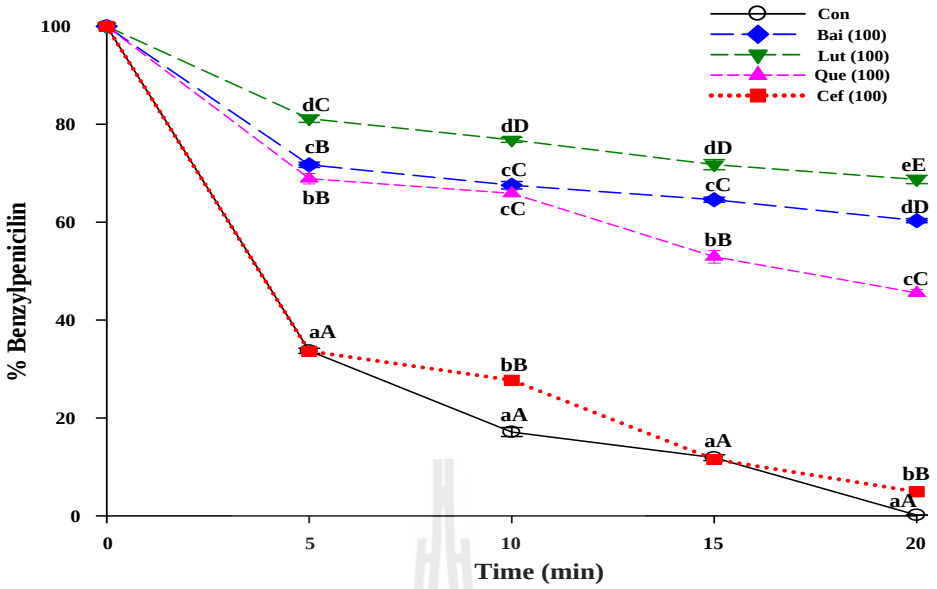

Figure 4.5 The inhibitory effect of baicalein, luteolin, quercetin and ceftazidime against β-lactamase enzyme in hydrolyzing β-lactamase used from *E. cloacae*. Con = Control (drugs free), Bai (100) = 100 µg/ml baicalein, Lut (100) = 100 µg/ml luteolin, Que (100) = 100 µg/ml quercetin, Cef (100) = 100 µg/ml ceftazidime. The graph shows the remaining benzylpenicillin at the same time. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, a = p<0.01, A = p<0.05).

Table 4.5 Benzylpenicillin remaining after treated with combination of β-lactamase enzyme and ceftazidime or each flavonoid. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, a = p<0.01, A = p<0.05).

Groups of treated/ %Benzylpenicillin	Time (min)				
	0	5	10	15	20
Control	100	33.72^{aA}	17.13^{aA}	11.9^{aA}	0.16^{aA}
Ceftazidime 100 µg/ml	100	33.64^{aA}	27.71^{bB}	11.54^{aA}	4.9^{bB}
Baicalein 100 µg/ml	100	71.71^{cB}	67.54^{cC}	64.59^{cC}	60.30^{dD}
Luteolin 100 µg/ml	100	81.12^{dC}	76.80^{dD}	71.74^{dD}	68.71^{eE}
Quercetin 100 µg/ml	100	68.88^{bB}	65.87^{cC}	52.92^{bB}	45.54^{cC}

The results displayed that ceftazidime exhibited a scarcely higher of benzylpenicillin remainder than controls. All tested flavonoids illustrated significantly higher β-lactamase inhibition than those of ceftazidime and control ($p<0.01$). The significant level of benzylpenicillin remainder from higher to lower was luteolin>baicalein>quercetin>ceftazidime>control start from 10 min and throughout the 20 min ($p<0.01$). Some flavonoids showed inhibitory activity to β-lactamase enzyme activity such as galangin and apigenin (Eumkeb et al., 2010). This result suggests that flavonoids play the role in β-lactamases enzyme inhibition (Denny et al., 2002; Eumkeb et al., 2010). So, these findings provide evidence that luteolin, baicalein and quercetin in combination with β-lactam antibiotic may useful to inhibit mixed β-lactamase-producing bacteria and *S. pyogenes* in oropharyngeal infections (Boon and Beale, 1987; Brook, 2009).

4.5 Transmission electron microscopy

Electron micrographs of log phase of ArSE cells in the presence of amoxicillin or quercetin alone and in combination are shown in Figures 4.6 to 4.9.

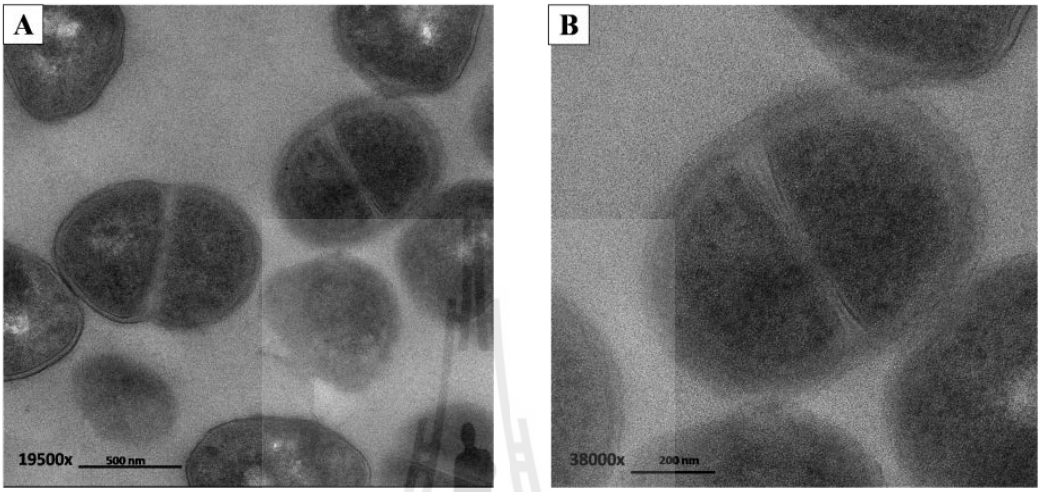

Figure 4.6 Ultrathin sections of log phase of ArSE grown in drug free. Original magnification, 19,500x; bar 500 nm (A), 38,000x; bar 200 nm (B).

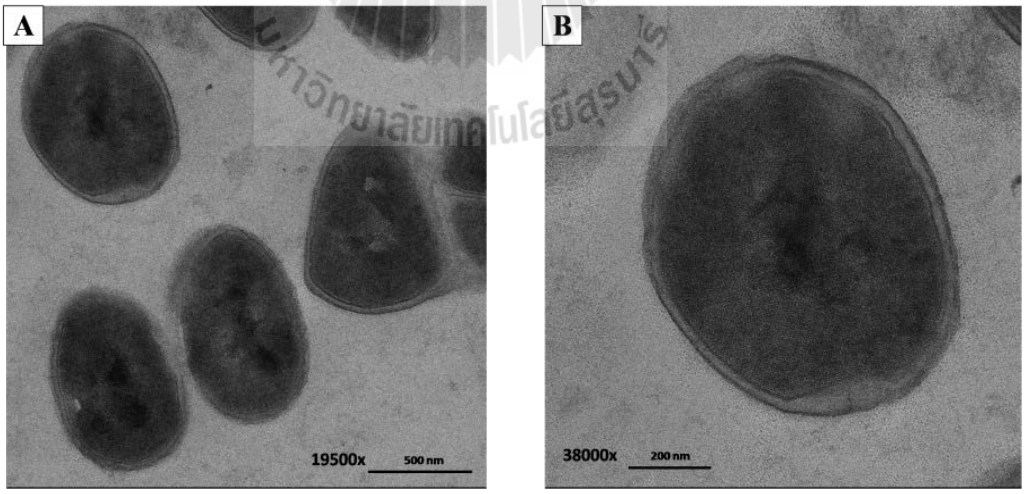

Figure 4.7 Ultrathin sections of log phase of ArSE containing amoxicillin (8 µg/ml). Original magnification, 19,500x; bar 500 nm (A), 38,000x; bar 200 nm (B).

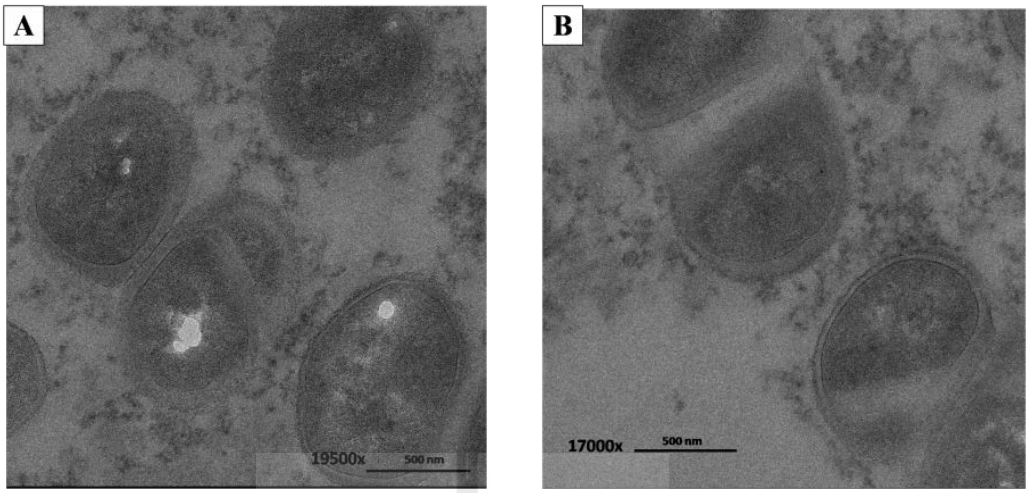

Figure 4.8 Ultrathin sections of log phase of ArSE containing quercetin (128 µg/ml). Original magnification, 19,500x; bar 500 nm (A), 17,000x; bar 500 nm (B).

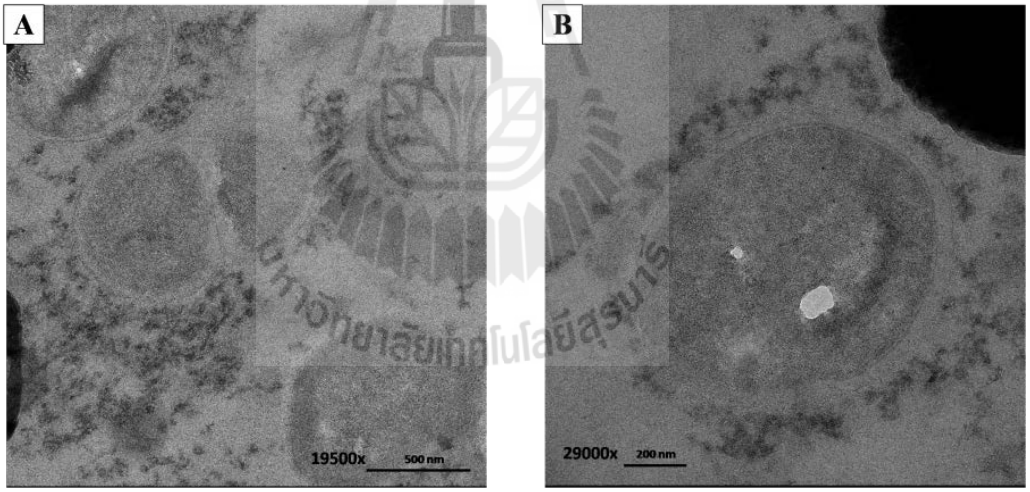

Figure 4.9 Ultrathin sections of log phase of ArSE containing combination of amoxicillin (3 µg/ml) plus quercetin (48 µg/ml). Original magnification, 19,500x; bar 500 nm (A), 29,000x; bar 200 nm (B).

To explore the antibacterial mechanisms, the effect of quercetin or amoxicillin alone and in combination on morphological changes of ArSE was studied by TEM. Electron micrographs of thin sections of ArSE cells grown for log phase in the

presence of amoxicillin or quercetin alone and combination of amoxicillin plus quercetin are investigated (Figures 4.6 to 4.9). Figure 4.6 displayed that peptidoglycan and cytoplasmic membrane on drug free cells can be distinguished. The normal cell shape was apparent in these untreated cells. The log phase cells after treatment with amoxicillin alone is shown in Figure 4.7. Amoxicillin is known to exert their antimicrobial effect by inhibition of the synthesis of peptidoglycan, which is a heteropolymeric component of the cell wall (Samaha-Kfoury and Araj, 2003). This result displayed damage peptidoglycan and few cytoplasmic membranes hurt around 5-10% of these cells. The average cross-sectional cell areas of these cells were slightly smaller than controls, but not significantly different (p<0.01) (Figure 4.10). However, the average cell division boundaries were a little bigger than controls, despite not significantly different (p<0.01) (Figure 4.11). Whereas, the micrograph of these cells after exposure to quercetin alone is shown in Figure 4.8. The result exhibited that about 40-50% of these cells revealed peptidoglycan and cytoplasmic membrane damage, devoid of the ribosome in cytoplasm and have a larger gap between peptidoglycan and cytoplasmic membrane. The average cell areas of these cells were hardly smaller than the controls, despite not significantly different (p<0.01) (Figure 4.10). Clearly, the average cell division boundary of this treatment was significantly larger than other groups (p<0.01) (Figure 4.11). Figure 4.9 reveals the amoxicillin plus quercetin treated cells. The figure demonstrated that approximately 70-80% of these cells exhibited marked morphological damage or cell shape distortion, noticeable peptidoglycan and cytoplasmic membrane damage, larger gap between peptidoglycan and cytoplasmic membrane, electron transparent areas devoid of ribosome. Obviously, these average cell areas were somewhat larger than the controls,

nevertheless, not a significant difference (p<0.01) (Figure 4.10). The average cell division boundary of this treated cell was significantly larger than controls (p<0.01) (Figure 4.11).

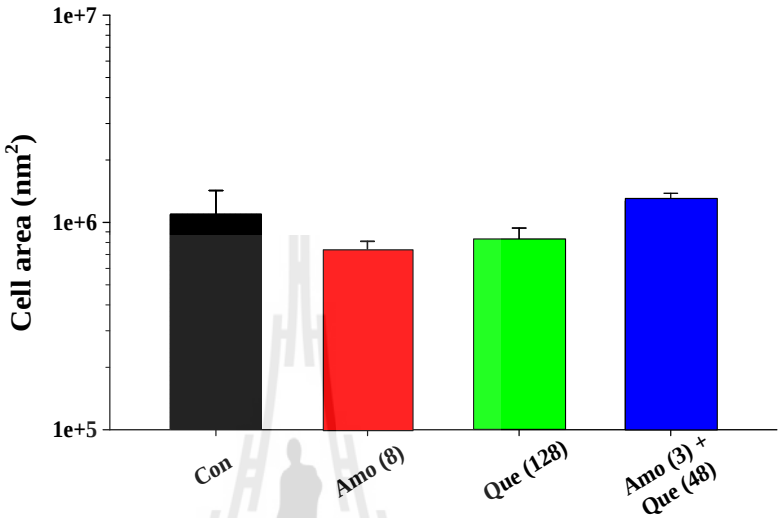

Figure 4.10 The cell area of ArSE after treatment with amoxicillin or quercetin alone and in combination. Con = Control (drugs free), Amo (8) = 8 µg/ml amoxicillin, Que (128) = 128 µg/ml quercetin, Amo (3) + Que (48) = 3 µg/ml amoxicillin plus 48 µg/ml quercetin. The mean ± S.E.M (n≥12 in all groups) for three replicates are illustrated. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, A = p<0.05; a = p<0.01).

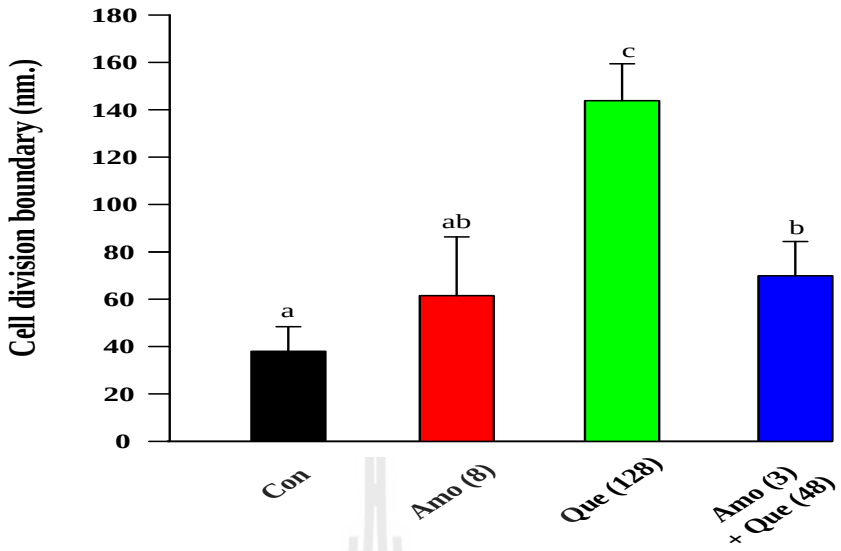

Figure 4.11 The effect of amoxicillin or quercetin alone and in combination on boundaries between the cell division of ArSE. Con = Control (drugs free), Amo (8) = 8 µg/ml amoxicillin, Que (128) = 128 µg/ml quercetin, Amo (3) + Que (48) = 3 µg/ml amoxicillin plus 48 µg/ml quercetin. The mean ± S.E.M (n≥12 in all groups) for three replicates are illustrated. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, a = $p<0.01$).

