ฤทธิ์ต้านเชื้อแบคทีเรียของแอลฟา-แมงโกสตินสกัดจากเปลือกผลมังคุดต่อแบคทีเรียดื้อยา

นางศิณีวัณย์ พิทักษ์ทิม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
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ANTIBACTERIAL ACTIVITY OF α-MANGOSTIN FROM THE PERICARP EXTRACT OF *GARCINIA MANGOSTANA* L. AGAINST DRUG RESISTANT BACTERIA

Sineewan Phitaktim

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

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ANTIBACTERIAL ACTIVITY OF α-MANGOSTIN FROM THE PERICARP EXTRACT OF *GARCINIA MANGOSTANA L.* AGAINST DRUG RESISTANT BACTERIA

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ศิณีวัณย์ พิทักษ์ทิม : ฤทธิ์ต้านเชื้อแบคทีเรียของแอลฟา-แมงโกสตินสกัดจากเปลือกผลมังคุดต่อแบคทีเรียดื้อยา (ANTIBACTERIAL ACTIVITY OF α-MANGOSTIN FROM THE PERICARP EXTRACT OF *GARCINIA MANGOSTANA* L. AGAINST DRUG RESISTANT BACTERIA) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เกรียงศักดิ์ เอื้อมเก็บ, 112 หน้า.

ในปัจจุบันนี้ อุบัติการณ์ดื้อยาแบบหลายชนิดของเชื้อแบคทีเรียก่อโรคและฉวยโอกาสมีมากขึ้นเรื่อยๆ งานวิจัยที่จะหาสารต้านแบคทีเรียใหม่ๆ ที่ทำให้ยาต้านเชื้อแบคทีเรียชนิดเบตาแลคแทมเหล่านั้นนำกลับมาใช้ได้เหมือนเดิมจึงเป็นเป้าหมายที่สำคัญและความต้องการอย่างเร่งด่วน ดังนั้นวัตถุประสงค์ของการศึกษาครั้งนี้เพื่อทำการทดสอบฤทธิ์ในการต้านเชื้อแบคทีเรียของสารสกัดจากเปลือกผลมังคุด เมื่อใช้แบบเดี่ยวๆและร่วมกับยาต้านเชื้อแบคทีเรียชนิดเบตาแลคแทม ผลของมังคุดแก่ถูกนำมาสกัดและพิสูจน์เอกลักษณ์ การใช้เครื่องสกัดแบบซอกเลท ทำให้ได้สารสกัดหยาบไดคลอโรมีเทน สารสกัดย่อยที่ 3 และแอลฟา-แมงโกสติน สารหลักถูกนำไปพิสูจน์เอกลักษณ์โดยเอนเอมอาร์ ได้แก่สารแอลฟา-แมงโกสติน ค่ายับยั้งต่ำสุดของสารสกัดหยาบไดคลอโรมีเทน สารสกัดย่อยที่ 3 แอลฟา-แมงโกสติน และออกซาซิลลิน ต่อเชื้อสแตปฟิโลคอคคัสซาโพลไฟติคัส ที่ดื้อออกซาซิลลิน มีค่า 50, 31, 8 และ 128 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ อย่างไรก็ตาม ค่ายับยั้งต่ำสุดของสารสกัดเหล่านี้เมื่อใช้เดี่ยวๆหรือผสมกับออกซาซิลลิน พบว่า เชื้ออี โคไลและอี โคลเอเซที่ดื้อต่อเซพตาซิดีม มีค่าดื้อต่อสารเหล่านี้ในทุกกลุ่มของสารที่ทดลอง ดังนั้นผลการทดสอบบ่งชี้ว่า สารสกัดจากเปลือกผลมังคุดเหล่านี้ มีความแรงในการต้านเชื้อโออาร์เอสเอสเหนือกว่าออกซาซิลลินเดี่ยวๆ ผลการทำเชคเคอบอร์ดบ่งชี้ว่า ค่าเอฟไอซีอินเดก ของสารสกัดหยาบไดคลอโรมีเทน สารสกัดย่อยที่ 3 และแอลฟา-แมงโกสตินเมื่อผสมกับออกซาซิลลิน ในการต้านเชื้อโออาร์เอสเอส มีผลเสริมฤทธิ์กันที่ค่า 0.25, 0.138, และ 0.375 ตามลำดับ กราฟยับยั้งการเจริญเติบโตของแบคทีเรีย เมื่อได้รับสารสกัดจากเปลือกผลมังคุดเหล่านี้ผสมกับออกซาซิลลิน พบว่าการเจริญของเชื้อนี้ที่ 6-24 ชั่วโมงลดลงอย่างมาก ผลจากการตรวจสอบด้วยกล้องจุลทรรศน์อิเลคตรอน พบว่าสารสกัดเหล่านี้ เมื่อผสมกับออกซาซิลลิน ที่ค่าต่ำกว่าค่าการยับยั้งต่ำสุดต่อเชื้อนี้ พบว่า ทำให้เซลล์จำนวนมากขนาดเล็กกว่าเซลล์กลุ่มควบคุม รูปร่างเซลล์บิดเบี้ยวและเยื่อหุ้มเซลล์ได้รับความเสียหายในเซลล์จำนวนมาก นอกจากนั้นแล้วยังพบว่า ไม่ว่าจะใช้สารสกัดเดี่ยวๆหรือผสมกับออกซาซิลลินที่ค่าต่ำกว่าค่าการยับยั้งต่ำสุดต่อเชื้อนี้ ทำให้เพิ่มการซึมผ่านของเยื่อหุ้มเซลล์ชั้นนอกและชั้นในได้ นอกจากนั้นแล้ว การทดสอบเอสดีเอส-เพจ พบว่า สารผสมเหล่านี้เมื่อใช้เดี่ยวหรือผสมกับออกซาซิลลิน ทำให้แบนของโปรตีนที่หนักกว่าหายไป ในขณะที่พบแบนที่เบากว่าเข้มขึ้น เมื่อเทียบกับกลุ่มควบคุม

จากผลการศึกษานี้ สามารถสรุปได้ว่า ออกซาซิลลิน มีฤทธิ์ต้านเชื้อโออาร์เอสเอสน้อยมาก ในขณะที่สารสกัดจากเปลือกผลมังคุดเหล่านี้ มีความแรงสูงกว่ายาเมื่อใช้เดี่ยวๆนี้มาก ยิ่งไปกว่านั้น ส่วนผสมของสารสกัดเหล่านี้ โดยเฉพาะแอลฟา-แมงโกสตินและออกซาซิลลิน แสดงฤทธิ์เสริมกัน ในการต้านเชื้อนี้อย่างชัดเจน ดังนั้น การค้นพบนี้ เป็นเครื่องพิสูจน์ว่า สารสกัดจากเปลือกผลมังคุด เหล่านี้ ออกฤทธิ์เสริมกับออกซาซิลลินเพื่อให้ออกซาซิลลินสามารถนำกลับมาใช้ใหม่กับเชื้อที่ดื้อต่อ ยาตัวนี้แล้ว

กล่าวโดยสรุป การเสริมฤทธิ์ที่เกิดขึ้น อาจมาจากกลไกการยับยั้งการสังเคราะห์ผนังเซลล์ หรือการลดการซึมผ่านของเยื่อหุ้มเซลล์ชั้นนอกและชั้นใน สารสกัดจากเปลือกผลมังคุดเหล่านี้มี ความปลอดภัยสูงสำหรับการรักษา ด้วยเหตุนี้ อาจสามารถพัฒนาสารเหล่านี้ โดยนำมาผสมกับออก ซาซิลลินในการต้านเชื้อโออาร์เอสเอส ซึ่งในปัจจุบันดื้อต่อยาในกลุ่มเพนนิซิลลินแทบทุกตัว การทดสอบนี้ ยังคงต้องทดสอบในสัตว์ทดลองและในมนุษย์ต่อไป

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา_____

SINEEWAN PHITAKTIM : ANTIBACTERIAL ACTIVITY OF α-MANGOSTIN FROM THE PERICARP EXTRACT OF *GARCINIA MANGOSTANA* L. AGAINST DRUG RESISTANT BACTERIA. THESIS ADVISOR : ASST. PROF. GRIANGSAK EUMKEB, Ph.D. 112 PP.

GARCINIA MANGOSTANA L./β-LACTAM ANTIBIOTICS/ANTIBACTERIAL ACTIVITY/*STAPHYLOCOCUS SAPROPHYTICUS*

In the recent years, incidence of multidrug resistance in pathogenic and opportunistic bacteria has been increasingly documented. The search for novel antibacterial agents that can reverse the resistance to *β*-lactam antibiotics are research objectives of far reaching importance and urgently needed. Thus, the objective of this study was to investigate the activity of bioactive compounds from the pericarp extract of *Garcinia mangostana* L. (GML) against drug resistant bacteria, when use alone and in combination with *β*-lactams antibiotic. The mature GML fruits extraction and identification methods were accomplished. The CH_2Cl_2 crude extract, Fr_3 extract, and α-mangostin were extracted by Soxhlet extraction. Main compound structure is identified as α-mangostin using NMR. MIC values of CH_2Cl_2 crude extract, Fr3 extract, α-mangostin, and oxacillin against oxacillin-resistant *S. saprophyticus* (ORSS) revealed 50, 31, 8, and 128 µg/mL, respectively. However, the MIC values of GML extracts either alone or in combination with oxacillin exhibited high resistant against both ceftazidime-resistant *E. coli* and ceftazidime-resistant *E. cloacae* strains in all treated compounds. So that, these results indicate that these compounds revealed a higher potency against ORSS than oxacillin alone. The checkerboard displayed that the FICs index of CH_2Cl_2 crude extract, Fr_3 and α-mangostin plus oxacillin revealed

synergistic effects at 0.25, 0.138, and 0.375 respectively against ORSS strain. The killing curves proved that the combination of these extracts plus oxacillin caused a marked decrease of ORSS cells within 6 h and throughout 24 h period. The TEM method exhibited that the effect of the combination of oxacillin plus these compounds at sub-MIC value on ORSS revealed great deal smaller than the control cells, cell shape distortion and cell envelope damage in most of these cells. In addition, either compound alone or in combination with oxacillin at sub MIC value steady increased the OM and CM permeability of this strain. Besides, the SDS-PAGE results exhibited that there was an absence of protein bands at higher MW whereas appeared darker at lower MW of these compounds treated cells compared to control.

From these results, it can be concluded that GML compounds showed rather higher potency than oxacillin alone against this strain. Moreover, the combination of oxacillin and these compounds, especially α-mangostin, obviously showed great synergism activity against this strain. So, our findings provide evidence that these compounds have the synergistic effect with oxacillin to reverse bacterial resistance to oxacillin against this resistant strain.

To conclude, this activity may be involved two mechanisms of action, including the cell wall synthesis inhibition and steady increase OM and CM permeabilization. These compounds have a sufficient margin of safety for therapeutic use. For this reason, these compounds offer for the development of a valuable adjunct to oxacillin against ORSS, which currently almost penicillins resistance. These in vitro results have to be still confirmed in an animal or in humans test.

School of Biology Student's Signature_____

Academic Year 2012 Advisor's Signature_____

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LIST OF ABBREVIATIONS

ATCC	=	American Type Culture Collection
CFU	=	Colony Forming Unit
CH_2Cl_2	=	Dichloromethane
CM	=	Cytoplasmic Membrane
CREC	=	ceftazidime–Resistant *Escherichia coli*
CREnC	=	Ceftazidime-Resistant *Enterobacter cloacae*
CSEC	=	Ceftazidime-Sensitive *Escherichia coli*
Da	=	Dalton
DMSO	=	Dimethylsulfoxide
DNA	=	Deoxyribonucleic Acid
DNP	=	Dinitropyrene
EDTA	=	Ethylenediaminetetraacetic Acid
EMCV	=	Enceplalomycarditis Virus
FIC	=	Fractional Inhibitory Concentration
Fr_3	=	Fraction 3
g	=	Gram
GML	=	*Garcinia mangostana* Linn.
h	=	Hour
HEPES	=	N-2-Hydroxyethyl Piperazine-N'-Ethanesulphonic Acid
HPLC	=	High-performance liquid chromatography

LIST OF ABBREVIATIONS (Continued)

HSVI	=	Herpes Virus Type I
IFN	=	Interferon
IV	=	Intravenous
KDa	=	Kilo Dalton
KV	=	Kilo Volts
LPS	=	Lipopolysaccharide
MHA	=	Mueller Hueller-Hinton agar
MHB	=	Mueller Hueller-Hinton broth
MIC	=	Minimum Inhibitory Concentration
ml	=	Millilitre
mM	=	Millimolar
MRSA	=	Methicillin-Resistant *Staphylococcus aureus*
MW	=	Molecular Weight
NaCl	=	Sodium Chloride
NAT	=	N-Acetyltransferase
NMR	=	Nuclear magnetic resonance
NO	=	Nitric oxide
NT	=	Nitropyrene
ODC	=	Ornithine Decarboxylase
OM	=	Outer Membrane
BM-PG	=	Bacterial Membrane and Peptidoglycan

LIST OF ABBREVIATIONS (Continued)

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

CHAPTER I

INTRODUCTION

1.1 Introduction

Multidrug resistance in pathogenic bacteria at an alarming rate increase. Thus, the search for new antibiotics and new approaches to treat these bacterial infections are urgently needed. In recent years, incidence of multidrug resistance in pathogenic and opportunistic bacteria has been increasingly documented (Jones *et al*., 2004). These multidrug resistant bacteria have also created immense clinical problems in immune compromised patients. Most important multidrug-resistant bacteria on the global scale include Gram-positive (methicillin-resistant *Staphylococcus aureus*, vancomycin resistant *enterococci*) and Gram-negative bacteria (members of enterobacteriaceae producing plasmid mediated extended spectrum β-lactamase (ESβL)) and others like *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis* (Medeiros, 1997; Sajduda *et al*., 1998). Around 90-95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin (Casal *et al*., 2005) and in most of the Asian countries, 70-80% of the same strains are methicillin-resistant (Chambers, 2001). Staphylococcal resistance to wide spectrum of β-lactam antibiotics, such as methicillin, oxacillin and flucloxacillin, emerged soon after the introduction of the first drug in this class and there has been a steady rise in the incidence of methicillin resistant *S. aureus* (MRSA) clinical isolates (Bush, 2004). Staphylococci show a strong tendency to accumulate antibiotic-resistant genes and the majority of MRSA

isolates are now resistant to a range of antibiotics (Firth, 2003). Reports on vancomycin-resistant Enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) infections in hospitals have increased worldwide in recent years (Emori and Gaynes, 1993; Leclercq and Courvalin, 1997; Moellering, 1998; Murry, 1997). There are a considerable number of reports on valuable trials carried out to control the infections caused by VRE (Garner, 1996; Montecalvo *et al*., 1999a; Nourse *et al*., 2000; Slaughter *et al*., 1996) and MRSA (Cookson, 1995; Cox *et al*., 1995; Kotilainen *et al*., 2001; Voss *et al*., 1994). Strains of ceftazidime-resistant *Escherichia coli* (CREsC), ceftazidime-resistant *Enterobacter cloacae* (CREnC) including β-lactam-resistant *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) have been posing serious problems to hospitalized patients and their care providers (Liu *et al*., 2000; Maharat Nakhon Ratchasima hospital, 2012; Mulligan *et al*., 1993).

MRSA are the most common organisms causing infections of the urinary tract, surgical wounds, skin respiratory and gastrointestinal tract including *Escherichia coli* and *Enterobacter* spp. (Isogai *et al*., 2001; Sundaram *et al*., 1983; Wang *et al*., 2003). In addition, antibiotics are sometimes associated with adverse effects on the host, which include depletion of beneficial gut and mucosal microorganisms, immunosuppression, hypersensitivity and allergic reaction. The drugs-resistant bacteria have further complicated the treatment of infectious diseases in immonocompromised, AIDS and cancer patients, especially in the case of nosocomial infections (McGaw *et al*., 2000). There is not only the loss of an effective antibiotic against multidrugs-resistant bacteria, but also a global problem for the loss of budget for treatment of infectious diseases.

Plant-derived antibacterials are an interesting source of novel therapeutics. For example, an active ingredient called galangin which was synergism with penicillin for MRSA inhibition (Eumkeb *et al*., 2010)

Many plants have interesting biological activities with potential therapeutic applications. Mangosteen (*Garcinia mangostana* Linn.) (GML), belonging to the family Guttiferae, is a tropical evergreen tree. Its origin is in Southeast Asia, India, Myanmar, Malaysia, Philippines, Sri Lanka, and Thailand. (Morton, 1987). GML (Figure 1.1A) is commonly known as Mang Kut, and is referred to as ‘‘the queen of fruits’’ in Thailand, which is very popular due to its delicious taste. The pericarp, 6–10 mm in thickness, dark purple or reddish (Figure 1.1B), has been used as a traditional medicine in Southeast Asia for the treatment of diarrhea, inflammation, and ulcers (Farnsworth and Bunyapraphatsara, 1992; Peres *et al*., 2000; Suksamrarn *et al*., 2006). Mangosteen fruit is a rich source of phenolic compounds such as xanthones, condensed tannins and anthocyanins (Fu *et al*., 2007; Jung *et al*., 2006; Mahabusarakam *et al*., 1987).

Xanthone derivatives, as the major secondary metabolites of *G. mangostana* fruits (Ji *et al*., 2007; Mahabusarakam *et al*., 1987), exhibited antibacterial (Sakagami *et al*., 2005; Suksamarn *et al*., 2003), antifungal (Gopalakrishnan *et al*., 1997), anti-inflammatory (Nakatani *et al*., 2004), antioxidant (Jung *et al*., 2006; Yu *et al*., 2007), antiplasmodial (Mahabusarakam *et al*., 2006), and cytotoxic activities (Matsumoto *et al*., 2005; Suksamrarn *et al*., 2006).

The xanthones, α- and γ-mangostin, are major bioactive compounds found in the pericarp of the mangosteen (Figure 1.1C) (Chairungsrilerd *et al*., 1996a, b, c ; Jinsart *et al*., 1992). The biological activities of α-mangostin have been confirmed to

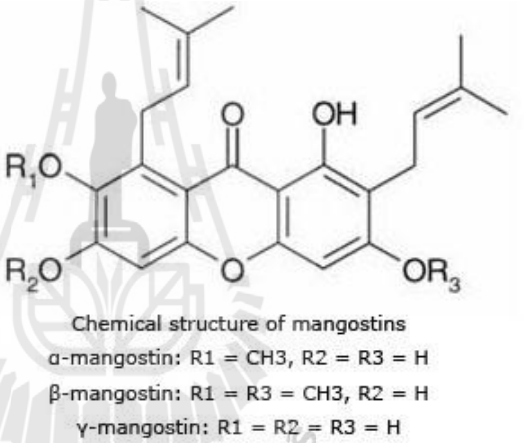
consist of a competitive antagonism of the histamine H1 receptor (Chairungsrilerd *et al*., 1996a; Iikubo *et al*., 2002), antibacterial activity against *Helicobacter pylori*, anti-inflammatory activities, inhibition of oxidative damage by human low-density lipoproteins (LDL) (Iikubo *et al*., 2002), antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (Chomnawang *et al*., 2009; Iinuma *et al*., 1996), weak antioxidant activity (Chairungsrilerd *et al*., 1996a), and inhibition of alveolar duct formation in a mouse mammary organ culture model and suppression of the carcinogen induced formation of aberrant crypt foci in a short-term colon carcinogenesis model (Jung *et al*., 2006; Nabandith *et al*., 2004). The other xanthone derivative, γ-mangostin has also been reported to have several pharmacological activities, such as being a potent inhibitor of animal Cdk activating kinases (Cak), plant Ca^{2+}-dependent protein kinases (CDPK) (Jinsart *et al*., 1992), and a selective antagonist for 5-HT2A receptors in smooth muscle cells and platelets (Chairungsrilerd *et al*., 1996b, 1998). Moreover, α- and γ-mangostin can inhibit both human immunodeficiency virus (HIV) infection (Chen *et al*., 1996; Vlietinck *et al*., 1998), and topoisomerases I and II (Tosa *et al*., 1997).

(A)

(B)

Garcinia mangostana L.

(C)



Chemical structure of mangostins
α-mangostin: R1 = CH3, R2 = R3 = H
β-mangostin: R1 = R3 = CH3, R2 = H
γ-mangostin: R1 = R2 = R3 = H

Figure 1.1 The *Garcinia mangostana* L. tree (A), the appearance of mangosteen fruit (B), and the chemical structures of xanthones included in the pericarps (C).

Source: Akao *et al*. (2008).

Maharat Nakhon Ratchasima hospital is the largest hospital in Nakhon Ratchasima province, northeast of Thailand. The problems of drug-resistant bacteria are found in high level in many sections of this hospital and other hospitals in

Thailand and worldwide (Jungthirpanich *et al*., 2000; Kusum and Dejsirilert, 2003; Maharat Nakhon Ratchasima hospital, 2012).

However, no works have investigated the effect of GML extract on some drug resistant bacteria such as *Staphylococcus saprophyticus* (*S. saprophyticus*), *Enterobacter cloacae* (*E. cloacae*), and *Escherichia coli* (*E. coli*). The purpose of this thesis was to investigate the activity of bioactive compound from the pericarp extract of *Garcinia mangostana* against drug resistant bacteria, when used alone and in combination with β-lactam antibiotics. Thus, the development of a new class of antibacterial agent may be developed.

1.2 Research objectives

(1) To test the effectiveness of α-mangostin or other bioactive compounds from the pericarp of GML extract on drug resistant bacteria when use alone.

