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ปีการศึกษา 2556

ANTIBACTERIAL ACTIVITY OF *STEPHANIA SUBEROSA* FORMAN AND *PLUCHEA INDICA* (L.) LESS EXTRACTS AGAINST ANTIBIOTIC RESISTANT BACTERIA

Nongluk Autarkool

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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ANTIBACTERIAL ACTIVITY OF *STEPHANIA SUBEROSA* FORMAN AND *PLUCHEA INDICA* (L.) LESS EXTRACTS AGAINST ANTIBIOTIC RESISTANT BACTERIA

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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นงลักษณ์ อยู่แท้กูล : ฤทธิ์ต้านแบคทีเรียของสารสกัดจากบอระเพ็ดพุงช้างและขลู่ (ANTIBACTERIAL ACTIVITY OF *STEPHANIA SUBEROSA* FORMAN AND *PLUCHEA INDICA* (L.) LESS EXTRACTS AGAINST ANTIBIOTIC RESISTANT BACTERIA) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เกรียงศักดิ์ เอื้อมเก็บ, 113 หน้า.

อุบัติการณ์การดื้อยาปฏิชีวนะหลายขนานในแบคทีเรียก่อโรคได้สร้างความจำเป็นอย่าง เร่งด่วนสำหรับค้นหายาปฏิชีวนะขนานใหม่รวมถึงแนวทางใหม่ๆ เพื่อรักษาโรคติดเชื้อแบคทีเรีย ยาต้านแบคทีเรียที่ได้จากพืชที่สามารถต้านการดื้อยาของแบคทีเรียต่อยาปฏิชีวนะที่ได้สูญเสีย ประสิทธิภาพดั้งเดิมเป็นวัตถุประสงค์ของการวิจัยที่มีความสำคัญอย่างมาก วัตถุประสงค์ของ การศึกษาครั้งนี้เพื่อศึกษาฤทธิ์ต้านแบคทีเรียและการเสริมฤทธิ์ของสารสกัดจากบอระเพ็ดพุงช้าง ต้านเชื้อสแตปฟิโลคอคคัส ออเรียส ที่ดื้อต่อยาแอมพิซิลิน (เออาร์เอสเอ) และสารสกัดจากขลู่ ต้านเชื้อสแตปฟิโลคอคคัส อิพิเดอร์มิดิส ที่ดื้อต่อยาคลอกซาซิลิน (ซีอาร์เอสอี) เมื่อใช้เดี่ยวๆ และใช้ร่วมกับยาปฏิชีวนะกลุ่มบีตาแลคแทม ค่าความเข้มข้นในการยับยั้งต่ำสุดสำหรับยาแอมพิ ซิลินและสารสกัดจากบอระเพ็ดพุงช้างในการต้านเชื้อเออาร์เอสเอ มีค่ามากกว่า 512 ไมโครกรัม/ มิลลิลิตร และ 4 มิลลิกรัม/มิลลิลิตร ตามลำดับ นอกจากนั้น ค่าความเข้มข้นในการยับยั้งต่ำสุด ของยาคลอกซาซิลินและสารสกัดจากขลู่ในการต้านเชื้อซีอาร์เอสอี มีค่ามากกว่า 1,024 ไมโครกรัม/มล. และ 4 มก./มล. ตามลำดับ ผลการศึกษาด้วยวิธีเชคเกอร์บอร์ดพบว่า มีการเสริม ฤทธิ์กันระหว่างสารผสมแอมพิซิลิน (0.15 ไมโครกรัม/มล.) และสารสกัดจากบอระเพ็ดพุงช้าง (0.5 มก./มล) มีค่าดัชนี เอฟไอซี น้อยกว่า 0.13 ในการต้านเชื้อเออาร์เอสเอ ขณะที่ไม่มีปฏิกิริยา ระหว่างยาหรือไม่มีการเสริมฤทธิ์กันระหว่างสารผสมสารสกัดจากขลู่และยาคลอกซาซิลินที่ดัชนี เอฟไอซี เท่ากับ 0.78 ในการต้านเชื้อซีอาร์เอสอี กราฟแสดงการตายของเชื้อเออาร์เอสเอได้ยืนยัน ว่าการรอดชีวิตของเชื้อเออาร์เอสเอ มีการลดลงอย่างเห็นได้ชัดจาก 5 x 10^5 ซีเอฟยู/มิลลิลิตร เหลือเพียง 10^3 ซีเอฟยู/มิลลิลิตร ภายในช่วง 6 ชั่วโมงและตลอด 24 ชั่วโมง หลังจากได้รับสาร ผสมระหว่างสารสกัดจากบอระเพ็ดพุงช้าง (0.5 มก./มล.) และยาแอมพิซิลิน (0.15 ไมโครกรัม/ มล.) การศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านแสดงให้เห็นอย่างชัดเจนว่า เชื้อเอ อาร์เอสเอที่ได้รับสารผสมระหว่างยาแอมพิซิลินผสมกับสารสกัดจากบอระเพ็ดพุงช้างมีรูปร่าง ผิดปกติ แพปทิโดไกลแคนและเยื่อหุ้มไซโทพลาสมิคได้รับความเสียหายชัดเจนรวมไปถึงพื้นที่ เฉลี่ยของเซลล์มีขนาดเล็กกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ นอกจากนั้น การซึมผ่านเยื่อหุ้มเซลล์ ชั้นในของเชื้อเออาร์เอสเอเพิ่มขึ้นหลังจากการได้รับสารผสมดังกล่าวเช่นเดียวกัน การศึกษาการ ทำงานของเอนไซม์แสดงให้เห็นว่าสารสกัดจากบอระเพ็ดพุงช้างมีฤทธิ์ยับยั้งการทำงานของ

เอนไซม์บีตาแลคแทมเมส อย่างชัดเจนและออกฤทธิ์แปลผันตามความเข้มข้นสารสกัดที่เพิ่มขึ้น ผลการศึกษาที่ได้ดังกล่าวเป็นหลักฐานที่ชี้ให้เห็นว่าสารสกัดจากบอระเพ็ดพุงช้างมีศักยภาพสูงที่ สามารถเปลี่ยนจากแบคทีเรียดื้อยาให้กลับมาเป็นแบคทีเรียที่ไวต่อยาปฏิชีวนะดั้งเดิมของมัน และกลไกการออกฤทธิ์ของสารสกัดจากบอระเพ็ดพุงช้างอาจเกี่ยวข้อง 3 กลไก (1) ยับยั้งการ สังเคราะห์แพปทิโดไกลแคนซึ่งส่งผลทำให้รูปร่างเซลล์เสียหาย (2) ยับยั้งการทำงานของ เอนไซม์บีตาแลคแทมเมส และ (3) เพิ่มการซึมผ่านของเยื่อหุ้มเซลล์ชั้นใน ดังนั้น สารสกัดจาก บอระเพ็ดพุงช้างเป็นสารที่มีศักยภาพโดดเด่นในการนำพัฒนาเป็นยาสูตรผสมพฤกษเภสัชตัว ใหม่กับยาแอมพิซิลินสำหรับใช้รักษาการติดเชื้อเออาร์เอสเอ การศึกษาเพิ่มเติมเพื่อหาสารออก ฤทธิ์ การศึกษาความเป็นพิษ และการศึกษาการเสริมฤทธิ์ในระดับเลือดและเนื้อเยื่อควรจะได้รับ การทดสอบยืนยันเพิ่มเติมในสัตว์ทดลองหรือในมนุษย์

สาขาวิชาเภสัชวิทยา   ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2556   ลายมือชื่ออาจารย์ที่ปรึกษา_____

                                        ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

NONGLUK AUTARKOOL : ANTIBACTERIAL ACTIVITY OF
STEPHANIA SUBEROSA FORMAN AND *PLUCHEA INDICA* (L.) LESS
EXTRACTS AGAINST ANTIBIOTIC RESISTANT BACTERIA. THESIS
ADVISOR : ASST. PROF. GRIANGSAK EUMKEB, Ph.D. 113 PP.

B-LACTAM ANTIBIOTIC/AMPICILLIN/AMPICILLIN-RESISTANT *S. AUREUS*
(ARSA)/*STEPHANIA SUBEROSA* FORMAN/ *PLUCHEA INDICA* (L.) LESS/
SYNERGISTIC ACTIVITY

The emergence of multidrug resistance in pathogenic bacteria has created an
urgent need for new antibiotics and new approaches for the treatment of bacterial
infections. Plant-derived antibacterial that can against the resistance to well-tried
agents which have lost their original effectiveness are the research objectives of far
reaching importance. The purpose of the present work was to investigate
antibacterial and synergistic activities of *Staphania suberosa* extracts (SSE) against
Ampicillin resistant *Staphylococcus aureus* (ARSA) and *Pluchea indica* crude
extract (PIE) against Cloxacillin resistant *S. epidermidis* (CRSE) when used singly
and in combination with β-lactams antibiotic. The Minimum Inhibitory
Concentration (MIC) for ampicillin (AMP) and SSE against ARSA strains
were >512 µg/ml and 4 mg/ml respectively. Besides, the MICs of cloxacillin
(CLX) and PIE against CRSE were >1024 µg/ml and 4 mg/ml, respectively.
Checkerboard assay revealed synergistic activity in the combination of AMP
(0.15 µg/ml) and SSE (0.5 mg/ml) with fractional inhibitory concentration
index (FICI) <0.13 against ARSA. Whereas, no interaction or synergistic effect

of PIE plus CLX with FICI <0.78 against CRSE was observed. The killing curve assay had confirmed that the viability of ARSA was a dramatic reduction from 5×10^5 cfu/ml to 10^3 cfu/ml within 6 h and throughout 24 h after exposure to SSE (0.5 mg/ml) combined with AMP (0.15 µg/ml). Electron microscopic study clearly revealed that ARSA cells treated with AMP plus SSE caused marked cell morphological damage, peptidoglycan and cytoplasmic membrane damage, and average cell areas were significantly smaller than the control. Furthermore, the CM permeability of ARSA was also increased by this combination. The enzyme assay showed marked inhibitory activity of SSE against β-lactamase in a concentration dependent manner. These findings provide an evidence that SSE has the high potential to reverse bacterial resistance to originate traditional drug susceptibility of it and may relate to three modes of actions of SSE: (1) inhibit peptidoglycan synthesis, resulting in cell morphological damage, (2) inhibit β-lactamases activity, and (3) increase CM permeability. Therefore, this SSE offers the prominent potential to develop a novel adjunct phytopharmaceutical to ampicillin for the treatment of ARSA. Further active ingredients study, toxicity and the synergistic effect on blood and tissue should be performed and confirmed in animals or in humans.