Electron micrographs of log phase of SP cells in the presence of ceftazidime, luteolin, quercetin or baicalein either alone or in combination are shown in Figures 4.12 to 4.19.

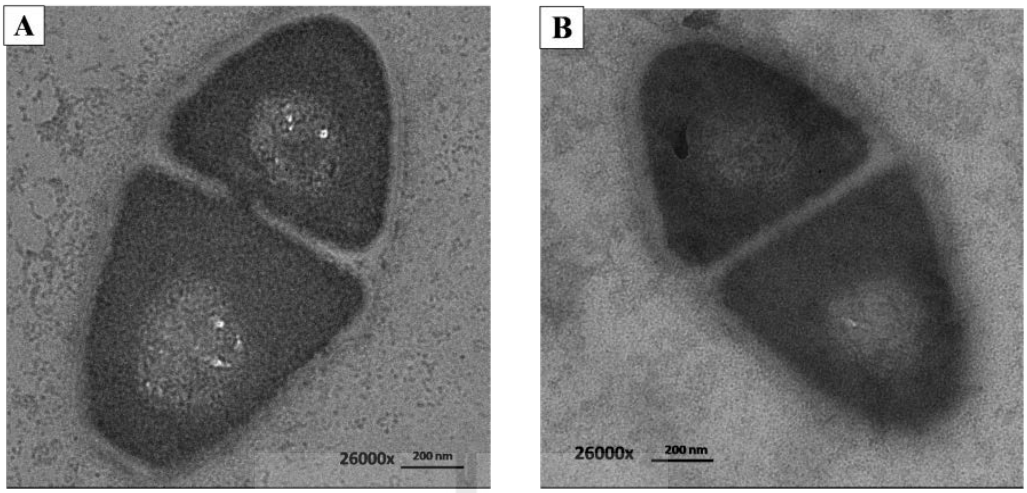

Figure 4.12 Ultrathin sections of log phase of SP grown in drug free. Original magnification, 26,000x; bar 200 nm (A), 26,000x; bar 200 nm (B).

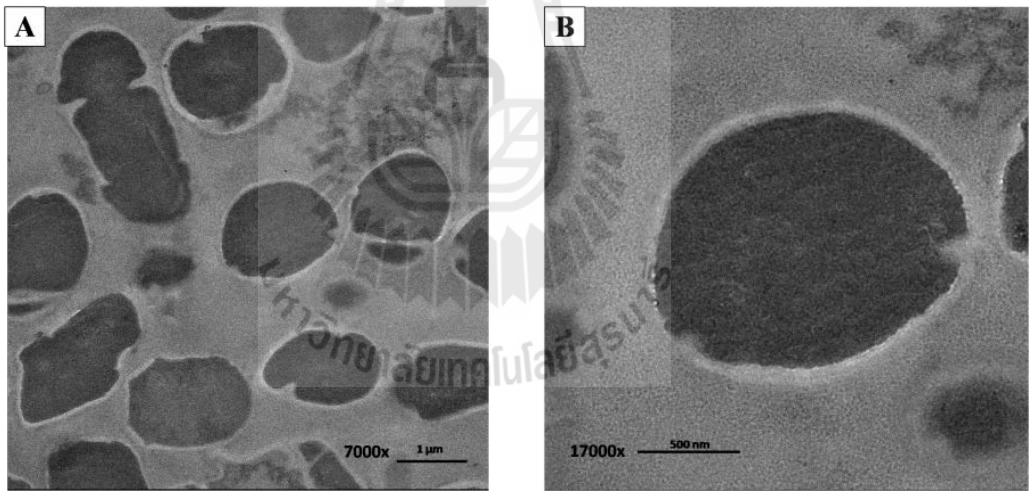

Figure 4.13 Ultrathin sections of log phase of SP containing ceftazidime (0.13 µg/ml). Original magnification, 7,000x; bar 1 µm (A), 17,000x; bar 500 nm (B).

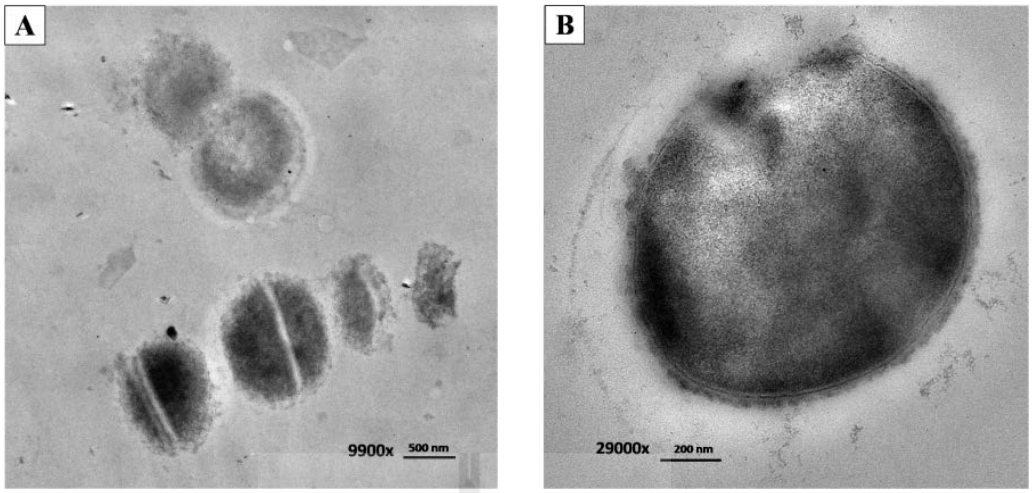

Figure 4.14 Ultrathin sections of log phase of SP containing luteolin (64 µg/ml). Original magnification, 9,900x; bar 500 nm (A), 29,000x; bar 200 nm (B).

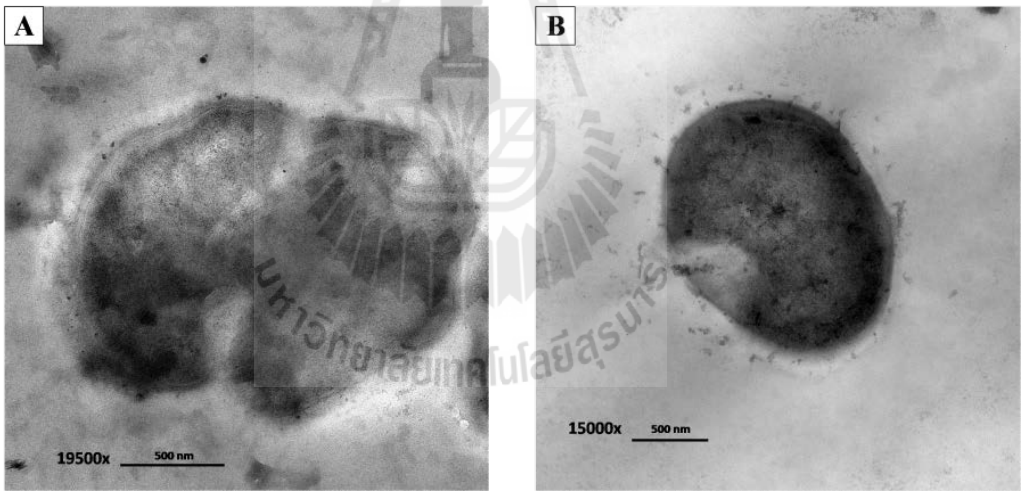

Figure 4.15 Ultrathin sections of log phase of SP containing baicalein (128 µg/ml). Original magnification, 19,500x; bar 500 nm (A), 15,000x; bar 500 nm (B).

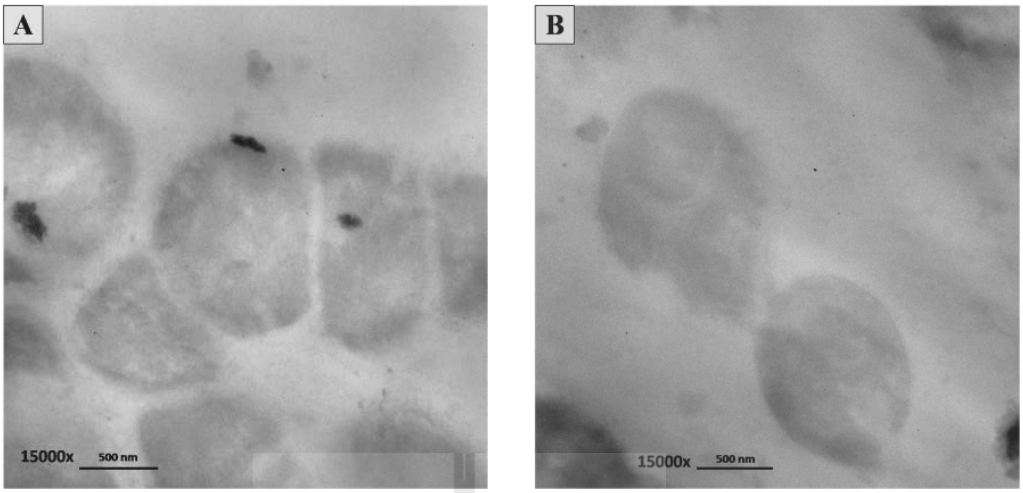

Figure 4.16 Ultrathin sections of log phase of SP containing quercetin (64 µg/ml). Original magnification, 15,000x; bar 500 nm (A), 15,000x; bar 500 nm (B).

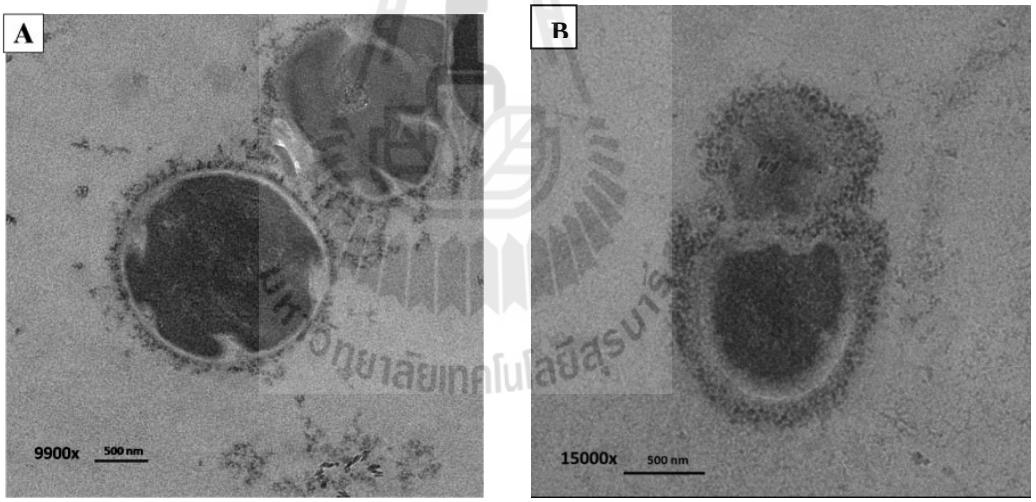

Figure 4.17 Ultrathin sections of log phase of SP containing ceftazidime (0.1 µg/ml) plus luteolin (12 µg/ml). Original magnification, 9,900x; bar 500 nm (A), 9,900x; bar 500 nm (B).

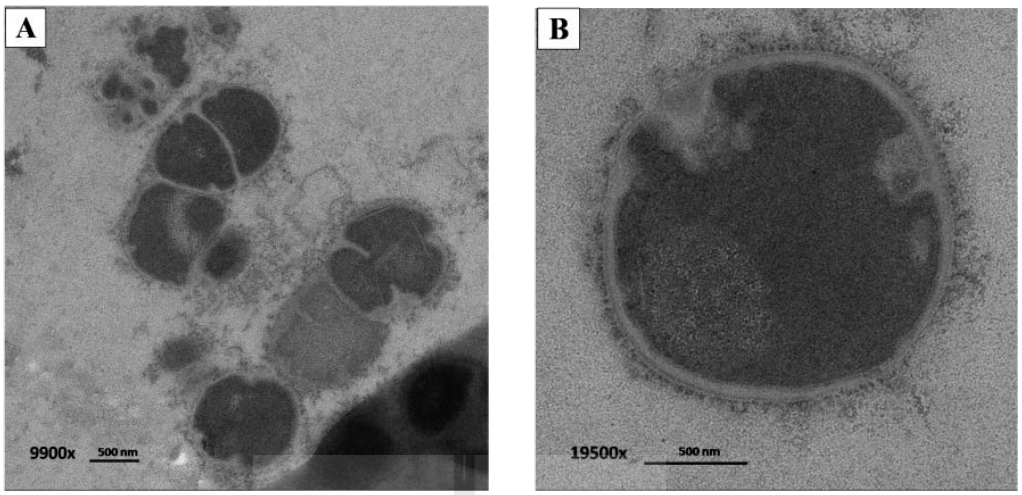

Figure 4.18 Ultrathin sections of log phase of SP containing ceftazidime (0.1 µg/ml) plus baicalein (24 µg/ml). Original magnification, 9,900x; bar 500 nm (A), 19,500x; bar 500 nm (B).

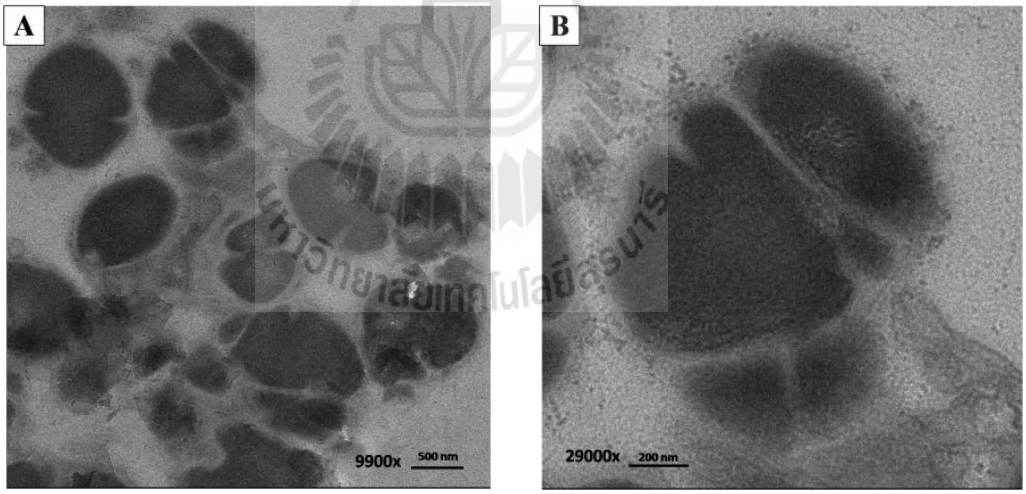

Figure 4.19 Ultrathin sections of log phase of SP containing ceftazidime (0.1 µg/ml) plus quercetin (3 µg/ml). Original magnification, 9,900; bar 500 nm (A), 29,000x; bar 200 nm (B).