(2) To test the effectiveness of α-mangostin or other bioactive compounds from the pericarp of GML extract on drug resistant bacteria in combination with antibacterial drugs.

(3) To investigate the elementary mechanism of action of α-mangostin or other bioactive compounds from the pericarp of GML extract on drug resistant bacteria when used alone or in combination with antibiotic drugs by examining morphology with transmission electronmicroscopy (TEM), outer and cytoplasmic membrane permeability and electrophoresis methods.

1.3 Research hypothesis

(1) α-Mangostin or other bioactive compounds from the pericarp of GML extract on drug resistant bacteria can show antibacterial activity against drug resistant bacteria.

(2) α-Mangostin or other bioactive compounds from the pericarp of GML extract on drug resistant bacteria in combination with antibiotic drugs can show synergistic activity against drug resistant bacteria.

1.4 Scope and limitation of the study

(1) The mangosteen fruits (*Garcinia mangostana* L.) were purchased from a local market in Nakhon Ratchasima, Thailand. The pericarps of mangosteen fruits were extracted and α-mangostin or other bioactive compounds were isolated with a little modification method.

(2) Clinical isolates of *Staphylococcus saprophyticus* (*S. saprophyticus*), clinical isolates of *Enterobacter cloacae (E. cloacae)* and clinical isolates of *Escherichia coli* (*E. coli*) were obtained from Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand and the American Type Culture Collection (ATCC), USA.

(3) Oxacillin and ceftazidime were obtained from Sigma, Bristol-Myers.

(4) Checkerboard assay of combinations that showed the lowest MIC were selected for further investigations such as viability counts, transmission electronmicroscopy (TEM), outer and cytoplasmic membrane permeability and electrophoresis methods.

1.5 Expected results

(1) Additional scientific data on synergism antimicrobial activity of the combination of oxacillin and α-mangostin or other bioactive compounds from the pericarp of GML extract and antibiotic drugs on drug-resistant bacteria will be obtained.

(2) Novel knowledge for further investigations such as mechanism of action of α-mangostin or other bioactive compounds from the pericarp of GML extract on drug resistant bacteria in animals and human will be known.

(3) The results may be useful for the development of new drug combination against drug-resistant bacteria.

(4) The results of the study will be a benefit to physicians and patients in case of tackling most of the dangerous drug-resistant bacteria by using new antibacterial combination drugs.

CHAPTER II

LITERATURE REVIEW

2.1 Overview of mangosteen

Mangosteen (*Garcinia mangostana* L.) is classified in family Guttiferae, genus Garcinia and Thai people call mangosteen as Mang Kut (Smitinand, 2001). Mangosteen is one of praised tropical fruits. This kind of fruit is usually eaten as dessert. Furthermore, they can be made as processed products such as juice, jam, jelly and sugar. The seeds are sometimes eaten alone after boiling or roasting (Figure 1B).

2.1.1 Pericarp active ingredients and their pharmacological data

Asai *et al*. (1995) reported that chemical constituents in pericarp of mangosteen consisted of xanthone derivatives such as alpha-mangostin, beta-mangostin, gamma-mangostin, gartanin, garcinone E, 1,5-dihydroxy-2-(3-methylbut-2-enyl)-3-methoxy, 1,7-dihydroxy-2-(3-methylbut-2-enyl)-3-methoxyxanthone and mangostinone (Figure 1.1C).

Chairungsrilerd *et al*. (1996a) described that the fruit hull (pericarp) of mangosteen was used as an anti-inflammatory agent, astringent and in the treatment of diarrhea. They reported that the yellowish excretion of the fruit hull, alpha-mangostin, gamma-mangostin and mangostanol, showed an inhibitory effect on cAMP phosphodiesterase.

Moreover, extracts of the pericarp of fruit have been used as a traditional medicine in Thailand for the treatment of trauma, diarrhea, and skin

infections (Nakatani *et al*., 2002). The biological activities of α-mangostin have been confirmed to consist of a competitive antagonism of the histamine H_1 receptor (Chairungsrilerd *et al*., 1996a; Iikubo *et al*., 2002), antibacterial activity against *Helicobacter pylori*, anti-inflammatory activities, inhibition of oxidative damage by human low-density lipoproteins (LDL) (Iikubo *et al*., 2002), antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (Chomnawang *et al*., 2009; Iinuma *et al*., 1996), and weak antioxidant activity (Chairungsrilerd *et al*., 1996a). In addition, α- and γ-mangostin can inhibit both human immunodeficiency virus (HIV) infection (Chen *et al*., 1996; Vlietinck *et al*., 1998), and topoisomerases I and II (Tosa *et al*., 1997). The mangosteen has long been widely used as an anti-inflammatory, anti-diarrhea, and anti-ulcer agent in Southeast Asia (Harbborne and Baxter, 1993; Lu *et al*., 1998).

2.1.2 Current use and importance of α-mangostin

α-Mangostin, the first xanthone isolated from mangosteen fruit (Schmid, 1855), is a yellow coloring matter that can also be obtained from bark and dried sap of *Garcinia mangostana*. In fact, for a long time people in many countries have used the pericarp (peel, rind, hull or ripe) of *Garcinia mangostana* as a traditional medicine for the treatment of abdominal pain, diarrhea, dysentery, infected wound, supporation and chronic ulcer (Chopra *et al*., 1956; Pedraza-Chaverri *et al*., 2008). Several properties have been reported for α-mangostin. For instance, Williams *et al*. (1995) found that α-mangostin decreased the human low-density lipoprotein (LDL) oxidation induced by copper or peroxyl radical. Besides, Mahabusarakam *et al*. (2000) found that α-mangostin prevented the decrease in α-tocopherol consumption induced by LDL oxidation. As well as, Jung *et al*. (2006) exhibited that several xanthones isolated from

the pericarp of *Garcinia mangostana*, including α-mangostin, were able to scavenge peroxynitrite anion. In addition, Suksamrarn *et al*. (2003) revealed that xanthones, isolated from the fruit hulls, the edible arils and seeds of *Garcinia mangostana*, such as alpha- and beta-mangostin and garcinone B exhibited strong inhibitory effect against *Mycobacterium tuberculosis* (TB). In the same way, Lin *et al*. (1996) demonstrated that xanthone derived compounds had potent antitumor activities against human cancer cells and some of them possessed strong anti-inflammatory properties. What is more, Iinuma *et al*. (1996) displayed that extracts of fruit of *Garcinia mangostana*, possessed inhibitory effects against the growth of *S. aureus*. So, the strong *in-vitro* antibacterial activity of xanthone derivatives against both methicillin-resistant and methicillin-sensitive *Staphylococcus aureus* suggested that the compounds might find wide pharmaceutical use. Apart from this, Chomnawang *et al*. (2005) indicated that the active compounds in *Garcinia mangostana* could be mangostin, a xanthone derivative, which had a strong inhibitory effect on *Propionibacterium acnes* and *Staphylococcus epidermidis*. Furthermore, Chomnawang *et al*. (2007) investigated that the activity of Thai medicinal plants on inflammation reduction caused by *Propionibacterium acnes* may be due to free radical scavenging and cytokine reducing properties. They found that *Garcinia mangostana* possessed the most significant antioxidant activity and reduced reactive oxygen species production. *G. mangostana* was highly effective in scavenging free radicals and was able to suppress the production of proinflammatory cytokines. This study has identified the promising source of anti-inflammatory agent which could be useful in treatment of acne vulgaris. Also, Chomnawang *et al*. (2009) showed that *G. mangostana* and its constituent, α-mangostin, had potent inhibitory effect against

methicillin-resistant *S. aureus*. In the same way, Sakagami *et al*. (2005) expressed that synergism between alpha-mangostin and gentamicin (GM) against vancomycin resistant Enterococci (VRE), alpha-mangostin and vancomycin hydrochloride (VCM) against methicillin resistant *Staphylococcus aureus* (MRSA) has occurred. These findings suggested that alpha-mangostin alone or in combination with GM or VCM against VRE or MRSA repectively might be useful in controlling VRE and MRSA infections.

2.1.3 Method for purification of active ingredient

Currently, the common methods that have been developed to purify the active ingredients from mangosteen generally involved using silica gel column chromatography. Chairungsrilerd *et al*. (1996a, b, c) extracted fresh fruit hull (pericarp) of *G. mangostana* with *n*-butanol and purified the extract on silica-gel column by eluting with CH_2Cl_2, CH_2Cl_2-MeOH (9:1), CH_2Cl_2-MeOH (4:1) and CH_2Cl_2-MeOH (1:1), respectively. Relatively less polar fractions gave known compounds as alpha mangostin, gamma mangostin and gartanin etc. They identified known compounds from their MS, IR, ^{1}H and ^{13}C NMR spectra. Successive purification of polar fraction by reverse phase HPLC using aq. MeOH as an elutent gave mangostanol. Besides, Govindachari *et al*. (1971) used silica gel column to purify active ingredients of different parts of mangosteen with various elutents. For example, partially ripe fruit was chromatographed using silica gel and eluted with benzene. The first 200 mL of elutate gave no material. The next 250 mL gave beta mangostin and the later 300 mL gave mangostin. In addition Sakagami *et al*. (2005.) extracted and isolated alpha-mangostin and beta-mangostin from stem bark of *G. mangostana* which was dried, powdered and extracted with hexane, methylene

chloride and methanol, respectively then used silica gel column chromatography with hexane, methylene chloride and methanol as solvent. The hexane extract and methylene chloride extract gave two major compounds, alpha mangostin and beta-mangostin as yellow needles. Apart from this, Chi *et al*. (2002) described isolation method in which the hull (pericarp) of mangosteen fruit was first dried at 60 ºC in an oven for 24 hour and 3 kg batches of the dried hulls were extracted with ethanol (25l) at 50 °C for about 16 hours. The extracts were pooled, concentrated by evaporation and then allowed to partition into aqueous and organic (EtOAc) phase. The EtOAc soluble fraction was then loaded into a silica gel column and eluted with gradient solvent system consisting of 10:l aliquots of each *n*-hexane-EtOAc (20:1, 10:1 and 5:1), 7 L aliquots of each CH_2Cl_2-Me_2CO (10:1, 5:1 and 0:1) and aliquots of each CH_2Cl_2-MeOH (10:1, 5:1 and 0:1) to yield 22 (~3 L) fractions. Fraction 13, the CH_2Cl_2-Me_2CO (10:1) eluted fraction, was further chromatographed on a silica gel column equilibrated and eluted with *n*-hexane –EtOAc (5:1) to yield alpha mangostin or in Sephadex LH20 column equilibrated with MeOH-H_2O (5:1) to yield gamma-mangostin.

For the isolation method in this research, those of previous described methods were followed with little modifications.

2.2 Overview of microorganisms

2.2.1 Bacterial structure

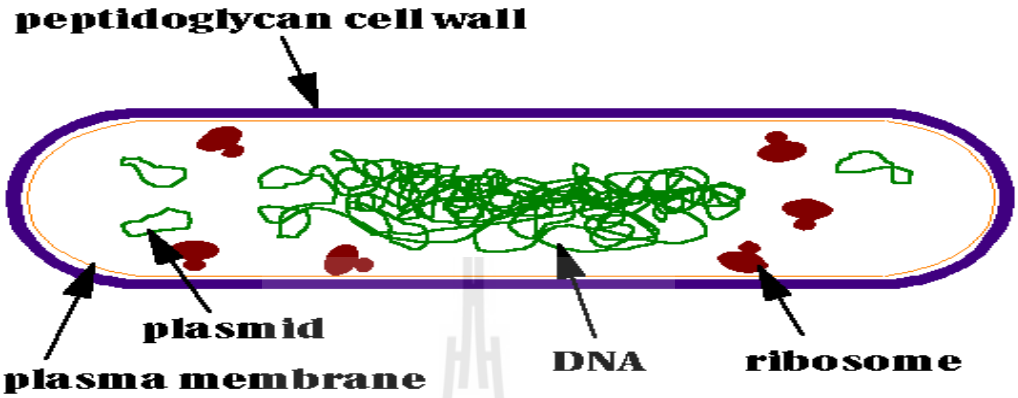


Figure 2.1 Show structure of bacterial cell.

Source: http://www. microbeworld.org/img/aboutmmicro/bacteria/bactdiag.gif.

The cytoplasmic membranes of gram-positive and gram-negative bacteria are indistinguishable. Each is composed of protein, lipids, phospholipids and a small amount of carbohydrate (Figure 2.1). It acts as an osmotic barrier, synthesizes the cell wall and provides a site to implant the chromosome. The other important functions are serving as the site of selective permeability, cytochrome activity, carrier-mediated transport and generation of proton motive force (PMF).

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

The cell wall is a web-like structure that is sometimes called the murien sacculus. It is composed of peptidoglycan. The cell wall provides the cell with its

sharp and osmotic stability. The cell wall constituents are peptidoglycan, teichoic acids and lipoteichoic acids.

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

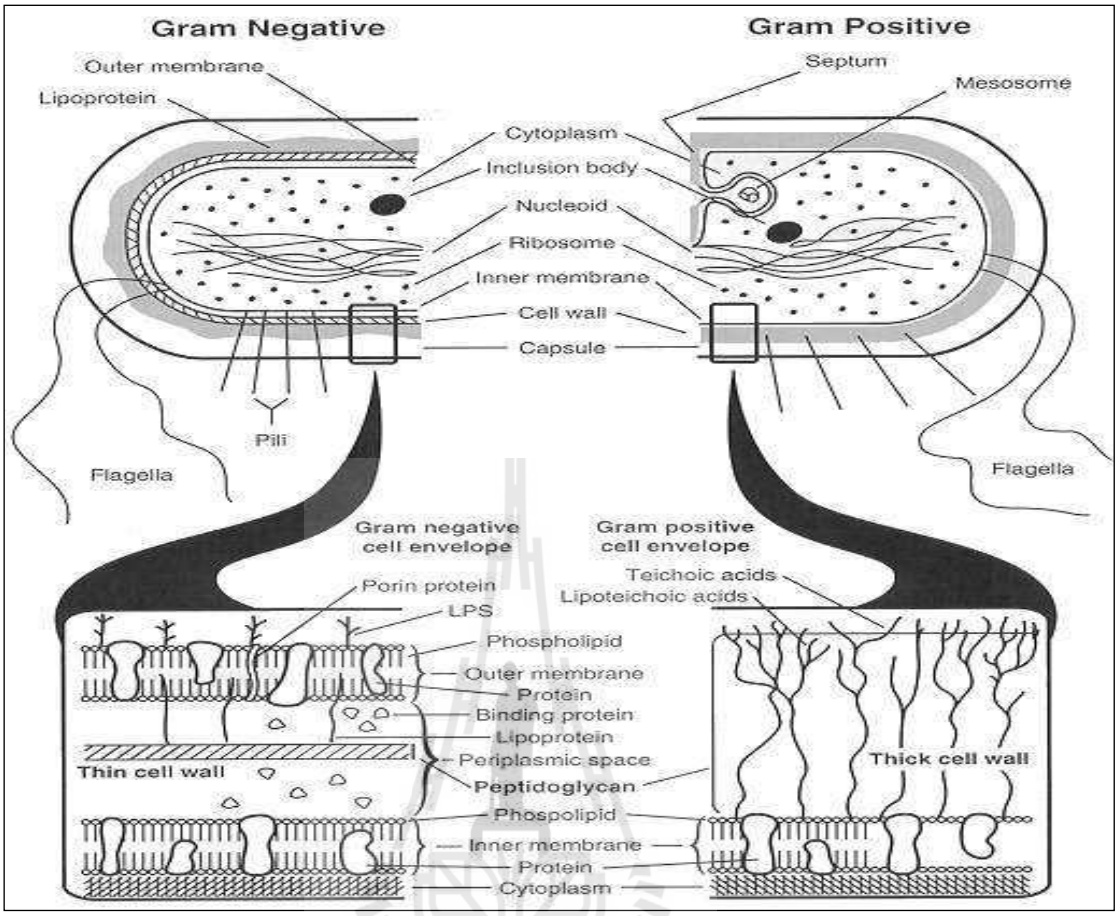


Figure 2.2 Comparison of the thick cell wall of gram-positive bacteria with the comparatively thin cell wall of gram-negative bacteria. Note the complexity of the gram-negative cell envelope (outer membrane, its hydrophobic lipoprotein anchor; periplasmic space).

Source: http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=mmed.figgrp.294.

2.2.2 Bacteria types

2.2.2.1 *Staphylococcus saprophyticus (S. saprophyticus)*

Family: Staphylococcaceae

General characteristics

S. saprophyticus is a coagulase-negative species of *Staphylococcus* bacteria. It is often implicated in urinary tract infections. *S. saprophyticus* is resistant to antibiotic novobiocin, a characteristic that is used in laboratory identification to distinguish it from *S. epidermidis,* which is also coagulase-negative. The bacterium has a capacity for selective adherence to human urothelium. It causes direct hemagglutination. The adhesin for *S. saprophyticus* is a lactosamine structure. This staphylococcal species produces an extracellular enzyme complex that can inhibit growth of both gram-positive and gram-negative bacteria. It is rarely found in healthy humans but is commonly isolated from animals and their carcasses (Shimeld and Rodgers, 1999).

Clinical significance

S. saprophyticus is implicated in 10-20% of urinary tract infections (UTI). In females between the ages of 17-27, it is the second most common cause of UTIs. It may also reside in the urinary tract and bladder of sexually active females. Some of the symptoms of this bacteria are burning sensation when passing urine, the urge to urinate more often than usual, the ‘‘dripping effect’’ after urination, weak bladder, bloated feeling with sharp razor pains in the lower abdomen around the bladder and ovary areas and razor-like pains during sexual intercourse. (Shimeld and Rodgers, 1999).

Antibiotic susceptibility characteristics

S. saprophyticus is usually susceptible to antibiotics commonly prescribed for patients with UTI, with the exception of nalidixic acid. Quinolones are commonly used in treatment of *S. saprophyticus* urinary tract infections (Shimeld and Rodgers, 1999) and resistance to penicillins such as oxacillin (Maharat Nakhonratchasima hospital, 2012).

2.2.2.2 *Enterobacter cloacae (E. cloacae)*

Family: Enterobacteriaceae

General characteristics

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

Clinical significance

E. cloacae generally does not cause disease in healthy individuals but significantly causes infections in immunocompromised or otherwise debilitated patients. This species is an opportunistic pathogen causing burn, wound and urinary tract infection and occasionally septicemia and meningitis (Shimeld and Rodgers, 1999).

Antibiotic susceptibility characteristics

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

2.2.2.3 *Escherichia coli (E. coli)*

Family: Enterobacteriaceae

General characteristics:

E. coli is a gram-negative, straight rod (1.1-1.5 μm wide x2.0-6.0 μm long) that occurs singly or in pairs. It is facultatively anaerobe and chemoorganotrophs. The optimal temperature for *E. coli* is 37 °C. It occurs as normal flora in the lower part of the intestine of warm-blooded animals (Shimeld and Rodgers, 1999).

Clinical significance

E. coli normally colonizes an infant's gastrointestinal tract within 40 hours of birth, arriving with food or water or with the individuals handling the child. In the bowel, it adheres to the mucus of the large intestine. It is the primary facultative organism of the human gastrointestinal tract. As long as these bacteria do not acquire genetic elements encoding for virulence factors, they remain benign commensals. Virulent strains of *E. coli* can cause gastroenteritis, urinary tract infections, and neonatal meningitis. In rarer cases, virulent strains are also responsible for haemolytic-uremic syndrome (HUS), peritonitis, mastitis, septicemia and gram-negative pneumonia (Shimeld and Rodgers, 1999).

Antibiotic susceptibility characteristics

Antibiotics that generally have strong activity against *E. coli* include sulfonamides, ampicillin, cephalosporins, chloramphemicol, tetracyclines and aminoglycosides. Sulfamethoxazole-trimethoprim and ciprofloxacin are usually very effective when treatment is started early (Shimeld and Rodgers, 1999) and resistance

to penicillins and cefalosporins such as ceftazidime (Maharat Nakhonratchasima hospital, 2012).

2.3 Overview of antibiotics

2.3.1 β -Lactam

The β-lactam antibiotics are useful and frequently prescribed antimicrobial agents that share a common structure and mechanism of action-inhibition of synthesis of the bacterial peptidoglycan cell wall (Laurence *et al*., 2006).

The basic structure of β-lactam antibiotics consists of a five-membered thiazolidine ring fused to the β-lactam portion (Figure 12). Different types of antibiotics are determined side chain (R-group) (Tenover, 2006).

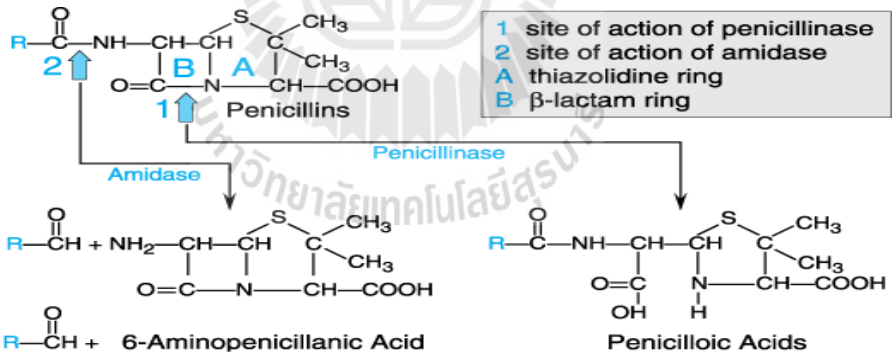


Figure 2.3 Common structure of penicillin and site of cleavage by penicillinase (http://www.acessmedicine.com)

2.3.1.1 Beta-Lactam antibiotics

2.3.1.1.1 Penicillins

They are the most widely effective antibiotics and are among the least toxic drugs known; the major adverse reaction to penicillins is hypersensitivity. The members of this family differ from one another in the *R* substituent attached to the 6-aminopenicillanic acid residue. The nature of this side chain affects their antimicrobial spectrum, stability to stomach acid, and susceptibility to bacterial degradative enzymes (β-lactamases). Figure 2.4 shows the classification of agents affecting cell wall synthesis (Mycek *et al*., 2000).