School of Pharmacology   Student's Signature_____

Academic Year 2013   Advisor's Signature_____

                                     Co-Advisor's Signature_____

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Nongluk Autarkool

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LIST OF ABBREVIATIONS

ATCC	=	American Type Culture Collection
CAMHB	=	Cation-Adjust Mueller-Hinton Broth
OD	=	Optical Density
HPLC	=	High Performance Liquid Chromatography
MHB	=	Mueller Hueller-Hinton Broth
MHA	=	Mueller Hueller-Hinton Agar
SSE	=	*Staphania suberosa* Forman Crude Extract
PIE	=	*Pluchea indica* Crude Extract
CFU	=	Colony Forming Unit
CM	=	Cytoplasmic Membrane
DMSO	=	Dimethylsulfoxide
FIC	=	Fractional Inhibitory Concentration
g	=	Gram
h	=	Hour
HEPES	=	N-2-Hydroxyethyl Piperazine-N'-ethanesulphonic Acid
LPS	=	Lipopolysaccharide
mM	=	Millimolar
MIC	=	Minimum inhibitory concentration
ml	=	Millilitre
MRSA	=	Methicillin-Resistant *Staphylococcus aureus*

LIST OF ABBREVIATIONS (Continued)

ARSA	=	Ampicillin-Resistant *Staphylococcus aureus*
MSSA	=	Methicillin-susceptible *Staphylococcus aureus*
MW	=	Molecular Weight
NaCl	=	Sodium Chloride
OsO_4	=	Osmium Tetroxide
s	=	Second
TEM	=	Transmission Electron Microscopy
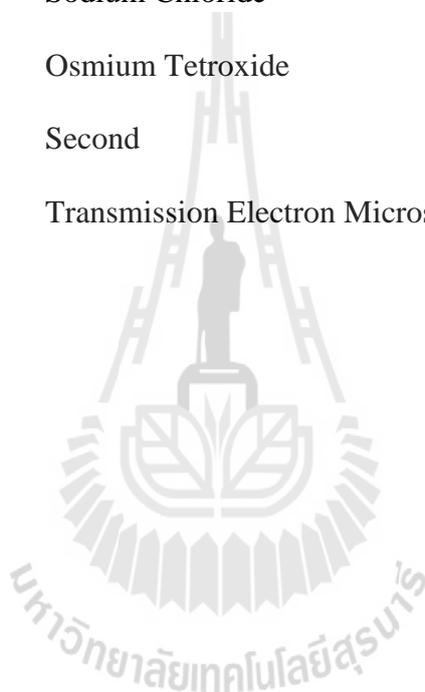
CHAPTER I

INTRODUCTION

1.1 Introduction

In recent times, several reports from the scientific community have raised concerns that antibacterial drug development more slowly developed than the problems posed by antibiotic resistance among important bacterial pathogens. The emergence of multidrug resistance in pathogenic bacteria has created an urgent need for new antibiotics and new approaches to conquer these bacteria. The prevalence of multidrug resistance in pathogenic and opportunistic bacteria has been increasingly reported (Buynak, 2006; Lee et al., 2013). During 1958-1968, *Staphylococcus aureus* strains are resistant to penicillin has been increased in St. Thomas's hospital, London (Ridley et al., 1970). In 2010 to 2011 *S. aureus* was isolated from patients swabbed in nine European countries. *S. aureus* isolated showed the highest resistance to penicillin, with the highest resistance in France (den Heijer et al., 2013). Methicilin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen causing health problems worldwide. Increasing antimicrobial resistance in *Staphylococcus aureus* has led to major concerns about the future of antimicrobial therapy of its infections (Havaei et al., 2012). Around 90–95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin and in most of the Asian countries, 70–80% of the same strains are methicillin resistant (Chambers, 2001). Staphylococcal resistance to wide spectrum beta-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).

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 (Wang, 2003). Although resistance was mainly a problem in north Africa, Asia and Europe bacteria are in food production animals (e.g., pigs), superbugs being in food animal (Collignon, 2013). However, in recent years these bacteria have emerged as an opportunistic pathogen, significant causative agents of bacteriemia, and the major cause of nosocomial infectiousness particularly associated with indwelling medical devices (e.g., prosthetic joints and heart valves) and in individuals with a compromised immune system (e.g., cancer patients and neonates) (Viale and Stefani, 2006; von Eiff et al., 2002). In addition to methicillin resistance, *S. epidermidis* strains have acquired resistance to several other antibiotics. Most antibiotic resistance genes are plasmid encoded and are more often found in methicillin resistant than methicillin susceptible strains (Miragaia et al., 2002). The apparent increase in the antibiotic resistance of *staphylococci*, one of the most common and dangerous bacterial pathogens, observed worldwide, encourages the development of new alternative, effective, and inexpensive agents for the treatment of this group of microorganism. Some of the alternative therapies in *S. aureus* diseases, such as bacteriophages, plant (e.g., stilbenoids and flavonoids) and animal derived (e.g., chitosan and propolis) compounds, have been recently discussed by (Kurlenda and Grinholc, 2012). From the situation of infections in Maharat NakhonRatchasima hospital, the result of drug sensitivity testing in the microbiology

section of the medical technique department showed that methicillin resistant *Staphylococcus aureus* (MRSA) has resisted to ceftazidime in many wards, including surgical ICU (85%), pediatric ICU (65%), premature (88%) and sepsis (74%). The ceftazidime-resistant *Escherichia coli* (CREC) has been found almost 100% of all wards (Maharat Nakhonratchasima Hospital, 2013).

In addition, ceftazidime-resistant *Enterobacter cloacae* (CREnC) was discovered about 100% in many wards. These bacteria cause infection to the patient at a high rate and a large number. It is found in the blood and secretions from the patients, such as phlegm, mucus, saliva, urine and feces, etc. This problem is a major problem of NakhonRatchasima province and Thailand (Maharat Nakhonratchasima Hospital, 2013). These problems lead to an invaluable economic wasteland.

The high growth rate of antibiotic resistance of microorganisms has been developed. So, the research for new antimicrobial agents or strategies that can conquer these microorganisms are urgently needed. Medicinal plant derived compounds have increased prevailing interest in the search of alternative antibacterial agents because of the understanding that they are safe and have a long history of use in traditional medicine for the treatment of infectious diseases (Guarrera, 2005). Natural products of plants may possess a new source of antimicrobial agents with possibly novel mechanisms of action (Barbour et al., 2004). In addition, they are effective in the treatment of infectious diseases while simultaneously decreasing many of the side effects that are often participatory with conventional antimicrobials. Systematic and methodical screening of them may result in the discovery of novel active compounds (Gibbons, 2004). Plants are widely used traditionally for the treatment of microbial infections. A quick look at the nature way, especially plants is

tackling the issue of infection will be provided a deeper understanding of the methodology, which needs to be adopted for the design and development of novel, highly effective anti-infectious agents in general, and antimycobacterials in particular (Hemaiswarya, 2006). Many plants have interesting biological activities with potential therapeutic applications.   There has been much interested.

In recent years, a compound derived from plants and herbs occupy their medicinal property or biological activities. Different reports from different countries have been published, showing the antimicrobial activities of various medicinal plants (Chomnawang et al., 2009; Moghadam et al., 2010; Sakagami et al., 2005). One of the most attractive approach is to search and develop for new antimicrobial agents with inexpensive and effective drugs from other source, including plants, for possible antimicrobial properties that will be able to act for longer periods before resistance.

In order to find bioactive substances that can replace medicine in the beta-lactam antibiotics or to find the synergistic effect of these substances with drugs, these studies were investigated. In the present work, two plant species *Stephania suberosa* Forman and *Pluchea indica* (L.) Less, were selected to test their antimicrobial activity.

The genus *Stephania*, belonging to Menispermaceae family that is a rich source of alkaloids (Patra, 1987; Patra et al., 1986; Patra et al., 1988; Schiff Jr, 1999; Schiff, 1991). More than 150 alkaloids have been isolated from *Stephania* genus and many of them are bioactive constituents (Semwal et al., 2010). *Stephania*, is the native of Africa, India, South-East Asia and the northern and eastern parts of Australia. These plants have recognized medicinal values and have been traditionally used for treatment of asthma, tuberculosis, dysentery, hyperglycemia, cancer, fever,

intestinal complaints, sleep disturbances and inflammation. Accordingly, these could be a potential source of biologically active compounds which might be used for the development of new drugs (Blanchfield et al., 2003; Semwal et al., 2010).

Pluche indica (L.) Less. is a genus of flowering plant in the Compositae family. The genus *Pluchea* (Asteraceae) comprises of 80 species distributed mainly in North and South America, Africa, Asia and Australia (Sen et al., 2002). This plant have been traditionally used as anti-inflammatory (Sen and Chaudhuri, 1990). Many biological activities of *Pluchea indica* have been studied as well as anti-ulcer, anti-tuberculosis, antimicrobial and anti-cancer property (Cho et al., 2012; Mohamad et al., 2011; Pal and Chaudhuri, 1989; Sittiwet, 2009).

However, most of these plants were not previously screened against multi-drugs resistant pathogenic organisms. Therefore, this is the first study to demonstrate the antibacterial activity of their extracts. The purpose of this thesis was to investigate the antibacterial activity of Thai medicinal plants such as *Stephania suberosa* Forman and *Pluchea indica* (L.) Less. extract against drug resistant bacteria when use either alone or in combination with antibacterial drugs. This study may lead to the development of a new class of antibacterial agent. Moreover, using natural products would also help to diminish the toxicity of the drugs when they are used on humans.

1.2 Research objectives

The purpose of this study was aimed as the following;

1.2.1 To test the antibacterial activity of crude extracts from *S. suberosa* Forman and *Pluchea indica* (L.) Less. against drug-resistant bacteria.

1.2.2 To test the antibacterial activity of the crude extracts from *S. suberosa* Forman and *Pluchea indica* (L.) Less. in combination with selected β-lactam penicillins on drug resistant bacteria.

1.2.3 The positive results of crude extracts plus selected β-lactam penicillins that show a synergistic effect against these bacteria were selected for further investigation of the preliminary mechanism of action by examining cytoplasmic membrane permeability methods, transmission electron microscopy (TEM) and enzyme assay.

1.3 Research hypothesis

1.3.1 The crude extract of these plants could show antibacterial activities against drug resistant bacteria when use singly.

1.3.2 These plant crude extracts in combination with selected β-lactam penicillins could show synergistic antibacterial activity against drug resistant bacteria.

1.3.3 The elementary mechanisms of action of these combinations against resistant bacteria should be elucidated.

1.4 Scope and limitation of the study

1.4.1 The *S. suberosa* Forman was purchased from Lamtakhong Research Station, Nakhon Ratchasima, Thailand. *Pluchea indica* (L.) Less was purchased from private companies in Nakhon Ratchasima. The crude extract of tuberous root of *S. suberosa* and *Pluchea indica* leaves were evaluated.

1.4.2 Clinical isolates of Ampicillin- resistant *Staphylococcus aureus* DMST 20651, 20652, 20653 (ARSA) and Cloxacillin resistant *S. epidermidis* DMST 15505, 15506, 15507 (CRSE) were obtained from the Department of Medical Sciences Thailand, Ministry of Public Health, Thailand.

Staphylococcus aureus ATCC 29213 were obtained from the American Type Culture Collection (ATCC), USA.

1.4.3 Ampicillin and cloxacillin were purchased from Sigma Aldrich, UK.

1.4.4 Checkerboard assay of the combinations that show synergistic effect was selected for further investigation of the elementary mechanism of action such as cytoplasmic membrane permeability methods, transmission electron microscopy (TEM), and enzyme assay.

1.5 Benefit of the study

1.5.1 Providing   information data on synergism antimicrobial   activity between crude extracts and selected β-lactam penicillins against drug resistant bacteria

1.5.2 Providing novel knowledge for further investigations such as investigate mechanisms of action of crude extracts from these plant extracts in combination with selected β-lactam penicillins on drug resistant bacteria.

1.5.3 Providing new source of antimicrobial agents, which could be used against drug resistant bacteria that caused infectious diseases.

1.5.4 The results may useful for development of new combination drugs against resistant bacteria for the clinical treatment in the future.

CHAPTER II

LITERATURE REVIEW

2.1 Overview of medicinal plants

2.1.1 Overview of *Stephania suberosa* Forman

Stephania suberosa Forman is a native herbal plant found in Thailand and commonly used for treatment of a variety of aliments under the local name 'boraphet phungchang'. This genus belonging to the Menispermaceae family, comprises about 65 genera and 350 species, distributed mainly warmer parts of the world (Semwal et al., 2010). A characteristic feature of the plant is its large tuber from which the long stem of the climber emerges. The tubers can grow to 0.4 m in diameter, and are partly submerged (Figure 2.1). It is mostly found growing in forests in northern, eastern and northeastern regions of Thailand. Furthermore, *S. suberosa* and *S. venosa* are used in Thailand for the treatment of medical conditions (Patra, 1987).