The thin sections of SP growth at log phase after exposing to ceftazidime alone and in combination with flavonoids are examined. The untreated cells (drug free) are shown in Figure 4.12. These cells looked normal in their appearance and could be

distinguished between peptidoglycan and cytoplasmic membrane. Some divided cells were shown. Figure 4.13 shows the log phase cells after exposure to ceftazidime alone. The cell division of about 70-80% these cells were interrupted, delayed and peptidoglycan was damaged resulting in cell shape distortions. The average cross-sectional cell areas of these cells were slightly larger than the control, although not significant difference (p<0.05). Besides, these cells treated with flavonoids alone are presented in Figures 4.14 to 4.16. The flavones, luteolin and baicalein, treated groups alone displayed thicker peptidoglycan, a little repaired cytoplasmic membrane, and cell shape distortion or broken cells in approximately 60-70% of these cells compared to the control (Figures 4.14 and 4.15). The average cross-sectional cell areas of these flavones treated cells were also slightly larger than the control, but not a significant difference (p<0.05) (Figure 4.20). The flavonol, quercetin, treated cells alone is presented in Figure 4.16. These treated cells revealed thinner or disappeared peptidoglycan, cytoplasmic membrane damage, cell shape distortion and broken in around 65-75% of these cells compared with controls. The average cell areas of these cells were a bit larger than the control, despite not significantly different (p<0.05) (Figure 4.20). The thin section of SP after treatment with the combination of ceftazidime and tested flavonoids are illustrated in Figures 4.17 to 4.19. The micrographs of these cells after exposure to ceftazidime plus the flavone, luteolin or baicalein, are shown in Figures 4.17 and 4.18 respectively. The result exhibited that around 70-80% of these cells revealed peptidoglycan and cytoplasmic membrane damage: cell shape was irregular, distortion or broken and cytoplasm devoided of ribosome in transparent area. Obviously, the average cell areas of baicalein combination treated cells were significantly larger than controls (p<0.05) (Figure

4.20). In addition, Figure 4.19 reveals the ceftazidime plus quercetin treated cells. Around 75-85% of these cells displayed marked peptidoglycan damage, irregular and distorted cell shape, cytoplasmic membrane damage, and a minority of transparent area devoid of ribosomes. These average cell areas were larger than the control, in spite of not significantly (p<0.05) (Figure 4.20). The bigger cells of these tested flavonoids plus ceftazidime showed clearly bigger than control may due to the cell division was interrupted (Duggirala et al., 2014).

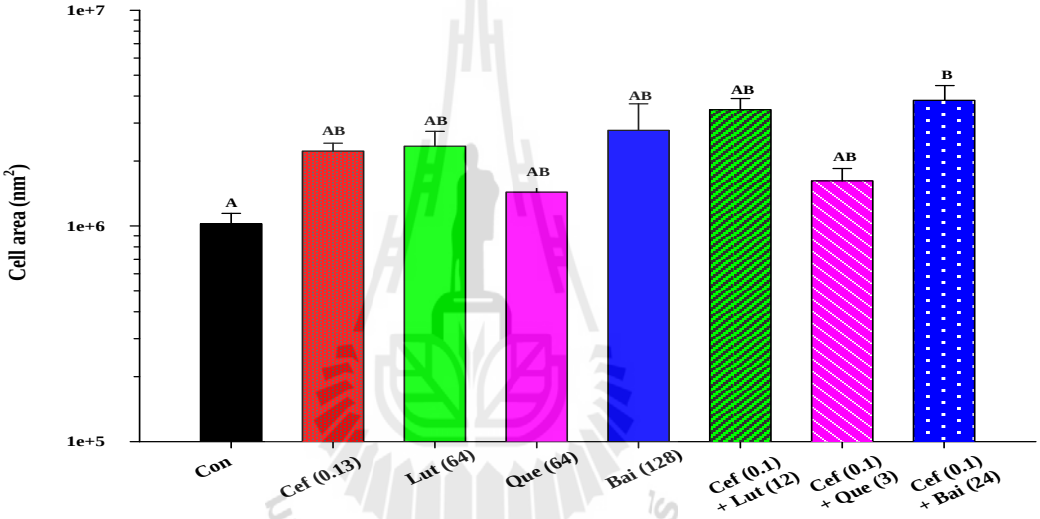

Figure 4.20 The effect of ceftazidime and flavonoids alone and in combination on average cross-section of SP cell areas from TEM. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Lut (64) = 64 µg/ml luteolin, Que (64) = 64 µg/ml quercetin, Bai (128) = 128 µg/ml baicalein, Cef (0.1) + Lut (12) = 0.1 µg/ml ceftazidime plus 12 µg/ml luteolin, Cef (0.1) + Que (3) = 0.1 µg/ml ceftazidime plus 3 µg/ml quercetin, Cef (0.1) + Bai (24) = 0.1 µg/ml ceftazidime plus 24 µg/ml baicalein. The mean ± S.E.M (n≥12 in all groups) for three replicates are illustrated. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, A = p<0.05).

Transmission electron microscope revealed the effect of flavonoids on not only cell shape, but also cell size. The TEM findings suggest that the cell division process may be interrupted leads to irregular and distorted cell shape. The cells were heterogeneous in size and form septa localization seemed to occur randomly. These results agree with Limsuwan and coworkers, they found the extracted from *Rhodomyrtus tomentosa* caused irregular shape and different sizes during binary fission on *S. pyogenes* (Limsuwan et al., 2012). These results indicate that flavonoids could effect on the integrity of septum formation, cell division and peptidoglycan (Hardoko and Yuliana, 2014; Ulanowska et al., 2006).

4.6 FT-IR microspectroscopy

The ArSE strain was grown in CAMHB medium in the presence of 8 μg/ml amoxicillin (½ MIC), 128 μg/ml quercetin (½ MIC) and 3 μg/ml amoxicillin plus 48 μg/ml quercetin (¾ FIC) and examined by FT-IR microspectroscopy. The results are presented in Figures 4.21 to 4.25.

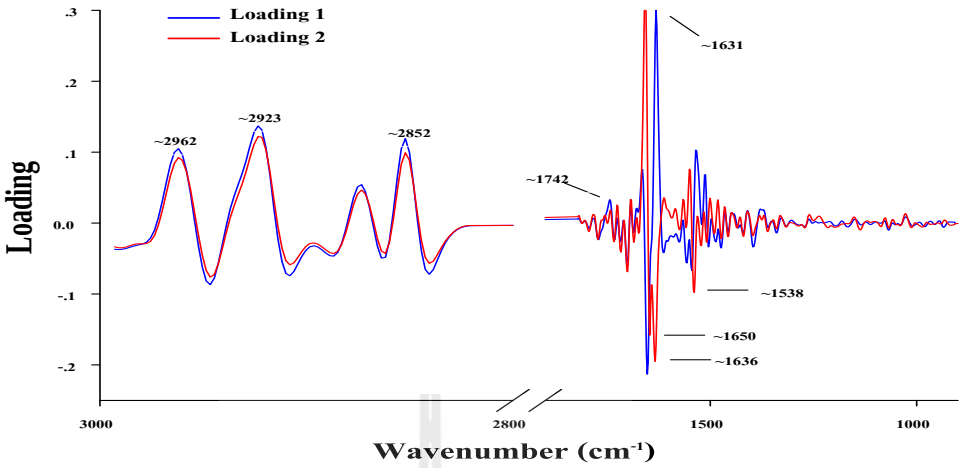

Figure 4.21 Loading plot of the PC1 (1st) and the PC2 (2nd) obtained from PCA of ArSE untreated cell and after treatment with amoxicillin alone and in combination with quercetin.

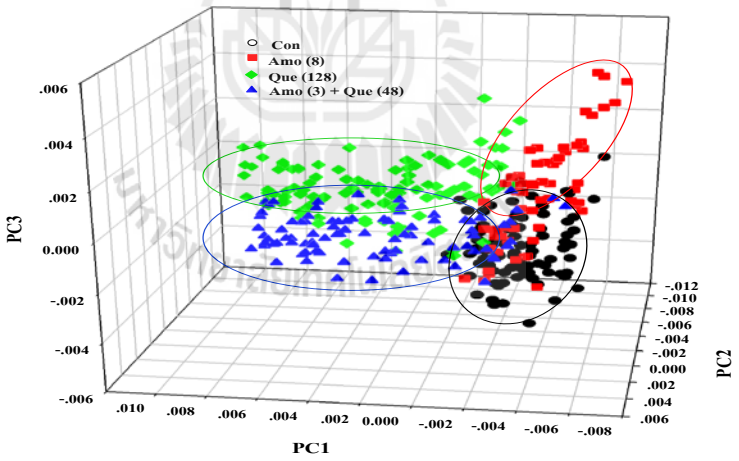

Figure 4.22 PCA results constructed using spectra of ArSE after treatment with amoxicillin or quercetin alone and in combination. Con = Control (drugs free), Amo (8) = 8 µg/ml amoxicillin, Que (128) = 128 µg/ml quercetin, Amo (3) + Que (48) = 3 µg/ml amoxicillin plus 48 µg/ml quercetin. PCA analysis was constructed by using second derivative, vector normalized spectra, over the spectral ranges from 3000 - 2800 cm^{-1} and from 1750 - 900 cm^{-1}.

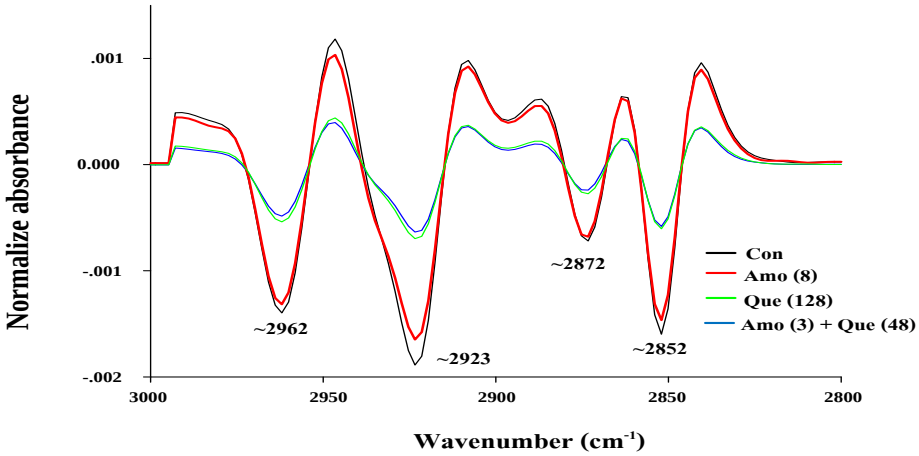

Figure 4.23 Representative 2nd derivative transformation spectra (3000 - 2800 cm^{-1}) of ArSE untreated cell and after treatment with ceftazidime alone and in combination with tested flavonoids. Con = Control (drugs free), Amo (8) = 8 µg/ml amoxicillin, Que (128) = 128 µg/ml quercetin, Amo (3) + Que (48) = 3 µg/ml amoxicillin plus 48 µg/ml quercetin.

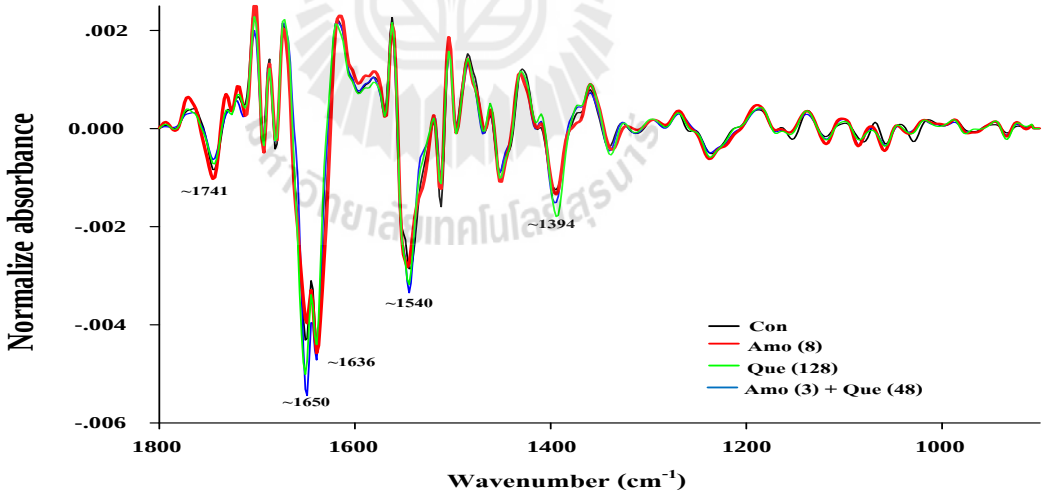

Figure 4.24 Representative 2nd derivative transformation spectra (1800 - 900 cm^{-1}) of ArSE untreated cell and after treatment with ceftazidime alone and in combination with tested flavonoids. Con = Control (drugs free), Amo (8) = 8 µg/ml amoxicillin, Que (128) = 128 µg/ml quercetin, Amo (3) + Que (48) = 3 µg/ml amoxicillin plus 48 µg/ml quercetin.

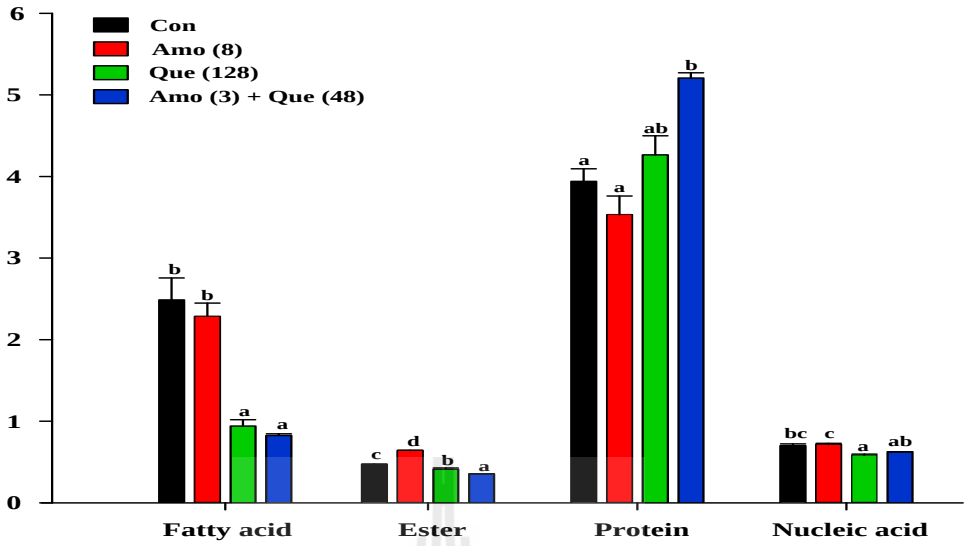

Figure 4.25 Alteration of biochemical structures and amount observed by peak position, intensity and areas of ArSE after treatment with amoxicillin alone or in combination with quercetin. Con = Control (drugs free), Amo (8) = 8 µg/ml amoxicillin, Que (128) = 128 µg/ml quercetin, Amo (3) + Que (48) = 3 µg/ml amoxicillin plus 48 µg/ml quercetin. Means sharing the same superscript are not significantly different from each other (Turkey's HSD, a = p<0.01).

FT-IR spectra measured for intact cells of bacteria are usually complex and the peaks are broad due to superposition of contributions from all the biomolecules present in a bacterial cell (Naumann, 2000).

The ArSE was grown in medium in a presence of amoxicillin or quercetin alone and in combination, and then was examined by FT-IR microspectroscopy. The 1st principal component (PC1) is characterized the maximum percentage of bacterial spectral variation follow by the PC2 (Al-Qadiri, et al., 2008). Loading from PC1 of ArSE after treatment with quercetin or amoxicillin alone and in combination were accounted for 80% of the total variability and the case of treating group loading PC2 was accounted for 66% of the total variability (Figure 4.21).

The statistical analysis based on PCA was clearly shown the separation between each group corresponding to the results of loading (Figure 4.22). The 1st loading represents an average from treatment group of amoxicillin or quercetin alone and in combination indicated that obvious the region at 3000-2800 cm^{-1} and ~1742 cm^{-1} were assigned to the stretching mode of CH_2 and CH_3 (~2962, ~2923, ~2852 cm^{-1}) of fatty acids on the various membrane amphiphiles and ester bond (~1742 cm^{-1}) (Figure 4.23 and 4.24) (Garip et al., 2009; Naumann, 2000).

The 2nd loading displays 3 region coefficients at ~1650 cm^{-1}, ~1636 cm^{-1} and ~1538 cm^{-1}. These regions related to average bands are shown in Figure 4.24. The treated group of quercetin or amoxicillin alone and in combination exhibited high intensity peak at ~1650 $cm^{-1}$and ~1638 cm^{-1} that are corresponding with an absorption peak of secondary structure of protein amide I (α-helix and β-sheet) and amide II (Naumann, 2000). The main spectra position ~1650 cm^{-1} is assigned to the C=O stretching vibrations of amide I group of proteins. The amide II band at ~1540 cm^{-1} represents the amide N-H deformation (Garip et al., 2009; Naumann, 2000; Naumann et al., 1991; Rodriguez-Saona et al., 2001). The variations of spectral patterns of ArSE after treatment with amoxicillin or quercetin alone and in combination were apparent and reflected how amoxicillin plus quercetin effects on ArSE.