2.3.1.1.2 Cephalosporins

Cephalosporins are similar to penicillins, but more stable to many bacterial β-lactamases and therefore have a broader spectrum of activity. However, strains of *E. coli* and *Klebsiella* species expressing extended-spectrum β-lactamases that can hydrolyze most cephalosporins are becoming a problem. Cephalosporins are not active against enterococci and *L. monocytogenes* (Katzung, 2006).

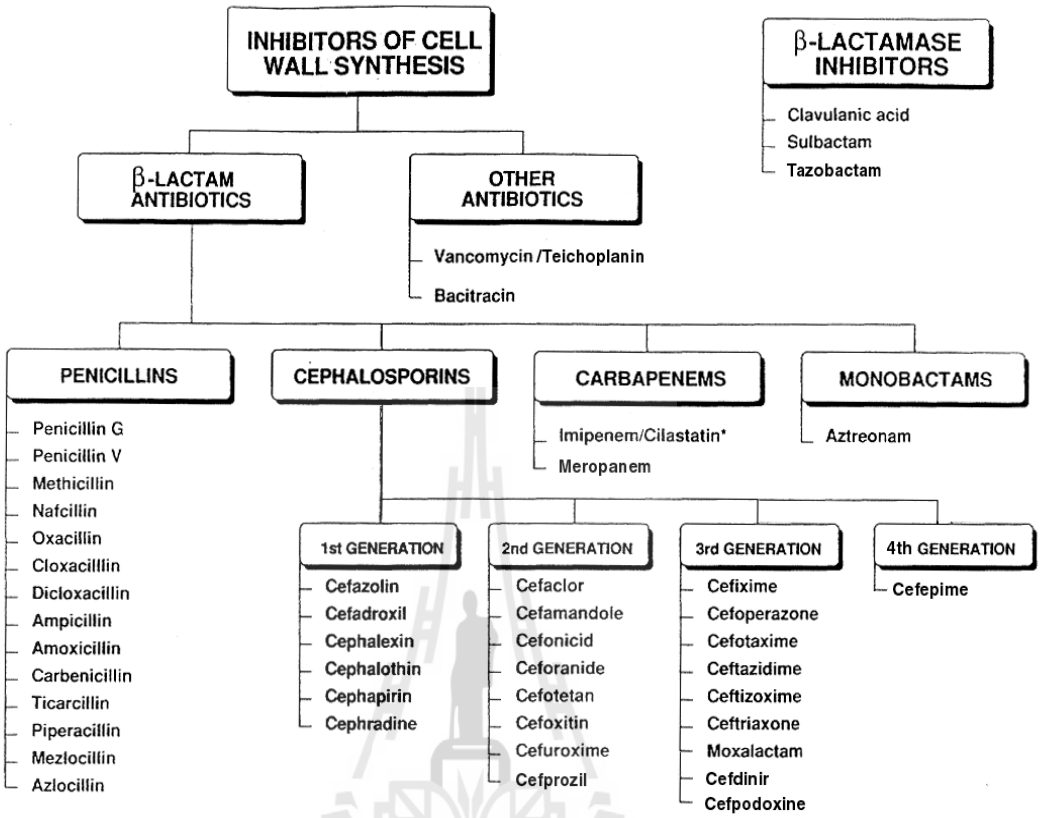
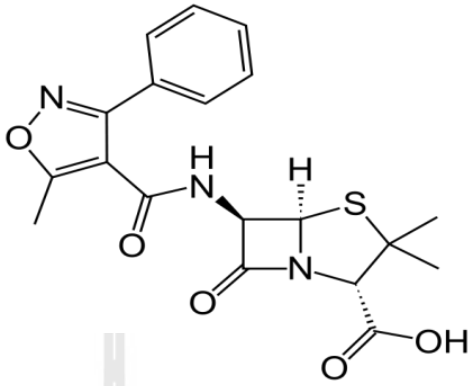


Figure 2.4 Summary of antimicrobial agents that inhibit cell wall synthesis.

Source: Mycek *et al*. (2000).

2.3.1.2 The use of β-lactam antibiotics

2.3.1.2.1 Oxacillin

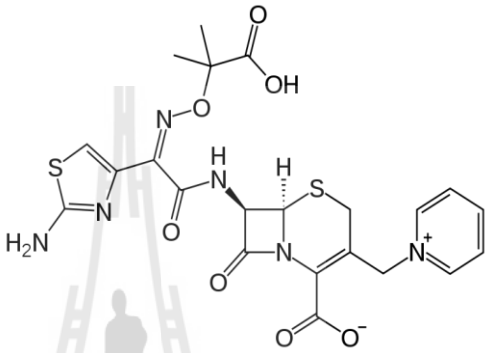


Molecular formular: $C_{19}H_{19}N_3O_5S$
Molecular mass: 401.436 g/mol
Solubility: Soluble in water and methanol
Appearance: Fine white crystalline powder
(http://en.wikipedia.org/wiki/Oxacillin)

Oxacillin is a penicillin beta-lactam antibiotic used in the treatment of bacterial infections caused by susceptible, usually gram-positive, organisms. The name "penicillin" can either refer to several variants of penicillin available, or to the group of antibiotics derived from the penicillins. Oxacillin has in vitro activity against gram-positive and gram-negative aerobic and anaerobic bacteria. The bactericidal activity of oxacillin results from the inhibition of cell wall synthesis and is mediated through oxacillin binding to penicillin binding proteins (PBPs). Oxacillin is stable against hydrolysis by a variety of beta-lactamases, including penicillinases, and cephalosporinases and extended spectrum beta-lactamases. Machanism of action by binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall, oxacillin inhibits the third and last stage of bacterial cell wall synthesis. Cell lysis is then mediated by bacterial cell wall autolytic enzymes such as autolysins; it is

possible that oxacillin interferes with an autolysin inhibition (http://www.onlinepharmacycatalog.com/category/common-drugs-and-medications /antibiotics/oxacillin/).

2.3.1.2.2 Ceftazidime



Molecular formular: $C_{22}H_{22}N_6O_7S_2$
Molecular mass: 546.58 g/mol
Solubility: Soluble in water
Appearance: white or almost white powder
(http://en.wikipedia.org/wiki/Ceftazidime)

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

gram-negative organisms *Pseudomonas* and Enterobacteriaceae. Its activity against *Pseudomonas* is a distinguishing feature of ceftazidime among the cephalosporins. The bactericidal activity of ceftazidime results from the inhibition of cell wall synthesis via affinity for penicillin-binding proteins (PBPs) (Goodman and Gillman, 2005).

2.3.1.3 Mechanisms of bacterial resistance to penicillins and cephalosporins

Although all bacteria with cell walls contain penicillin-binding protein (PBPs), β-lactam antibiotics cannot kill or even inhibit all bacteria because by various mechanisms bacteria can be resistant to these agents. The microorganism may be intrinsically resistant because of structural differences in the PBPs that are the targets of these drugs. Furthermore, a sensitive strain may acquire resistance of this type by the development of high-molecular-weight PBPs that have decreased affinity for the antibiotic. Because the β-lactam antibiotics inhibit many different PBPs in a single bacterium, the affinity for β-lactam antibiotics of several PBPs must decrease for the organism to be resistant. Altered PBPs with decreased affinity for β-lactam antibiotics are acquired by homologous recombination between PBP genes of different bacterial species. Four of the five high-molecular-weight PBPs of the most highly penicillin-resistant *Streptococcus pneumoniae* isolates have decreased affinity for β-lactam antibiotics as a result of interspecies homologous recombination events (Laurence *et al*., 2006).

2.3.1.4 Mechanisms of antimicrobial resistance

Bacteria can have an intrinsic immunity to a particular antibiotic because of biochemical structure and/or function. They may simply not respond to the

antibiotic mechanism of action. Bacteria can also acquire resistance to a drug in one of two ways. Primary resistance occurs through spontaneous mutation, which is a rare event. Resistance is then transferred to progeny. Bacteria replicate asexually, so all off spring of a resistant bacteria will inherit the resistant gene. Secondary resistance requires a transfer of genetic material between same or different species of bacteria through transduction, transformation, although reproduction in or conjugation (Table 2.1). Bacteria are asexual; they can share genetic material by forming a conjugation bridge with bacteria from the same or different species (Figure 2.5). Genetic material is carried on plasmids or transporans. Genetic transfer may confer multidrug-resistance and cross-resistance, where by resistance to one drug in a class translates into resistance to other antimicrobial drugs in that class. The ease with which bacteria share genetic material accounts for most antimicrobial resistance.

Table 2.1 How bacteria transfer genetic material.

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

Source: Roe (2008).

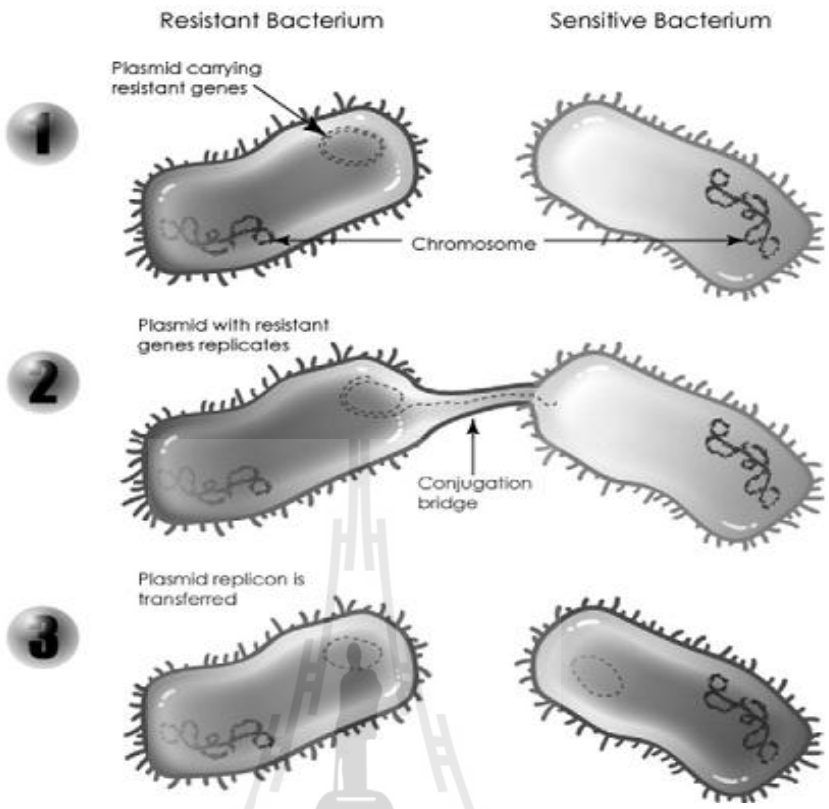


Figure 2.5 Bacterial conjugation.

Source: Roe (2008).

Once an antimicrobial enters the bacteria, it must bind to a specific biochemical target site in order to interfere with cell metabolism. Genetically altered bacteria can combat antibiotics in several ways (Figure 2.6). They may synthesize enzymes that destroy the drug or chemically modify it so that it becomes inactive. For example, some β-lactamase, which destroys β-lactam ring common to the structure of β-lactam antibiotics. Resistance to the β-lactam is widespread in both gram-positive (Staphylococci) and gram-negative (*Neisseria gonorrhoea, Escherichia coli*, and *Haemophilus influenza*) species. Some β-lactam antimicrobials, the antistaphylococcal penicillins (methicillin, nafcillin, oxacillin, and dicloxacillin), were developed specifically to avoid the effects of the β-lactamase

producing bacteria. Dicloxacillin is the only member of this group of antibiotics available in oral form.

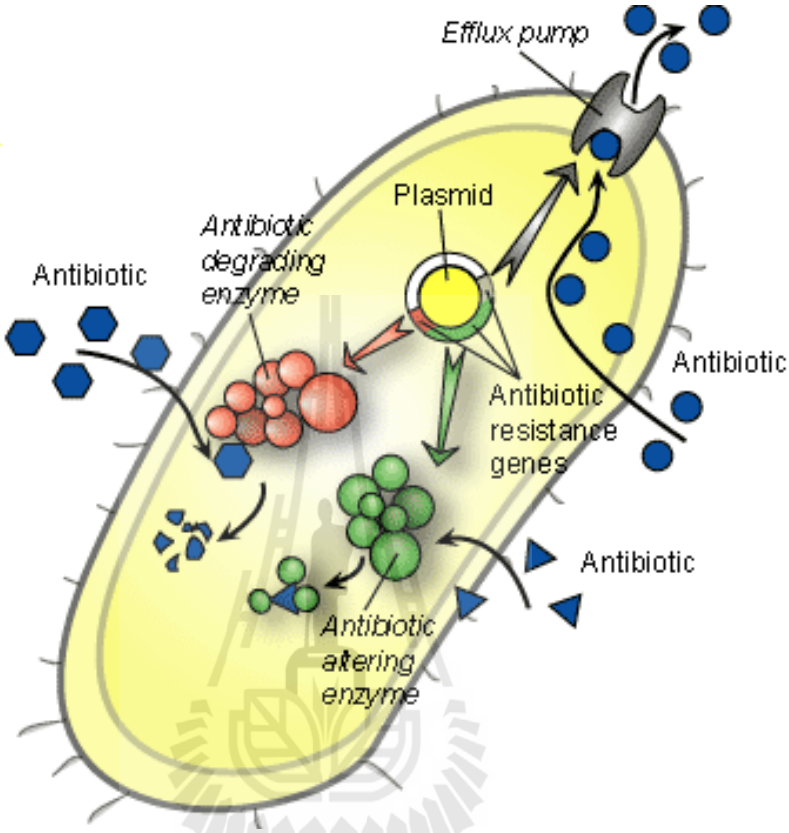


Figure 2.6 Mechanisms of antibiotic resistance.

Source: Roe (2008).

2.3.1.4.1 Enzyme-based resistance

Bacterial resistance to antibiotics is most often mediated by the bacterial synthesis of β-lactamase enzymes. These are the main cause of bacterial resistance to penicillins and cephalosporins. Definitive identification of these enzymes is only possible by gene or protein sequencing (Livermore and Brown, 2001).

2.3.1.4.2 Ribosomal modifications

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

2.3.1.4.3 Protein modifications

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

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

2.3.1.4.4 Metabolic resistance

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

2.3.1.4.5 Effluxing the toxin

Antibiotic efflux pumps are nowadays believed to significantly contribute to acquire bacterial resistance because of the very broad variety of substrates they recognize, their expression in important pathogens, and their cooperation with other mechanisms of resistance. Their existence also explains many situations of apparent intrinsic resistance of specific organisms (Seral *et al*., 2003).

For example, a strain of *Enterococcal* bacteria can pump out tetracycline. This type of pumping is called an “efflux phenomenon” (Tenover, 2006).

2.3.1.4.6 Acquired resistance

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

2.3.1.4.7 Transduction

A virus (bacteriophage) serves as the agent of transfer DNA segment between bacterial strains (McManus, 1997). When a phage is being replicated inside a host cell, the new viruses self-assemble from proteins and viral nucleic acid (genetic material) that the host cell has produced. Sometimes some of the DNA of the host, which had been chopped up during the lytic replication process, gets inside a new virus during viral self-assembly. When that phage then infects another cell, the new host may incorporate the donated DNA into its chromosome by recombination (Bauman, 2005).

2.3.1.4.8 Transformation

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

2.3.1.4.9 Conjugation

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

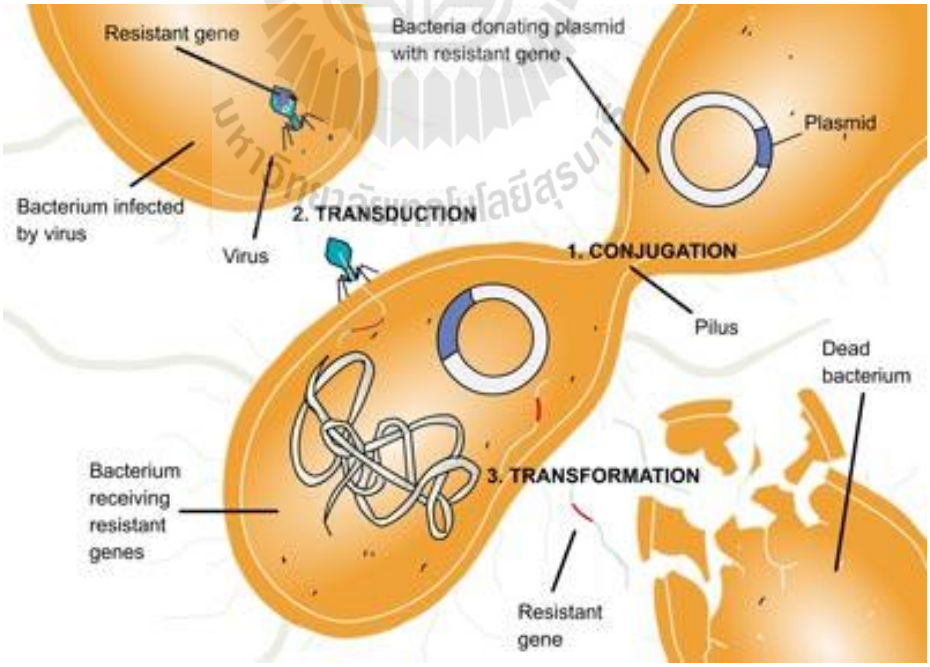


Figure 2.7 Bacteria transfer genetic material.

(http://www.wiley.com/college/)

2.4 Laboratory methods used for antimicrobial susceptibility testing

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

2.4.1 Susceptibility test method

Dilution Testing:

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

2.4.2 Checkerboard results

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

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

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

3. Antagonism; The combined action is less than that of more effective agent when used alone (FIC index >4.0) (American Society for Microbiology, 2004; John, 2004; Odds, 2003).

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

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

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant species

The mangosteen fruits (*Garcinia mangostana* L.) were purchased from a local market in Nakhon Ratchasima, Thailand. The specimen of this plant was authenticated with a voucher specimen at the Forest Herbarium, Bangkok, Thailand. The pericarps of mature mangosteen fruits were extracted to get α-mangostin or other bioactive compounds. The extraction and identification methods are mentioned in this chapter.

3.1.2 Test organisms

3.1.2.1 Bacterial strains

Clinical isolates of *Staphylococcus saprophyticus* DMST 27055 (*S. saprophyticus*), clinical isolates of *Enterobacter cloacae* DMST 21394 (*E. cloacae*) and clinical isolates of *Escherichia coli* DMST 19629 (*E. coli*), Clinical isolates of *Staphylococcus aureus* ATCC 29213 (*S. aureus*) and clinical isolates of *Escherichia coli* ATCC 25922 (*E. coli*) were obtained from the Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand and the American Type Culture Collection (ATCC), USA.

3.1.2.2 Preparation and maintenance of stock cultures

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

3.1.3 β-lactam antibiotics

Oxacillin and ceftazidime were obtained from Sigma, Bristol-Myers.

3.1.4 Culture media

Nutrient agar, Mueller-Hinton broth and agar were obtained from Oxiod. Apigenin, α-mangostin were obtained from Indofine chemical company (The Flavonoid Company, USA).

Approximate formula per liter of each medium was as following:

3.1.4.1 Nutrient agar

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

The formula was:

	g/L
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	1.5

pH (at 25 °C) 7.4 ± 0.2

3.1.4.2 Mueller-Hinton broth (MHB)

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

The formula was:

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

pH 7.4 ± 0.2 at 37 °C

Mueller-Hinton had been cation-adjusted that had the corrected concentrations of the divalent cations of Ca^{2+} 20 mg/L and Mg^{2+} 10 mg/L.

All culture media were dissolved by water.

3.1.4.3 Mueller-Hinton agar (MHA)

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

The formula was:

	g/L
Agar	17.0
Beef heart infusion	2
Casien acid hydrolysate	17.5
Soluble starch	1.5

pH 7.3 ± 0.2 at 25 °C

3.1.5 Chemicals

All chemicals used were laboratory grade otherwise specified.

Ethanol absolute	Lab grade
Hexane	Lab grade
Dichloromethane	Lab grade
Acetone	Lab grade
Ethyl acetate	Lab grade

Silica gel

Merck silica gel 60 Art. 7734 (70-230 mesh ASTM) was used as adsorbent for normal column chromatography.

Merck silica gel 60 G Art. 7731 and 60 GF_{254} Art. 7730 were applied as adsorbent for preparative TLC.

Merck TLC aluminum sheet, silica gel 60 F_{254} precoated 20 cm x 20 cm in size with layer thickness of 0.2 mm was used to identify the identical fractions.