Moreover the *Stephania suberosa* plants have been shown a rich source of interested phytochemical substances. Many interesting compounds have been isolated (Table 2.1).
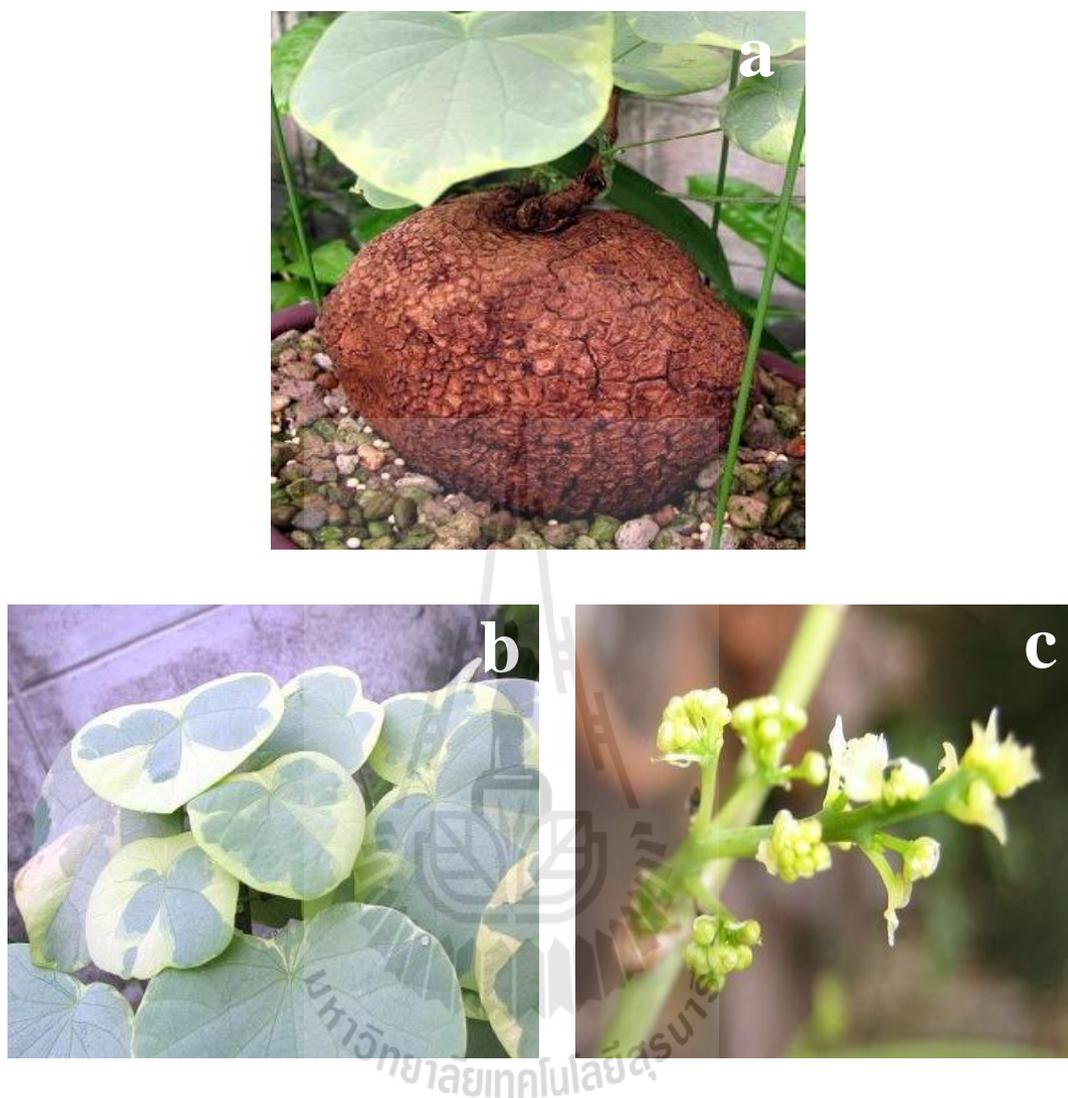
Figure 2.1 Photo of *Stephania suberosa* Forman (Menispermaceae), a, tuberous root;

b, leaves; c, fluorescent flowers (http://www.bihrmann.com/caudiciforms/subs/ste-

sub-sub.asp).

Chemical Constituents:

Table 2.1: Some of the chemicals found in *S. suberosa*.
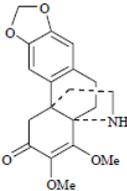
Compounds	References
 N-Nordelavaine	(Patra, 1987)
 Stephasubine	(Patra, 1987)
 Cepharanthine	(Patra et al., 1986)
 Cepharanthine 2′-β-N-oxide	(Patra et al., 1986)
 Stephasubimine	(Patra et al., 1986)
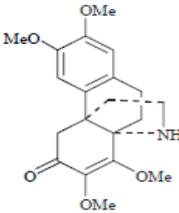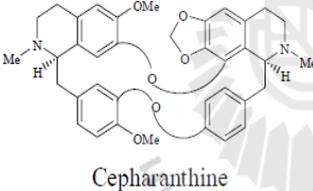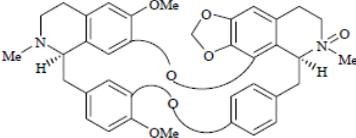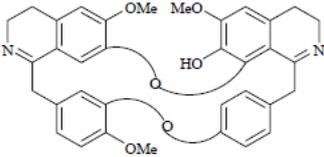
Table 2.1 (Continued).
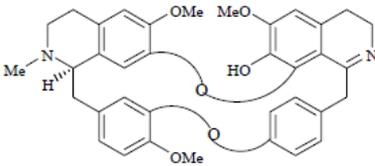
Compounds	References
Stephasubine	(Patra et al., 1986)
Norstephasubine	(Patra et al., 1986)
Isostephodeline	(Patra, 1987)
Capaurimine	(Patra et al., 1987)
Coreximine	(Patra et al., 1987)
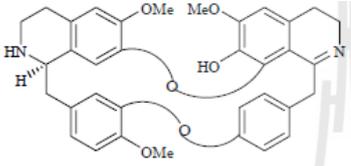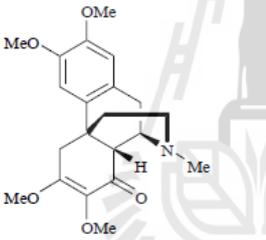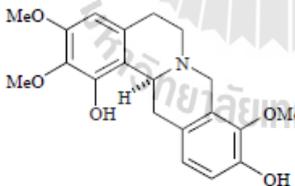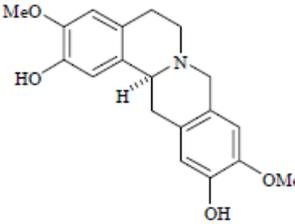
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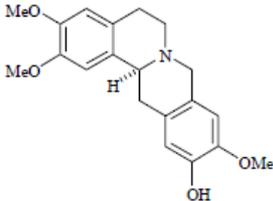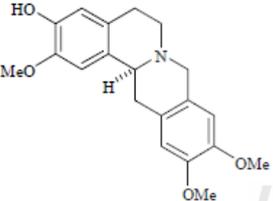
Compounds	References
Corytenchine	(Patra et al., 1987)
Discretine	(Patra et al., 1987)
Pseudopalmatine	(Patra et al., 1987)
8-Oxopseudopalmatine	(Patra et al., 1987)
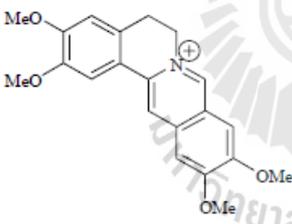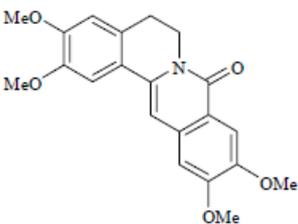
Table 2.1 (Continued).
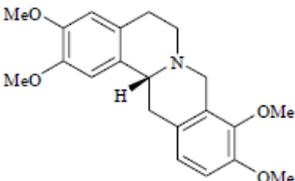
Compounds	References
 Tetrahydropalmatine	(Patra, 1987)
 Stephabinamine	(Patra et al., 1987)
 Stephabine	(Patra et al., 1987)
 Tetrahydrostephabine	(Patra et al., 1987)
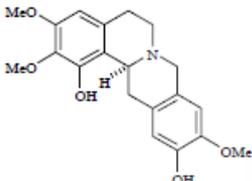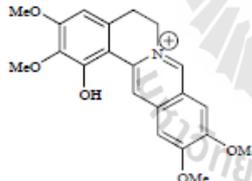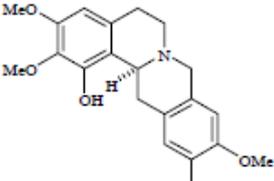
Table 2.1 (Continued).
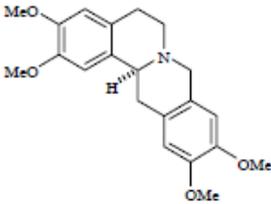
Compounds	References
Xylopinine	(Patra et al., 1987)
cis-Xylopinine N-oxide trans-Xylopinine N-oxide	(Patra et al., 1987)
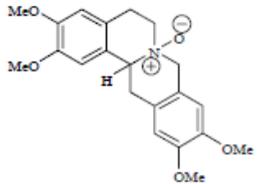
Moreover, these plants are major sources of bioactive alkaloids such as morphines, hasubanans, aporphines, berberines and protoberberine, that have been shown to possess antiplasmodial, antibacterial (Baghdikian et al., 2013; Deng et al., 2011). The biological activities of alkaloids from *Stephania* genus has also been extensively reviewed (Table 2.2).

The majority of chemical compounds has been reported on *S. tetrandra*, *S. cepharantha* Hayata, *S. glabra* (Roxb.) Miers, *S. japonica* (Thunb.) Miers and *S. venosa* (Blume) Spreng. Furthermore, many of these plants from *Stephania* genus have shown their biological activities including antitumor and emetine type activity (Gupta et al., 1980; Schiff, 1991; Kuroda et al., 1976). So, these chemical compounds has been traditionally used that are interested by researchers.

Protoberberine alkaloids display a great variety of biological and pharmacological activities. These activities include the inhibition of DNA and protein synthesis, inhibition of membrane permeability (Schmeller et al., 1997). These processes likely contribute to the allelochemical and toxic effects observed against bacteria and fungi. In addition, the protoberberines alkaloids found in plant families such as Papaveraceae, Berberidaceae, Fumariaceae, Menispermaceae, Ranunculaceae, Rutaceae and Annonaceae (Grycová et al., 2007).

Ingkaninan et al. (2003) found that the methanolic extracts from roots of *S. suberosa* and *Tabernaemontana divaricata* showed significant acetylcholinesterase inhibitory activity.

Putalun et al. (2009) investigated that the production of dicentrine from a hairy roots culture of *S. suberosa* by using *Agrobacterium rhizogenes* ATCC 15834 had the highest yield at 10 mM of tyrosine.

Semwal et al. (2010) reported that cepharanthine showed the antimalarial against *Plasmodium falciparum* W2 with IC_{50} values of 0.61 µM. In addition, cepharanthine showed significant effect on proliferation of culture cells from the murine skin in the range of 0.01–0.1µg/mL. Moreover, cepharanthine showed antioxidant activity with 93.3% inhibition on lipid peroxidation of linoleic acid emulsion at 30 µg/mL.

Table 2.2 Biological activities of *Stephania* genus.

Compounds	Plants	Biological activity	Reference
Sinomenine	*Stephania cepharantha*	Anti-inflammatory	(Hojo et al., 1985)
Cepharanthine	*Stephania cepharantha*	Anti-inflammatory	(Okamoto et al., 2001)
Crebanine Stephanine	*Stepkania dielsiana*	Antimicrobial	(Deng et al., 2011)
Cepharanthine	*Stephania epigaea*	Cytotoxicity against cancer cell	(Lv et al., 2013)
Glabradine Hasubanalactam	*Stephania glabra*	Antimicrobial	(Semwal et al., 2009)
(-)-Stepholine	*Stephania glabra*	Antipsychotic	(Jin et al., 2002)
Isochondodendrine	*Stephania hernadifolia*	Antiplasmodial	(Mambu et al., 2000)
Stepholine Thalrugosine	*Stephania japonica*	Antibacterial	(Kuroda et al., 1976)
Pseudopalmatine	*Stephania rotunda*	Antiplasmodial	(Baghdikian et al., 2013)
Cepharanthine	*Stephania rotunda*	Antimalarial	(Chea et al., 2007)

Table 2.2 (Continued).

Compounds	Plants	Biological activity	Reference
cycleanine	*Stephania rotunda*	Anti-inflammatory	(Angerhofer et al., 1999)
2-norcepharanthine	*Stephania rotunda*	Cytotoxic	(Angerhofer et al., 1999)
Fangchinoline	*Stephania rotunda*	Antioxidant	(Mambu et al., 2000) (Chea et al., 2010) (Bun et al., 2009)
fangchinoline	*Stephania rotunda*	Antioxidant	(Gulcin et al., 2010)
demethylaristofolin	*Stephania succifera*	Antibacterial	(Yang et al., 2013)
Fangchinoline	*Stephania tetrandra*	Antidiabetic	(Choi et al., 2000)
Tetrandrine	*Stephania tetrandra*	Anesthetic	(Wang and Lemos, 1995)
Tetrandrine	*Stephania tetrandra*	Antiarrythmic	(Wang and Lemos, 1995)

Table 2.2 (Continued).