Integral areas of absorbance between 3000-2800 and 1750-900 cm^{-1} used as estimate contents of fatty acid, ester, protein and nucleic acid are shown in Figure 4.25. After treatment with amoxicillin plus quercetin, lipid contents were reduced (p<0.01). The reduction of these band areas could be suggested a change in properties of cytoplasmic membrane or damage of cytoplasmic membrane (Bizani et al., 2005). Significantly increased of the protein (p<0.01) compared to control might be due to

bigger cell size after treatment (Lu et al., 2011). The peak at 1085 cm^{-1}, which represent nucleic acid was little decreased but not significantly (p<0.01). The changes in the nucleic acid region could indicate a reduction in viable counts, prevention of cell growth (Booyens and Thantsha, 2014).

The *S. pyogenes* strain was grown in BHIB medium in the presence of 0.13 µg/ml ceftazidime, ceftazidime at 0.10 µg/ml plus luteolin at 12 µg/ml, ceftazidime at 0.10 µg/ml plus baicalein at 24 µg/ml, and ceftazidime at 0.10 µg/ml plus quercetin at 3 µg/ml combination (¾ FIC) and examined by FT-IR microspectroscopy. Results are presented in Figures 4.26 to 4.38.

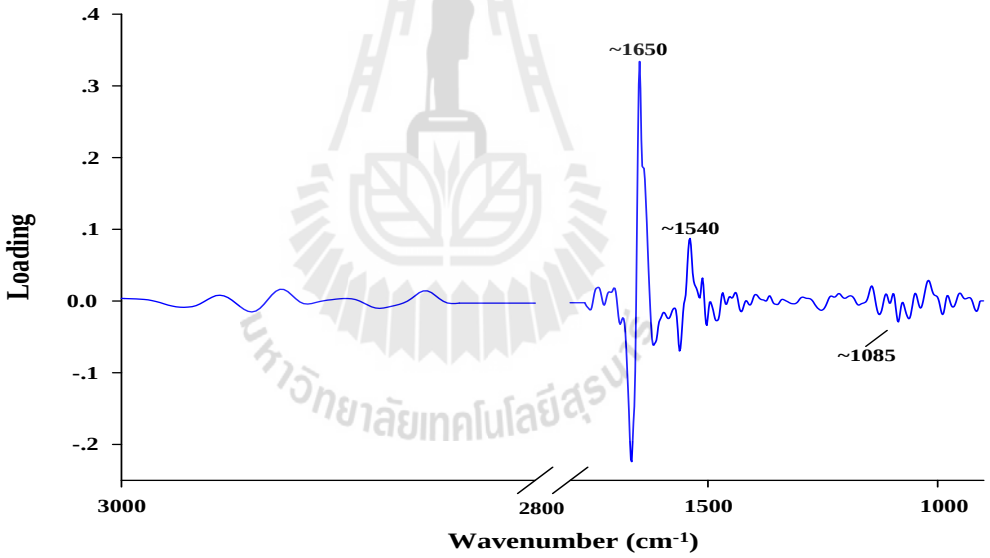

Figure 4.26 The loading plot of the PC1 obtained from PCA of SP untreated cell and after treatment with ceftazidime alone and in combination with baicalein.

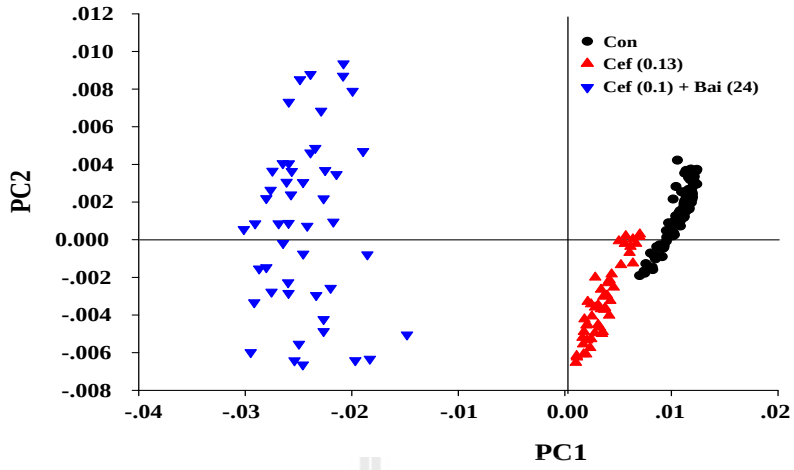

Figure 4.27 The PCA results constructed using spectra of SP after treatment with ceftazidime alone and in combination with baicalein. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Cef (0.1) + Bai (24) = 0.1 µg/ml ceftazidime plus 24 µg/ml baicalein. PCA analysis was constructed by using second derivative, vector normalized spectra, over the spectral ranges from 3000 - 2800 cm^{-1} and from 1750 - 900 cm^{-1}.

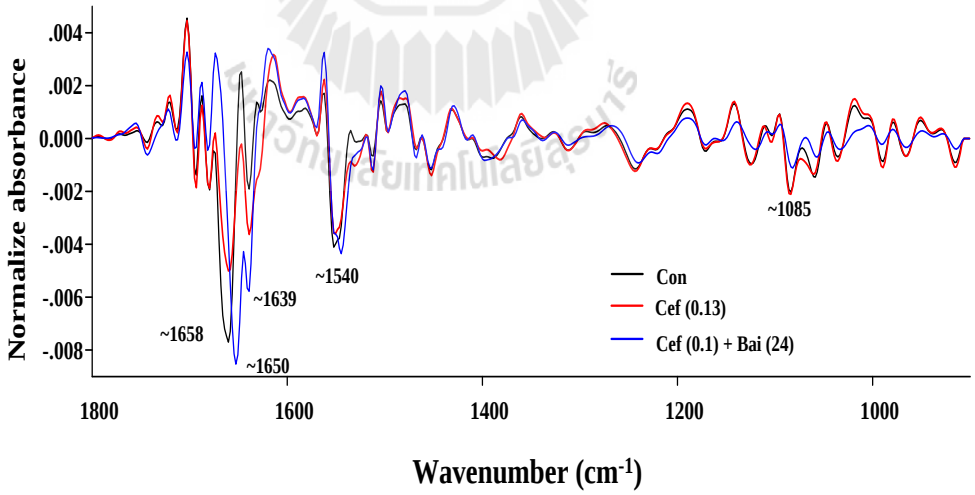

Figure 4.28 The 2nd derivative transformation spectra (~1750-900 cm^{-1}) of SP after treatment with ceftazidime alone and in combination with baicalein. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Cef (0.1) + Bai (24) = 0.1 µg/ml ceftazidime plus 24 µg/ml baicalein.

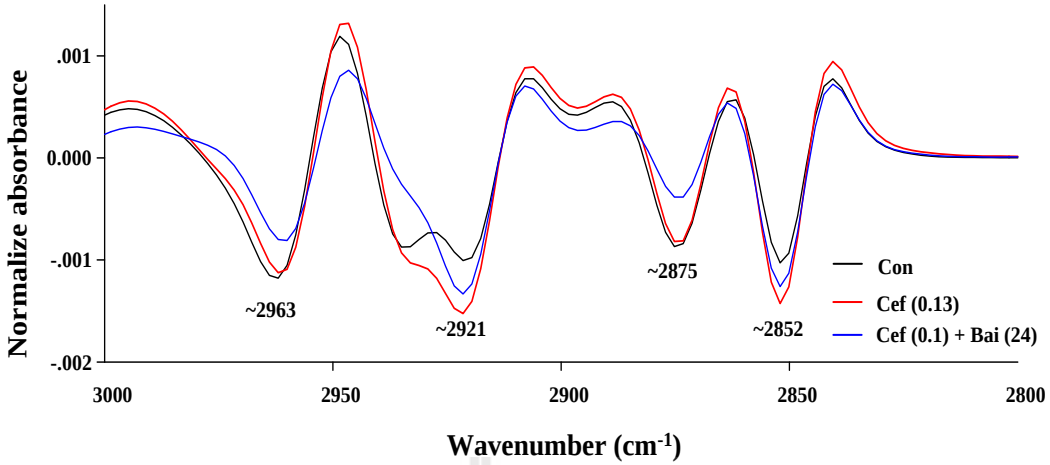

Figure 4.29 The 2nd derivative transformation spectra (~3000-2800 cm^{-1}) of SP after treatment with ceftazidime alone and in combination with baicalein. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Cef (0.1) + Bai (24) = 0.1 µg/ml ceftazidime plus 24 µg/ml baicalein.

Loading plot study of SP after treatment with ceftazidime alone and in combination with baicalein is shown in Figure 4.26. The major bands on the PC1can explain approximately 93% discrimination. PCA result also presents the separation of each group of treatment (Figure 4.27). The loading of PC1 indicates that obviously of 3 regions at ~1650, ~1639 and ~1540 cm^{-1} were assigned to the absorption peaks of the protein amide I and amide II, which separate cells after treatment with combination of ceftazidime plus baicalein from other groups. The ~1085 cm^{-1} was assigned to nucleic acid (stretching mode of P=O) of these treated groups. The results revealed that the higher intensity and area of protein amide I and II were observed in ceftazidime plus baicalein and higher intensity and area of nucleic acid on ceftazidime treated groups compared to others (Figure 4.28). The intensity and area of fatty acid region are shown in Figure 4.29. The intensity and integral area results exhibited that fatty acid and nucleic acid of this combination was significantly decreased (p<0.01)

compared to controls (Figure 4.38). This might be associated with a decrease in viable counts, cessation of cell growth (Booyens and Thantsha, 2014).

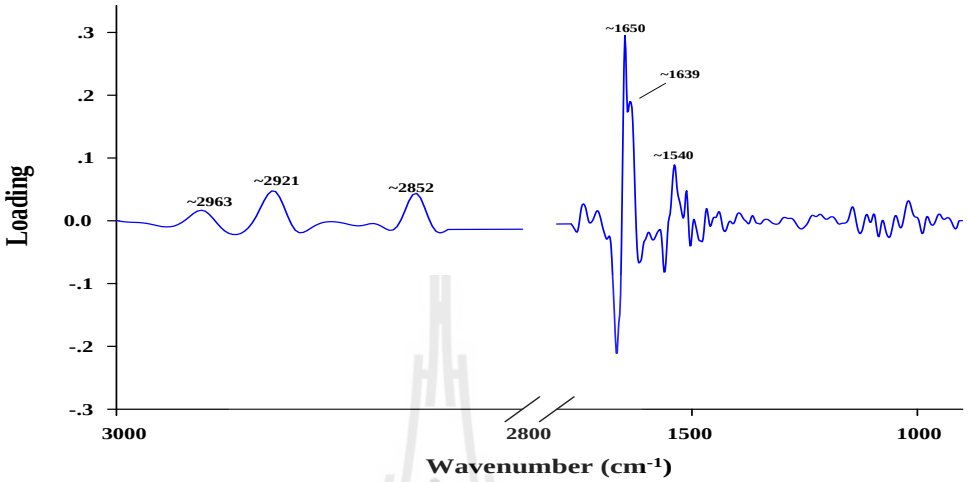

Figure 4.30 The loading plot of the PC1 obtained from PCA of SP untreated cell and after treatment with ceftazidime alone and in combination with luteolin.

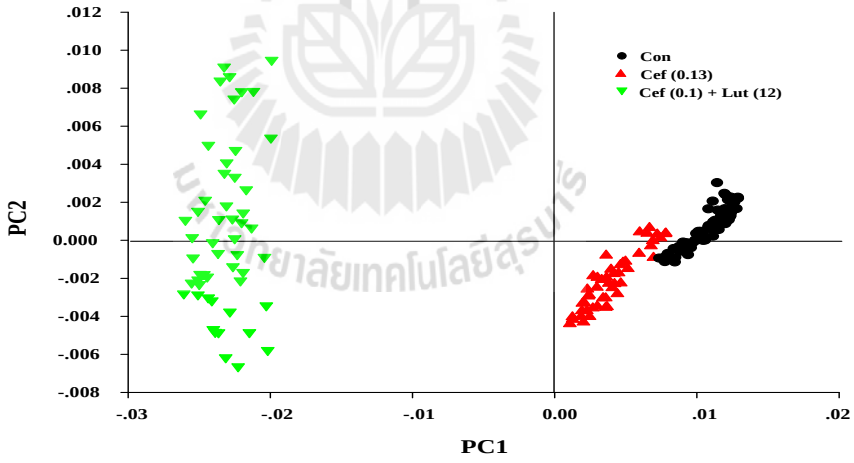

Figure 4.31 The PCA results constructed using spectra of SP after treatment with ceftazidime alone and in combination with luteolin. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Cef (0.1) + Lut (12) = 0.1 µg/ml ceftazidime plus 12 µg/ml luteolin. PCA analysis was constructed by using second derivative, vector normalized spectra, over the spectral ranges from 3000 - 2800 cm^{-1} and from 1750 - 900 cm^{-1}.

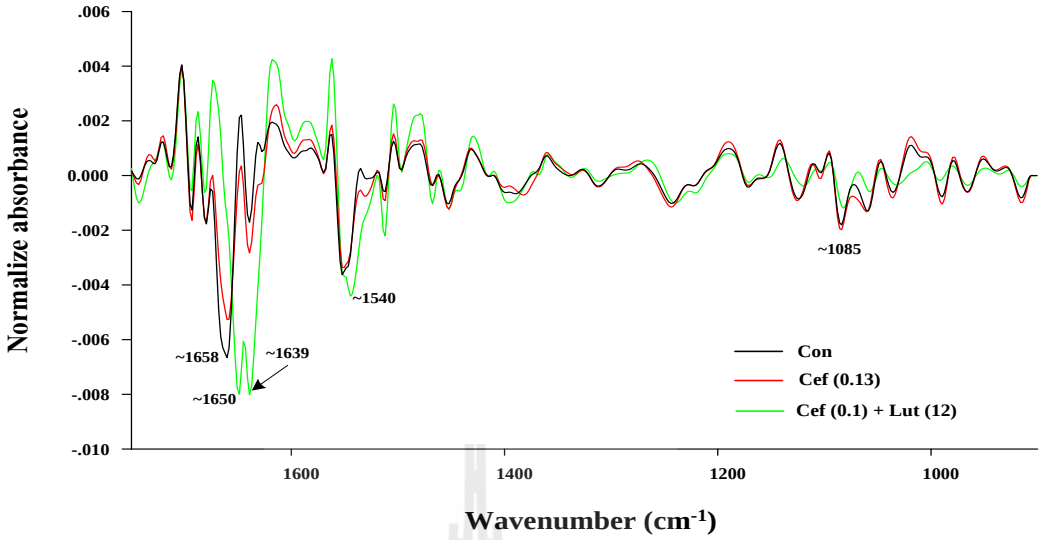

Figure 4.32 The 2nd derivative transformation spectra (~1750-900 cm^{-1}) of SP after treatment with ceftazidime alone and in combination with luteolin. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Cef (0.1) + Lut (12) = 0.1 µg/ml ceftazidime plus 12 µg/ml luteolin.

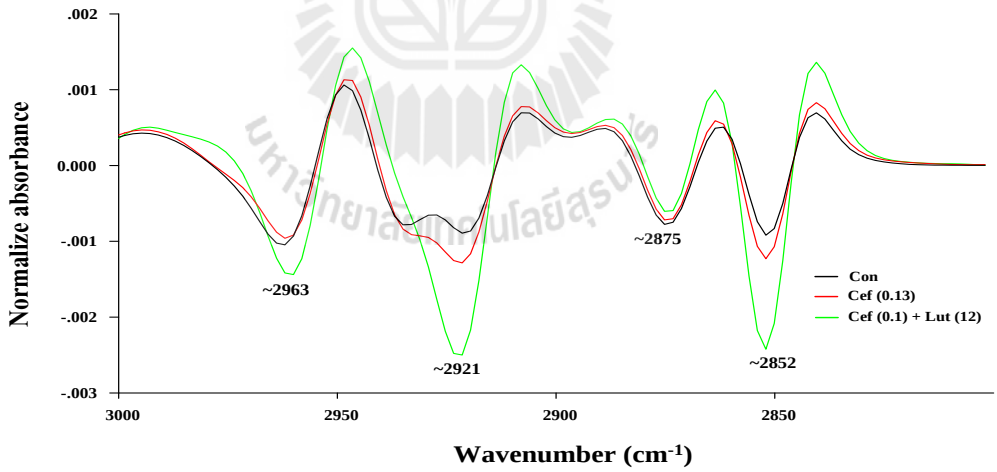

Figure 4.33 The 2nd derivative transformation spectra (~3000-2800 cm^{-1}) of SP after treatment with ceftazidime alone and in combination with luteolin. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Cef (0.1) + Lut (12) = 0.1 µg/ml ceftazidime plus 12 µg/ml luteolin.

The loading plot of SP after treatment with ceftazidime alone and in combination with luteolin and PCA are shown in Figure 4.30 and 4.31.