Dimethylsulfoxide (DMSO)	AR grade
Disodium tetraborate	Lab grade
Sodium chloride	AR grade
Sodium phosphate	Lab grade
Sodium hydroxide	Lab grade
95% Ethanol	Lab grade
Ammonium acetate	AR grade
Acetronitrile	Lab grade
Albumin	AR grade
Lecithin	Lab grade
Tween 80	Lab grade

Paraformaldehyde	Lab grade
Glutaraldehyde	Lab grade
Osmiun tetroxide	Lab grade
Methanol	Lab grade
Araldite	Lab grade
Agarose	Lab grade
Uranyl acetate	Lab grade
Lead acetate	Lab grade
HEPES buffer	Lab grade
Phosphate	Lab grade
PMSF	Lab grade
Triton X-100	Lab grade
Ceftazidime	AR grade
Oxacillin	AR grade
α-mangostin	AR grade

3.1.6 Equipments

3.1.6.1 Apparatus

Rotary evaporator	Büchi
Heating bath: Büchi heating bath B-490	
Rotavapor: Büchi rotavapor R-200	
Controller: Büchi vacuum controller V-800	
UV-Cabinet II	Camag
NMR Spectrometer INOVA 300	Varian
Soxhlet apparatus	Büchi

Mixer (Model 5000)	Büchi
Hot air oven (Memmert-600)	Shellab
Column chromatography	Merck
Filter paper	Whatman
Tank of TLC analysis	Merck
Spectronic 21	Milton Roy
Labofuge	400R Heraeus
Autoclave	Yamato
Laminar air flow	Woerden
Hot air oven	Shellab
Shaking incubator	Heto
Hot plate	VELP scientifica
Refrigerated incubator	VELP scientifica
Ultramicrotome	JEM
Micropipettors (2-20 μL)	Witeg
Micropipettors (100-1000 μL)	Witeg
Centrifuge tubes	Pyrex
Spectraphysics	Agilent

3.1.6.2 Glasswares

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

Pipettes (1, 5, 10 μL)

Measuring cylinder (10, 20 μL)

Petri dishes

Test tubes

3.2 Methods

3.2.1 Extraction of compounds from the mangosteen fruit

Pericarps of mangosteen fruit were dried under a hot air oven and were powdered by mixer then were extracted using Soxhlet extractor. Hexane, acetone, dichloromethane, and Ethanol were used as solvent systems (1 kg dried mangosteen pericarp powder/ 3000 mL solvent/ extraction). The temperature of the extraction process was set at 75 °C for 8 hours. Each extract was concentrated in a rotary evaporator to give a brown sticky semi-solid for ethanol (262 g) and acetone (130 g) and yellowish powder for *n*-hexane (84 g) and dichloromethane (106 g). The crude extracts were isolated using column chromatography and thin layer chromatography.

3.2.2 Isolation of active compounds

The method for isolating the compounds from pericarp of mangosteen fruit procedure was based on those by Chairungsrilerd *et al*. (1996a, b, c), Chi *et al*. (2002) and Sakagami *et al*. (2005) with little modifications. The dichloromethane crude extract was subjected to silica gel column chromatography to yield eleven fractions, 1-11 on the successive elution with ethyl acetate: hexane system, of 10:90, 20:80, 30:70, 35:65, 40:60, 45:55, 50:50, 60:40, 70:30, 80:20 and 100:0, respectively. Every fraction of 1000 mL was collected and concentrated to a small volume and then was separated by monitoring with TLC (2 x 5 cm^2 in size with ethyl acetate: hexane, 1:1 as developing solvent). Fraction 3 was confirmed by HPLC to contain α-mangostin and further purified by preparative thin layer chromatography (hexane:acetone 2:1) to give the main component, a yellowish crystalline compound. The purification of this compound was confirmed by Thin layer chromatography with dichloromethane: hexane system (50:50) under UV condition and its structure continued to be elucidated

by NMR at the Center for Scientific and Technological Equipment (CSTE), Suranaree University of Technology and compared with the structure spectrum data of previous papers.

3.2.3 Preparation of test solution and inoculums

Antibiotic test solutions were prepared by dissolving Oxacillin and Ceftazidime (1 mg/mL) in sterile water.

A solution (10% dimethylsulfoxide in water) of crude extract and/or α-mangostin or other bioactive compounds (CH_2Cl_2 crude extract, fr_3 extract and α-mangostin) from the pericarp of GML extract alone and in combination with selected drugs were prepared by the doubling dilution method with sterilized water and adjustment to give the required test concentrations.

Test organisms were incubated in 100 mL nutrient broth for 18 h at 37 °C. The cell cultures were centrifuged at 4,000 r.p.m for 10 min. The cell pelletes were washed with saline, recentrifuged and resuspended in saline. The cell concentrations were adjusted with saline to give 5 x 10^8 CFU/mL using a predetermined calibration curve of absorbance at 500 nm against viable count (Liu *et al*., 2000). The MICs of crude extract, fr_3 extract, α-mangostin and the selected antibiotics alone and there extraction in combination with each antibiotic were examined.

3.2.4 Bacterial suspension standard curve

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

A separate loopful of each bacterium was used to inoculate in 100 mL of Mueller Hinton broth. The cultures were incubated at 37 °C for 18 h. The bacterial cells were pelletted by centrifugation at 4,000 r.p.m. for 10 min. The cells were

washed twice by resuspending and centrifuging at 4,000 r.p.m/min for 10 min in 10 mL of 0.9% NaCl. The cells were resuspended in 50 mL of sterile 0.9% NaCl. The cell suspensions were diluted so that 5-6 spectrophotometer readings could 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 overdried agar plate counting method. (Eumkeb, 1999; Richards *et al*., 1993).

3.2.5 MICs determination

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

MICs were determined using an agar dilution method. The sterile wire loop test organism from a slope culture was inoculated into Mueller Hinton broth and was incubated for 18 h at 37 $^{\circ}$C. Then, preparation of a bacterial suspension, the density of the bacterial suspension in normal saline was adjusted to approximately 1 x 10^8 CFU/mL by using the absorption of bacterial suspension viable count standard curve. The inoculum of 0.1 mL of standard of suspension (18 h culture) of each strain of the test bacteria was added to triplicate tubes containing 0.90 mL (MHB for the

tested strains), plus serial dilutions of the antibacterials, to give approximately 1 x 10^7 CFU/mL. Tubes of broth without antibacterials were used as the control for each of the test bacteria. Applied an aliquot of each inoculums to the agar surface which deliver 2 µL by using replicators with 3 mm pins. The final inoculum on the agar was approximately 10^4 CFU/spot. Incubation was at 37 °C for 16-24 h. The MICs were defined as the lowest concentration of antibiotic at which there is no visible growth in the triplicate spots (CLSI, 2013; Eumkeb, 1999; Wikler *et al*., 2006).

3.2.6 Checkerboard determination

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

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

An 18 h culture of each of the test bacteria was prepared. The test bacterial suspensions were adjusted to 1 x 10^8 CFU/mL using the absorption of bacterial suspension from the previously determine standard curve. The inoculum of 0.1 mL of standard of suspension (18 h culture) of each strain of the test bacteria was added to

triplicate tubes containing 0.90 mL (MHB for the tested strains), plus serial dilutions of the antibacterials, to give approximately 1 x 10^7 CFU/mL. Tubes of broth without antibacterials were used as the controls for each of the test bacteria. Applied an aliquot of each inoculums to the agar surface which deliver 2 μL by using replicators with 3 mm pins. The final inoculum on the agar was approximately 10^4 CFU/spot. The culture was incubated for 18-24 h at 37 °C. The test was carried out in triplicate. MICs were determined for each antibacterial combination and the isobolograms were plotted. The calculation of the FIC (Minimum Inhibitory Concentration) index for each antibacterial combination was undertaken as follows:

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

FIC (A+B)	≤ 0.5	Synergy
FIC (A+B)	> 0.5-4.0	No interaction
FIC (A+B)	> 4.0	Antagonism

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

3.2.7 Killing curve determinations

Viable counts for the determination of the killing curve were performed as previously described (Richards and Xing, 1996) with slight modification (Eumkeb, 1999) using a culture medium volume of 100 mL. Inocula of 5 x 10^6 CFU/mL of drug resistant bacteria were exposed to the antibacterials either singly or in combination with antibiotic drugs at concentrations ¼ of their MICs of CH_2Cl_2 crude extract, fr_3 extract and α-mangostin (bioactive compounds from the pericarp of GML extract) an incubation temperature of 37 °C. After contact time of 0, 0.5, 1, 2, 4, 6, and 24 h.

Subsequent dilution plating on overdried Mueller Hinton agar plates in quadruplicate and incubation at 37 °C for 18 h allowed counting of growing colonies. The lowest detectable limit for counting is 10^3 CFU/mL. Positive controls were used containing similar cell and solvent concentrations (Wikler *et al*., 2006).

3.2.8 Transmission electronmicroscopy (TEM) method

Preparation of cultures

To examine the effect of drugs, CH_2Cl_2 crude extract, fr_3 extract and α-mangostin on the cell structure of oxacillin-resistant *S. saprophyticus*, the following mehtods were performed.

S. saprophyticus was incubated in 10 mL Mueller Hinton broth for 18 h at 37 °C. A 2.0 mL volume of 18 h culture was inoculated into a 250 mL conical flask containing 98 mL Mueller Hinton broth which was placed in a water bath shaking at 100 oscillations/min for 4 h at 37 °C. The cells were then washed two times by suspending and centrifuging at 4,000 r.p.m. for 10 min in 0.9% NaCl. Volume of 10 mL of 5 x 10^7 of CFU/mL in 0.9% NaCl was inoculated into 250 mL conical flasks each containing 90 mL Mueller Hinton broth plus antibiotic drugs at concentrations ¼ of their MICs of CH_2Cl_2 crude extract, fr_3 extract and α-mangostin plus selected antibacterial to give approximately 5 x 10^6 CFU/mL final concentration. A flask containing 90 mL MHB for *S. saprophyticus* without any antibiotics was used as the control. The cultures together with the appropriate bioactive compounds from the pericarp of GML extract plus antibacterial and control cultures were incubated for 4 h shaking at 100 oscillations/min in a water bath at 37 °C (Richards *et al*., 1993; Xing, 1994).

The *S. saprophyticus* cultures were centrifuged at 6,000 g for 15 min at 4 °C and the supernate was removed. The pellets were fixed in glutaraldehyde 8% v/v in 0.1 M phosphate buffer (pH 7.2), for 1 h at 4 °C and then were fixed in 4%v/v glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h at 4 °C. After washing in the buffer the bacteria were suspended in osmium tetroxide (OsO_4, Emscope, Watford), 1% w/v, for 1 h at room temperature. They were then washed three times by centrifugation and resuspended in distilled water. The final pellet were resuspended in a small volume of warm agarose 2% w/v, poured onto a glass slide and were allowed to cool. When set, small pieces of gel containing suspended bacteria were cut out and dehydrated through a grade series of ethanol solutions. After embedding in resin, thin sections were cut with a diamond knife on an RMC ultramicrotome model MTX, stained with uranyl acetate and lead citrate, and examined in a JEOL, JEM 2010 electron microscope at 80-100 kV (Eumkeb, 1999; Richards *et al*., 1993).

3.2.9 Outer and Cytoplasmic membrane permeability

3.2.9.1 Outer membrane (OM) permeability

To examine the effect of antibacterial characteristic of bioactive compounds from the pericarp of GML extract (CH_2Cl_2 crude extract, fr_3 extract and α-mangostin) alone or in combination with antibiotics on the function of the OM as a permeability barrier, the following method was performed.

The cell cultures were incubated in 100 mL MHB for 18 h at 37 °C. Inocula of 10 mL of quantities of 18 h culture were added to 250 mL conical flasks containing 90 mL MHB in shaking water bath at 37 °C and shaking at 100 oscillations/min for 4 h. Inocula of 5 x 10^6 of CFU/mL for 10 mL of the 4 h log phase

culture were added to 250 mL conical flasks each containing 90 mL MHB plus CH_2Cl_2 crude extract, fr_3 extract and α-mangostin alone or in combination with antibiotic drugs at concentrations ¼ of their MICs against bacteria to give final concentration approximately 5 x 10^5 CFU/mL. The control flasks containing 90 mL MHB without antibiotics were used as control. The flasks were incubated at 37 °C for 4 h in a shaking water bath (Eumkeb, 1999; Richards *et al.*, 1993). The resulting cultures were harvested by centrifuging at 4,000 rpm for 10 min followed by washing the cells two times in 10 mL 0.9% NaCl. The cells were resuspended in sodium phosphate buffer and adjusted to an absorbance reading of 0.1-0.2 at 500 nm. The cell suspensions were treated with Triton X-100 (TTX-100) at a final concentration of 300 µg/mL in each tube. Cell suspensions without TTX-100 were used as control (Eumkeb, 1999).

3.2.9.2 Cytoplasmic membrane (CM) permeability

Cytoplasmic membrane permeability was determined by the ability of the peptides to unmask cytoplasmic β-galactosidase activity in bacteria by using ortho-nitrophenylgalactoside (ONPG) as the substrate. The method of sample preparation was prepared the same as for the OM permeability determinations. Then, 0.5 mL of culture were transferred from each flask at 0, 1, 2, 3, 4, and 5 h. These samples were diluted to 1.0 mL with buffer. Beta-galactosidase activity was measured using ONPG (Marri *et al.*, 1996; Miller, 1972). The method to assay of beta-galactosidase was as follows:

The cell density of a diluted culture in assay buffer was determined by measuring the absorbance at 600 nm. Volume of 0.2 mL of ONPG (4 mg/mL) was added to each tube and shaken for a few seconds. The tubes were

placed in a water bath at 37 °C. The time of the reaction was determined with a stop watch and the reaction stopped by adding 0.5 mL of a 1 M Na_2CO_3 solution after sufficient yellow colour had developed. The absorption was recorded at 420 nm for each tube. Toluene 50 µl/mL was used as a positive control (Eumkeb, 1999).

3.2.10 Electrophoresis

3.2.10.1 Extraction of bacterial membrane peptidoglycan-associated protein

To examine the effect of antibacterial characteristic from bioactive compounds from the pericarp of GML extract (CH_2Cl_2 crude extract, fr_3 extract and α-mangostin) alone and in combination with antibiotic drugs on the bacterial membrane and peptidoglycan associated protein (BMPG). The following method was practiced.

S. saprophyticus was incubated in 100 mL quantities of Mueller Hinton broth for 18 h at 37 °C. An 8.0 mL volume of 18 h culture was inoculated into a 250 mL conical flask containing 192 mL Mueller Hinton broth which was place in a water bath shaking at 100 oscillations/min for 4 h at 37 °C. Volumes of 100 mL of 1×10^6 CFU/mL for the 4 h log phase culture were inoculated into 250 mL conical flasks each containing 100 mL Mueller Hinton broth plus concentrations ¼ of their MICs of CH_2Cl_2 crude extract, fr_3 extract and α-mangostin plus selected antibacterial. A flask containing 100 mL Mueller Hinton broth without any antibacterials was used as the control. The log phase cultures together with the concentrations ¼ of their MICs of bioactive compounds from the pericarp of GML extract plus antibacterial and log phase control culture were incubated for 4 h shaking at 100 oscillations/min in a water bath at 37 °C. Bioactive compounds from the

pericarp of GML extract and drugs were used singly or in combination at ¼ MIC for clinical isolates of *S. saprophyticus* (Eumkeb, 1999; Richards *et al*., 1993; Xing, 1994).

A 200 mL of bacterial culture was harvested by centrifugation (15 min, 6,000 g, 4 °C) and washed twice with N-2-hydroxyethyl piperazine-N,-ethanesulphonic acid (HEPES) buffer (10 mM, pH 6.8). The bacteria were resuspended in 10 mL diluted water and disintegrated by sonication (3 x 60 s with a 30 s cooling period between each burst) at 4 °C. Unbroken cells were removed by centrifugation at 5,000 g, 4 °C for 5 min and the pellet was discarded.

The bacterial membrane and peptidoglycan complex was recovered by centrifugation at 40,000 g for 60 min, washed twice in distilled water containing 2 mg/mL phenyl methyl sulphonyl fluoride (PMSF). Then, the same precise weight (25 mg) of BMPG extract of each sample from *S. saprophyticus* was resuspended in 0.5 mL of (distilled water + 2 mL/mL PMSF). Therefore, the same quatity of BMPG extract of each sample (50 mg/mL) from *S. saprophyticus* could be investigated. These extract proteins were then stored frozen at -70 °C (Eumkeb, 1999; Richards and Xing, 1996; Williams and Gledhill, 1991).

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

3.2.10.2 SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used a gel system having a 4% stacking gel and a 15% separating gel. A volume of the

BMPG extract was mixed with a volume of sample buffer containing 0.125 M Tris-HCl buffer, pH 6.8, containing 0.04 M Na_2EDTA (Sigma), 4% w/v SDS, 10% w/v β-mercaptoethanol, 20% v/v glycerol and 0.1% w/v bromophenol blue (Sigma) and boiled for 5 min. The electrophoresis was performed at 8 mA per gel for stacking gel and 15 mA per gel for separating gel to maximize the resolution at the important subtyping areas of the gel. After electrophoresis the separating gel was stained with a Coomassie Brilliant Blue stain for 2 h at room temperature with gentle mixing. The gel was initially destained with 45% v/v ethanol, 10% v/v acetic acid solution followed by final destaining with 7% v/v acetic acid solution. The following standard proteins (BDH) was used as molecular mass markers: myoglobin (17,200), carbonic anhydrase (30,000), ovalbumin (42,700), albumin (66,250) and ovotransferrin (76,000-78,000) (Eumkeb, 1999).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Properties of bioactive compounds and yield from the pericarp of *Garcinia mangostana* L. (GML) extract

Soxhlet extraction was chosen to extract the bioactive compounds from pericarp of *G. mangostana* L. fruit. Hexane, acetone, dichloromethane and ethanol were used as extractants due to their safety and free solubility of the compounds. Also, good efficiency of these solvents in degrading plant cell walls promotes a greater amount of endocellular material in the extract. The percent yield of CH_2Cl_2 crude extract, Fr_3 extract, and α-mangostin at 10.6 (106g), 0.235 (2.35g), and 0.0162 (0.01623g) % w/w of dried powder respectively were obtained. The purity of CH_2Cl_2 crude extract, Fr_3 extract, and α-mangostin was analyzed by HPLC and the structures of the compounds were identified by NMR spectra compare to the reference.

4.1.1 Identification of bioactive compound present in the GML extract by HPLC

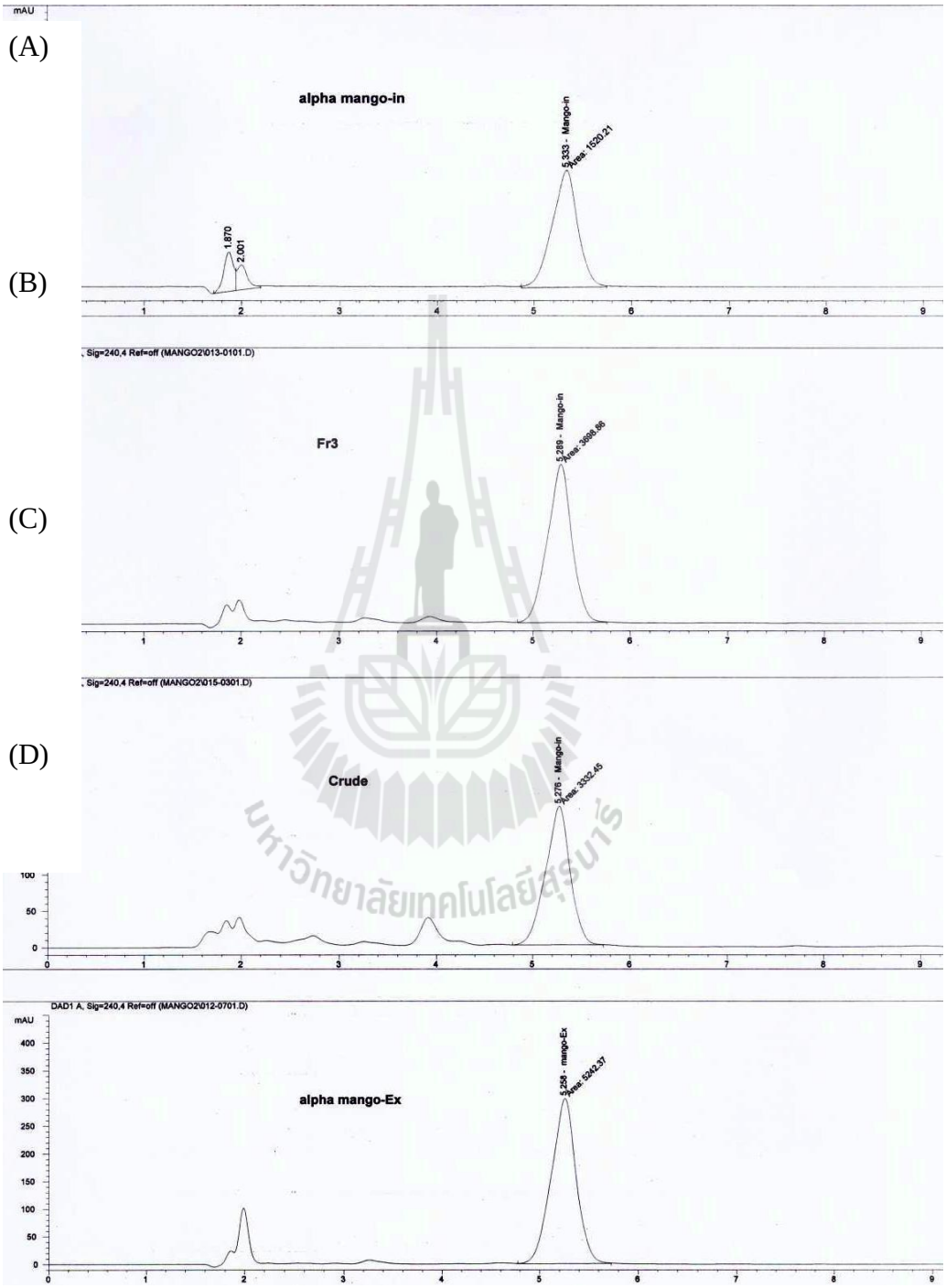


Figure 4.1 HPLC chromatograms of standard α-mangostin obtained from Indofine chemical (A), Fr_3 extract (B), CH_2Cl_2 crude extract (C) and α-mangostin extract (D).