Compounds	Plants	Biological activity	Reference
Tetrandrine	*Stephania tetrandra*	Anti-inflammatory	(Shen et al., 2001)
Tetrandrine	*Stephania tetrandra*	Antimicrobial	(Liu et al., 1995) (Lee et al., 2013)
Tetrandrine	*Stephania tetrandra*	Antitumor	(Cheng et al., 2010)
Tetrandrine	*Stephania tetrandra*	Synergistic anti-candidal	(Zhang et al., 2010)
Dehydrocrebanine	*Stephania venosa*	Cytotoxic	(Makarasen et al., 2011)
Oxostephanine	*Stephania venosa*	Toxicity	(Makarasen et al., 2011)
Crebanine	*Stephania venosa*	Anti-proliferative effects on human cancer cells	(Gomuttapong et al., 2012) (Wongsirisin et al., 2012)
Methylbulbocapnine	*Stephania venosa*	Anti-invasive	(Yodkeeree et al., 2013)

2.1.2 Overview of *Pluchea indica* (L.) Less

Figure 2.2 Photo of *Pluchea indica* (L.) Less. (http://www.phargarden.com)

Pluchea indica is a genus of flowering plant in the Compositae family is locally known as "Khlu". The genus *Pluchea* (Compositae) comprises of 80 species distributed mainly in North and South America, Africa, Asia and Australia. The height is more than one meter and it normally grows in wet-sandy soil. The leaves are obovate, 3-6 cm long and 1.5-2.5 cm wide, green and with an aromatic aroma, these are native to tropical and warm temperate areas. The plants of *Pluchea* genus have been traditionally used as astringent, antipyretic, anti-inflammatory, hepatoprotective, diaphoretic in fevers, smooth muscle relaxant, nerve tonics, laxatives and for the treatment of dysentery, lumbago, leucorrhoea, dysuria, hemorrhoids, gangrenous ulcer and disorders causing cachexia (Thongpraditchote et al., 1996).

Chemical Constituents: Terpenic glycosides, linaloyl glucoside, linaloyl apiosyl glucoside, 9-hydroxylinaloyl glucoside, plucheosides A and B, were found (Uchiyama et al., 1989). Moreover, new monoterpene glycoside, plucheoside C, three new eudesmane-type sesquiterpenes, plucheols A, B, plucheoside E and three new lignan glycosides, plucheosides D_1, D_2, D_3 were discovered (Uchiyama et al., 1991).

This genus has shown the presence of eudesmane-type sesquiterpenoids, monoterpenes, lignan glycosides, triterpenoids and flavonoids (Sharma and Goyal, 2011). However, to answer this, more research is needed to investigate the relationship of the specific phytochemicals and their contribution to human health.

Pal and Chaudhuri, (1989) showed that the methanolic fraction of *P. indica* root extract was found to possess significant antiulcer activity. *P. indica* extracted have been reported to possess anti-inflammatory (Sen and Chaudhuri, 1990; Buapool et al., 2013). Sittiwet, (2009) investigated that the aqueous extract of *P. indica* showed inhibition zone against *E. coli* and *K. pneumoniae*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) results showed that the possibility of using *P. indica* as an alternative therapy in the treatment of urinary tract infections was possible. Sharma and Goyal, (2011) reported that *P. indica* root extract showed the bacteriostatic against *S. flexneri* 2a NK 307.

However, no work has been investigated the effect of these medicinal plant extracts on drug resistant bacteria such as *S. aureus, S. epidermidis.* The purpose of this thesis was to investigate the activity of crude extract of these selected plant extracts against drug resistant bacteria when use either alone or in combination with selected penicillins. This study may lead to the development of a new class of antibacterial combination agent in the near future.

2.2 Literature review of microorganisms

2.2.1 Bacterial structure

Bacterial cell wall is located outside the plasma membrane and gives the cell its shape and provides rigid structural support for the cell. The cell wall constituents are peptidoglycan, teichoic acid and lipoteichoic acid. In addition, protects the cell from its environment. The cell membranes or cytoplasmic membranes of gram-positive and gram-negative bacteria are indistinguishable. Each is composed of protein, lipids, phospholipids and carbohydrate. The composition of their cell wall are distinguishing. Peptidoglycan layers joined together forming a thick and rigid structure is the unique of gram-positive, no outer membrane is present. By contrast, bacteria have only a thin peptidoglycan layer as known as gram-negative bacteria (Figure 2.3) (Tenover, 2006).
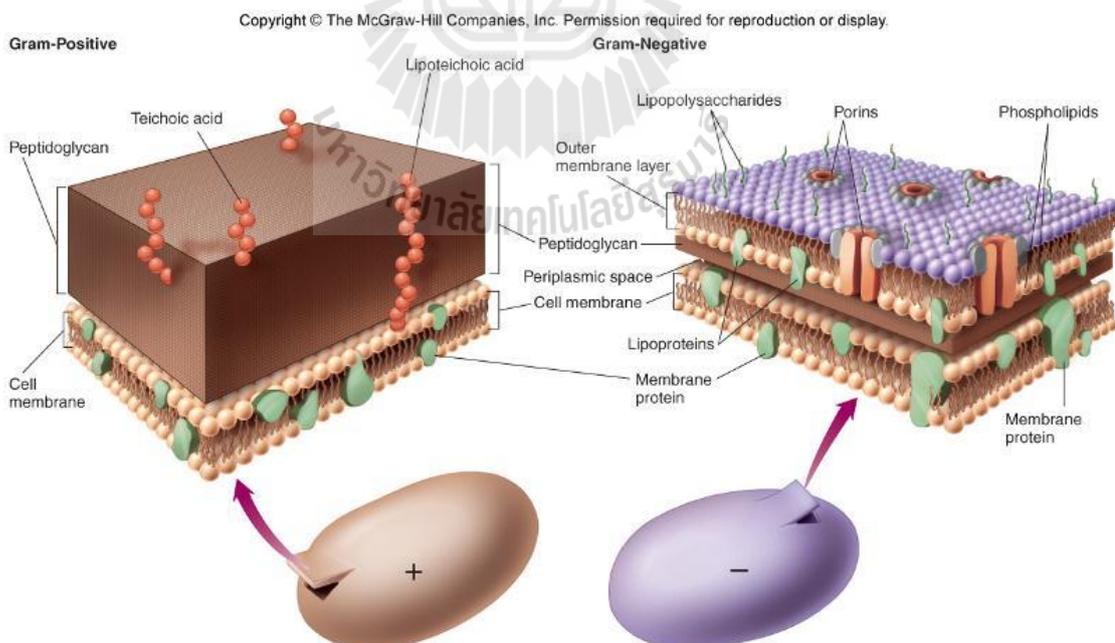
Figure 2.3 Comparison of the cell wall of Gram-positive and Gram-negative bacteria.

(http://www.pc.maricopa.edu/Biology/rcotter/BIO20205/LessonBuilders0.jpg)

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 outer membrane is a characteristic component of a Gram-negative cell wall. It is anchored to the peptidoglycan layer. The outer membrane is a bilayer consisting of a phospholipid layer on the inner side, and a lipopolysaccharide (LPS) layer towards the outer side. This LPS comprises side chains anchored to a core lipopolysaccharide. The side chains are made up of oligosaccharide units, and are often the basis to distinguish and classify these bacteria. The outer membrane is selectively permeable owing to the presence of specialized membrane proteins called porins (Tenover, 2006).

2.2.2 Bacterial types

2.2.2.1 *Staphylococcus aureus (S. aureus)*

Domain	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Staphylococcaceae
Genus	*Staphylococcus*
Species	*aureus*
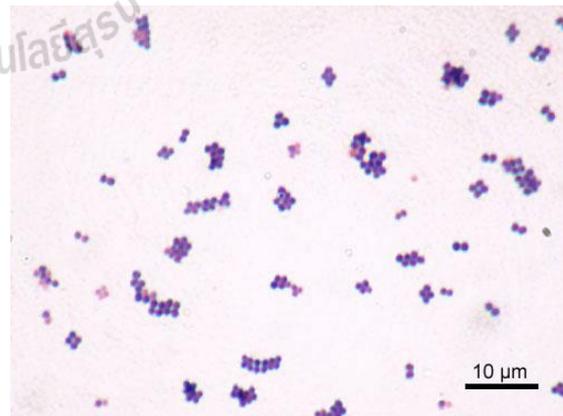
Figure 2.4 Gram stain of *S. aureus* cells.

(http://commons.wikimedia.org/wiki

File:Staphylococcus_aureus_Gram.jpg)

Staphylococcus aureus, members of the genus Staphylococcus (staphylococci) are grams positive cocci bacteria. Staphylococci are spherical cells about 1 μm in diameter, arranged in irregular clusters. Single cocci, pairs, tetrads, and chains are also seen in liquid cultures. Young cocci stain strongly gram-positive; an aging, many cells become gram-negative. Staphylococci are non-motile and do not form spores which have long been recognized as one of the most important agents of food poisoning worldwide. The primary habitat of *S. aureus* is the mucous membranes of the human nasopharynx and animal skin (Genigeorgis, 1989). The micro-organism can exist as a persistent or a transient member of the normal flora without causing any symptoms in humans. Skin infections are the most common type of disease produced by Staphylococcus. Staphylococcus infections of the skin can progress to impetigo (a crusting of the skin) or cellulitis (inflammation of the connective tissue under the skin, leading to swelling and redness of the area). The presence of *S. aureus* in foods is often related to improper manipulation by personnel, who are frequently contaminated with these micro-organisms (Hatakka, 2000). *S. aureus* infection is especially difficult to treat because of the evolved resistance to antimicrobial drugs. Treatment of *S. aureus* infections has been complicated by the persistent rise in rates of *S. aureus* isolates with methicillin resistance.

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first described in 1961 and has since become endemic in many hospitals. In some institutions, MRSA accounts for more than 40-70 % of *S. aureus* infections (Pfaller et al., 1999; Zetola et al., 2005). MRSA, is a type of *Staphylococcus aureus* that is resistant to beta-lactamase drugs, including penicillin, amoxicillin, and oxacillin (Naimi et al., 2003). When the majority of nosocomial staphylococcal isolated were already penicillin-

resistant, the introduction of semi-synthetic beta-lactamase resistant penicillins was a major therapeutic break through. (Voss and Doebbeling, 1995). Methicillin resistance is associated with the acquisition of a particular resistance island-staphylococcal cassette chromosome mec (*SCCmec*). The most important element of *SCCmec* is a gene *mecA* coding for beta-lactam-insensitive enzyme of cell wall synthesis called penicillin binding protein 2a or 2′ (PBP 2a or PBP 2′), a 78-kDa protein with low affinity for beta-lactam antibiotics. The methicillin-resistant *S. aureus* (MRSA) strains are very often classified as multidrug resistant (MDR), which is a consequence of incorporation of many other determinants of resistance into the sequence of *SCCmec* (Lowy, 2003).

MRSA quickly spread around the world and are now a major nosocomial and emerging community pathogen (Zetola et al., 2005). Community acquired MRSA has been isolated from children and adults with skin and soft tissue infections, septic arthritis, bacteraemia, toxic shock syndrome, necrotising fasciitis, and necrotizing pneumonia (Lina et al., 1999; Miller et al., 2005; Gillet et al., 2002; Lina et al., 1999). Community acquired MRSA has been reported most often from indigenous populations (Groom et al., 2001).