Loading PC1 displayed 94% isolation and was shown coefficient at ~2963, ~2923, ~2852 cm^{-1} and ~1650, ~1639, ~1540 cm^{-1} that were in correspondence to the fatty acid and protein intensity of SP after treatment with ceftazidime plus luteolin. These prominent peaks displayed separated cell after treatment with ceftazidime plus luteolin from other groups. The average spectrum of these is shown in Figure 4.32 and 4.33 that display high intensity and area in the region of protein and fatty acid. These results illustrated that the ceftazidime plus luteolin treated cells displayed significantly higher peak intensity and areas in fatty acid and protein but lower in nucleic acid areas compared with controls (p<0.01) (Figures 4.32, 4.33 and 4.38). The lipid alteration might indicate the conformation of the acyl chains of cytoplasmic membrane that is disordered or changed in properties of the cell membrane of treated cell (Scherber et al., 2009; Bizani et al., 2005). Moreover, the decrease in spectral intensity and areas of nucleic acid might associate with prevention of cell growth and induction of cell death (Booyens and Thantsha, 2014).

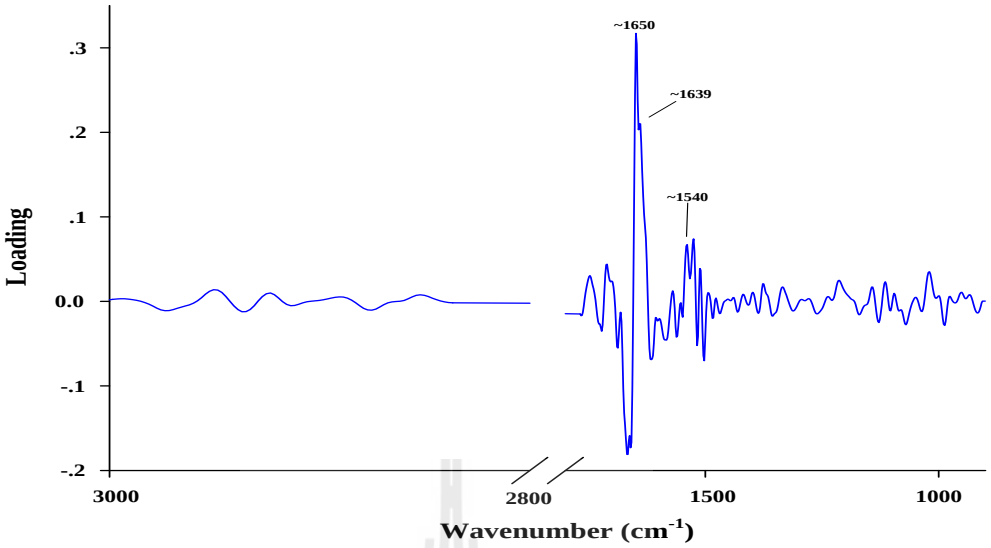

Figure 4.34 The loading plot of the PC1 obtained from PCA of SP untreated cell and after treatment with ceftazidime alone and in combination with quercetin.

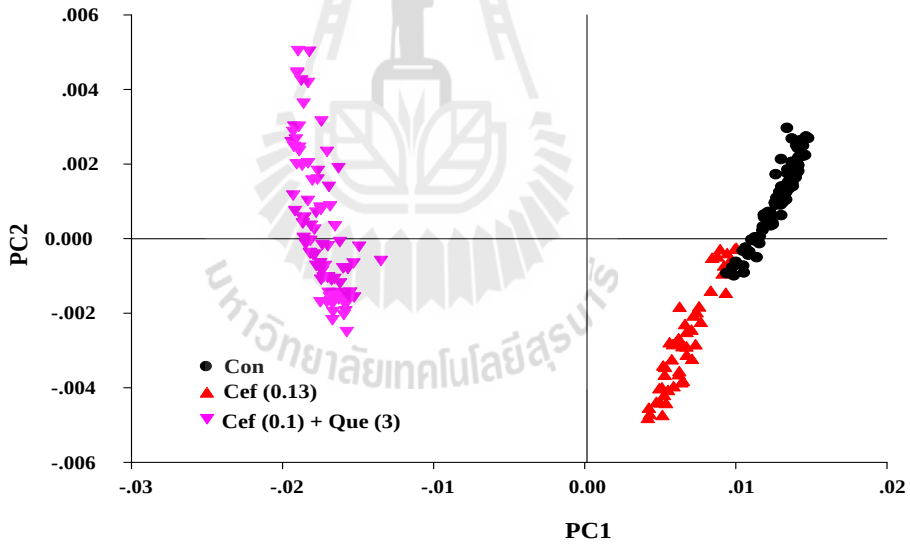

Figure 4.35 The PCA results constructed using spectra of SP after treatment with ceftazidime alone and in combination with quercetin. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Cef (0.1) + Que (3) = 0.1 µg/ml ceftazidime plus 3 µg/ml quercetin. PCA analysis was constructed by using second derivative, vector normalized spectra, over the spectral ranges from 3000 - 2800 cm^{-1} and from 1750 - 900 cm^{-1}.

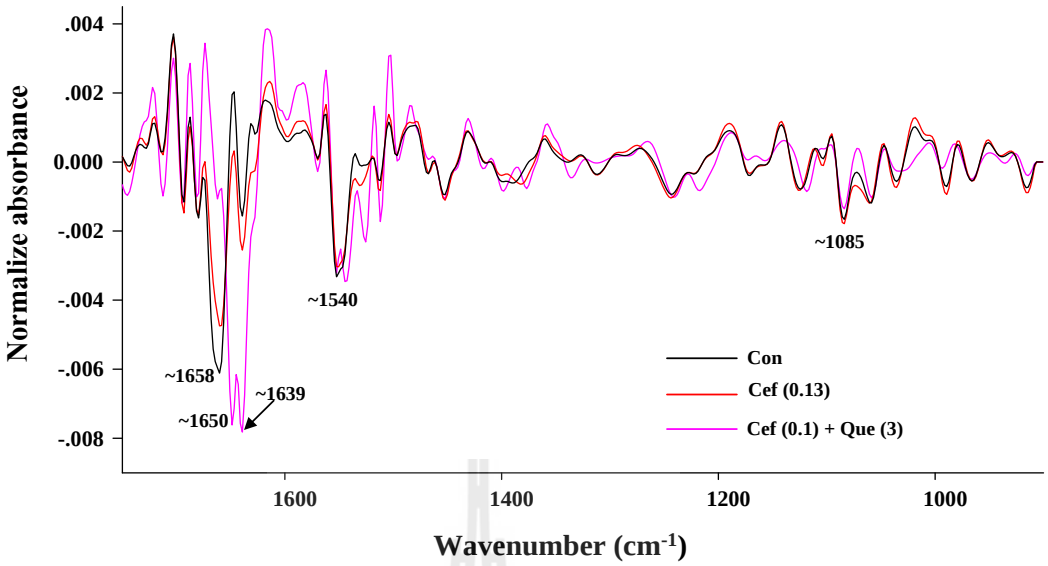

Figure 4.36 The 2nd derivative transformation spectra (~1750-900 cm^{-1}) of SP after treatment with ceftazidime alone and in combination with quercetin. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Cef (0.1) + Que (3) = 0.1 µg/ml ceftazidime plus 3 µg/ml quercetin.

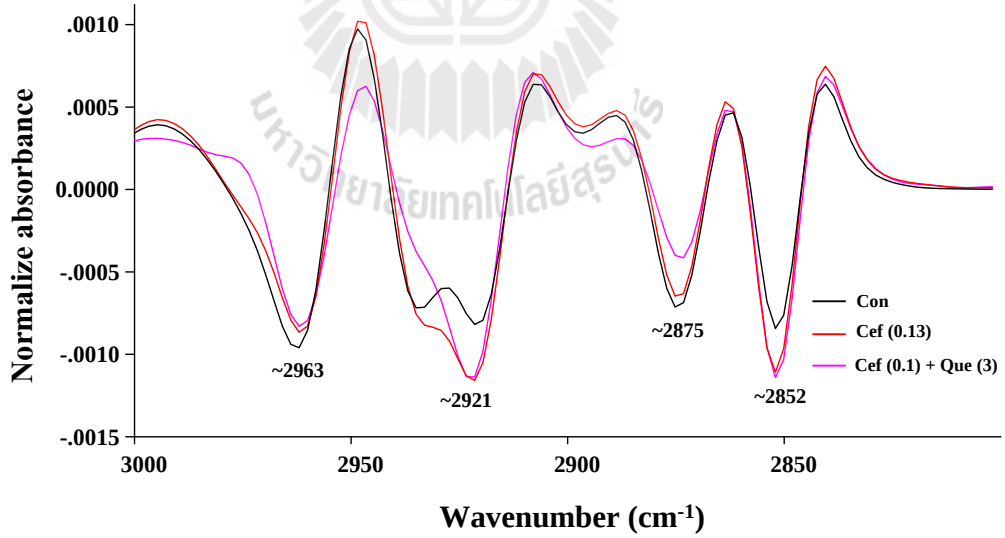

Figure 4.37 The 2nd derivative transformation spectra (~3000-2800 cm^{-1}) of SP after treatment with ceftazidime alone and in combination with quercetin. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Cef (0.1) + Que (3) = 0.1 µg/ml ceftazidime plus 3 µg/ml quercetin.

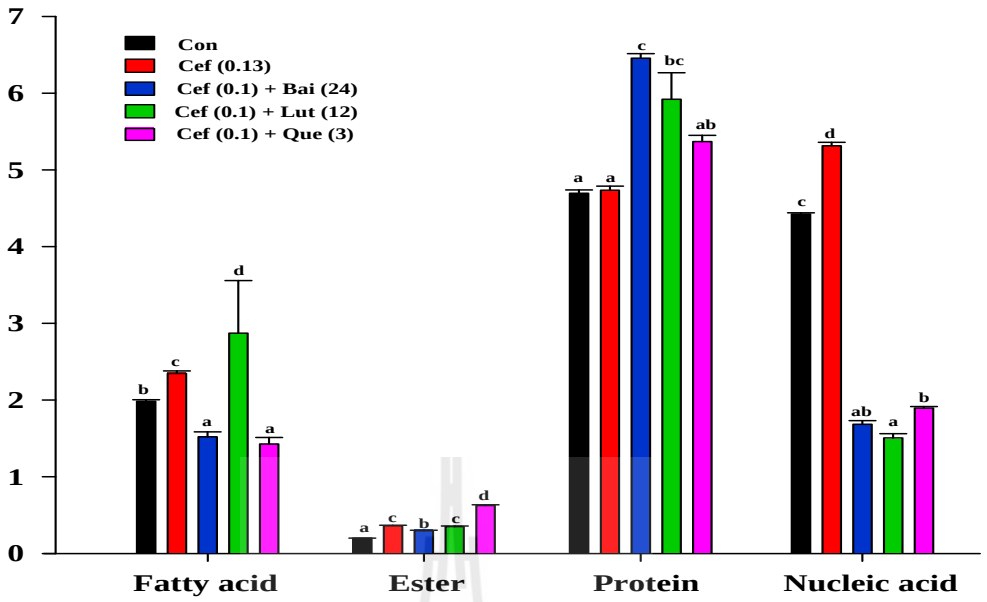

Figure 4.38 Alteration of biochemical structures and amount observed by peak position, intensity and areas of SP after treatment with ceftazidime alone or in combination with baicalein, luteolin and quercetin. Con = Control (drugs free), Cef (0.13) = 0.13 µg/ml ceftazidime, Cef (0.1) + Bai (24) = 0.1 µg/ml ceftazidime plus 24 µg/ml baicalein, Cef (0.1) + Lut (12) = 0.1 µg/ml ceftazidime plus 12 µg/ml luteolin, Cef (0.1) + Que (3) = 0.1 µg/ml ceftazidime plus 3 µg/ml quercetin. Means sharing the same superscript are not significantly different from each other (Turkey's HSD, a = $p<0.01$).

The biochemical components of SP after treatment with ceftazidime alone and in combination with quercetin showed clearly different. The loading plot and PCA are shown in Figure 4.34 and 4.35, respectively. The loading PC1 of SP after treatment with ceftazidime plus quercetin displayed 96% classification and PCA showed clear separation compared to other groups. The major spectrum differences were observed at position ~1650, ~1639, ~1540 cm^{-1} that were correspondence to the protein amide I and II (Figure 4.36). The high intensity of the average spectral region of fatty acid is

shown in Figure 4.37. These results presented that the ceftazidime plus quercetin appeared significantly lower peak intensity and areas of fatty acid and nucleic acid while higher of protein in comparison with the control (p<0.01) (Figures 4.36 to 4.38). These changes might associate with reduction of cell viability (Lu et al., 2011).

Our findings provide evidence that both ArSE and SP treated with amoxicillin plus quercetin and ceftazidime plus quercetin respectively display entirely the significant reduction of fatty acid and nucleic acid peak intensity and areas whereas an increase in protein compared with controls (p<0.01).

The integrated peak intensity and area of biomolecules shows correlation between bacterial cell damage after treatment with combination of antibiotic plus flavonoids and change of bimolecular structure and content. Our findings suggest that the selected flavonoids in combination with amoxicillin or ceftazidime significantly alter nucleic acid, fatty acid and protein of these treated cells. Obviously, the regions of the protein amide I and II were shifted after treatment the SP with ceftazidime plus selected flavonoids. These results imply that the conformation of the protein structure of amide was changed (Al-Qadiri, et al., 2008). In addition, these findings are in substantial agreement with those of Lu and coworkers that the bacterial cells storage at 4°C showed significantly decrease in the band area of lipid content compared to controls. This may associate with a decrease in viable counts or cell death (Lu et al., 2011). So, the flavonoids may effect on phospholipid in cytoplasmic membrane or fatty acid synthesis results in reduction in the viability of the cells. Furthermore, the nucleic acid contents are reduced by these combinations may be because of cell wall damage and nucleic acid inside the cells is leaked (Booyens and Thantsha, 2014).

CHAPTER V

CONCLUSION

The aims of this research were to investigate the inhibitory and synergistic effect of tested flavonoids using singly and combined with β-lactam antibiotic drugs on drug-resistant bacteria, ArSE and SP. Then, the combinations that showed synergistic activity were chosen to investigate the elementary mechanism of action. The bacterial suspension standard curves were performed to determine actual bacterial numbers. The MICs, checkerboard assay, and viability curves were operated to test the sensitivity and synergistic effect of the combinations. The cytoplasmic membrane permeability, enzyme assay, TEM, and FT-IR microspectroscopy were performed to determine the primary mechanism of action.

Flavonoids have inhibitory activity against a variety of bacteria. The structure of flavonoids was isolated and identified. Many researchers described that flavonoids possessed antibacterial activity, including quercetin, baicalein and luteolin (Brown et al., 2011; Hossion et al., 2010).

The MIC results revealed that these testing ArSE strains were resistant to amoxicillin alone because of the standard value of the sensitivity of amoxicillin against this strain is ≤0.25 μg/ml (Matthew et al., 2013). As well as, luteolin, quercetin and baicalein demonstrated little bacteriostatic effect against these strains. However, the ceftazidime was still sensitive to SP because of the standard value of the

sensitivity of cefotaxime, which is also third-generation cephalosporins, against this strain is ≤0.50 µg/ml (Matthew et al., 2013). Whereas, luteolin, quercetin and baicalein demonstrated little bacteriostatic effect against this strain with MICs 128, 128 and >256 µg/ml, respectively.

The checkerboard determination revealed synergistic effects of amoxicillin plus quercetin against ArSE strain with FIC index at 0.25 (Wagner and Ulrich-Merzenich, 2009). Also, this checkerboard determination exhibited synergistic effects of ceftazidime plus all tested flavonoids (luteolin, quercetin or baicalein) against SP strain with FIC indexes between <0.63 to 0.63 (Wagner and Ulrich-Merzenich, 2009). The killing curve results were also confirmed synergistic effect of amoxicillin plus quercetin against ArSE by reduction of ≥2 log10 cfu/ml compared to amoxicillin alone treatment.