HPLC method with gradient elution was developed for the qualification of bioactive compounds from the pericarp of GML extract. The mixture of 0.2% formic acid in acetonitrile gave optimum chromatographic separation of α-mangostin with other peaks in the extract (Figure 4.1). The wavelength at 240 nm was used for all measurements due to its maximum absorption. HPLC chromatograms of CH_2Cl_2 crude extract, Fr_3 and α-mangostin extracted showed similar pattern with a major peak of α-mangostin at retention time of 5.333 min (Figure 4.1(A)), 5.289 min (Figure 4.1(B)), 5.276 min (Figure 4.1(C)) and 5.258 min (Figure 4.1(D)), respectively. The identity of the peak of α-mangostin in the sample chromatograms was confirmed by spiking with the standard α-mangostin obtained from Indofine chemical company (Figure 4.1(A)).

HPLC method can be used for quantitative determination of α-mangostin in the extract from *G. mangostana* (Yodhnu *et al.*, 2009). In addition, HPLC method was proven to be precise, specific, sensitive, and accurate for routine quantity assessment of raw material of mangosteen fruit rind, its extract, and products (Pothitirat *et al.*, 2009).

4.1.2 Structural elucidation of α-mangostin from NMR

This compound, α-mangostin, is yellow a powder classified in the xanthone group. The structure contains two double bonds susceptible to hydrogenation, one methoxyl group and three hydroxyl groups as shown in Figure 4.2, Table 4.1, and 4.2 show ^{1}H NMR and ^{13}C NMR data of α-mangostin extracted compared to the reference.

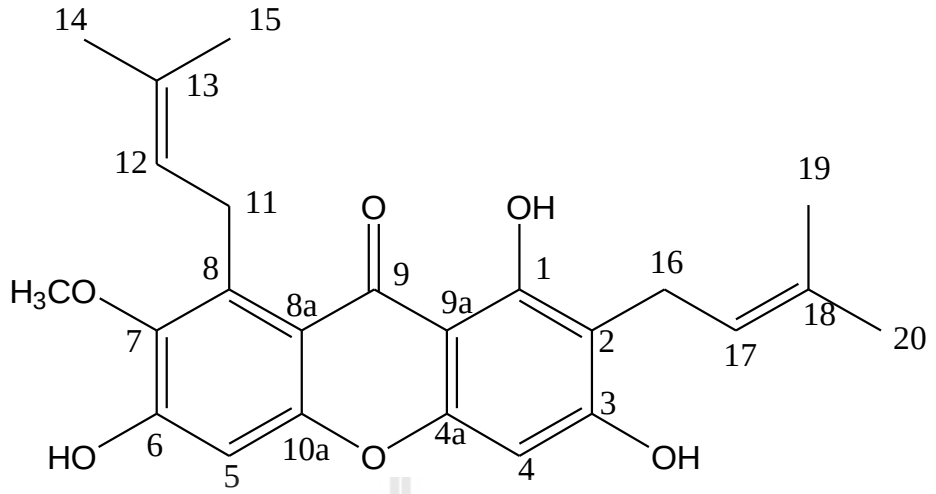


Figure 4.2 Structure of α-mangostin from NMR chromatogram reported by Ee *et al*. (2006).

Table 4.1 The 300 MHz ^{1}H NMR (acetone-d6) spectral data of α-mangostin.

Chemical Shift (δ, ppm)	Assignment	Chemical Shift (δ, ppm) from reference (Ee *et al*., 2006)
13.78	singlet, OH-1	13.72
6.82	singlet, H-5	6.72
6.40	singlet, H-4	6.25
5.27	triplet, H-12, H-17	5.26
4.13	doublet, H-11	4.10
3.80	singlet, 7-OMe	3.78
3.35	doublet, H-16	3.37
2.07	singlet, H-20	1.83
2.05	singlet, H-15	1.82
1.81	singlet, H-14	1.71
1.65	singlet, H-19	1.68

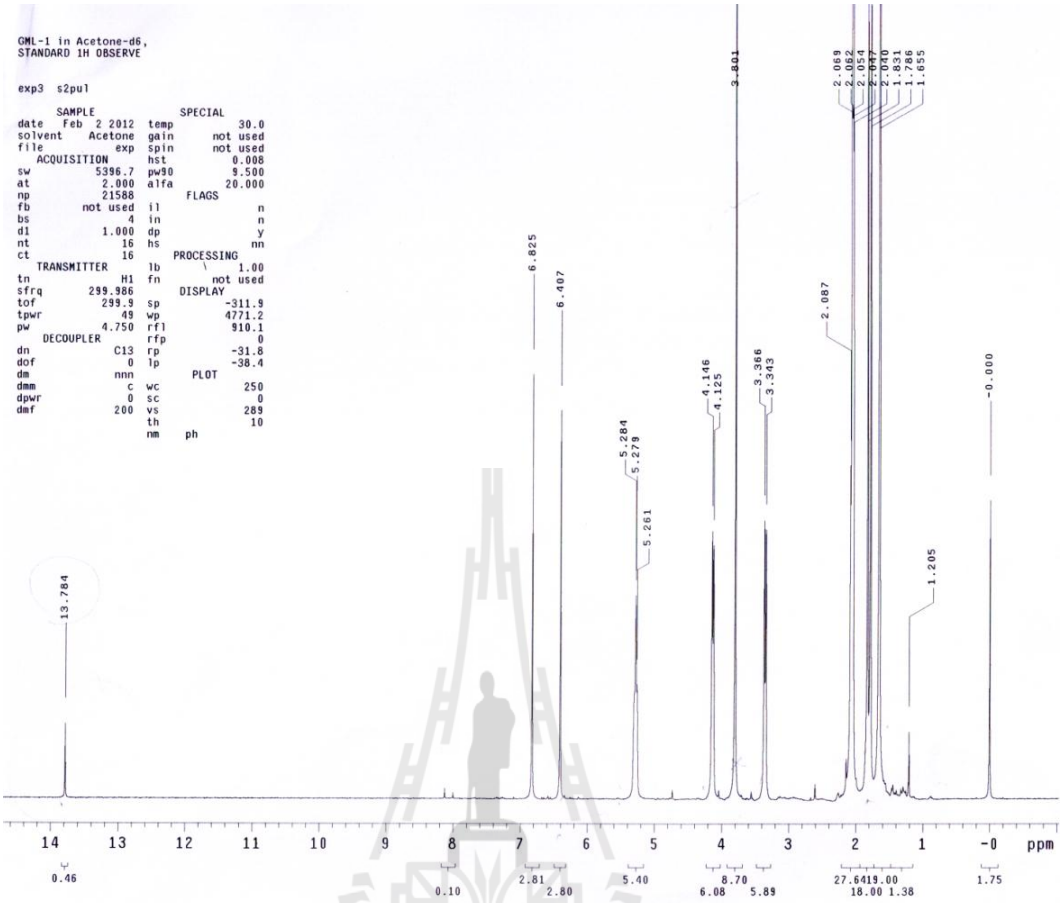


Figure 4.3 ^{1}H NMR spectroscopic data of α-mangostin extracted.

Table 4.2 The 300 MHz ^{13}C NMR (acetone-d6), spectral data of α-mangostin.

Chemical Shift (δ, ppm)	Assignment	Chemical Shift (δ, ppm) from reference (Ee *et al*., 2006)
182.81	C-9	181.8
162.92	C-3	161.6
161.40	C-1	160.2
157.39	C-6	155.4
156.23	C-10a	155.2
155.65	C-4a	154.8
144.51	C-7	142.7
138.14	C-8	137.2
131.39	C-13	131.7
124.82	C-17	123.4
123.50	C-12	122.1
112.06	C-8a	111.7
111.00	C-2	109.7
103.63	C-9a	103.1
102.67	C-5	101.6
93.15	C-4	92.4
61.31	7-OCH_3	61.2
26.89	C-11	26.3
25.92	C-14	25.7
25.88	C-19	20.7
22.00	C-16	21.3
18.29	C-20	18.1
17.90	C-15	17.7

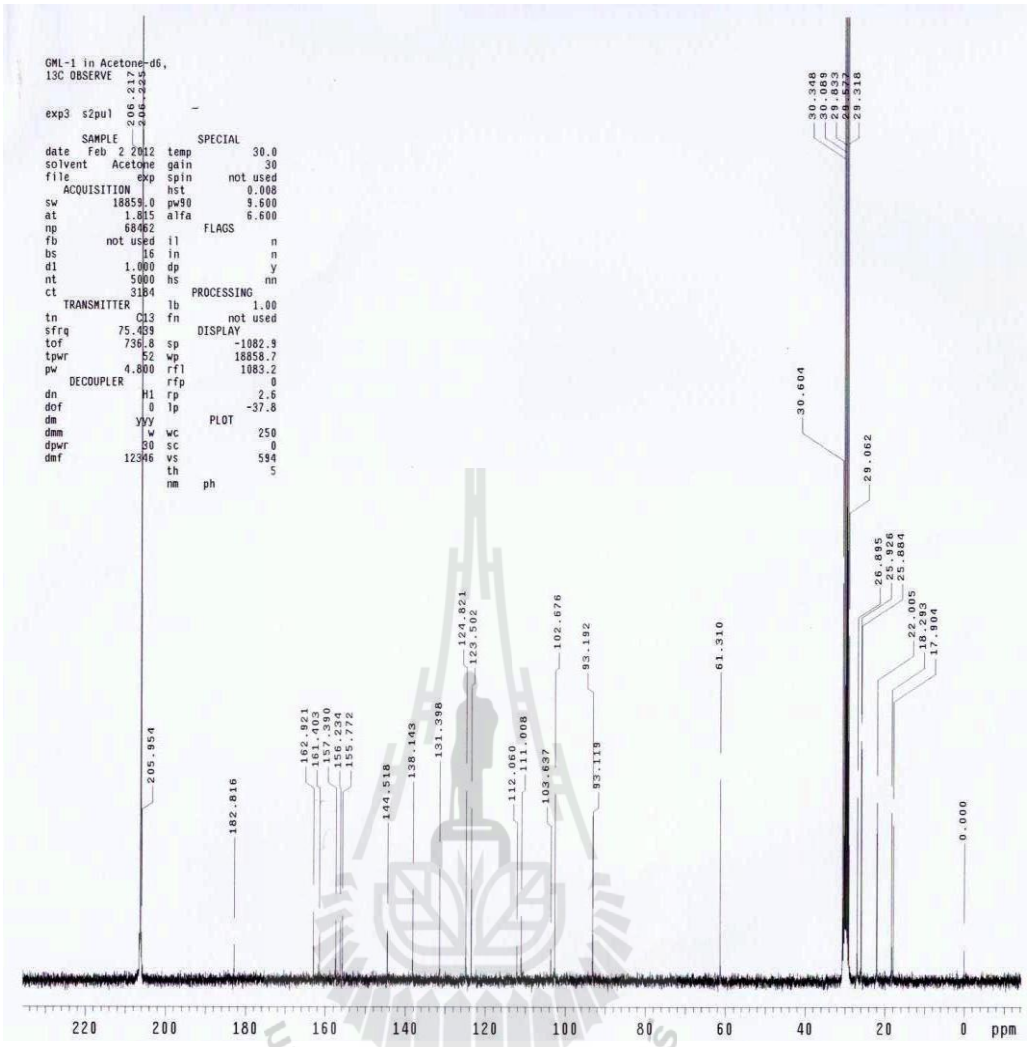


Figure 4.4 ^{13}C NMR spectroscopic data of α-mangostin extracted.

4.2 Bacterial suspensions viable count absorption standard curve

The results of the bacterial suspensions viable count standard curve for oxacillin-resistant *Staphylococcus saprophyticus* DMST 27055 (ORSS), ceftazidime–resistant *Escherichia coli* DMST 19629 (CREC), ceftazidime-resistant *Enterobacter cloacae* DMST 21394 (CREnC), oxacillin-sensitive *Staphylococcus aureus* ATCC 29213 (OSSA) and ceftazidime-sensitive *Escherichia coli*. ATCC 25922 (CSEC) are shown in Figures 4.5 to 4.9.

Figures 4.5 to 4.9 indicate that approximately $1X10^8$ CFU/mL of ORSS, CREC, CREnC, OSSA, and CSEC have absorption at 500 nm of 0.19, 0.15, 0.14, 0.12, and 0.10 respectively. This method was performed to count the exact bacterial number.

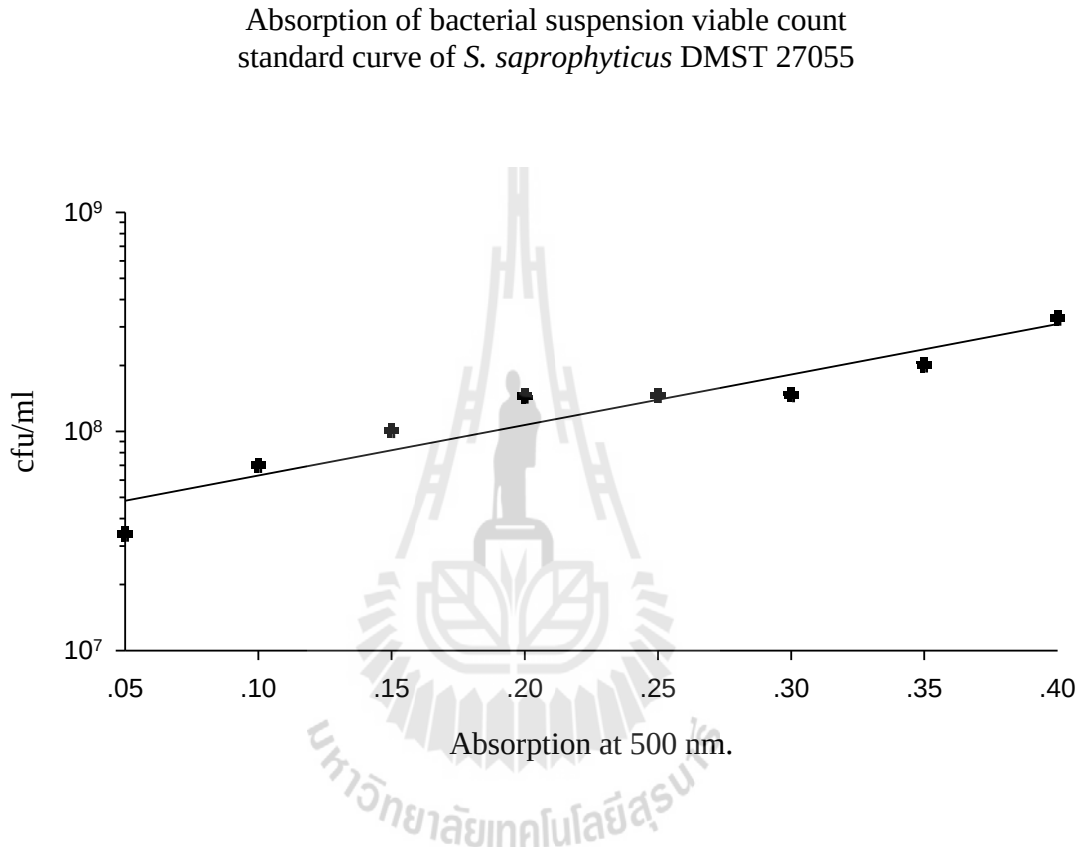


Figure 4.5 Standard curves for suspensions of oxacillin-resistant *Staphylococcus saprophyticus* DMST 27055.

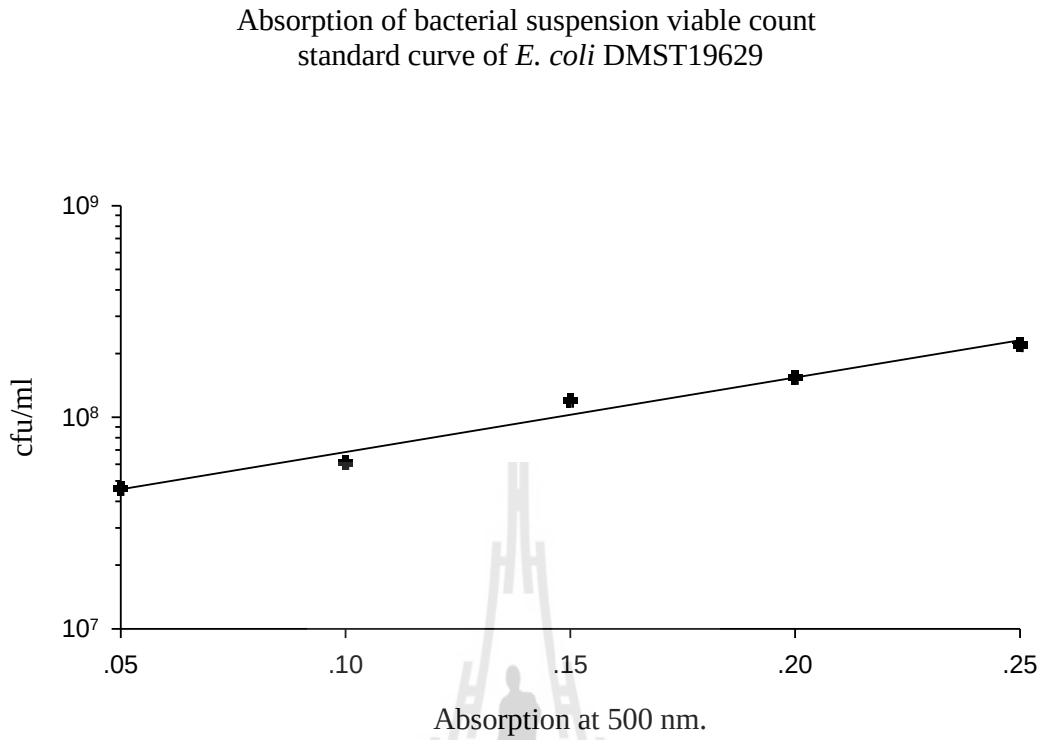


Figure 4.6 Standard curves for suspensions of ceftazidime-resistant *Escherichia coli* DMST 19629.

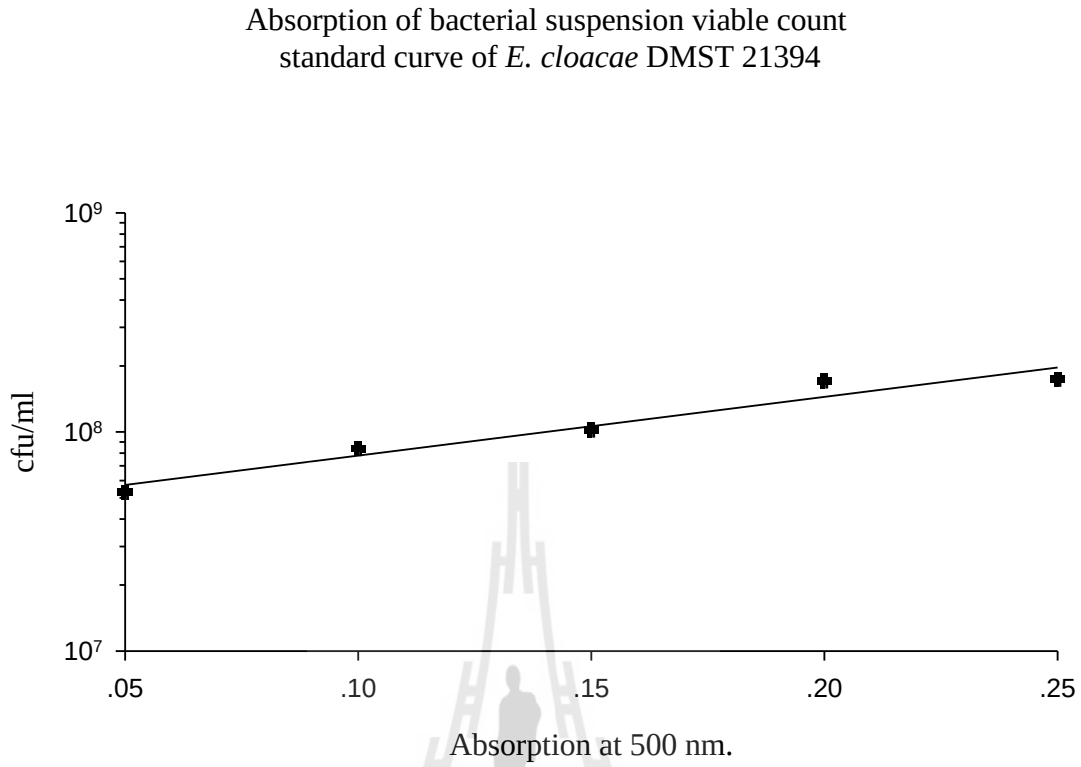


Figure 4.7 Standard curves for suspensions of ceftazidime-resistant *Enterobacter cloacae* DMST 21394.