2.2.2.2 *Staphylococcus epidermidis* (*S. epidermidis*)

Domain	Bacteria
Phylum	Firmicutes
Class	Cocci
Order	Bacillales
Family	Staphylococcaceae
Genus	*Staphylococcus*
Species	*S. epidermidis*
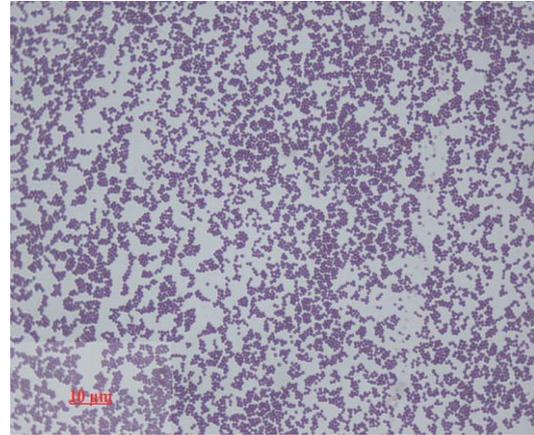
Figure 2.5 Gram stain of *S. aureus* cells. (http://en.wikipedia.org/wiki/File:Staphylococcus_epidermids.jpg)

Staphylococcus epidermidis, members of the genus Staphylococcus (staphylococci) is a gram positive cocci bacteria. *S. epidermidis* is approximately 0.5 to 1.5 micrometres, arranged in grape-like clusters. *Staphylococcus epidermidis* is primarily a normal inhabitant of the healthy human skin and mucosal microflora and has emerged as a common cause of numerous nosocomial infections. *S. epidermidis,* as one of the most often isolated bacterial pathogens in hospitals in general and as the most important pathogen involved in nosocomial bloodstream infections, cardiovascular infections, and infections of the eye, ear, nose, and throat. *S. epidermidis* very often becomes the major infective agent in compromised patients, such as drug abusers and immuno-compromised patients (patients under immunosuppressive therapy, AIDS patients, and premature newborns). The port of entry into the human body in all of these infections is usually an intravascular catheter

(Lim and Webb, 2005). *Staphylococcus epidermidis* is part of the human normal flora. It has developed resistance to many common antibiotics such as methicillin, novobiocin, clindamycin, and benzyl penicillin. As a result, vancomycin or rifampin is used to treat an infection. Approximately 80% of *S. epidermidis* isolates from device-associated infections are resistant to methicillin. Nosocomial *S. epidermidis* isolates were characterized by their pronounced resistance against many of today's commonly used antibiotics including methicillin. The *mecA* gene and its regulators are located on large DNA elements that are termed staphylococcal cassette chromosome *mec* (SCC*mec*). In addition to the methicillin resistance determinant, SCC*mec* carry a set of recombinase and a wide variety of mobile DNA elements such as transposons, insertion sequences or integrated plasmids (Kozitskaya et al., 2004). To date, five major SCC*mec* types have been identified, ranging in size from 21 kb to 67 kb, and a recent study of SCC*mec* distribution has provided evidence that *S. epidermidis* can harbor all types of SCC*mec* (Wisplinghoff et al., 2003). Interestingly, SCC*mec* have been shown to be transferable among staphylococcal species. Genome sequencing of the methicillin-resistant *S. epidermidis* RP62A revealed recently the presence of a SCC*mec* type II cassette (Hanssen et al., 2004). Antibiotic resistance and the ability of many nosocomial *S. epidermidis* isolate to form biofilms on inert surfaces made these infections harder to treat (Ziebuhr et al., 2006).

2.3 Overview of antibiotics

Antibiotics are molecules that kill, or stop the growth of, microorganisms, including both bacteria and fungi also known as antibacterials. Antibiotics that kill bacteria are called "bactericidal", antibiotics that stop the growth of bacteria are called

"bacteriostatic", used to treat infections caused by bacteria. In general, any chemical made by one microorganism that is used to destroy or hinder the spread of other microorganisms is known as an antibiotic. The most widely know antibiotic is perhaps penicillin. The first antibiotic activity of penicillin was discovered by Alexander Fleming, a Scottish bacteriologist in 1929 (Kong et al., 2010). Between the various classes of beta-lactam antibiotics, such as penicillin, cephalosporins are the most frequently used agent of antibacterial infection (Levy, 2002). An antibiotic may be classified basically as "narrow-spectrum" or "broad-spectrum" depending on the range of bacterial types that it affects. Narrow-spectrum antibiotics are active against a selected group of bacterial types (gram positive and gram negative bacteria). Broad-spectrum antibiotics are active against a wider number of bacterial types and, thus, may be used to treat a variety of infectious diseases (Tenover, 2006; Richard Harvey et al., 2012).
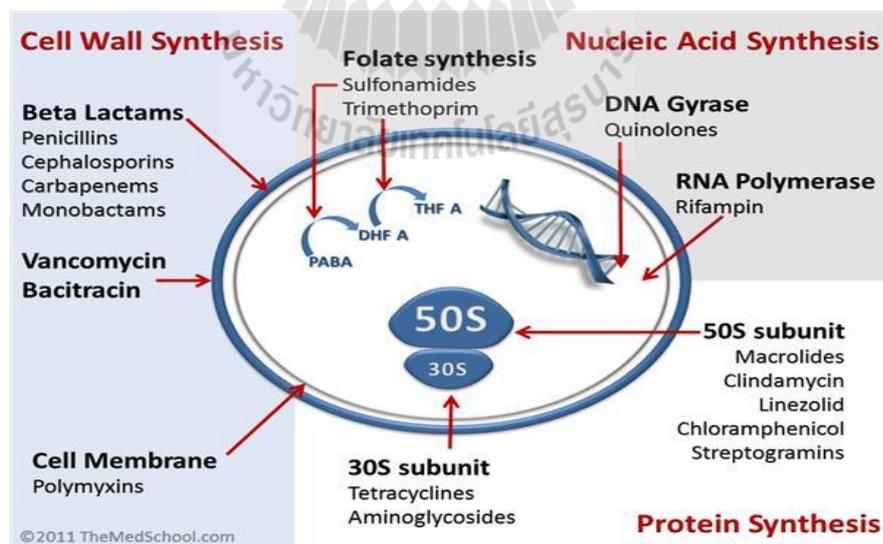
Figure 2.6 Classification and mechanism of antibiotic drugs.

(http://www.orthobullets.com/basic-science/9059/)

2.3.1 Antibiotic resistance

A main problem in antimicrobial therapy is the breakthrough of antibiotic resistant strains of bacteria and, which are a major cause of serious infections. Unnecessary overuse of antimicrobial agents speeds up the evolution of resistant strains. The antibiotics associated with a high resistance potential such as ampicillin, cefamandole, ceftazidime, imipenem, ciprofloxacin, and vancomycin. The use of these antibiotics should be restricted to prevent generalized resistance problems. Antimicrobial resistance is the resistance of a microorganism to an antibiotic to which it was originally sensitive. Resistant organisms are able to combat attacks by antibiotic, so that standard treatments become ineffective and increasing risk of spread to others. The evolution of resistant strains is a natural phenomenon that happens when microorganisms are exposed to antimicrobial drugs, and resistant traits can be exchanged between certain types of bacteria. The misuse of antimicrobial medicines accelerates this natural phenomenon (Cunha, 2000).

Resistance can be described in two ways:

a) Intrinsic or nature whereby microorganisms naturally do not posses target sites for the drugs and therefore the drug does not affect them or they naturally have a low permeability to those agents because of the differences in the chemical nature of the drug and the microbial membrane structures especially for those that require entry into the microbial cell in order to effect their action.

b) Acquired resistance, whereby a naturally susceptible microorganism acquires ways of not being affected by the drug (Byarugaba, 2010).

In addition, bacteria can develop resistance to antibiotics by mutating existing genes (vertical evolution) (Crumplin and Odell, 1987; Martinez and Baquero, 2000)

or by acquiring new genes from other strains or species (Hegstad et al., 2010; Palmer et al., 2010). The drugs for which most resistances have developed are beta-lactam antibiotics. The continued search for effective antibacterial drugs is therefore still very important (Tenover, 2006).

2.3.2 Inhibition of cell wall synthesis

The bacterial cell wall is a highly complex structure consisting of multiple classes of polymers. These polymers include the peptidoglycan, teichoic acid, attached and secreted polysaccharides or proteins, or both, and in gram negative organism, lipopolysaccharide. The state of knowledge regarding these structures has been ably discussed in previous reviews, which cover cell wall. In general, most bacteria contain a cell wall made of peptidoglycan which provides maintenance and support. The peptidoglycan layer is formed by two linear chains of alternating amino sugars; N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). These sugars are connected by a peptide bond; a chemical bond formed between two molecules. Bacterial enzymes aid the bonds that link one or more polymer chain to another to form a cell wall (Bos et al., 2007; DiRienzo et al., 1978; Gupta et al., 2010; Murray et al., 1965; Nikaido, 1979; Osborn and Wu, 1980).

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

synthesis of the bacterial cell wall by interfering with the enzymes required for the synthesis of the peptidoglycan layer (Tenover, 2006). Some drugs such as penicillin and cephalosporin bind to specific proteins and cause inhibition of transpeptidase, an enzyme that links the peptidoglycan chain to form a cell wall. The result of the drugs weakens the cell wall and can no longer provide protection and is subject to lysis or rupture. This mechanism of drug action is the least harmful to humans since they do not have cell walls. A cell that has a damaged cell membrane dies from lysis, disruption in metabolism, or inability to prevent chemicals that are harmful. Polymyxin antibiotics damage the cell membrane function by dissolving the membrane phospholipids or interfere with the movement of molecules in or out of the cell (Ghooi and Thatte, 1995; Silver, 2003).

2.3.3 Beta-Lactam

Beta-lactam antibiotics, which are named for the beta-lactam ring in their chemical structure, include the penicillins, cephalosporins. These agents are active against many gram-positive, gram-negative and anaerobic organisms. The beta-lactam antibiotics exert their effect by interfering with the structural crosslinking of peptidoglycans in bacterial cell walls. Because many of these drugs are well absorbed after oral administration, they are clinically useful in the outpatient setting (Mandell and Perti, 1996). The beta-lactams are ineffective against the pathogens which cause chronic disease as they can't enter into human cells (Thwaites and Gant, 2011). Beta-lactamases are enzymes that prevent bacteria from the fatal effects of beta-lactam antibiotics, and are therefore of significant clinical importance (Herzberg and Moult, 1987). The resistant strains of bacteria have quickly increased in hospitals,

leading to nosocomial infections which are hard to treat. In a study performed in a hospital in England, 14% of the isolates were resistant to penicillin in 1946, whereas 59% of the isolates beta-lactam antibiotics have been in clinical used for more than 50 years. The susceptibility of various pathogenic bacteria to the beta-lactam antibiotics has changed dramatically over the years due to their widespread and liberal usage. It is remarkable that before the wide spread of clinical used penicillin, cases of resistant Staphylococci have been already in subsistence. Onward, penicillinase (now more commonly referred to as beta-lactamase) was isolated from the clinical strains of penicillin-resistant *Staphylococcus aureus* and from clinical isolate of *Pseudomonas aeruginosa* (Shannon et al., 1982).

2.3.4 Beta-lactam compounds
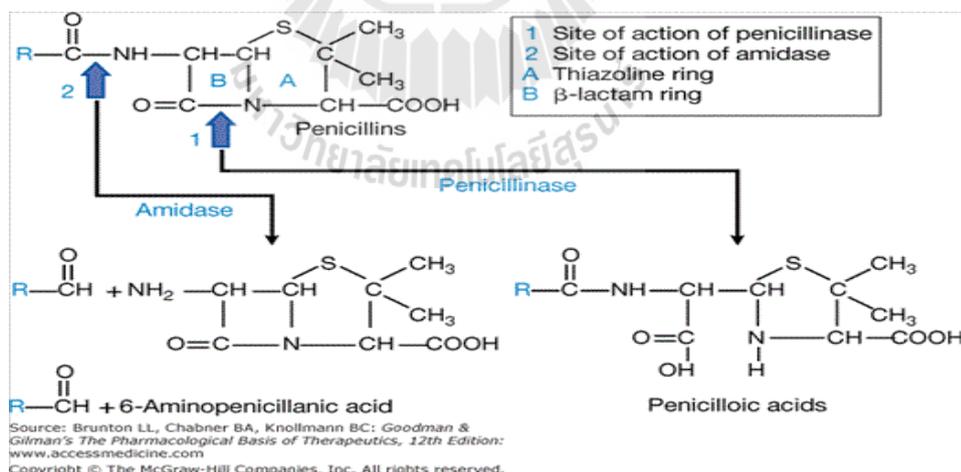
Figure 2.7 Structure of penicillins and products of their enzymatic hydrolysis.

(http://accessmedicine.mhmedical.com/content)

Penicillins

Penicillin antibiotics are historically important because they were the first drugs that were proficient against many previously serious diseases such as tuberculosis, syphilis, and staphylococcus infections. Penicillins are still widely used today, though many types of bacteria are now resistant (Mursic et al., 1996). Penicillins are one of a group of antibiotics that kill bacteria or prevent their growth. There are several types of penicillins, each used to treat different kinds of infections, such as skin, dental, ear, respiratory tract, urinary tract, and other bacterial infections. They are the most widely effective antibiotics and are among the least toxic drugs now, 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 enzyme beta-lactamases (Petri Wa, 2011; Wright, 1999). Several natural penicillins can be produced depending on the chemical composition of the fermentation medium used to culture *Penicillium.* Penicillin G (benzylpenicillin) has the greatest antimicrobial activity of these and is the only natural penicillin used clinically. For penicillin G, the side chain is a phenyl-methyl substituent (Kong et al., 2010).