Naturally, the isolated *S. epidermidis* strains can produce β-lactamase (Baldwin et al., 1969) and the result from enzyme assay found that quercetin, luteolin and baicalein had an inhibitory activity against β-lactamase type IV from *E. cloacae.* Hence, these results provide evidence that one mode of action of these tested flavonoids against ArSE may involve in β-lactamase inhibition and may cause reverse the resistance of bacterial strains to its sensitivity to an antibiotic (Eumkeb et al., 2012). Although, whether *S. pyogenes* produces β-lactamase or not, previous study exhibited that the β-lactamase produced by other bacteria in the pharynx could potentially inactivate the penicillin, resulting in increased treatment failures or infection relapses (Reed et al., 1991). Besides, additional previous research revealed that amoxicillin alone therapy failed to eliminate *S. pyogenes* from a wound infection in the presence of a β-lactamase-producing strain of *S. aureus* and suggested the

potential of β-lactamase inhibitor combination in the treatment of mixed bacterial skin infections involving β-lactamase producing organisms (Boon and Beale, 1987). Moreover, β-lactamase producing bacteria (BLPB) may not have only survived penicillin therapy but can also have protected other penicillin susceptible bacteria from penicillin by releasing the free enzyme into their environment (Brook, 2009). So, these findings provide evidence that baicalein, luteolin and quercetin in combination with β-lactam antibiotic may be useful to inhibit mixed β-lactamase producing bacteria and *S. pyogenes* in oropharyngeal infections.

The CM permeability revealed that quercetin plus amoxicillin increased cytoplasmic membrane permeability of ArSE strain. In the same way, the CM permeability of SP exhibited that luteolin, quercetin and baicalein combined with ceftazidime significantly dramatic increased CM permeability compared to controls ($p<0.01$). The increase in CM permeability may be one of the synergistic actions of these combinations against both ArSE and SP strains. This result could be explained that the formation of pores in the plasma membrane might be disrupted (Zhou et al., 2008).

The TEM results of amoxicillin plus quercetin treated cells demonstrated that these cells exhibited marked morphological damage, clear peptidoglycan and cytoplasmic membrane damage and average cell areas significantly bigger than controls. As well as, TEM results of SP cells after exposure to ceftazidime plus tested flavonoids exhibited that cell division of around 70-80% of these cells were interrupted lead to twisted and irregular cell shape, revealed peptidoglycan and cytoplasmic membrane damage. Clearly, the average cell areas of these ceftazidime combination treated cells were larger than other groups including controls. These

results can be explained by assuming that these tested flavonoids may insert synergistic action with β-lactam antibiotics to inhibit peptidoglycan synthesis leads to marked morphological damage and delay cell division.

In general, previous findings revealed that the bactericidal effect of chlorine caused changes in the second derivative spectra because of alteration in bacterial ester functional groups of lipids, structural proteins and injured bacterial cells (Al-Qadiri, et al., 2008). Our FT-IR microspectroscopy results exhibited that ArSE cells treated with quercetin either alone or in combination with amoxicillin decreased fatty acid and nucleic acid, but increased protein amide I and amide II in bacterial cells compared to control. These results are in substantial agreement with the previous finding that the effect of baicalein or quercetin plus ceftazidime also caused a decrease in fatty acid and nucleic acid in SP (Booyens and Thantsha, 2014). These results could affect the prevention of cell growth. The combination of ceftazidime plus either quercetin or baicalein against SP are inconsistent with amoxicillin plus quercetin against ArSE that these combinations decreased fatty acid and nucleic acid despite increased protein amide I and II, whereas luteolin plus ceftazidime increased fatty acid compared to control. These findings could be explained that the combination of quercetin or baicalein plus β-lactam antibiotics cause disruption on membrane function or increase of membrane fluidity resulting from the conformation of acyl chains disorder of cell membrane phospholipid (Alverez-Ordonez et al., 2010; Bizani et al., 2005). Moreover, SP treated with a combination of flavonoids and ceftazidime showed that the proteins were shifted between amide I of α-helical structures and amide I of β-pleated sheet. These results suggest that the combination of flavonoids and ceftazidime may affect protein structure on SP (Beekes et al., 2007).

These results lend us to believe that these tested flavonoids (luteolin, baicalein and quercetin) in combination with β-lactam antibiotics may affect the content of fatty acid chains on the various membrane amphiphiles, resulting in cytoplasmic membrane damage, increase in cytoplasmic membrane permeabilization and release of nucleic acid to cell envelope (Scherber et al., 2009; Booyens et al., 2014).

In summary, this study provides evidence that quercetin, luteolin and baicalein have the extraordinary potential to reverse bacterial resistance to originate traditional drug susceptibility of it. This is the first report of the mechanism of synergistic action of the flavones and flavonol plus β-lactam antibiotic combination against ArSE and SP using FT-IR microspectroscopy. Four modes of actions would be implied that these combinations; (1) inhibit peptidoglycan synthesis, resulting in morphological damage, (2) inhibit β-lactamases activity, (3) increase CM permeability and (4) change biomolecular molecules such as fatty acid, protein amide I and II and nucleic acid in bacterial cells. Naturally, these tested flavonoids have restricted, limited toxicity. So, these flavonoids propose the high potential to develop a useful of novel adjunct phytopharmaceutical to β-lactam antibiotics for the treatment of ArSE and SP. Future studies should be investigated and confirmed in animals test or in humans, Also, the synergistic effect on blood and tissue would be evaluated and achieved.

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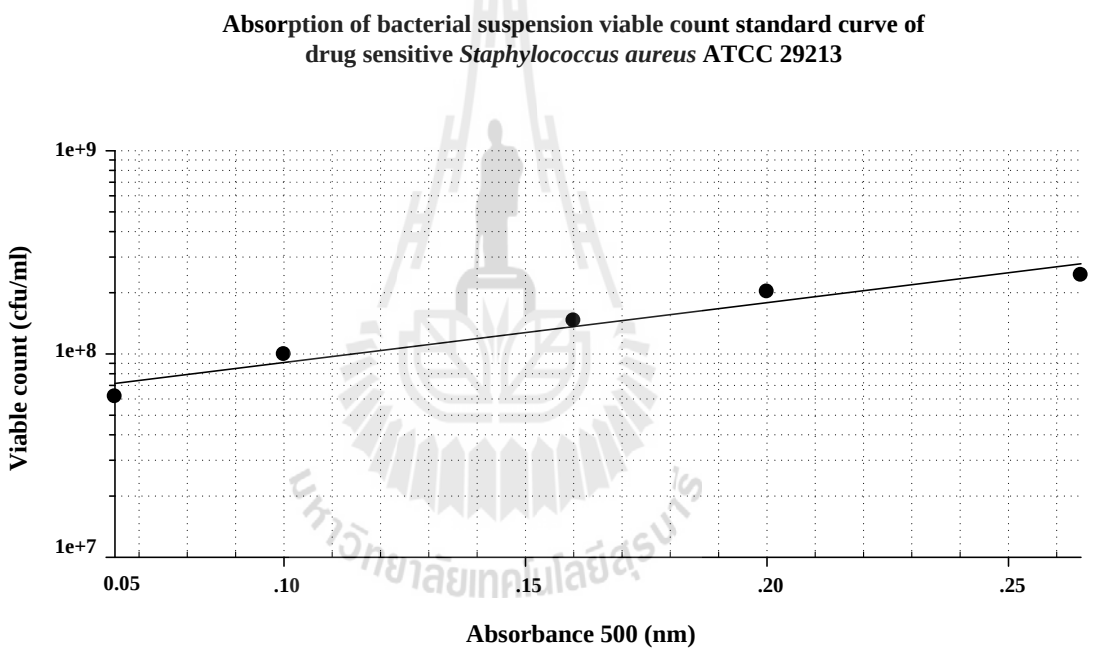
APPENDICES

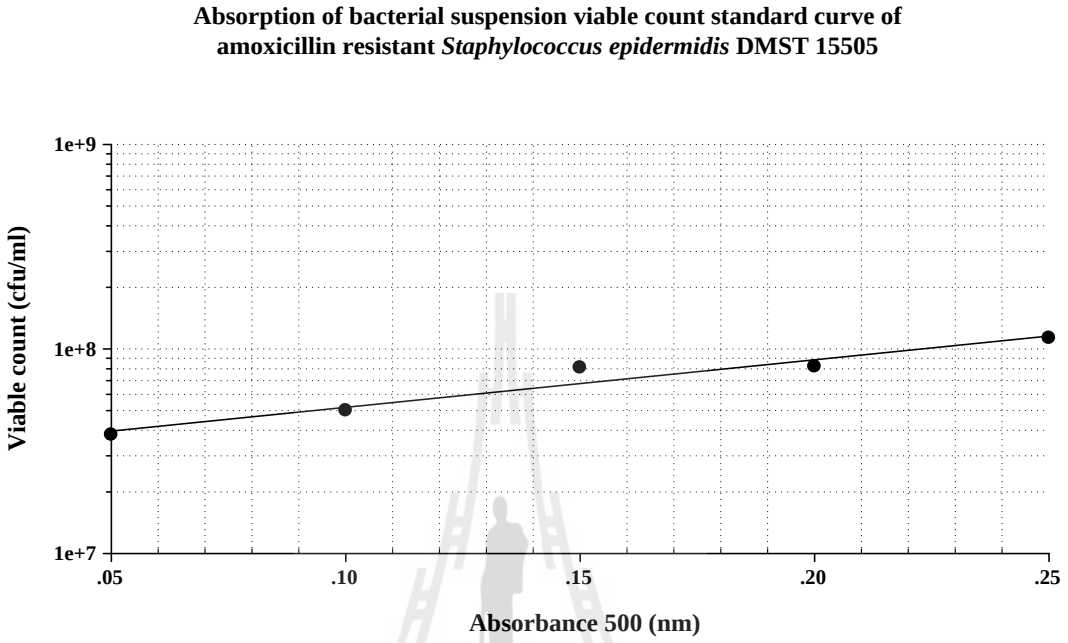
APPENDIX A

BACTERIAL SUSPENSION STANDARD CURVE

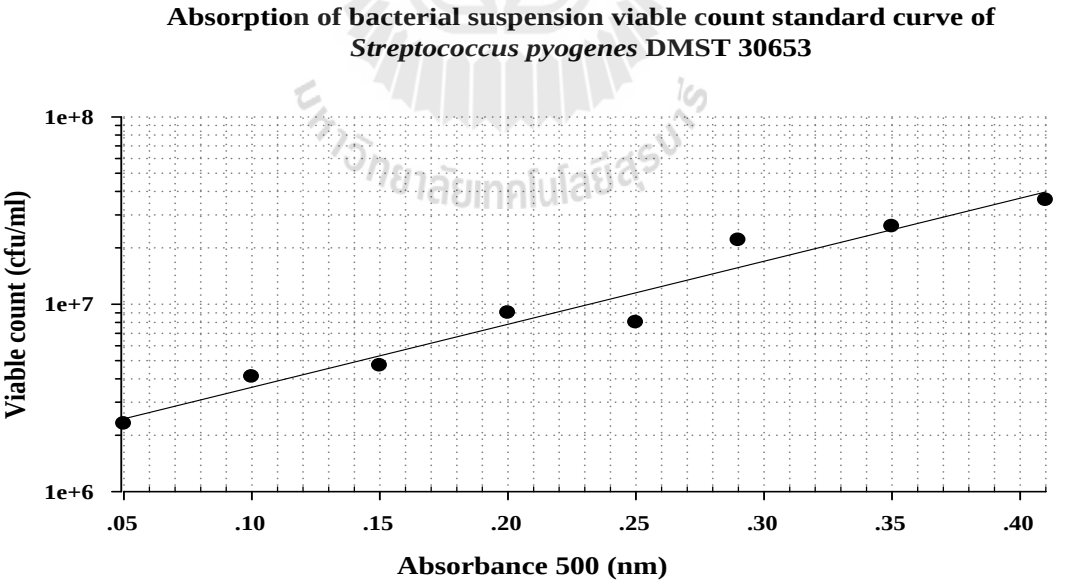
A1. Standard curve of suspension of drug sensitive *Staphylococcus aureus* ATCC 29213



A2. The standard curve of suspension of amoxicillin-resistant *Staphylococcus epidermidis* DMST 15505



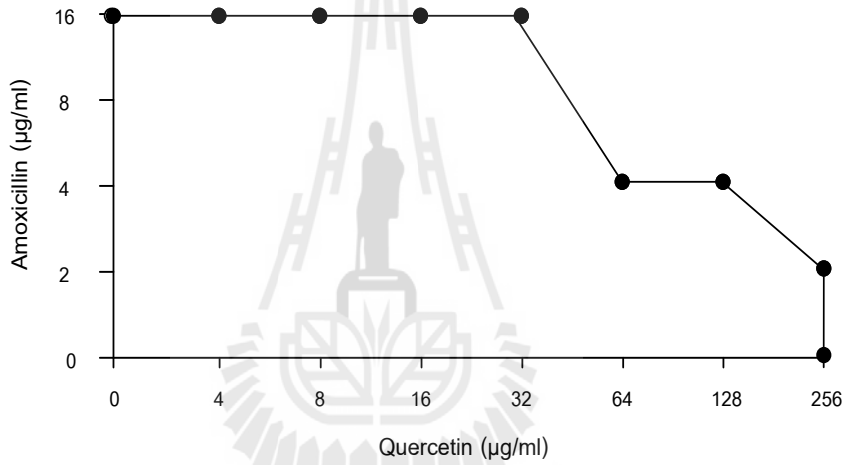
A3. The standard curve of suspension of *Streptococcus pyogenes* DMST 30653



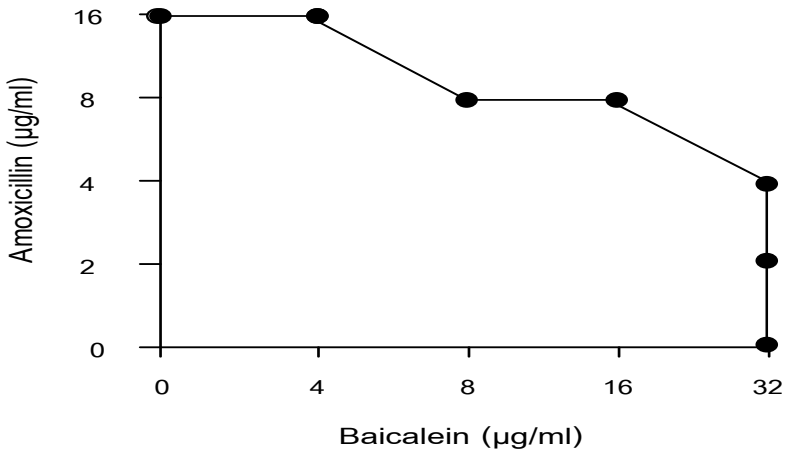
APPENDIX B

CHECKERBOARD ASSAY RESULTS

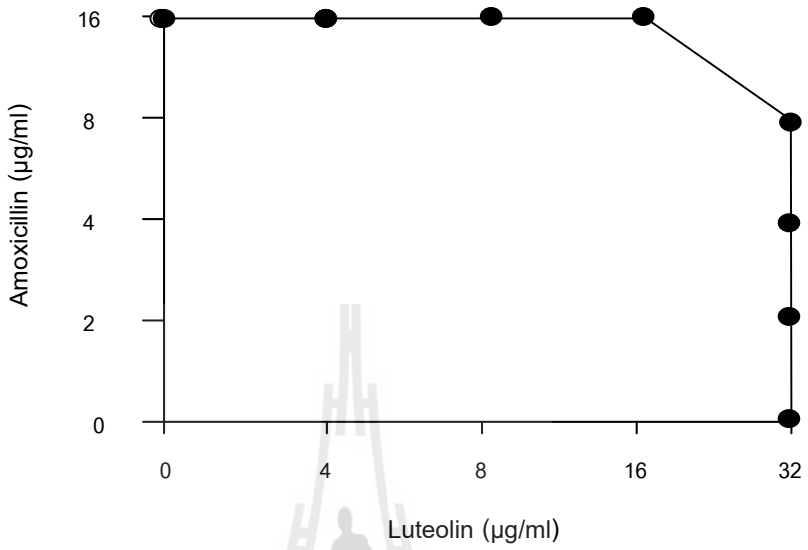
B1. Isobologram constructed from checkerboard MIC data showing a combination of amoxicillin plus quercetin against ArSE.



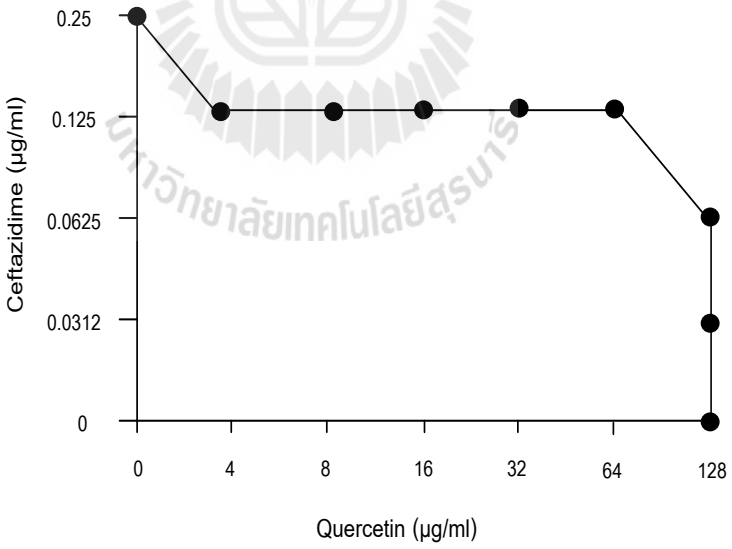
B2. Isobologram constructed from checkerboard MIC data showing a combination of amoxicillin plus baicalein against ArSE.