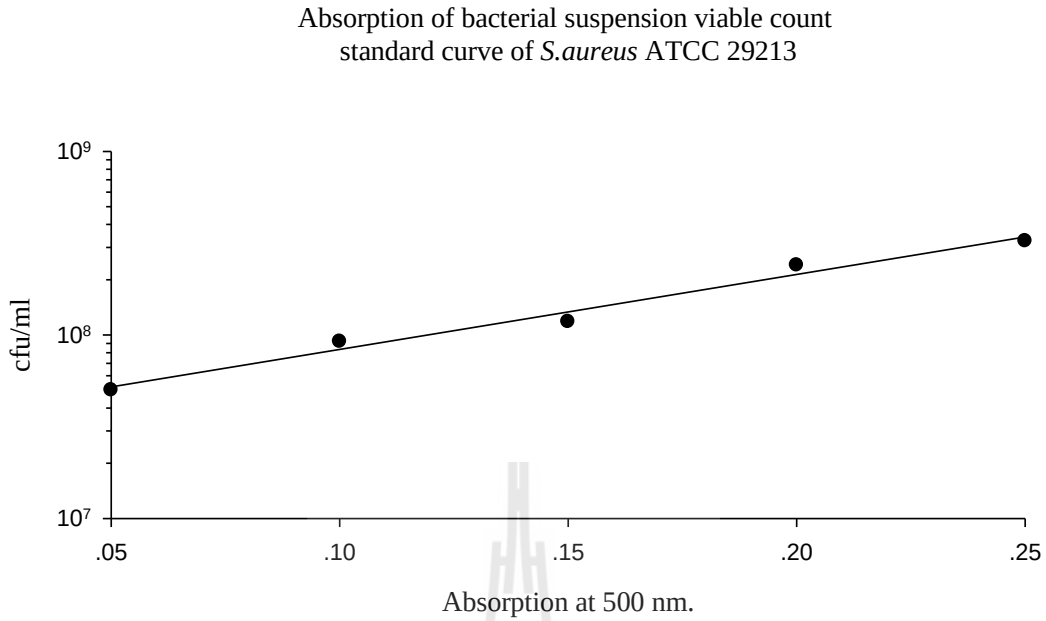


Figure 4.8 Standard curves for suspensions of oxacillin-sensitive *Staphylococcus aureus* ATCC 29213.

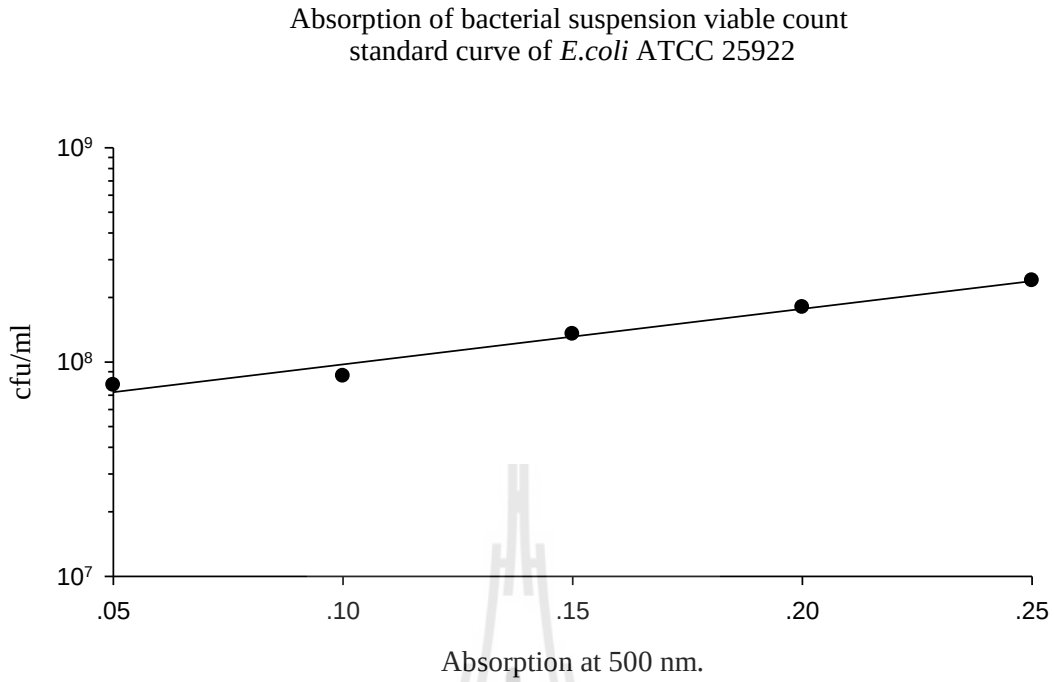


Figure 4.9 Standard curves for suspensions of ceftazidime-sensitive *Escherichia coli* ATCC 25922.

4.3 MIC determination

Table 4.3 Minimum inhibitory concentration of the following β-lactams and bioactive compounds from the pericarp of GML extract against clinical isolates of oxacillin-resistant *S. saprophyticus*, ceftazidime-resistant *E. coli*, ceftazidime- resistant *E. cloacae*, oxacillin-sensitive *S. aureus* and ceftazidime-sensitive *E. coli* determined by agar dilution method.

Bioactive compounds (μg/mL)	Susceptibility (MIC)					
	S. saprophyticus DMST 27055	*S. aureus* ATCC 29213 (sensitive strian)	*E. coli* DMST 19629	*E. coli* ATCC 25922 (sensitive strian)	*E. cloacae* DMST 21394	*E. coli* ATCC 25922 (sensitive strian)
Crude extract (CH_2Cl_2)	50	20	>10,000	>10,000	>10,000	>10,000
Fr_3	31	7	>10,000	>10,000	>10,000	>10,000
α-mangostin	8	4	>1,024	>1,024	>1,024	>1,024
Ceftazidime	-	-	>1,024	8	>1,024	8
Oxacillin	128	2	-	-	-	-

These results showed MIC values of bioactive compounds from the pericarp of GML extract and selected drugs against clinical isolates of oxacillin-resistant *S. saprophyticus*, ceftazidime–resistant *E. coli*, ceftazidime-resistant *E. cloacae*, oxacillin-sensitive *S. aureus* and ceftazidime-sensitive *E. coli* determined by agar dilution method. Oxacillin alone showed some antibacterial activities against clinical isolates of oxacillin-resistant *S. saprophyticus* and clinical isolates of oxacillin-sensitive *S. aureus* at MICs 128 and 2 μg/mL, respectively. The MIC of oxacillin against OSSA strain exhibited sensitive value (CLSI, 2013). Ceftazidime alone showed some antibacterial activities against clinical isolates of ceftazidime–resistant *E. coli*, clinical isolates of ceftazidime–resistant *E. cloacae* and clinical isolates of ceftazidime-sensitive *E. coli* at MICs >1024, >1024 and 8 μg/mL, respectively. In the same way, the sensitive MIC value of ceftazidime against CSEC strain was proved (CLSI, 2013).

The MIC values of CH_2Cl_2 crude extract, Fr_3 extract, and α-mangostin against clinical isolates of oxacillin-resistant *S. saprophyticus* were 50, 31 and 8 μg/mL, respectively. Whereas, the MIC values of CH_2Cl_2 crude extract, Fr_3 extract, and α-mangostin against both clinical isolates of ceftazidime-resistant *E. coli* and ceftazidime-resistant *E. cloacae* were at >10,000, >10,000 and >1,024 μg/mL, respectively. These MICs values against these sensitive strains were the same as those of resistant strains.

These results indicate that bioactive compounds from the pericarp of GML extract exhibit higher potency against ORSS than oxacillin alone. On the other hand, the ceftazidime and bioactive compounds from the pericarp of GML extract did not inhibit those of selected gram-negative bacteria. The results seem inconsistent with

those of Iikubo *et al*. (2002) that this extract showed antibacterial activity against *Helicobacter pylori*, which is a gram negative rod. Nevertheless, these results are in substantial agreement with those of Chomnawang *et al*. (2009) and Iinuma *et al*. (1996) that this extract inhibited MRSA, *Staphylococcus epidermidis* and *Propionibacterium* strain.

In general, gram-negative drug-resistant bacteria were greater resistant to β-lactam antibiotics. These were likely to be the result of the difference in cell wall structure between gram-positive and gram-negative bacteria. The gram-negative bacteria has a multi-layered and complex structure. The outer membrane can act as a barrier to many environmental substances including antibiotics (Eumkeb *et al*., 2004).

4.4 Checkerboard determination

The reasons to support the use of antimicrobial combinations are first the decreased emergence of resistant strains, secondly the decreased dose-related toxicity as a result of reduced dosage, and thirdly, polymicrobial infection (Lorian, 1999). Some previous researchers reported antibacterial activities of plant material combination with antibiotics against both gram-positive and gram-negative bacteria (Darwish *et al*., 2002; Liu *et al*., 2000). The oxacillin-resistant *S. saprophyticus* DMST 27055 was chosen to perform this method and crude extract, fraction 3, α-mangostin against oxacillin were investigated for synergistic effects. The isobolograms obtained from plotting of checkerboard MIC determination are shown in Figure 4.6 to 4.8. A summary of data from the isobolograms is given in Table 4.4.

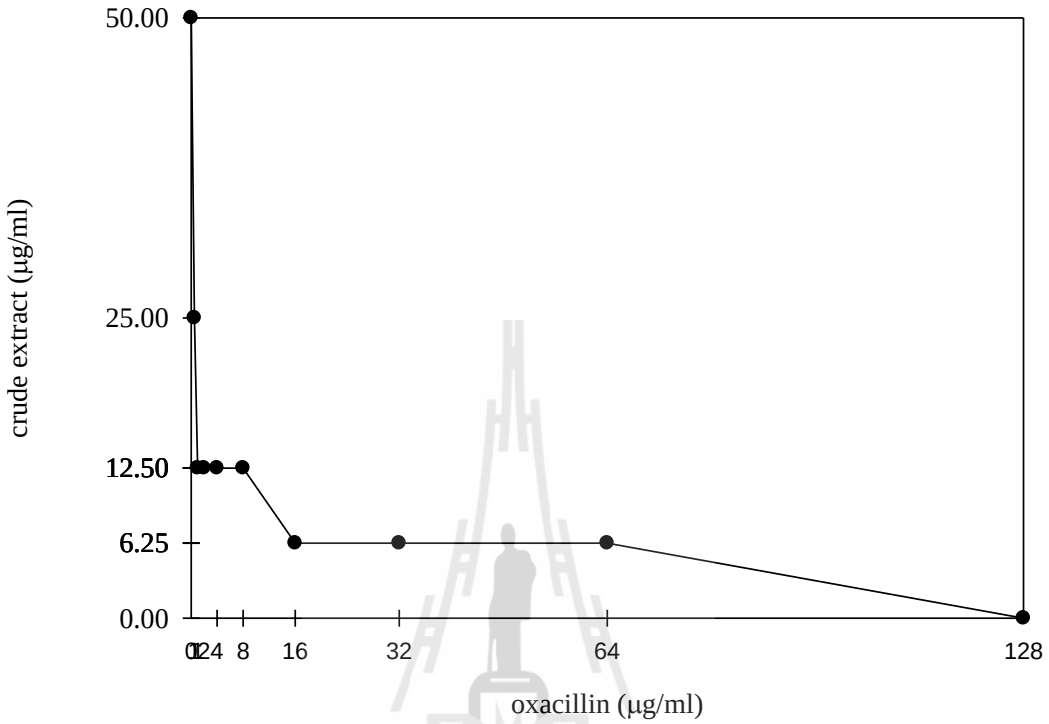


Figure 4.10 Isobologram constructed from checkerboard MIC data showing antibacterial combination of oxacillin plus CH_2Cl_2 crude extract against clinical isolates of oxacillin-resistant *S. saprophyticus* DMST 27055.

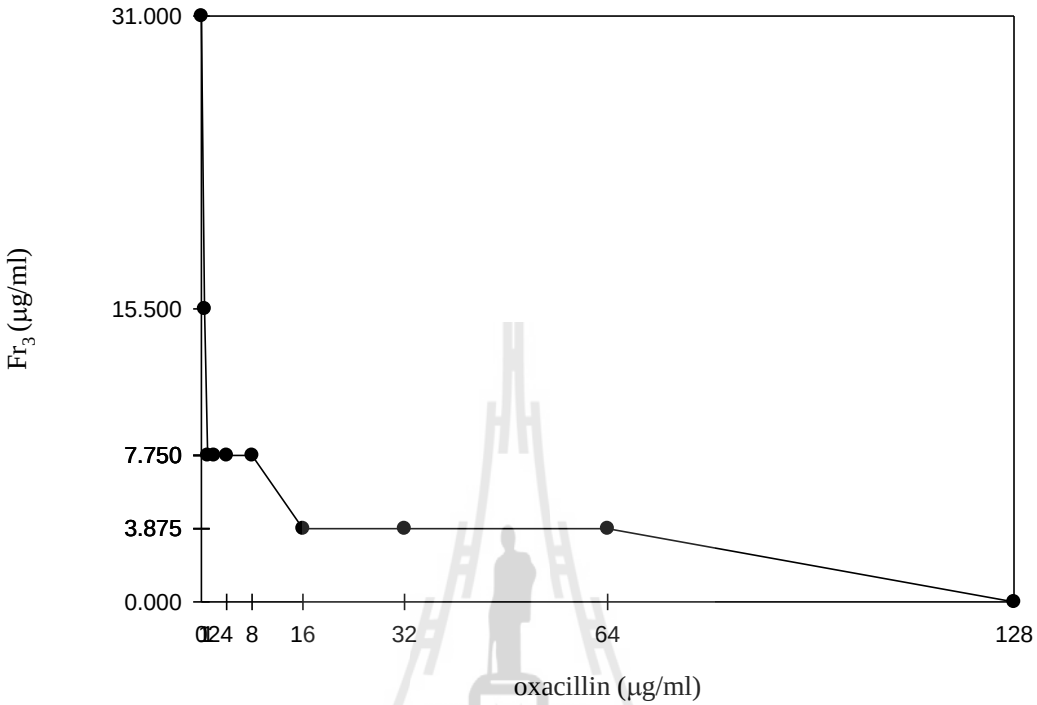


Figure 4.11 Isobologram constructed from checkerboard MIC data showing antibacterial combination of oxacillin plus Fr_3 extract against clinical isolates of oxacillin-resistant *S. saprophyticus* DMST 27055.

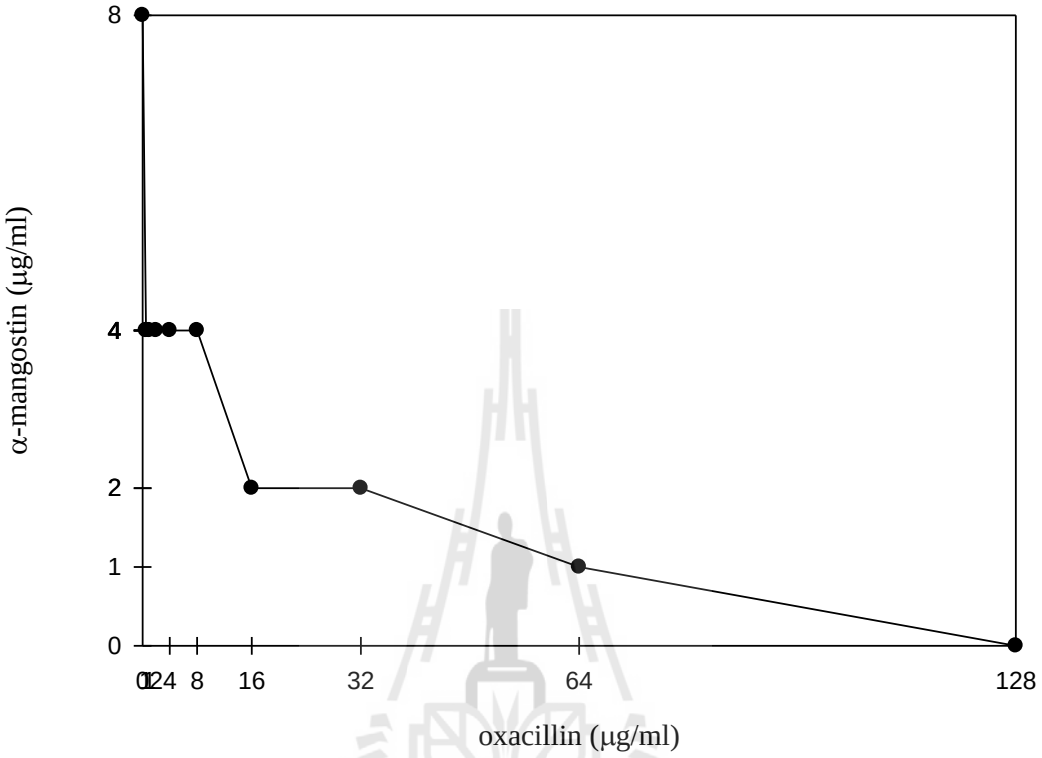


Figure 4.12 Isobologram constructed from checkerboard MIC data showing antibacterial combination of oxacillin plus α-mangostin against clinical isolates of oxacillin-resistant *S. saprophyticus* DMST 27055.

Figure 4.10 to 4.12 indicate the synergistic activity for combinations of bioactive compounds from the pericarp of GML extract and oxacillin against clinical isolates of oxacillin-resistant *S. saprophyticus* DMST 27055 (FIC ≤ 0.5) (Johnson *et al.*, 2004; Odds, 2003). The MICs of CH_2Cl_2 crude extract, Fr_3 extract, and α-mangostin plus oxacillin were reduced from 50, 31 and 8 μg/mL plus 128 μg/mL to 6.25 (1/8 MIC), 3.875 (1/8 MIC) and 2 (1/4 MIC) μg/mL plus 16 (1/8 MIC) μg/mL, respectively, against this strain.

The checkerboard results are shown in Tables 4.4. The synergistic activity for combination of bioactive compounds from the pericarp of GML extract and tested β-lactams against clinical isolates of oxacillin-resistant *S. saprophyticus*, ceftazidime-resistant *E. coli* and ceftazidime-resistant *E. cloacae* were evaluated. The lowest fractional inhibitory concentration (FIC) for each combination was calculated as described in Chapter III.

Table 4.4 Summary of the FICs for checkerboard assay of β-lactams used alone and in combination with bioactive compounds from the pericarp of GML extract against the drug resistant bacteria.

Test bacteria	Combination of agents	MIC (μg/mL)	MIC (A+B)	FIC index value	FIC index
S. saprophyticus	Oxacillin	128	16	0.25	synergism
	Crude extract	50	6.25		
	Oxacillin	128	16	0.138	synergism
	Fr_3	31	3.875		
	Oxacillin	128	16	0.375	synergism
	α-mangostin	8	2		
E. coli	Ceftazidime	>1,024	>1,024	>2	no interaction
	Crude extract	>10,000	>10,000		or antagonism
	Ceftazidime	>1,024	>1,024	>2	no interaction
	Fr_3	>10,000	>10,000		or antagonism
	Ceftazidime	>1,024	>1,024	>2	no interaction
	α-mangostin	>1,024	>1,024		or antagonism
E. cloacae	Ceftazidime	>1,024	>1,024	>2	no interaction
	Crude extract	>10,000	>10,000		or antagonism
	Ceftazidime	>1,024	>1,024	>2	no interaction
	Fr_3	>10,000	>10,000		or antagonism
	Ceftazidime α-	>1,024	>1,024	>2	no interaction
	mangostin	>1,024	>1,024		or antagonism

The checkerboard determinations demonstrated that the FICs index of CH_2Cl_2 crude extract, Fr_3 and α-mangostin plus oxacillin exhibited synergistic effects at 0.25, 0.138 and 0.375 respectively. In general, these results indicate a high level synergistic activities since values below 0.5 are widely accepted as representing synergism between two antibacterials (American Society for Microbiology, 2004; Johnson *et al*., 2004; Odds, 2003). These results are consistent with earlier finding that synergism between alpha-mangostin plus gentamicin (GM) against vancomycin resistant *Enterococci* (VRE) , alpha-mangostin and vancomycin hydrochloride (VCM) against methicillin resistant *Staphylococcus aureus* (MRSA) were revealed (Sakagami *et al*., 2005). Furthermore, these results are in substantial agreement with those of (Eumkeb *et al.*, 2010) that the combination of ceftazidime at 5 μg/mL and 5 μg/mL of test flavonoids (galangin, quercetin, and baicalein) exhibited synergistic effect by reduceing the CFU/mL of MRSA strain to 1 x 10^3 over 6 and throughout 24h. These results indicate that bioactive compounds from the pericarp of GML extract not only have weak activity of their own against ORSS but also have the ability to reverse the resistance of such bacterial strains to the activity of the primary antibiotics (Eumkeb *et al.*, 2010).

4.5 Killing curve determinations

Viable counts for the determination of the killing curves were performed as previously described by Richards and Xing (1996) with little modifications (Eumkeb, 1999).