2.3.5 Ampicillin
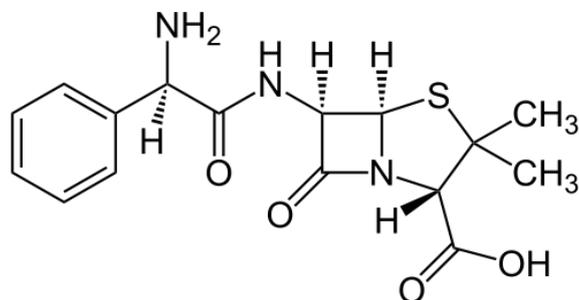
Figure 2.8 Ampicillin structure.

(http://openwetware.org/wiki/Image:Amp.png)

Ampicillin is a 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. Ampicillin has in vitro activity against gram-positive and Gram-negative aerobic and anaerobic bacteria. The bactericidal activity of ampicillin results from the inhibition of cell wall synthesis and is mediated through ampicillin binding to penicillin binding proteins (PBPs) (Figure 2.6). Ampicillin is stable against hydrolysis by a variety of beta-lactamases, including penicillinases, and cephalosporinases and extended spectrum beta-lactamases. By binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall, ampicillin 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 ampicillin interferes with an autolysin inhibitor.

Point mutations in PBP5, including the addition of aspartic acid or serine after position 466 and change of methionine to alanine or threonine at position 485, alanine or Isoleucine to threonine at position 499 and glutamate to Valine at position 629,

were found to be significantly associated with ampicillin resistance. A significant correlation was obtained for the combined mutation (alleles 10 and 11), suggesting that the combined mutation of PBP5 can be a marker for ampicillin resistance of *E. faecium* (Hsieh et al., 2006).

2.3.6 Cloxacillin
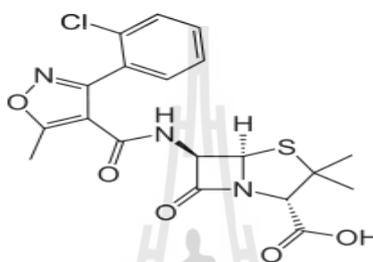
Figure 2.9 Cloxacillin structure.

(http://www.onlinepharmacycatalog.com/drugs/antibiotics/cloxacillin)

Cloxacillin sodium is a beta-lactam antibiotic commonly used to treat gram-positive infections, especially those caused by *Staphylococcus aureus*. Cloxacillin is a semisynthetic antibiotic in the same class as penicillin for use against staphylococci that produce beta-lactamase by binding to specific penicillin-binding proteins (PBPs) located inside the bacterial cell wall (Chan et al., 2013). Cloxacillin inhibits the third and last stage of bacterial cell wall synthesis. Cell lysis is then mediated by bacterial cell wall autolytic enzymes such as autolysins; it is possible that cloxacillin interferes with an autolysin inhibitor.
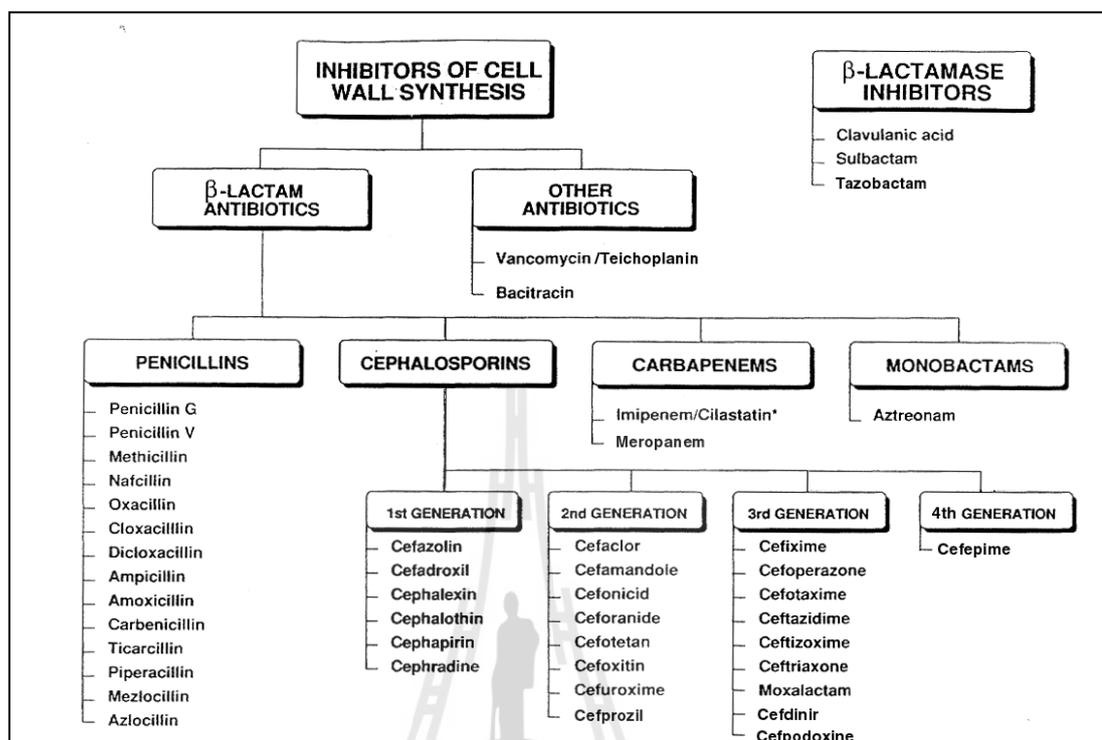
Figure 2.10 Summary of antimicrobial agents that inhibit cell wall synthesis.

(Richard A. Harvey et al., 2012).

2.3.7 Nisin
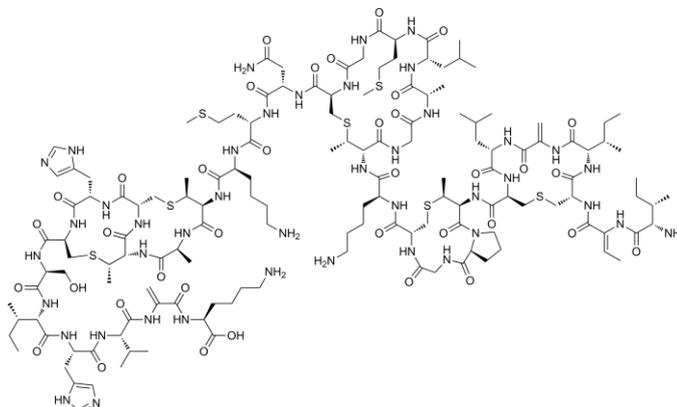
Figure 2.11 Nisin structure.

(http://en.wikipedia.org/wiki/Nisin)

Nisin is a polycyclic antibacterial peptide with 34 amino acid residues used as a food preservative. It contains the uncommon amino acids lanthionine (Lan), methyllanthionine (MeLan), didehydroalanine (Dha) and didehydroaminobutyric acid (Dhb). These unusual amino acids are introduced by posttranslational modification of the precursor peptide. In these reactions a ribosomally synthesized 57-mer is converted to the final peptide (Buchman et al., 1988). Nisin is produced by fermentation using the bacterium *Lactococcus lactis*. In the food industry, it is obtained from the culturing of *L. lactis* on natural substrates, such as milk or dextrose, and are not chemically synthesized (Buchman et al., 1988). While in general, most bacteriocins inhibit only closely related species, nisin is a rare example of a "broad-spectrum" bacteriocin effective against many Gram-positive organisms, including lactic acid bacteria (commonly associated with spoilage), *Listeria monocytogenes* (a known pathogen), *Staphylococcus aureus, Bacillus cereus, Clostridium botulinum,* etc. It is also particularly effective against spores. Gram-negative bacteria are protected by their outer membrane, but may become susceptible to nisin action after a heat shock or when this is coupled with the chelator EDTA. Nisin is soluble in water and can be effective at levels nearing the parts-per-billion range (Benkerroum and Sandine, 1988; Delves-Broughton et al., 1996; Harris et al., 1992). Nisin is used in processed cheese, meats, beverages, etc. during production to extend shelf life by suppressing Gram-positive spoilage and pathogenic bacteria. In foods, it is common to use nisin at levels ranging from ~1-25 ppm, depending on the food type and regulatory approval. As a food additive, nisin has an E number of E234 (Delves-Brougthon, 1996; Harris et al., 1992). In combination with miconazole it has been

studied as a possible treatment for infections of *Clostridium difficile* (Young and Thomas, 2013).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant species

The medicinal plants used were the roots of *S. suberosa* (BK257189) purchased from Lamtakhong Research Station, Nakhon Ratchasima, Thailand. Leaves of *Pluchea indica* (L.) Less. (QBG No.39171) were purchased from private companies in Nakhon Ratchasima. These plants were identified and authenticated by Dr Paul J. Grote, a lecturer and plant biologist at Institute of Science, Suranaree University of Technology, Thailand. The voucher specimens have been deposited at the Thailand National Herbarium.

3.1.2 Test organisms

3.1.2.1 Bacterial strains

Clinical isolates of Ampicillin- resistant *Staphylococcus aureus* DMST 20651, 20652, 20653 (ARSA) and Cloxacillin resistant *S. epidermidis* DMST 15505, 15506, 15507 (CRSE), were obtained from the Department of Medical Science Thailand, Ministry of Public Health, Thailand. *Staphylococcus aureus* ATCC 29213 were obtained from 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 4°C. Fresh slope cultures were prepared every 3-4 weeks (Eumkeb, 1999).

3.1.3 β-lactam antibiotics

Ampicillin, Cloxacillin, *o*-nitrophenol-*β*-D-galactoside (ONPG), nisin, and *β* -lactamase type IV from *Enterobacter cloacae* were obtained from Sigma Aldrich, UK.

3.1.4 Culture media

Nutrient agar, Cation-adjusted Mueller-Hinton broth and agar were obtained from Oxiod (Basingstoke, UK). Approximate formula per liter of each medium.

3.2 Methods

3.2.1 Extraction of *S. suberosa*

S. suberosa were dried under a hot air oven and powdered by mixer, and then were extracted using a Soxhlet extractor. Ethanol was used as solvent systems (50 g dried *S. suberosa* of root powder/500 mL ethanol). The temperature of extraction process was set at 75°C for 8 hours. Each extract was concentrated in a rotary evaporator using the freeze dryer to yield a brown powder of water extract and a dark brown sticky oil of ethanol extract (Patra et al., 1986).

3.2.2 Extraction of *Pluchea indica* (L.) Less

Pluchea indica (L.) Less were dried under a hot air oven and were powdered by mixer, then the Soxhlet extractor was used to extract and methanol was used as solvent systems. The temperature of extraction process was set at 75°C for 8 hours. Each extract was concentrated in a rotary evaporator at 40°C using freeze dryer to yield a powder of water extract and sticky oil of methanol extract (Biswas et al., 2007).

3.2.3 Preparation of test solution and inoculums

Antibiotic test solutions were prepared by dissolving Ampicillin, Cloxacillin, and nisin in sterile water.

S. suberosa crude extract (SSE) was prepared by dissolving in sterile water and *Pluchea indica* crude extract (PIE) were dissolved in 50% dimethylsulfoxide prepared by the doubling dilution method to obtain stock solutions.

Test organisms were incubated in 100 mL nutrient broth for 18 h at 37º C. The cell cultures were centrifuged at 4,000 r.p.m for 10 min. The cell pellets were washed with saline, recentrifuged and resuspended in saline. The cell concentrations were adjusted with saline to give 5×10^8 CFU/mL using a predetermine calibration curve of absorbance at 500 nm against viable count (Liu et al., 2000). The MICs of each crude extract, the antibiotics alone and crude extracts in combination with each antibiotic were examined (Table 3.1).

Table 3.1 The test of MICs of each crude extract with resistant bacteria.