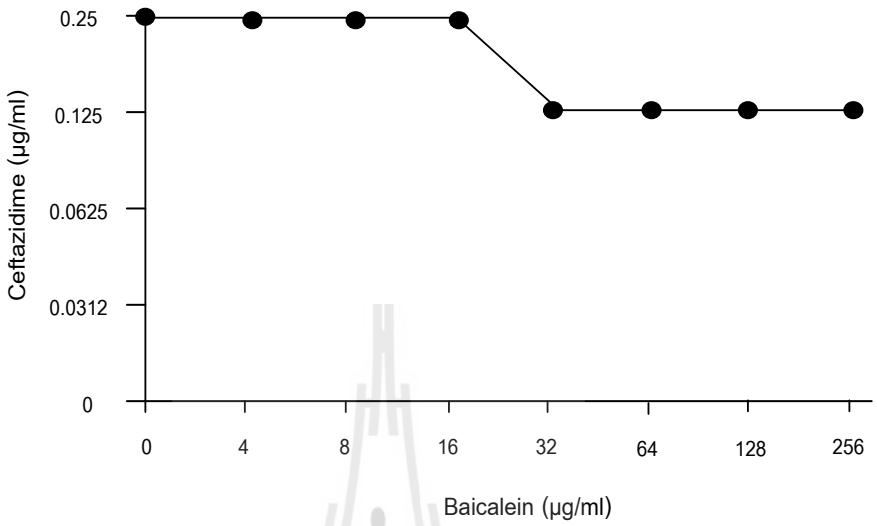
B3. Isobologram constructed from checkerboard MIC data showing a combination of amoxicillin plus luteolin against ArSE.



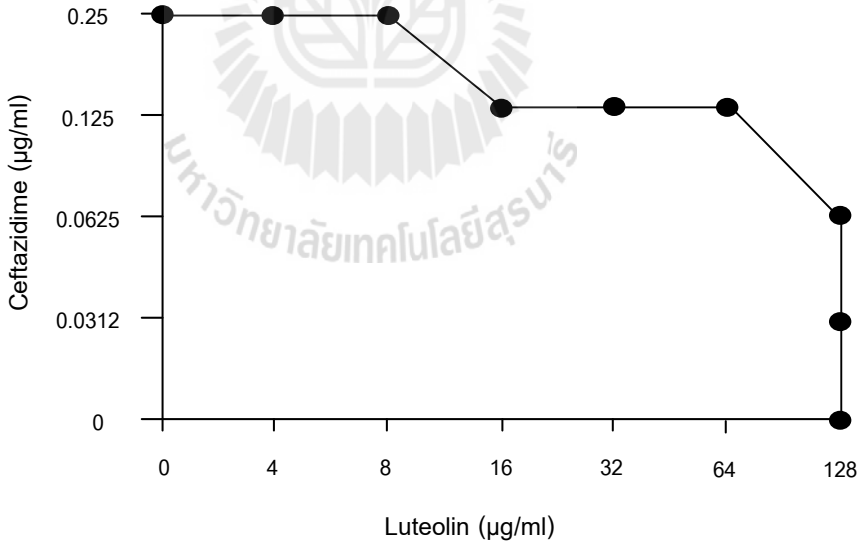
B4. Isobologram constructed from checkerboard MIC data showing a combination of ceftazidime plus quercetin against SP.



B5. Isobologram constructed from checkerboard MIC data showing a combination of ceftazidime plus baicalein against SP.



B6. Isobologram constructed from checkerboard MIC data showing combinations of luteolin plus luteolin against SP.



APPENDIX C

PREPARATION OF REAGENTS

C1. Nutrient agar

The Difco nutrient agar was used for cultivation of stock culture of *Staphylococcus* sp. on agar slopes.

The formula was:	Gms / Litre
Beef extract	3
Peptone	5
Agar	15

C2. Mueller-Hinton agar

Himedia Mueller-Hinton agar was used for cultivation of *Staphylococcus* sp. colony to determination of bacterial viability curve.

The formula was:	Gms / Litre
Beef, infusion from	300
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17

C3. Mueller-Hinton broth

Himedia Mueller-Hinton broth was recommended to determine the susceptibility of *Staphylococcus* sp. to antibacterial agents.

The formula was:	Gms / Litre
Beef, infusion from	300
Casein acid hydrolysate	17.5
Starch	1.5

Final pH 7.4±0.2 at 25°C

Calcium and magnesium ion concentrations are adjusted to provide the amounts recommended by CLSI to give the correct MIC values.

The formula was:	Gms/Litre
Sodium chloride	5
Disodium phosphate	2.5
Agar	15

Final pH 7.4±0.2 at 25°C

C4. Brain Hearth Infusion agar

Himedia Brain Hearth Infusion agar was used for cultivation of *S. pyogenes* to determination of bacterial viability curve.

The formula was:	Gms / Litre
Calf brain, infusion from	200
Beef heart, infusion from	250
Proteose peptone	10
Dextrose	2
Sodium chloride	5

Disodium phosphate	2.5
Agar	17

C5. Brain Hearth Infusion broth

Himedia Brain Hearth Infusion broth was recommended to determine the susceptibility of bacterial to antibacterial agents

The formula was:	Gms / Litre
Calf brain, infusion from	200
Beef heart, infusion from	250
Proteose peptone	10
Dextrose	2
Sodium chloride	5
Disodium phosphate	2.5

Final pH 7.4±0.2 at 25°C

C6. Chemicals

All chemicals used were laboratory grade otherwise specified.

Dimetheyl sulphoxide 99.5%	AR grade
Sodium chloride	AR grade
95% Ethanol	Lab grade
99.9% Ethanol	AR grade
Amoxicillin	AR grade
Penicillin	AR grade
Ceftazidime	AR grade
Nisin	AR grade
Apigenin	AR grade

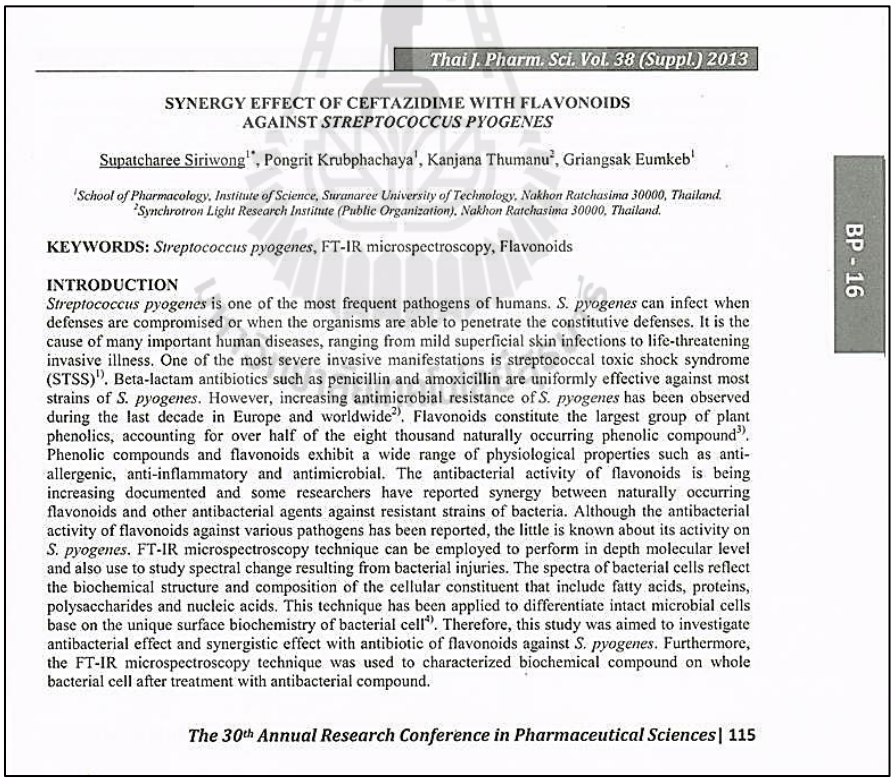
Baicalein	AR grade
Luteolin	AR grade
Kaempferol	AR grade
Quercetin	AR grade
Mg++	Lab grade
Ca++	Lab grade

APPENDIX D

PROCEEDINGS AND RESEARCH ARTICLES

D1. Proceedings

1) Siriwong S, Krubphachaya P, Thumanu K, Eumkeb G. 2013. Synergy effect of ceftazidime with flavonoids against *Streptococcus Pyogenes.* In: Proceedings of the 30th Annual Research Conference in Pharmaceutical Sciences. Abstract Book (Poster presentation, BP-16/pp115-118.) 6-8 December 2013, Bangkok, Thailand: Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.



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SYNERGY EFFECT OF CEFTAZIDIME WITH FLAVONOIDS AGAINST *STREPTOCOCCUS PYOGENES*

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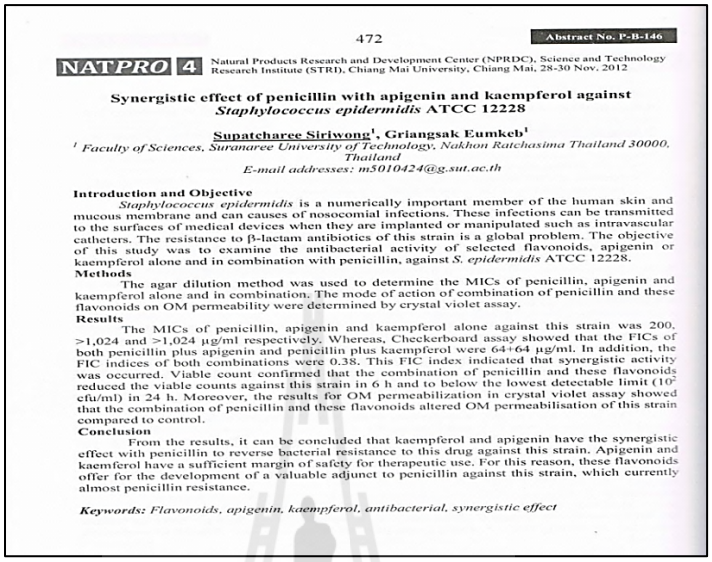
KEYWORDS: *Streptococcus pyogenes*, FT-IR microspectroscopy, Flavonoids

BP - 16

INTRODUCTION

Streptococcus pyogenes is one of the most frequent pathogens of humans. *S. pyogenes* can infect when defenses are compromised or when the organisms are able to penetrate the constitutive defenses. It is the cause of many important human diseases, ranging from mild superficial skin infections to life-threatening invasive illness. One of the most severe invasive manifestations is streptococcal toxic shock syndrome (STSS)[1]. Beta-lactam antibiotics such as penicillin and amoxicillin are uniformly effective against most strains of *S. pyogenes*. However, increasing antimicrobial resistance of *S. pyogenes* has been observed during the last decade in Europe and worldwide[2]. Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compound[3]. Phenolic compounds and flavonoids exhibit a wide range of physiological properties such as anti-allergenic, anti-inflammatory and antimicrobial. The antibacterial activity of flavonoids is being increasing documented and some researchers have reported synergy between naturally occurring flavonoids and other antibacterial agents against resistant strains of bacteria. Although the antibacterial activity of flavonoids against various pathogens has been reported, the little is known about its activity on *S. pyogenes*. FT-IR microspectroscopy technique can be employed to perform in depth molecular level and also use to study spectral change resulting from bacterial injuries. The spectra of bacterial cells reflect the biochemical structure and composition of the cellular constituent that include fatty acids, proteins, polysaccharides and nucleic acids. This technique has been applied to differentiate intact microbial cells base on the unique surface biochemistry of bacterial cell[4]. Therefore, this study was aimed to investigate antibacterial effect and synergistic effect with antibiotic of flavonoids against *S. pyogenes*. Furthermore, the FT-IR microspectroscopy technique was used to characterized biochemical compound on whole bacterial cell after treatment with antibacterial compound.

2) Siriwong, S. and Eumkeb, G. 2012. Synergistic effect of penicillin with apigenin and kaempferol against Penicillin-resistant *Staphylococcus epidermidis*. Natural Products Research and Development Center (NATPRO4) Chiang Mai Thailand.



472 Abstract No. P-B-146

NATPRO 4 Natural Products Research and Development Center (NPRDC), Science and Technology Research Institute (STRI), Chiang Mai University, Chiang Mai, 28-30 Nov. 2012

Synergistic effect of penicillin with apigenin and kaempferol against *Staphylococcus epidermidis* ATCC 12228

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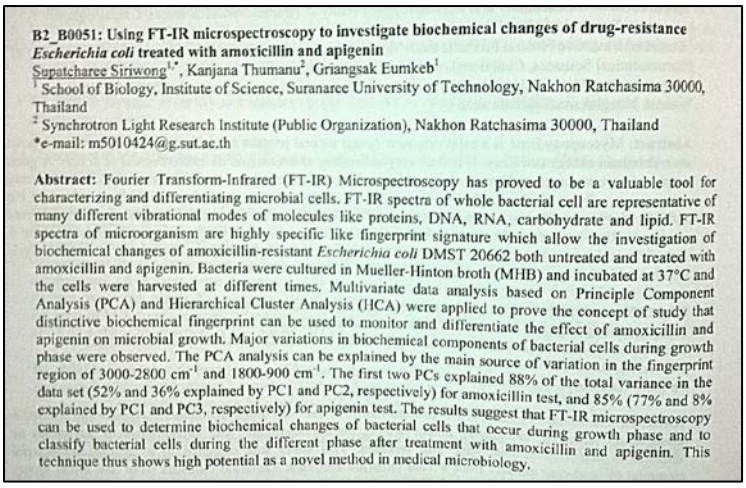
Introduction and Objective
Staphylococcus epidermidis is a numerically important member of the human skin and mucous membrane and can causes of nosocomial infections. These infections can be transmitted to the surfaces of medical devices when they are implanted or manipulated such as intravascular catheters. The resistance to β-lactam antibiotics of this strain is a global problem. The objective of this study was to examine the antibacterial activity of selected flavonoids, apigenin or kaempferol alone and in combination with penicillin, against *S. epidermidis* ATCC 12228.
Methods
The agar dilution method was used to determine the MICs of penicillin, apigenin and kaempferol alone and in combination. The mode of action of combination of penicillin and these flavonoids on OM permeability were determined by crystal violet assay.
Results
The MICs of penicillin, apigenin and kaempferol alone against this strain was 200, >1,024 and >1,024 µg/ml respectively. Whereas, Checkerboard assay showed that the FICs of both penicillin plus apigenin and penicillin plus kaempferol were 64+64 µg/ml. In addition, the FIC indices of both combinations were 0.38. This FIC index indicated that synergistic activity was occurred. Viable count confirmed that the combination of penicillin and these flavonoids reduced the viable counts against this strain in 6 h and to below the lowest detectable limit (10^2 efu/ml) in 24 h. Moreover, the results for OM permeabilization in crystal violet assay showed that the combination of penicillin and these flavonoids altered OM permeabilisation of this strain compared to control.
Conclusion
From the results, it can be concluded that kaempferol and apigenin have the synergistic effect with penicillin to reverse bacterial resistance to this drug against this strain. Apigenin and kaemferol have a sufficient margin of safety for therapeutic use. For this reason, these flavonoids offer for the development of a valuable adjunct to penicillin against this strain, which currently almost penicillin resistance.

Keywords: *Flavonoids, apigenin, kaempferol, antibacterial, synergistic effect*

3) Siriwong, S., Thumanu, K. and Eumkeb, G. 2010. Using FT-IR microspectroscopy to investigate biochemical changes of drug-resistance *Escherichia coli* treated with amoxicillin and apigenin. In: Proceeding of the 36th Congress on Science and Technology of Thailand (STT36). Abstract Book (Oral presentation, B2_0051/pp70.) 26-28 October 2010, Bangkok, Thailand: Bangkok International Trade & Exhibition Centre (BITEC), Thailand.