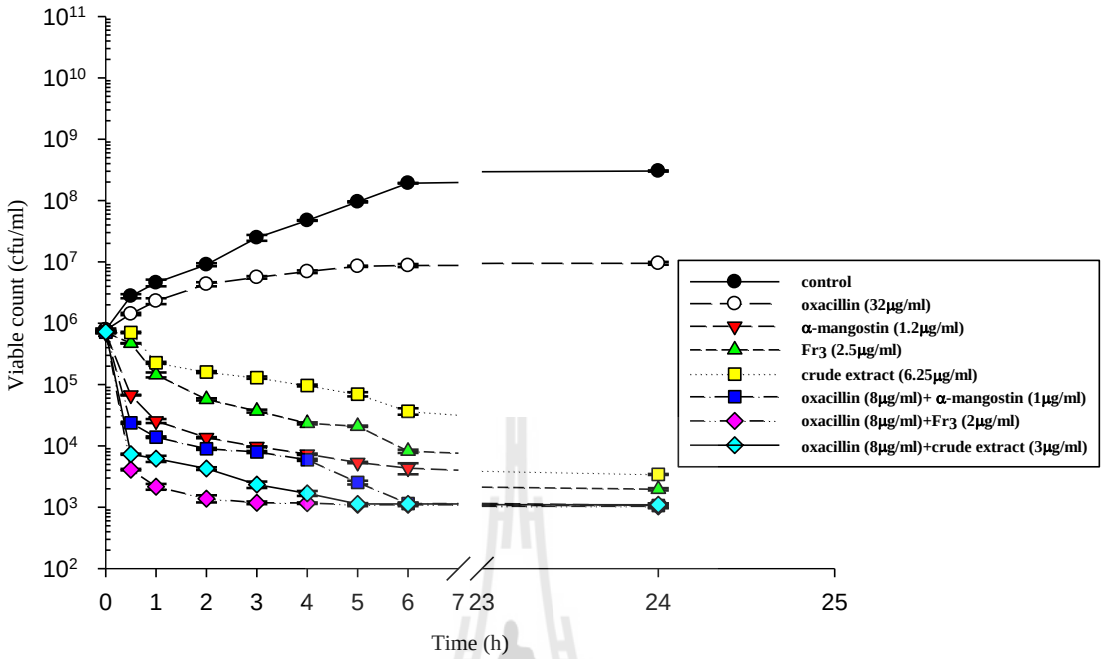


Figure 4.13 The effect of oxacillin combined with bioactive compounds from the pericarp of GML extract on the clinical isolates of oxacillin-resistant *S. saprophyticus* DMST 27055. The values plotted are the means of 3 observations, and the vertical bars indicate the standard errors of the means.

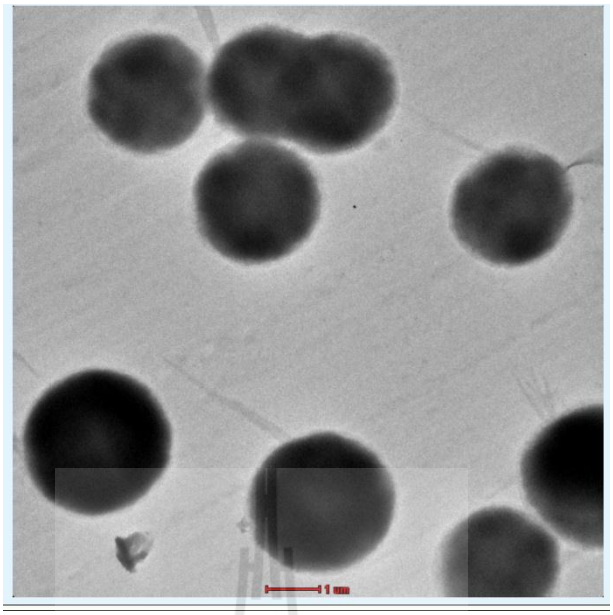
Sampling killing curves resulting from CH_2Cl_2 crude extract, Fr_3 and α-mangostin alone and in combination with oxacillin against clinical isolates of oxacillin-resistant *S. saprophyticus* are presented in Figure 4.13. The control showed no reduction in the counts of CFU from control inoculum. The results showed that the combination of CH_2Cl_2 crude extract (3.125 µg/mL) plus oxacillin (8 µg/mL), Fr_3 (1.94 µg/mL) plus oxacillin (8 µg/mL) and α-mangostin (1 µg/mL) plus oxacillin (8 µg/mL) caused a reduction of 5 x 10^5 CFU/mL of clinical isolates of oxacillin-resistant *S. saprophyticus* DMST 27055 to 10^3 CFU/mL within 6 h and throughout the

remainder of a 24 h period. These results seem consistent with earlier findings that Ceftazidime at 5 μg/mL in combination with 5 μg/mL of tested flavonoids reduced the CFU/mL of MRSA strain by 5 x 10^3 over 6 h. The reduced counts did not recover within 24 h (Eumkeb *et al.*, 2010).

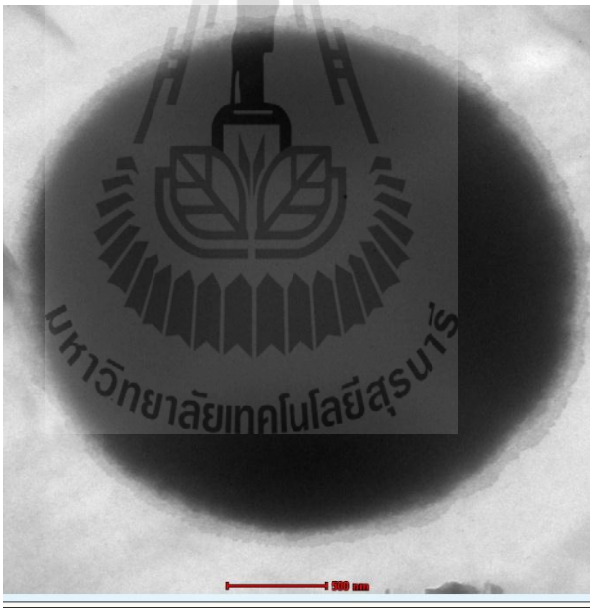
These results provide evidence that bioactive compounds from the pericarp of GML extract (CH_2Cl_2 crude extract, Fr_3 extract, and α-mangostin) in combination with oxacillin have synergistic activity against clinical isolates of oxacillin-resistant *S. saprophyticus*. In fact, the results of checkerboard assay, synergistic effects, are confirmed by this finding.

4.6 Transmission electronmicroscopy (TEM)

Electronmicroscope investigations clearly showed that the combination of oxacillin with bioactive compounds from the pericarp of GML extract caused damage to ultrastructures of clinical isolates of oxacillin-resistant *S. saprophyticus* (Figures 4.14-4.21).

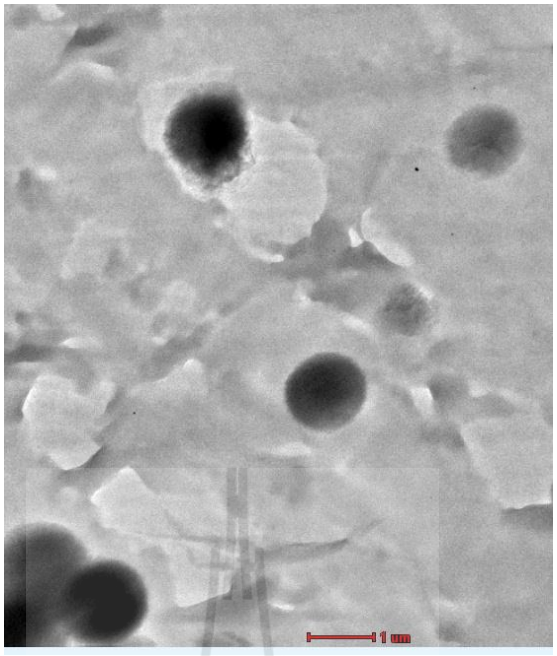


(a)



(b)

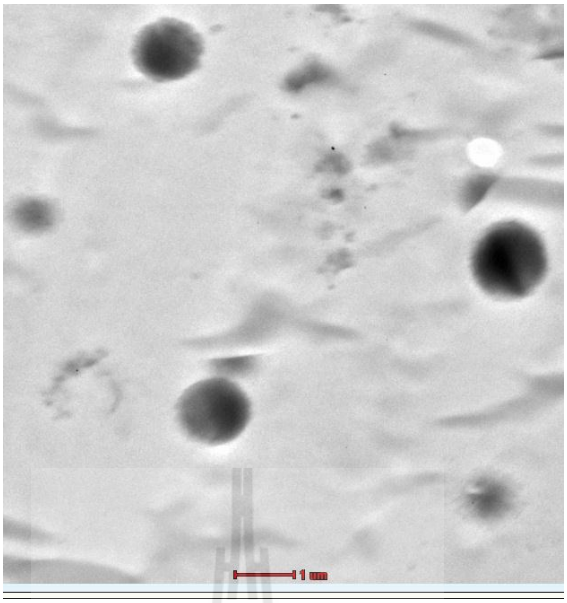
Figure 4.14 Utrathin sections of log phase of clinical isolates of oxacillin-resistant *S. saprophyticus* grown for 4 h in Mueller-Hinton broth: (a), (b), control (no antibacterial agent). x4,000, bar = 1 μm (a); x15,000, bar = 500 nm (b).



(a)

(b)

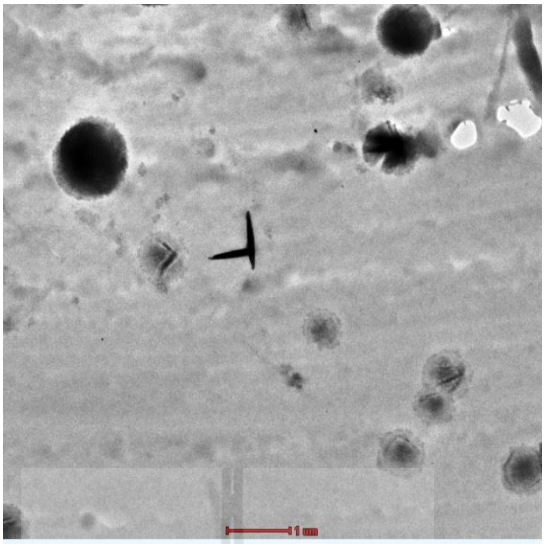
Figure 4.15 Utrathin sections of log phase of clinical isolates of oxacillin-resistant *S. saprophyticus* grown for 4 h in Mueller-Hinton broth: (a), (b), oxacillin (32 μg/mL). x4,000, bar = 1 μm (a); x19,500, bar = 200 nm (b).



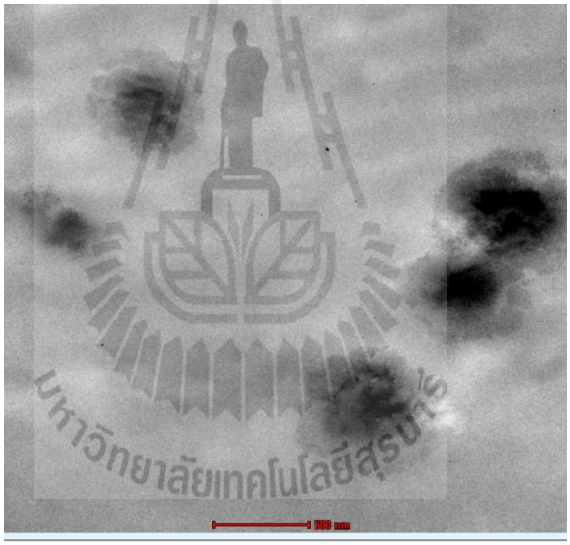
(a)

(b)

Figure 4.16 Utrathin sections of log phase of clinical isolates of oxacillin-resistant *S. saprophyticus* grown for 4 h in Mueller-Hinton broth: (a), (b), crude extract (6.25 µg/mL). x4,000, bar = 1 µm (a); x29,000, bar = 200 nm (b).

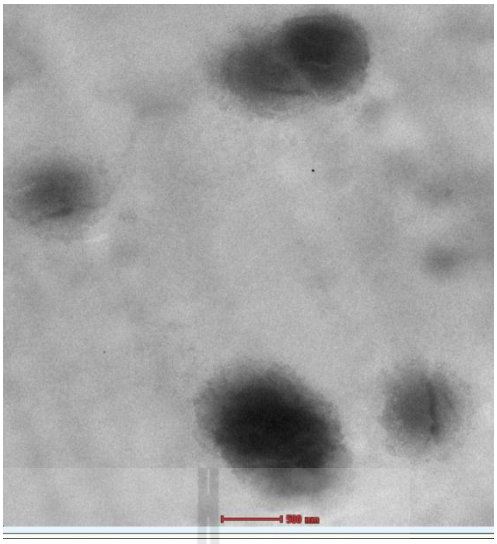


(a)



(b)

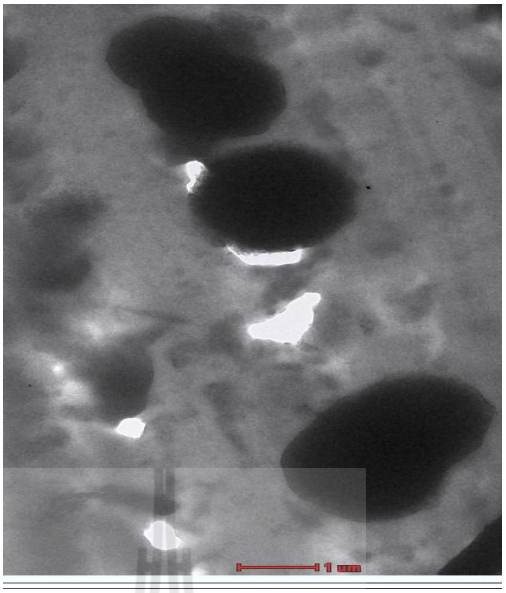
Figure 4.17 Utrathin sections of log phase of clinical isolates of oxacillin-resistant *S. saprophyticus* grown for 4 h in Mueller-Hinton broth: (a), (b), Fr_3 (2.50 µg/mL). x5,000, bar = 1 µm (a); x15,000, bar = 500 nm (b).



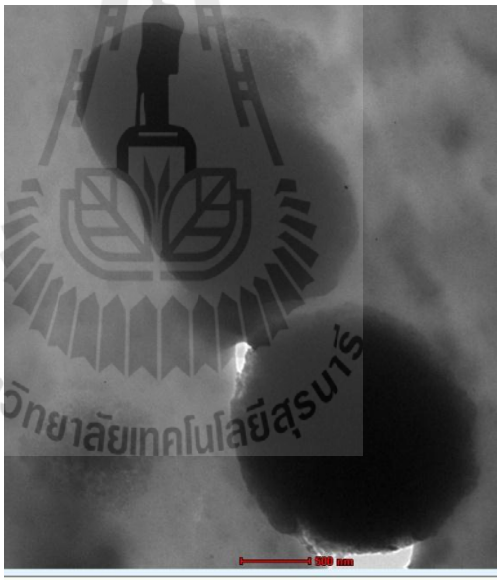
(a)

(b)

Figure 4.18 Utrathin sections of log phase of clinical isolates of oxacillin-resistant *S. saprophyticus* grown for 4 h in Mueller-Hinton broth: (a), (b), α-mangostin (1.2 μg/mL). x9,900, bar = 500 nm (a); x29,000, bar = 200 nm (b)

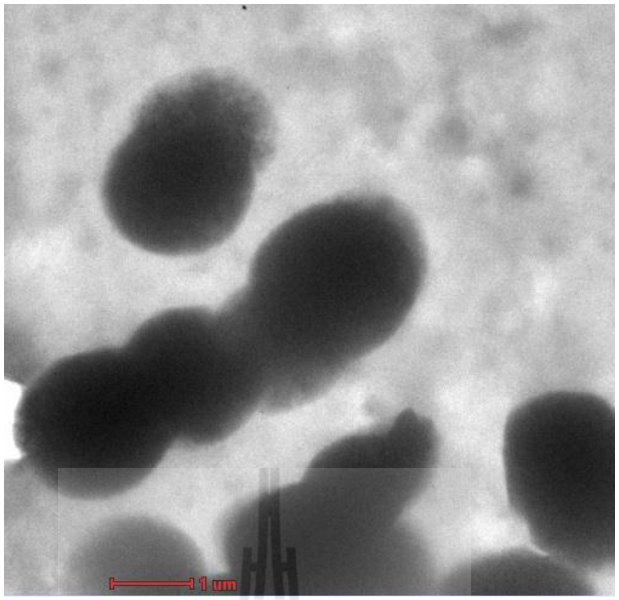


(a)

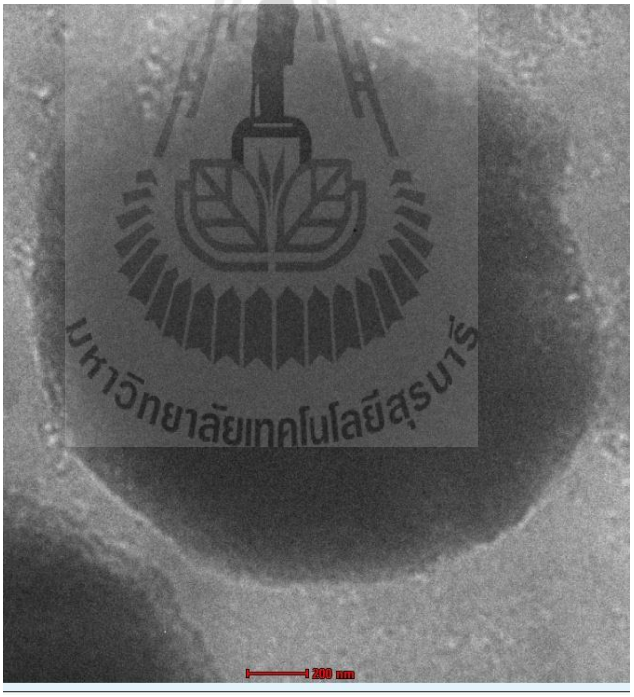


(b)

Figure 4.19 Utrathin sections of log phase of clinical isolates of oxacillin-resistant *S. saprophyticus* grown for 4 h in Mueller-Hinton broth: (a), (b), oxacillin (8 µg/mL) plus crude extract (3.125 µg/mL). x4,000, bar = 1 µm (a); x9,900, bar = 500 nm (b).

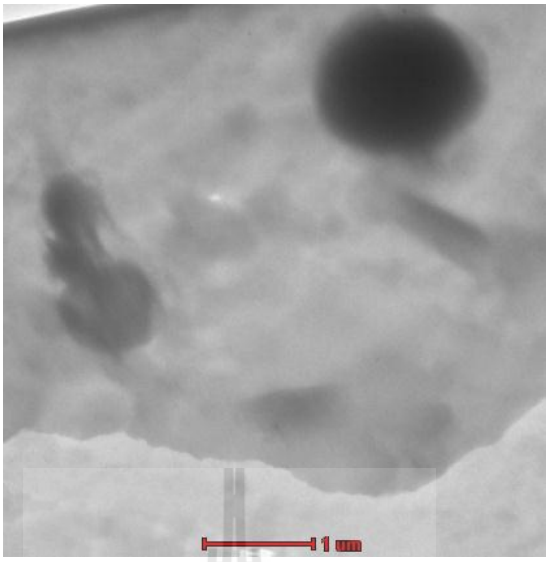


(a)



(b)

Figure 4.20 Utrathin sections of log phase of clinical isolates of oxacillin-resistant *S. saprophyticus* grown for 4 h in Mueller-Hinton broth: (a), (b), oxacillin (8 μg/mL) plus Fr_3 (1.938 μg/mL). x4,000, bar = 1 μm (a); x19,500, bar = 200 nm (b).



(a)

(b)

Figure 4.21 Utrathin sections of log phase of clinical isolates of oxacillin-resistant *S. saprophyticus* grown for 4 h in Mueller-Hinton broth: (a), (b), oxacillin (8 μg/mL) plus α-mangostin (1 μg/mL). x5,000, bar = 1 μm (a); x29,000, bar = 200 nm (b).

Figure 4.14 shows the appearance of normal log phase cells of clinical isolates of oxacillin-resistant *S. saprophyticus*. The cell wall and cytoplasmic membrane can be distinguished.

Figure 4.15 shows the effect of 32 µg/mL oxacillin on clinical isolates of oxacillin-resistant *S. saprophyticus* strain. Tested antibiotics showed no activity against the clinical isolates of oxacillin-resistant *S. saprophyticus.* These treated cell sizes seemed slightly smaller than those of control cell sizes.

Figure 4.16 shows the micrographs of log phase cells of clinical isolates of oxacillin-resistant *S. saprophyticus* after treatment with crude extract at 6.25 µg/mL. The crude extract treated cells showed rather smaller than those of normal cells. Most of these bacteria exhibited cell membrane damage.

Figure 4.17 shows the micrographs of log phase cells of clinical isolates of oxacillin-resistant *S. saprophyticus* after treatment with Fr_3 extract at 2.50 µg/mL. These treated cells were considerably smaller than those of control cells. Most of these bacteria exhibited cell membrane and morphological damage.

Figure 4.18 shows the micrographs of log phase cells of clinical isolates of oxacillin-resistant *S. saprophyticus* after treatment with α-mangostin at 1.2 µg/mL. The majority of these cells were substantially smaller than the control cells. Many of these bacteria exhibited change in morphology and cell membrane damage.

Figure 4.19 shows the effect of the combination of oxacillin 8 µg/mL plus crude extract 3.125 µg/mL on clinical isolates of oxacillin-resistant *S. saprophyticus*. The majority of these cells were rather smaller than the control cells. Some of these cells showed cell shape distortion and cell membrane damage.

Figure 4.20 shows the effect of the combination of oxacillin 8 μg/mL plus Fr_3 extract 1.938 μg/mL on clinical isolates of oxacillin-resistant *S. saprophyticus*. Most of these cells revealed cell shape distortion and cell membrane damage.