NO	Resistance Bacteria	Gram	Drug resist	Crude extract
1	ARSA 20651	+	Ampicillin	*S. suberosa*
	ARSA 20652			
	ARSA 20653			
2	CRSE 15505	+	Cloxacillin	*P. indica*
	CRSE 15506			
	CRSE 15507			

3.2.4 Bacterial suspension standard curve

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

1. A separate loopful of each bacterium was used to inoculate in 100 mL of Mueller Hinton broth.

2. The cultures were incubated at 37°C for 18 h. The bacterial cells were pelletted by centrifugation at 4,000 r.p.m. for 10 mins.

3. The cells were washed twice by resuspending and centrifuging at 4,000 r.p.m/min for 10 mins in 10 mL of 0.9% NaCl.

4. The cells were resuspended in 50 mL of sterile 0.9%NaCl.

5. The cell suspensions were diluted so that 5-6 spectrophotometer readings could obtained over the absorbance range of approximately 0.05-0.25 at a wavelength of 500 nm.

6. Viable counts for each absorbance reading were determined in triplicates using overdried agar plate counting method. (Eumkeb, 1999; Richards, Gregory, and Xing, 1993)

3.2.5 MICs determination

MICs were determined using a broth macrodilution method. The sterilized wire loop test organism from a slope culture were inoculated into a nutrient broth for 18 h at 37°C. Then, the preparation of a bacterial suspension, the density of the bacterial suspension in normal saline was adjusted to approximately 1 (10^8 CFU/mL by using the absorption of bacterial suspension viable count standard curve. The inoculums of 0.01 mL of standard suspension (18h culture) of each strain of the test bacteria was added to triplicate tubes containing 4.95 mL CAMHB, plus serial dilutions of the antibacterials, to give approximately 5×10^5 CFU/mL. Tubes of broth without antibacterials will be used as the control for each of the test bacteria. Incubation is at 35°C for 20 hours The MICs will be defined as the lowest concentration of antibiotic at which there is no visible growth in the triplicate tubes (Bonapace et al., 2002; CLSI, 2013; Eumkeb, 1999).

3.2.6 Checkerboard determination

Antimicrobial combinations were selected for various reasons, including minimized drug toxicity by using the lowest possible doses of two or more agents that have additive efficacies but independent toxicities, or to reduce the potential for development of resistance to one agent. 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 (Bonapace et al., 2002; Eumkeb, 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 to1/10 of their MICs along the ordinate and abscissa respectively.

An 18 h culture of each of the test bacteria were prepared. The test bacterial suspensions were adjusted to 1×10^8 CFU/mL using the absorption of bacterial suspension from the previously determine standard curve. 0.01 mL of the bacterial suspension was added to a series of 4.95 mL CAMHB, plus 10% serial dilutions of the antibacterial combinations, to give 5×10^5 CFU/mL. Tubes of broth without antibacterials were used as the controls for each of the test bacteria. The culture was incubated for 20 hours at 35°C. The test was carried out in triplicate. FIC values were calculated according to the equation below, where B is the crude extracts tested (SSE, PIE) in combination with A which is antibiotics (AMP, CLX). MIC(A) is defined as the minimum inhibitory concentration of A alone, and MIC(B) is defined as the minimum inhibitory concentration of B alone, Crude extract were tested in combination with the antibiotic to test for synergy. MIC and FIC values were determined by constructing isobolograms from the checkerboard assay. Furthermore, by assessing the fractional inhibitory concentrations of each component, a quantitative value can be assigned to the individual efficacies of each drug. By summing the fractional inhibitory concentrations, the fractional inhibitory concentration index (FIC index) can be calculated as shown in the equation below:

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

FIC (A+B) ≤ 0.5 Synergy

FIC (A+B) $>$ 0.5-4.0   No interaction

FIC (A+B) $>$ 4.0   Antagonism

(Odds, 2003)
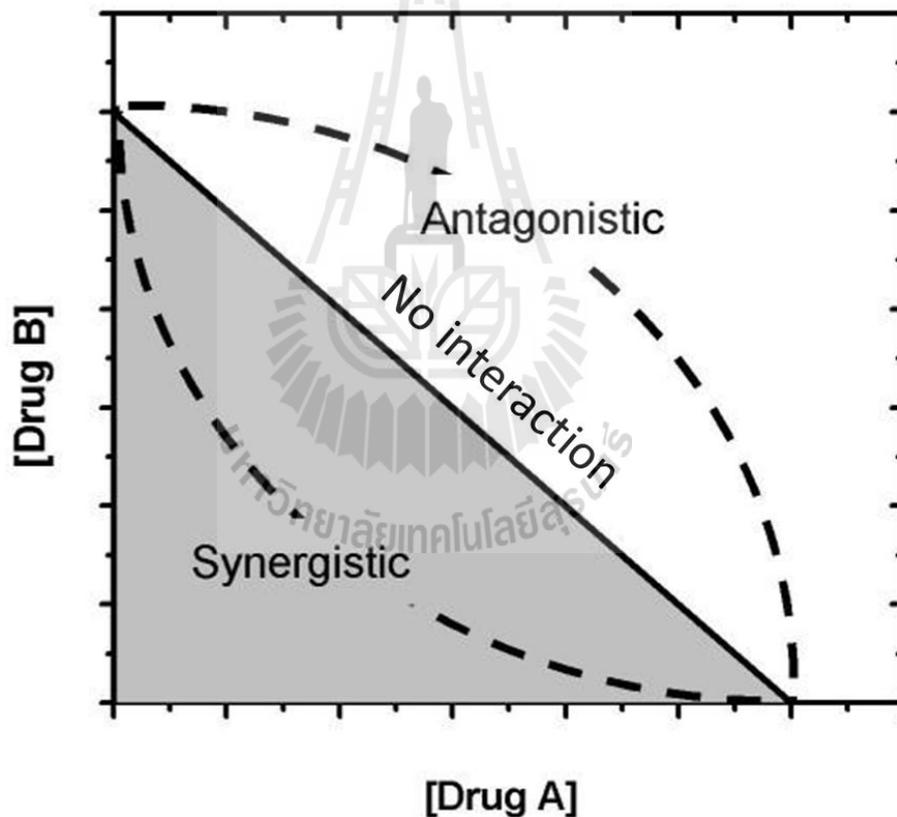
Figure 3.1 Isobologram of two drugs to evaluate possible combined effects.

(Van Dyke, 2003)

Synergism between two compounds can be identified qualitatively and quantitatively assessed by varying the concentrations of the two compounds of

interest and measuring their combined efficacy. Traditionally in an antimicrobial study, a checkerboard assay is used in which varying concentrations of Drug A and Drug B are used and percent activity assessed. Efficacy can then be plotted on a graph detailing the interaction between drugs called an isobologram, and based on the curvature; the effect of the combination of the drugs tested can be determined to be synergistic, no interaction, or antagonistic as shown in Figure 3.1. If the two drugs are no interaction there is a straight line between drug B and drug A. It the line or curve bends below the straight line the drugs are synergistic. If the line bends above the straight line the two drugs are antagonistic (Odds, 2003; Wagner, 2011).

3.2.7 Killing curve determinations

The checkerboard determinations that give synergistic effect was chosen to confirm by killing curve method. Viable counts for the determination of killing curve were performed as previously described using a culture medium volume of 100 mL (Richards et al., 1993). Inocula of 5×10^6 CFU/mL were exposed to the antibacterials either singly or in combination with crude extract from the *S. suberosa* to give a final concentration approximately 5 (10^5 CFU/mL and incubation in shaking water bath at temperature of 37°C. After a contact time of 0, 0.5, 1, 2, 4, 6 and 24 h, a 1 mL sample of each incubated mixture was inactivated by the addition of Muller-Hinton broth (MHB). Subsequent dilution plating on overdried Mueller-Hinton agar plates in quadruplicate and incubation at 35°C for 20 h were allowed the counting of growing colonies. The lowest detectable limit for counting was 10^3 CFU/mL. Positive controls containing similar cell and solvent concentrations were used (Eumkeb, 1999).

3.2.8 Transmission electron microscopy (TEM) method

Preparation of cultures

The checkerboard determinations that give synergistic effect was chosen to investigate preliminary mechanism of action. To examine the effect of crude extract of *S. suberosa* on the cell structure of ARSA 20651 the following methods were performed.

ARSA 20651 was incubated in 10 mL CAMHB for 20 h at 35°C. A 0.2 mL volume of 20 h culture was inoculated into a 250 mL conical flask containing 98 mL CAMHB which was placed in a water bath shaking at 100 oscillations/min for 4 h at 35°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 5×10^6 of CFU/mL in 0.9% NaCl, the 4 h log phase culture was inoculated into 250 mL conical flasks each containing 90 mL CAMHB plus *S. suberosa* crude extract and ampicillin to give approximately 5×10^5 CFU/mL final concentration. A flask containing 90 mL CAMHB for ARSA 20651 without any antibiotics was used as the control. The cultures together with the crude extract plus antibacterial and control cultures was incubated for 4 h shaking at 100 oscillations/min in a water bath at 35°C (Richards et al., 1993; Xing, 1994). The ARSA 20651 cultures were centrifuged at 6,000 g for 15 min at 4°C, and the supernate was removed. The pellets were fixed in glutaraldehyde 8% v/v in 0.1 M phosphate buffer (pH 7.2) for 1 h at 4°C and then were in 4% v/v in 0.1 M phosphate buffer (pH 7.2) for 4 h at 4°C. After washing in the buffer the bacteria 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 was 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 graded 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 et al., 2010; Richards et al., 1995).

Measurement of bacterial cell areas : The average cell area of bacteria in each group was calculated by sampling each square in each field that can be the most representative of bacterial cells in those groups. All of the bacterial cells in those squares were measured cell width x cell length using Milli-ruler. The average cell areas (mean ± S.E.M) were presented. Then, these data were compared using one-way analysis of variance (one-way ANOVA) and Tukey HSD post-hoc test was calculated as statistically significant difference.

3.2.9 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. SSE used either singly or in combination with ampicillin induced CM permeability were examined by the ability of these antimicrobial agents to disclose cytoplasmic β-galactosidase activity in bacteria by using ONPG as a substrate that can be cleaved by β-galactosidase localized within cytoplasm. The products of β-galactosidase-ONPG reaction were galactose (colorless) and *o*-nitrophenol (yellow). The assays were prepared in

according to the methods of Marri and Eumkeb's descriptions (Eumkeb et al., 2012; Marri, 1996). The method to assay of beta-galactosidase was as follows:

18 h cultured and adjusted bacteria were grown without antibacterial agents (control), with SSE alone, ampicillin alone and SSE plus ampicillin combination, at six different times (0,1,2,3,4 and 5 h) with shaking 110 oscillations/min in a water bath at 35ºC. Nisin (8 µg/mL) was used as a positive control. Aliquot (2 mL) of each treatment at each time were removed to tubes containing ONPG (4mg/mL) and Phosphate buffered saline (PBS). If presence of yellow was recorded as positive β-galactosidase activity (increased CM permeability), while the appearance of colorless was recorded as negative β-galactosidase activity (no effect on CM permeability).

3.2.10 Enzyme assay

Beta-Lactamase are bacterial enzymes that inactivate beta-lactam antibiotics. Beta-Lactamase test rapidly detects the presence of beta-Lactamase enzyme produced by strains of bacteria. This enzymes resistance to a number of penicillin antibiotics by attacking the common beta-lactam ring structure, resulting in inactivation of these drugs. This mode of action forms the basis of the beta-Lactamase test reaction (Livermore and Brown, 2001).

Beta-lactamaes type IV activity of *E. cloacae* was used in this study. Benzylpenicillin was determined as a substrate for beta-lactamaes type IV. Enzyme activities were adjusted to a concentration sufficient to hydrolyze 50-60% substrate within 5 minutes. SSE at 1, 2, and 4 mg/mL were preincubated with enzyme in 50 mM sodium phosphate buffer (pH 7.0) at 37°C for 5 minutes prior to adding a

substrate. Time-course assays were performed at 0, 5, 10, 15 and 20 minutes using methanol/acetic acid (100:1) as a stopping reagent. The remaining substrate was analyzed by reverse-phase HPLC. Acetronitrile/10mM ammonium acetate (25:75) was used as a mobile phase with flow rate 1 mL/min, UV detector at 200 nm and Ascentis C18 column (Eumkeb et al., 2010; Richards et al., 1995).