B2_B0051: Using FT-IR microspectroscopy to investigate biochemical changes of drug-resistance *Escherichia coli* treated with amoxicillin and apigenin

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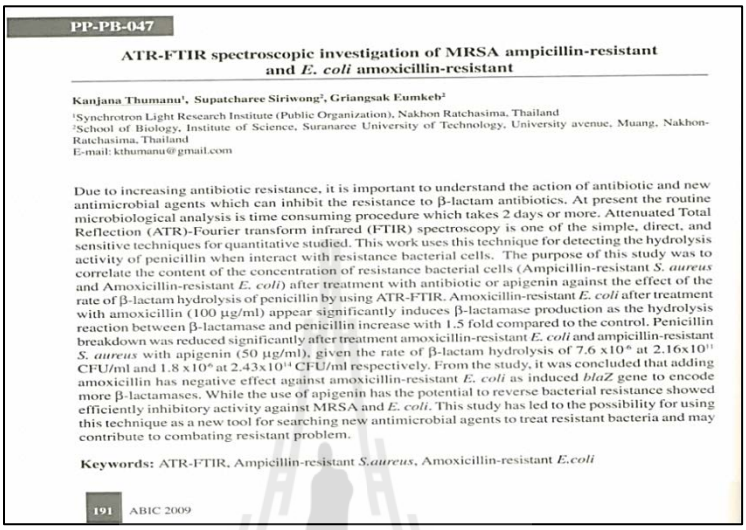
Abstract: Fourier Transform-Infrared (FT-IR) Microspectroscopy has proved to be a valuable tool for characterizing and differentiating microbial cells. FT-IR spectra of whole bacterial cell are representative of many different vibrational modes of molecules like proteins, DNA, RNA, carbohydrate and lipid. FT-IR spectra of microorganism are highly specific like fingerprint signature which allow the investigation of biochemical changes of amoxicillin-resistant *Escherichia coli* DMST 20662 both untreated and treated with amoxicillin and apigenin. Bacteria were cultured in Mueller-Hinton broth (MHB) and incubated at 37°C and the cells were harvested at different times. Multivariate data analysis based on Principle Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were applied to prove the concept of study that distinctive biochemical fingerprint can be used to monitor and differentiate the effect of amoxicillin and apigenin on microbial growth. Major variations in biochemical components of bacterial cells during growth phase were observed. The PCA analysis can be explained by the main source of variation in the fingerprint region of 3000-2800 cm^{-1} and 1800-900 cm^{-1}. The first two PCs explained 88% of the total variance in the data set (52% and 36% explained by PC1 and PC2, respectively) for amoxicillin test, and 85% (77% and 8% explained by PC1 and PC3, respectively) for apigenin test. The results suggest that FT-IR microspectroscopy can be used to determine biochemical changes of bacterial cells that occur during growth phase and to classify bacterial cells during the different phase after treatment with amoxicillin and apigenin. This technique thus shows high potential as a novel method in medical microbiology.

4) Thumanu, K., Siriwong, S. and Eumkeb, G. 2009. ATR-FTIR Spectroscopic investigation of MRSA ampicillin-resistant and *E.coli* amoxicillin-resistant. Agricultural Biotechnology International Conference 2009 (ABIC 2009). Bangkok Thailand.



PP-PB-047

ATR-FTIR spectroscopic investigation of MRSA ampicillin-resistant and *E. coli* amoxicillin-resistant

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Due to increasing antibiotic resistance, it is important to understand the action of antibiotic and new antimicrobial agents which can inhibit the resistance to β-lactam antibiotics. At present the routine microbiological analysis is time consuming procedure which takes 2 days or more. Attenuated Total Reflection (ATR)-Fourier transform infrared (FTIR) spectroscopy is one of the simple, direct, and sensitive techniques for quantitative studied. This work uses this technique for detecting the hydrolysis activity of penicillin when interact with resistance bacterial cells. The purpose of this study was to correlate the content of the concentration of resistance bacterial cells (Ampicillin-resistant *S. aureus* and Amoxicillin-resistant *E. coli*) after treatment with antibiotic or apigenin against the effect of the rate of β-lactam hydrolysis of penicillin by using ATR-FTIR. Amoxicillin-resistant *E. coli* after treatment with amoxicillin (100 μg/ml) appear significantly induces β-lactamase production as the hydrolysis reaction between β-lactamase and penicillin increase with 1.5 fold compared to the control. Penicillin breakdown was reduced significantly after treatment amoxicillin-resistant *E. coli* and ampicillin-resistant *S. aureus* with apigenin (50 μg/ml), given the rate of β-lactam hydrolysis of 7.6×10^{6} at 2.16×10^{11} CFU/ml and 1.8×10^{6} at 2.43×10^{11} CFU/ml respectively. From the study, it was concluded that adding amoxicillin has negative effect against amoxicillin-resistant *E. coli* as induced *blaZ* gene to encode more β-lactamases. While the use of apigenin has the potential to reverse bacterial resistance showed efficiently inhibitory activity against MRSA and *E. coli*. This study has led to the possibility for using this technique as a new tool for searching new antimicrobial agents to treat resistant bacteria and may contribute to combating resistant problem.

Keywords: ATR-FTIR, Ampicillin-resistant *S.aureus*, Amoxicillin-resistant *E.coli*

5) Eumkeb, G., Chaisena, N., Rojtinnakorn, N., Siriwong, S. and Duangkham, A. 2008. Reversing β-lactam antibiotic Resistant Bacteria with flavonoids. Thai Journal of Pharmacology. 29; 117-126.

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P14 Reversing β-Lactam Antibiotic Resistant Bacteria with Flavonoids

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Abstract

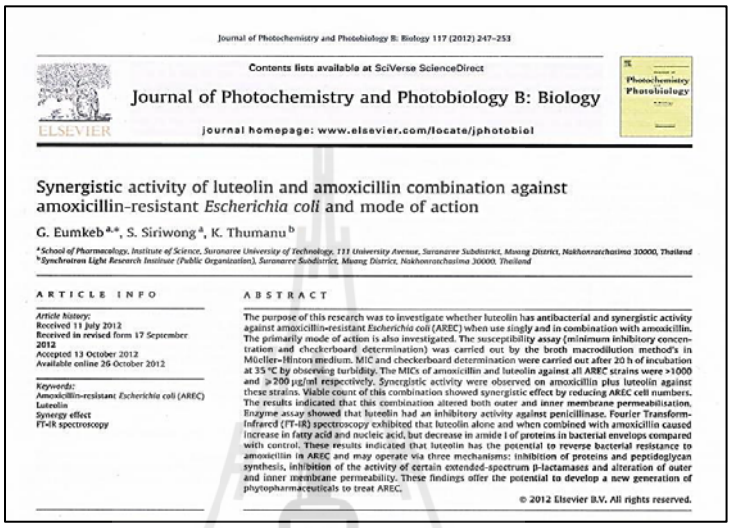
Resistance to β-lactam antibiotics is a global problem. Today over 90% of *Staphylococcus aureus* strains are β-lactamase positive. In addition, strains of methicillin-resistant *S. aureus* (MRSA) and ceftazidime-resistant *Enterobacter cloacae* (CREnC) are usually multiply resistant to many antibiotics 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, 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 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 galangin or quercetin also reduced the CFU/ml of MRSA to low levels (1×10^{3} CFU/ml) over 6 h. 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 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. Enzymes assays indicated that galangin, baicalein, and quercetin had inhibitory activity against β-lactamase I from *Bacillus cereus*. Apigenin showed marked inhibitory activity against penicillinase type IV from *Enterobacter cloacae*.

From the 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.

Keywords: β-lactam antibiotic, Resistant bacteria, Flavonoids

D2. Research articles

1) Eumkeb, G., Siriwong, S. and Thumanu, K. 2012. Synergistic activity of luteolin and amoxicillin combination against amoxicillin-resistant *Escherichia coli* and mode of action. Journal of Photochemistry and Photobiology B: Biology. 117; 247-253.



Journal of Photochemistry and Photobiology B: Biology 117 (2012) 247–253

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Synergistic activity of luteolin and amoxicillin combination against amoxicillin-resistant *Escherichia coli* and mode of action

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Amoxicillin-resistant *Escherichia coli* (AREC)
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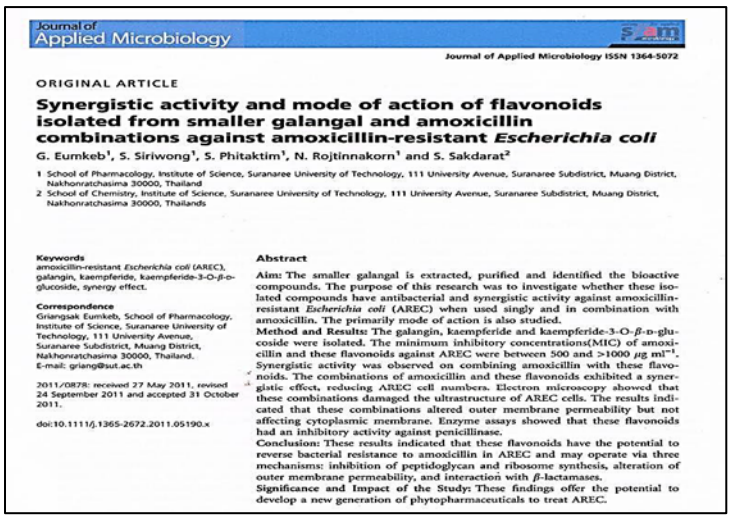
ABSTRACT

The purpose of this research was to investigate whether luteolin has antibacterial and synergistic activity against amoxicillin-resistant *Escherichia coli* (AREC) when use singly and in combination with amoxicillin. The primarily mode of action is also investigated. The susceptibility assay (minimum inhibitory concentration and checkerboard determination) was carried out by the broth macrodilution method's in Mueller–Hinton medium. MIC and checkerboard determination were carried out after 20 h of incubation at 35 °C by observing turbidity. The MICs of amoxicillin and luteolin against all AREC strains were >1000 and ≥200 μg/ml respectively. Synergistic activity were observed on amoxicillin plus luteolin against these strains. Viable count of this combination showed synergistic effect by reducing AREC cell numbers. The results indicated that this combination altered both outer and inner membrane permeabilisation. Enzyme assay showed that luteolin had an inhibitory activity against penicillinase. Fourier Transform-Infrared (FT-IR) spectroscopy exhibited that luteolin alone and when combined with amoxicillin caused increase in fatty acid and nucleic acid, but decrease in amide I of proteins in bacterial envelops compared with control. These results indicated that luteolin has the potential to reverse bacterial resistance to amoxicillin in AREC and may operate via three mechanisms: inhibition of proteins and peptidoglycan synthesis, inhibition of the activity of certain extended-spectrum β-lactamases and alteration of outer and inner membrane permeability. These findings offer the potential to develop a new generation of phytopharmaceuticals to treat AREC.

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



Journal of Applied Microbiology

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ORIGINAL ARTICLE

Synergistic activity and mode of action of flavonoids isolated from smaller galangal and amoxicillin combinations against amoxicillin-resistant *Escherichia coli*

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Keywords
amoxicillin-resistant *Escherichia coli* (AREC), galangin, kaempferide, kaempferide-3-O-β-D-glucoside, synergy effect.

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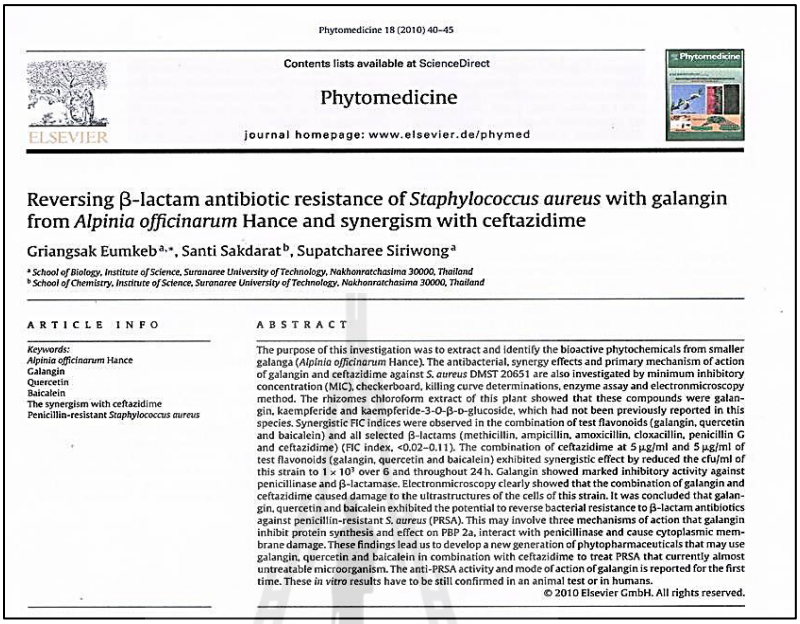
Abstract

Aim: The smaller galangal is extracted, purified and identified the bioactive compounds. The purpose of this research was to investigate whether these isolated compounds have antibacterial and synergistic activity against amoxicillin-resistant *Escherichia coli* (AREC) when used singly and in combination with amoxicillin. The primarily mode of action is also studied.
Method and Results: The galangin, kaempferide and kaempferide-3-O-β-D-glucoside were isolated. The minimum inhibitory concentrations(MIC) of amoxicillin and these flavonoids against AREC were between 500 and >1000 μg ml^{-1}. Synergistic activity was observed on combining amoxicillin with these flavonoids. The combinations of amoxicillin and these flavonoids exhibited a synergistic effect, reducing AREC cell numbers. Electron microscopy showed that these combinations damaged the ultrastructure of AREC cells. The results indicated that these combinations altered outer membrane permeability but not affecting cytoplasmic membrane. Enzyme assays showed that these flavonoids had an inhibitory activity against penicillinase.
Conclusion: These results indicated that these flavonoids have the potential to reverse bacterial resistance to amoxicillin in AREC and may operate via three mechanisms: inhibition of peptidoglycan and ribosome synthesis, alteration of outer membrane permeability, and interaction with β-lactamases.
Significance and Impact of the Study: These findings offer the potential to develop a new generation of phytopharmaceuticals to treat AREC.

3) Eumkeb, G., Sakdarat, S. and Siriwong, S. 2010. Reversing β-lactam antibiotic resistance of *Staphylococcus aureus* with galangin from *Alpinia officinarum* Hance and synergism with ceftazidime. Phytomedicine. 18; 40-45.



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Reversing β-lactam antibiotic resistance of *Staphylococcus aureus* with galangin from *Alpinia officinarum* Hance and synergism with ceftazidime

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Penicillin-resistant *Staphylococcus aureus*

ABSTRACT

The purpose of this investigation was to extract and identify the bioactive phytochemicals from smaller galanga (*Alpinia officinarum* Hance). The antibacterial, synergy effects and primary mechanism of action of galangin and ceftazidime against *S. aureus* DMST 20651 are also investigated by minimum inhibitory concentration (MIC), checkerboard, killing curve determinations, enzyme assay and electronmicroscopy method. The rhizomes chloroform extract of this plant showed that these compounds were galangin, kaempferide and kaempferide-3-O-β-D-glucoside, which had not been previously reported in this species. Synergistic FIC indices were observed in the combination of test flavonoids (galangin, quercetin and baicalein) and all selected β-lactams (methicillin, ampicillin, amoxicillin, cloxacillin, penicillin G and ceftazidime) (FIC index, <0.02–0.11). The combination of ceftazidime at 5 μg/ml and 5 μg/ml of test flavonoids (galangin, quercetin and baicalein) exhibited synergistic effect by reduced the cfu/ml of this strain to 1×10^1 over 6 and throughout 24 h. Galangin showed marked inhibitory activity against penicillinase and β-lactamase. Electronmicroscopy clearly showed that the combination of galangin and ceftazidime caused damage to the ultrastructures of the cells of this strain. It was concluded that galangin, quercetin and baicalein exhibited the potential to reverse bacterial resistance to β-lactam antibiotics against penicillin-resistant *S. aureus* (PRSA). This may involve three mechanisms of action that galangin inhibit protein synthesis and effect on PBP 2a, interact with penicillinase and cause cytoplasmic membrane damage. These findings lead us to develop a new generation of phytopharmaceuticals that may use galangin, quercetin and baicalein in combination with ceftazidime to treat PRSA that currently almost untreatable microorganism. The anti-PRSA activity and mode of action of galangin is reported for the first time. These *in vitro* results have to be still confirmed in an animal test or in humans.

CURRICULUM VITAE

Miss Supatcharee Siriwong was born in February 24, 1983 in Maung, Nakhon Ratchasima, Thailand. She attended in Boonwattana School and graduated in 2001. She received a Bachalors'degree in Microbiology in 2005 from the faculty of Sciences, Ubon Ratchathani University. She continued her Doctor of Philosophy in Biomedical Sciences in the School of Pharmacology at Suranaree University of Technology. While, she was studying. She had experiences of working as the teacher assistant in school of biology in Microbiology and Principle Biology I.