Figure 4.21 shows the effect of the combination of oxacillin 8 μg/mL plus α-mangostin 1 μg/mL on clinical isolates of oxacillin-resistant *S. saprophyticus*. Most of these cells showed a great deal smaller than the control cells. The majority of these cells undoubtedly exhibited cell shape and cell membrane.

These results are consistent with those of Eumkeb *et al*. (2010) that electronmicroscopy clearly showed that the combination of galangin and ceftazidime caused damage to the ultrastructures of the cells of MRSA strain. Apart from this, these results are in correspondence with those of (Oonmetta-aree *et al*., 2006) that transmission electron microscopy clearly demonstrated that galangal extract caused both outer and inner membrane damage and cytoplasm coagulation of *S. aureus* strain. From these results, it can be concluded that the oxacillin had little activity against clinical isolates of oxacillin-resistant *S. saprophyticus*. Whereas, biochemical compounds of GML extracts showed much higher potency than oxacillin against this strain. Moreover, the combination of oxacillin and these bioactive compounds obviously showed great synergism activity against this strain, as supported in Figure 4.21.

4.7 Outer membrane (OM) permeability

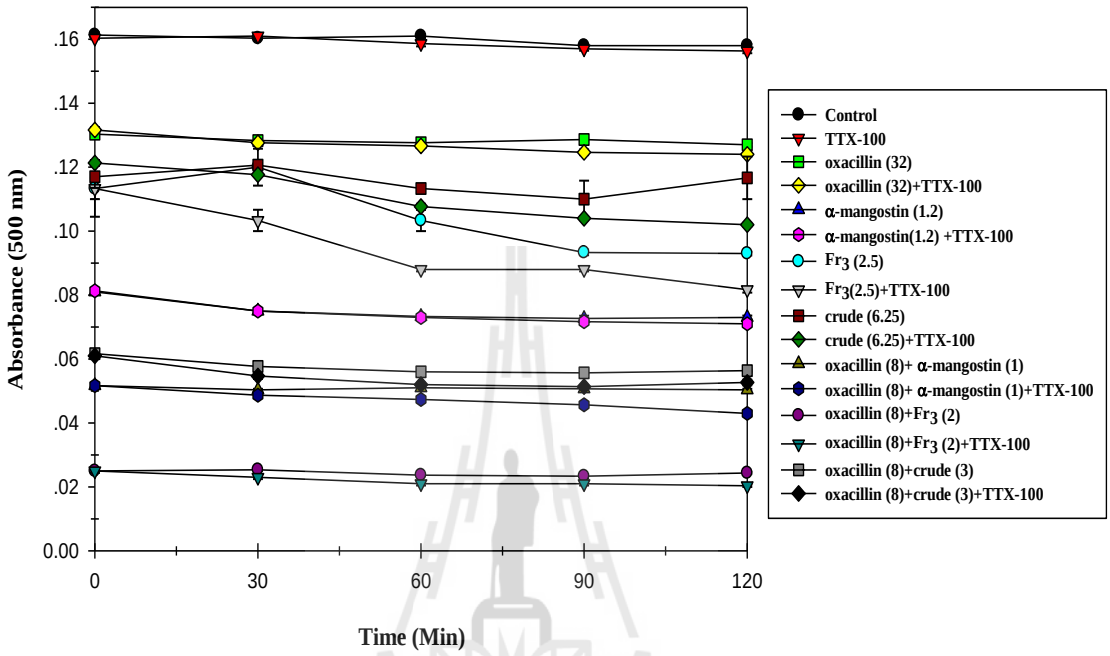


Figure 4.22 Permeabilization of clinical isolates of oxacillin-resistant *S. saprophyticus* by bioactive compounds from the pericarp of GML extract alone and in combination with oxacillin and lysis caused by subsequent treatment with 300 μg/mL TTX-100. The bars represent the standard deviations of 3 replicates.

Figure 4.22 shows the OM permeabilization of clinical isolates of oxacillin-resistant *S. saprophyticus* by oxacillin, CH_2Cl_2 crude extract, Fr_3 extract, and α-mangostin at concentrations of 32 μg/mL, 6.25 μg/mL, 2.50 μg/mL and 1.2 μg/mL, respectively, alone and in combination of oxacillin 8 μg/mL plus CH_2Cl_2 crude extract 3.125 μg/mL, oxacillin 8 μg/mL plus Fr_3 1.94 μg/mL and oxacillin 8 μg/mL plus α-mangostin 1 μg/mL. These results revealed that bioactive compounds from the pericarp of GML extract (CH_2Cl_2 crude extract, Fr_3 extract, and α-mangostin) and

oxacillin altered the OM permeability of clinical isolates of oxacillin-resistant *S. saprophyticus* when they were used alone and in combination at above concentrations. Triton X-100 was used as permeabilizing probe. These results are consistent with previous findings that D,L-1'-Acetoxychavicol acetate caused *S. aureus* outer membrane damage (Oonmetta-aree *et al*., 2006). Besides, the combination of both galangal extract plus amoxicillin and luteolin plus amoxicillin or apigenin plus ceftazidime also caused OM altered permeabilization of amoxicillin resistant *E. coli* or ceftazidime resistant *E. cloacae* respectively (Eumkeb and Chukrathok, 2013; Eumkeb *et al.*, 2011; Eumkeb *et al*., 2012). These findings indicate that OM permeability alteration this strain by either these bioactive compounds from the pericarp of GML extracts alone or in combination with oxacillin may play one of several important mechanism of actions and leads to cell lysis.

4.8 Cytoplasmic membrane (CM) permeability

Table 4.5 β-galactosidase activity results of clinical isolates of oxacillin-resistant *S. saprophyticus* after treatment with bioactive compounds from the pericarp of GML extract used alone and in combination with oxacillin 1 = Control, 2 = oxacillin 32 μg/mL, 3 = α-mangostin 1.2 μg/mL, 4 = Fr_3 2.50 μg/mL, 5 = crude extract 6.25 μg/mL, 6 = oxacillin 8 μg/mL plus α-mangostin 1 μg/mL, 7 = oxacillin 8 μg/mL plus Fr_3 1.94 μg/mL and 8 = oxacillin 8 μg/mL plus crude extract 3.125 μg/mL.

Time	1	2	3	4	5	6	7	8	Positive control Toluene 50 μL/mL	A_{420}
0 h	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg
1 h	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg
2 h	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg
3 h	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg
4 h	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
5 h	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Neg = No evidence of activity, Pos= have evidence of activity

The effect of either oxacillin and bioactive compounds from the pericarp of GML extract alone or in combination on the cytoplasmic membrane permeabilization of clinical isolates of oxacillin-resistant *S. saprophyticus* was investigated using the cytoplasmic enzyme β-galactosidase. The results showed that there was no increase in β-galactosidase activity with increasing time in the presence of either these agents alone or in combination within 0-3 h period. These finding suggest that it is likely that these compounds increase the permeability of the cytoplasmic membrane of *S. saprophyticus* after 4 h of treatment. In the same way, these results are consistent with previous findings that D,L-1'-Acetoxychavicol acetate caused *S. aureus* CM damage. Then, the cytoplasmic membranes impermeability properties was disrupted by determining the release of cell materials including nucleic acid which absorbed at 260 nm (Oonmetta-aree *et al*., 2006). In addition, the combination of luteolin plus amoxicillin or apigenin plus ceftazidime also caused CM altered permeabilization of amoxicillin resistant *E. coli* or ceftazidime resistant *E. cloacae* respectively (Eumkeb and Chukrathok, 2013; Eumkeb *et al*., 2012).

These results can be explained by assuming that the cytoplasmic membrane is also a highly selective barrier, enabling a cell to concentrate specific metabolites and excrete waste materials. The general structure of most biological membranes is a phospholipid bilayer. The major proteins of the cell membrane generally have very hydrophobic external surface in the regions of the protein that make intimate association with the highly non-polar fatty acid chains (Brock *et al*., 1997; Tropp, 1997).

4.9 Electrophoresis

The result of SDS-PAGE is shown in Figure 4.23.

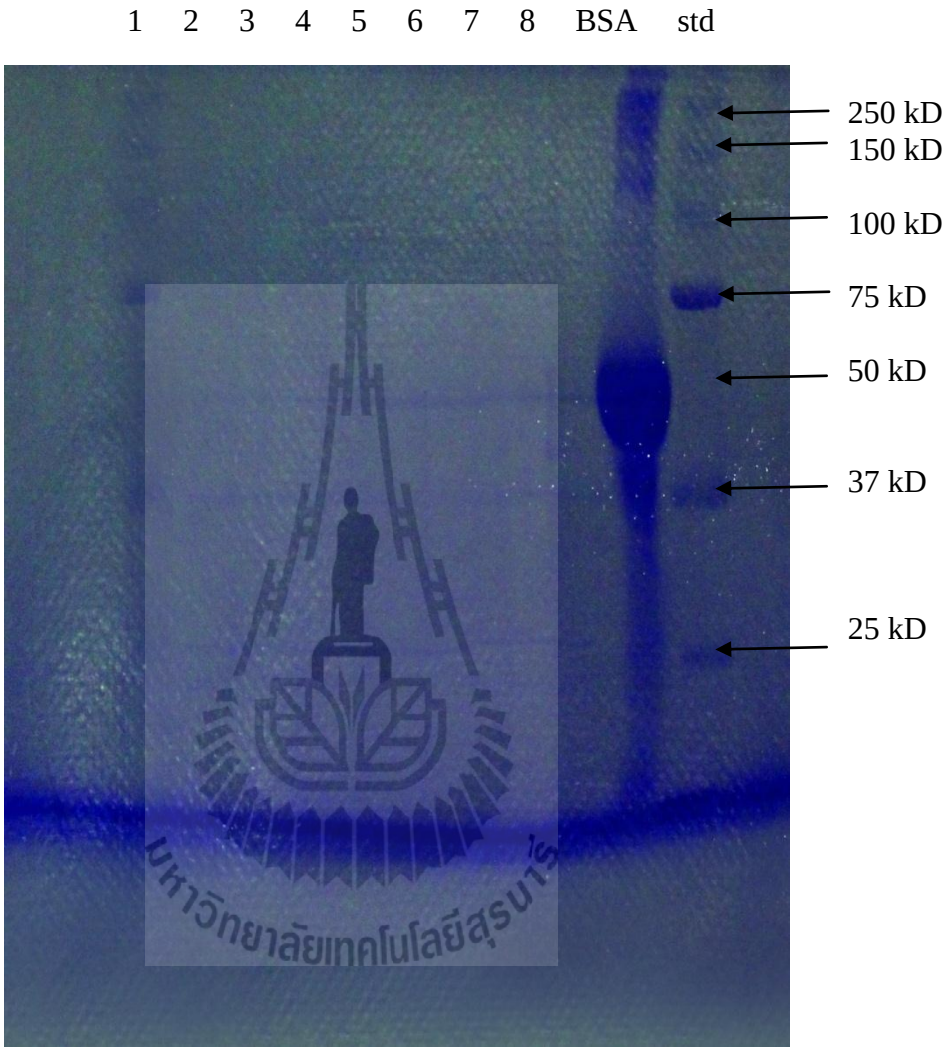


Figure 4.23 SDS-PAGE showing the bacterial membrane and peptidoglycan associated protein (BMPG) of clinical isolates of oxacillin-resistant *S. saprophyticus* DMST 27055 grown in the absence of drug (contron; lane 1), oxacillin 32 μg/mL (lane 2), α-mangostin 1.2 μg/mL (lane 3), Fr_3 2.50 μg/mL (lane 4), crude extract 6.25 μg/mL (lane 5), oxacillin 8 μg/mL plus α-mangostin 1 μg/mL (lane 6), oxacillin 8 μg/mL plus Fr_3 1.94 μg/mL (lane 7), oxacillin 8 μg/mL plus crude extract 3.125 μg/mL (lane 8), BSA; standard Bovine serum and std; molecular weight marker proteins (kDa).

Figure 4.23 shows that SDS-PAGE exhibited the BMPG of clinical isolates of oxacillin-resistant *S. saprophyticus* grown in the absence of drug (control; lane 1) and presence of bioactive compounds from the pericarp of GML extract; oxacillin alone at 32 μg/mL (lane 2), α-mangostin alone at 1.2 μg/mL (lane 3), Fr_3 extract alone at 2.50 μg/mL (lane 4), crude extract alone at 6.25 μg/mL (lane 5), oxacillin 8 μg/mL plus α-mangostin 1 μg/mL (lane 6), oxacillin 8 μg/mL plus Fr_3 extract 1.94 μg/mL (lane 7), and oxacillin 8 μg/mL plus crude extract 3.125 μg/mL (lane 8). Bovine serum albumin (BSA) was used as protein standard. Protein in kDa is represented for molecular weight marker protein.

The BM-PG associated protein bands of clinical isolates of oxacillin-resistant *S. saprophyticus* after treatment with bioactive compounds from the pericarp of GML extract are shown in Figure 4.23. It is noticed that there was an absence of protein band at MW 75, 100, 150 and 250 kDa of lane 2 to lane 8 of treated cells compared to control. On the contrary, the BMPG band of treated cells appeared darker than the control at MW 25 kDa. These findings lend support to the assumption that higher MW proteins of these treated cells were disrupted. Therefore, lower MW proteins were established. Thus, the BMPG associated protein synthesis may be disrupted by these GML extracts.

CHAPTER V

CONCLUSIONS

The emergence of multidrug resistance in pathogenic bacteria has increased. Thus, the search for new antibiotics and new approaches to treat these bacterial infections are urgently needed. Staphylococcal resistance to a wide spectrum of β-lactam antibiotics, such as methicillin, oxacillin and flucloxacillin, began to emerge soon after the introduction of the first drug in this class, and there has been a steady risen in the incidence of methicillin resistant *S. aureus* (MRSA) clinical isolates (Bush, 2004). An interesting source of novel therapeutics are plant-derived antibacterials, such as galangal from which there has been extracted on active ingredient called galangin which showed synergism with penicillin for MRSA inhibition (Eumkeb *et al*., 2010). The xanthones, α- and γ-mangostin, are major bioactive compounds found in the pericarp of the mangosteen. The biological activities of α-mangostin have been confirme in that it has antimicrobial activity against methicillin-resistant *Staphylococcus aureus* and *Helicobacter pylori* (Chomnawang *et al*., 2009; Iikubo *et al*., 2002; Iinuma *et al*., 1996). No works have been done on the effect of GML extract on drug resistant bacteria such as *Staphylococcus saprophyticus* (*S. saprophyticus*), *Enterobacter cloacae* (*E. cloacae*), and *Escherichia coli* (*E. coli*). The purpose of this study was to investigate the activity of a bioactive compound, α-mangostin, from the pericarp extract of *Garcinia*

mangostana against these drug resistant bacteria, when used alone and in combination with β-lactam antibiotics.

The mature mangosteen fruits (*Garcinia mangostana* L.) were collected from Nakhon Ratchasima. The extraction and identification methods were accomplished. The CH_2Cl_2 crude extract, Fr_3 extract, and α-Mangostin were extracted by Soxhlet extraction. The HPLC chromatograms of these extracts showed similar patterns with a major peak of α-mangostin at retention time around 5.33 min. Then, the main compound structure was identified as α-mangostin using NMR and compared with the reference. The bacterial suspension standard curves of all tested strains were executed to count the exact bacterial numbers. The MIC values of CH_2Cl_2 crude extract, Fr3 extract, α-mangostin and oxacillin against clinical isolates of oxacillin-resistant *S. saprophyticus* revealed 50, 31, 8 and 128 μg/mL, respectively. The MIC values of CH_2Cl_2 crude extract, Fr3 extract, α-mangostin and ceftazidime against both clinical isolates of ceftazidime-resistant *E. coli* and ceftazidime-resistant *E. cloacae* strains showed at >10,000, >10,000, >1,024 and >1,024 μg/mL respectively. Thus, these results indicate that bioactive compounds from the pericarp of GML extract revealed a great deal higher potency against ORSS than oxacillin alone. The high resistance to ceftazidime and bioactive compound extracts from the pericarp of GML of both *E. coli* and *E. cloacae* strains were demonstrated. These results are concistent with those of Chomnawang *et al*. (2009) and Iinuma *et al*. (1996) that this extract inhibited MRSA, *Staphylococcus epidermidis* and *Propionibacterium* strains. These findings lend support to the assumption that these seem to be the result of the difference in cell wall structure between gram-positive and gram-negative bacteria. The gram-negative bacteria has a multi-layered and complex structure. The outer

membrane can act as a barrier to many environmental substances including antibiotics (Eumkeb *et al*., 2004).

The checkerboard results displayed that the MICs of CH_2Cl_2 crude extract, Fr_3 extract, and α-mangostin plus oxacillin were reduced from 50, 31 and 8 μg/mL plus 128 μg/mL to 6.25 (1/8 MIC), 3.875 (1/8 MIC) and 2 (1/4 MIC) μg/mL plus 16 (1/8 MIC) μg/mL, respectively, against ORSS strain. For this reason, the FICs index of CH_2Cl_2 crude extract, Fr_3 and α-mangostin plus oxacillin revealed synergistic effects at 0.25, 0.138 and 0.375 respectively against ORSS strain.

These results are in substantial agreement with earlier findings that synergism between alpha-mangostin plus gentamicin (GM) against vancomycin resistant Enterococci (VRE), alpha-mangostin and vancomycin hydrochloride (VCM) against methicillin resistant *Staphylococcus aureus* (MRSA) were recorded (Sakagami *et al*., 2005). These results suggest that these compounds from the pericarp of GML extract not only have weak activity of their own against ORSS but also have the ability to reverse the resistance of such bacterial strain to the activity of the primary antibiotics (Eumkeb *et al*., 2010).

The killing curves proved that the combination of CH_2Cl_2 crude extract (3.125 μg/mL), Fr_3 (1.94 μg/mL) and α-mangostin (1 μg/mL) each plus oxacillin (8 μg/mL) caused a marked decrease of 5×10^5 CFU/mL of ORSS to 10^3 CFU/mL within 6 h and throughout the remainder of a 24 h period. These results imply that these bioactive compounds in combination with oxacillin have synergistic activity against this strain. In fact, the results of checkerboard assay, as synergistic effects, are confirmed by this finding.

Furthermore, the TEM method exhibited that the effect of the combination of oxacillin plus these compound from GML extracts including α-mangostin at sub-MIC value on ORSS revealed great deal smaller than the control cells. The majority of these cells undoubtedly exhibited cell shape distortion and cell envelope damage in most of these cells. These results are in substantial agreement with those of Eumkeb *et al*. (2010) that electronmicroscopy clearly exhibited the combination of galangin and ceftazidime caused damage to the ultrastructures of the cells of MRSA strain. These findings lend support to the assumption that either these GML extracts alone or in combination with oxacillin may inhibit cell wall synthesis leads to cell shape distortion and cell envelope damage.

In addition, the OM permeabilization results demonstrated that either bioactive compounds from this plant including α-mangostin alone or in combination with oxacillin at sub MIC value slow increased the OM permeability of ORSS strain. These results are correspondence with previous findings that D,L-1'-Acetoxychavicol acetate caused *S. aureus* outer membrane damage (Oonmetta-aree *et al*., 2006).

In the same way, the cytoplasmic membrane permeabilization results showed that there was no increase in β-galactosidase activity with increasing time in the presence of either bioactive compounds from this plant including α-mangostin alone or in combination with oxacillin at sub MIC value within 0-3 h whereas exhibited this enzyme positive activity from 4-5 h periods. These finding provide evidence that it is likely that these compounds markedly increase the permeability of the cytoplasmic membrane of ORSS strain after 4 h of treatment. These results are consistent with previous findings that D,L-1'-Acetoxychavicol acetate caused *S. aureus* CM damage (Oonmetta-aree *et al*., 2006). These results can be explained by assuming that the

cytoplasmic membrane is also a highly selective barrier, enabling a cell to concentrate specific metabolites and excrete waste materials. The general structure of most biological membrane is a phospholipid bilayer. Consequently, these extracts either alone or in combination with oxacillin take a long time to penetrate CM of this resistant strain.

Besides, the SDS-PAGE results exhibited that there was an absence of protein bands at MW 75, 100, 150 and 250 kDa of lane 2 to lane 8 of either bioactive compounds from this plant including α-mangostin alone or in combination with oxacillin at sub MIC value treated cells compared to control. Whereas, the BMPG band of these treated cells appeared darker than the control at MW 25 kDa. These findings lend support to the assumption that higher MW proteins of these treated cells were disrupted. Therefore, lower MW proteins were established. So, the BMPG associated protein synthesis me be disrupted by these GML extracts.

From these results, it can be concluded that the oxacillin had little activity against clinical isolates of oxacillin-resistant *S. saprophyticus*. Whereas, biochemical compounds of GML extracts showed rather higher potency than oxacillin alone against this strain. Moreover, the combination of oxacillin and these bioactive compounds, especially α-mangostin, obviously showed great synergic activity against this strain. So, our findings provide evidence that these plant extract compounds have the synergistic effect with oxacillin to reverse bacterial resistance to oxacillin against this resistant strain.

To conclude, this activity may be involved two mechanisms of action by these GML extract compounds in combination with oxacillin. The first is on the cell wall (peptidoglycan) synthesis inhibition. The second mode of action is a steady increased

OM and CM permeabilization. These GML extract compounds including α-mangostin have a sufficient margin of safety for therapeutic use. For this reason, these extract compounds offer for the development of a valuable adjunct to oxacillin against ORSS, which currently almost penicillins resistance. These in vitro results have to be still confirmed in an animal or in humans test. If possible, blood and tissue levels would be achievable to work synergistically.

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