3.2.11 Statistical analysis of data

All experiments were carried out in triplicate; data were expressed as mean±standard error of the mean (SEM). Significant differences of cell area in each treated group from Killing curve determination, TEM, and enzyme assay among each treated group at the same interval times were analyzed by one-way ANOVA. A p-valve <0.05 or <0.01 of the Games-Howell or Tukey's HSD post-hoc test was considered as statistically significant difference.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The percentage of extract obtained from each plant

The percent yield of *S. suberosa* crude extract (SSE) and *P. indica* crude extract (PIE) was 3 and 5 % (w/w) from dried powder respectively (Table 4.1).

Table 4.1 The % yield of two 95% ethanolic plant extracts calculated by using the weight of dried residue extract per weight of dried plant

Plants	% yield (w/w)
S. suberosa crude extract (SSE)	3
P. indica crude extract (PIE)	5

4.2 Bacterial suspensions viable count absorption standard curve

The results of the bacterial suspensions viable counts standard curves for *Staphylococcus aureus* ATCC 29213 (MSSA), Ampicillin- resistant *Staphylococcus aureus* DMST 20651, 20652, 20653 (ARSA) and Cloxacillin resistant *S. epidermidis* DMST 15505, 15506, 15507 (CRSE) are shown in figure 1 to 7 of Appendix C

Figure 1 to 7 of Appendix C illustrate that the absorptions of MSSA, ARSA DMST 20651, 20652, 20653 and CRSE DMST 15505, 15506, 15507 at 500 nm were approximately 1×10^{8} CFU/mL at the absorption of 0.10, 0.09, 0.11, 0.12, 0.22, 0.22 and 0.21 respectively.

4.3 MIC determinations

In present study, a total of two crude extracts from *S. suberosa* and *P. indica*, and selected antibiotics (ampicillin, cloxacillin and nisin) against clinical isolates of *Staphylococcus aureus* ATCC 29213 (MSSA), ampicillin- resistant *Staphylococcus aureus* DMST 20651, 20652, 20653 (ARSA) and Cloxacillin resistant *S. epidermidis* DMST 15505, 15506, 15507 (CRSE). The MIC value of the extracts was determined using the macrobroth dilution method as described previously (Bonapace et al., 2002; CLSI formerly, 2013; Eumkeb, 1999). The MICs of two medicinal plants in the present study are summarized in Table 4.2
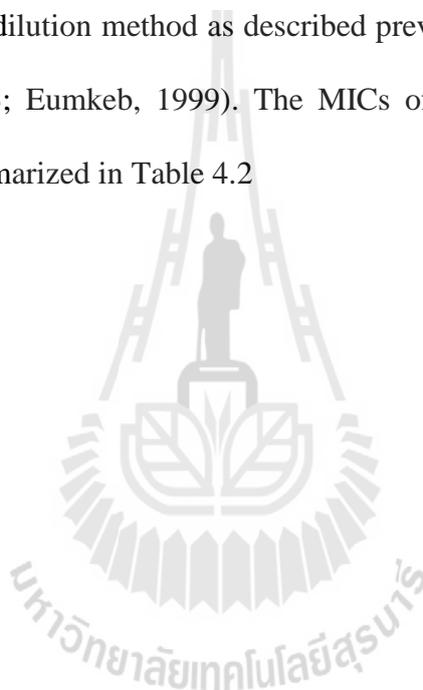
Strains	MIC					FIC		FIC index
	AMP (μg/ml)	CLX (μg/ml)	SSE (mg/ml)	PIE (mg/ml)	NIS (μg/ml)	AMP+SSE (μg/ml+mg/ml)	CLX+PIE (μg/ml+mg/ml)	
S. aureus DMST 20651	>512[R]	-	4.0[ND]	-	16	0.15+0.5	-	<0.13
S. aureus DMST 20652	>512[R]	-	4.0[ND]	-	16	0.15+0.5	-	<0.13
S. aureus DMST 20653	>512[R]	-	4.0[ND]	-	16	0.15+0.5	-	<0.13
S. epidermidis DMST 15505	-	4.0[R]	-	>1,024[ND]	16	-	3.0+30	<0.78
S. epidermidis DMST 15506	-	4.0[R]	-	>1,024[ND]	16	-	3.0+30	<0.78
S. epidermidis DMST 15507	-	4.0[R]	-	>1,024[ND]	16	-	3.0+30	<0.78
*S. aureus ATCC 29213	0.25[S]	0.25[S]	4.0[ND]	>1,024[ND]	0.5	NT	NT	NT

Table 4.2 Minimum inhibitory concentration (MICs) SSE, PIE, AMP, CLX against clinical isolates of ampicillin- resistant

Staphylococcus aureus DMST 20651, 20652, 20653 (ARSA) and Cloxacillin resistant *S. epidermidis* DMST 15505, 15506, 15507

(CRSE) determined by the macrobroth dilution method. [S] = Susceptible; [R] = resistant; [ND] = No data in CLSI; NT= not test; AMP =

Ampicillin; CLX = Cloxacillin; SSE = *S. suberosa* crude extract; PIE = *P. indica* crude extract; NIS = Nisin; **S. aureus* ATCC 29213

was used as a reference strain.

Table 4.2 represents the MICs of two medicinal plant extracts tested against MSSA, all ARSA strains and all CRSE strains. The results showed that *S. suberosa* exhibited little inhibitory effect with MICs 4.0 mg/mL against MSSA and all ARSA strains, while the MICs of ampicillin were 0.25 and >512 µg/mL against MSSA and all ARSA strains, respectively. According to CLSI guideline, these results suggested that all ARSA strains used in this study revealed a high resistance level to ampicillin and nisin because of the standard value for the sensitivity of ampicillin against these strains are ≤ 0.25 µg/ml, but the reference strain MSSA was susceptible to ampicillin at 0.25 µg/mL (CLSI, 2013). Likewise, these results are in substantial agreement with those of Eumkeb et al. (Eumkeb et al., 2010) that the MIC of ampicillin against *S. aureus* DMST 20651 was > 1,000 µg/ml. Also, the MIC result of nisin against MRSA strains seem consistent with previous finding that 90% MIC of nisin against MRSA was 16 µg/ml (Giacometti et al., 2000).

In addition, the MICs of *P. indica* against all CRSE strains and MSSA showed a same concentration against these strains at concentration of >1,024 mg/mL. While the MICs of cloxacillin against MSSA and all CRSE strains were 0.25 and 4.0 µg/mL, respectively. These results indicated that *P. indica* did not inhibit those of all CRSE strains and MSSA.

From this study, *S. suberosa* extracts were found to have a little antibacterial activity against reference strain and resistant strain bacteria. The MIC results suggest that all ARSA strains are resistant to SSE, ampicillin and nisin while the reference strain exhibits susceptible to ampicillin (CLSI, 2013). Similarly, previous reports also found that *Stephania* genus in Menispermaceae family showed that it possessed antibacterial activity (Deng et al., 2011; Lohombo-Ekomba et al., 2004). However,

these findings provide evidence that *P. indica* do not exhibit antibacterial activity against all CRSE strains and MSSA.

4.4 Checkerboard determination

The combinations of antibacterial agents were performed using a checkerboard assay in order to examine synergistic antibacterial activity of two crude extracts and antibiotic.

The checkerboard and isobologram results of the combination against these strains are shown in Table 4.2 and Figure 4.1 to 4.6 respectively. In checkerboard assay, based upon FICI calculation, the combination of SSE and ampicillin exhibited synergistic activity at FICI <0.13 against all ARSA strains (Table 4.2). Obviously, the concentration of ampicillin that can inhibit all ARSA strains growths had considerably reduced from >512 µg/ml to 0.15 µg/ml in combination with SSE. Whereas, the combination of PIE and cloxacillin exhibited no interaction or synergistic effect against all CRSE with FICI at <0.78 (Table 4.2). The FIC index (FICI) was calculated and interpreted in accordance with Odd's description as follows; FICI ≤ 0.5 denoting synergistic; FICI > 0.5-4.0 denoting no interaction; FICI > 4.0 denoting antagonism (Odds, 2003). The FIC of each agent and FIC index were calculated as above mentioned (MIC and checkerboard determinations). When ampicillin was combined with SSE, the fractional inhibitory concentrations (FICs) of ampicillin plus SSE were 0.15 µg/ml + 0.50 mg/mL against all of ARSA tested strains. So, the FIC indices of these combinations against all of ARSA tested strains were < 0.13 (Figures 4.1 to 4.3 and Table 4.2). In the same way, the FIC indices of cloxacillin plus PIE against all CRSE tested strains were calculated exactly the same

method. As a consequence, the FICI of these combinations against all CRSE strains were <0.78 which represent no interaction or synergistic effect (Figures 4.4 to 4.6 and Table 4.2). These results are in substantial correspondence with those of Eumkeb et al. that galangin, quercetin or baicalein plus ampicillin exhibited synergistic activity against penicillins-resistant *S. aureus* strains at FIC indices < 0.03 (Eumkeb et al., 2010). Besides, previous studies reported that a synergistic effect between quercetin and oxacillin against vancomycin-intermediate *S. aureus* displayed the lowest FIC index value of 0.0417 (Basri et al., 2008). Apart from this, the antibacterial activity of quercetin plus ampicillin or vancomycin against the sensitive MRSA strain were significantly increased compared to control (no any testing agent) ($p < 0.01$) (Hirai et al., 2010). As previously documented, drug combination approach by achieving a synergistic effect can eliminate and neutralize the adverse effects (Wagner, 2011).

Ampicillin- resistant *Staphylococcus aureus* DMST 20651 (ARSA)
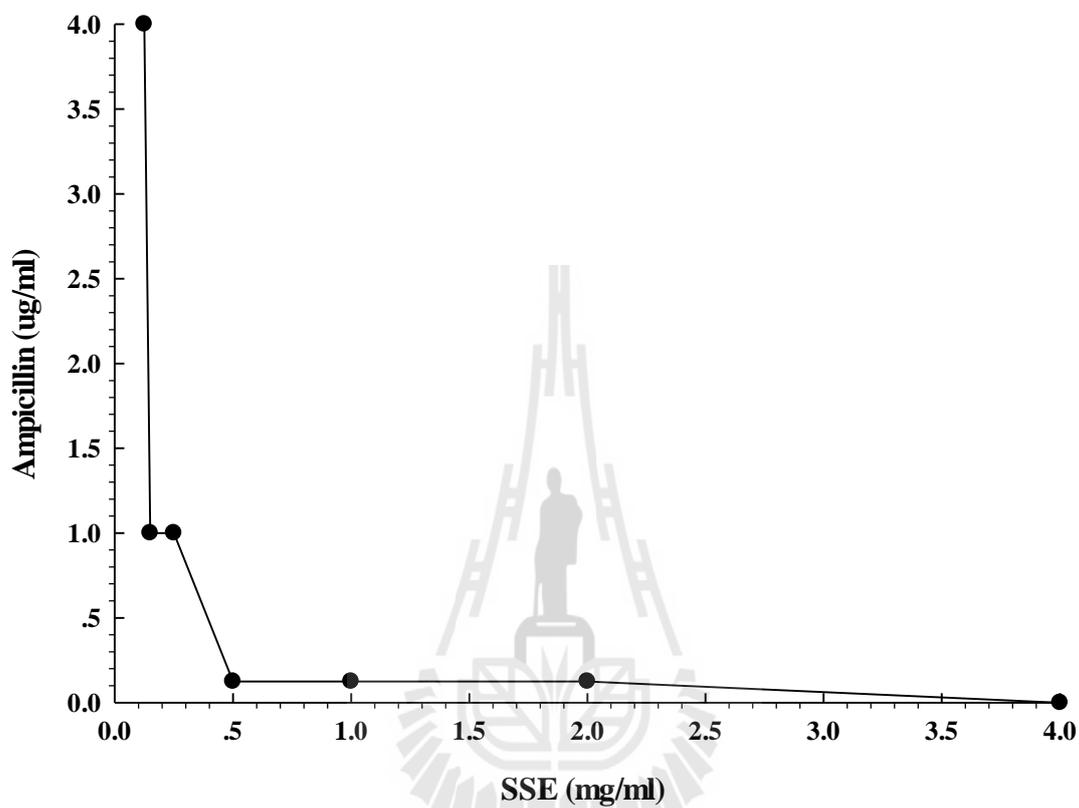
Figure 4.1 Isobologram constructed from checkerboard MIC data showingan antibacterial combinationn of ampicillin plus *S. suberosa* crude extract (SSE) against ARSA 20651.

Ampicillin- resistant *Staphylococcus aureus* DMST 20652 (ARSA)
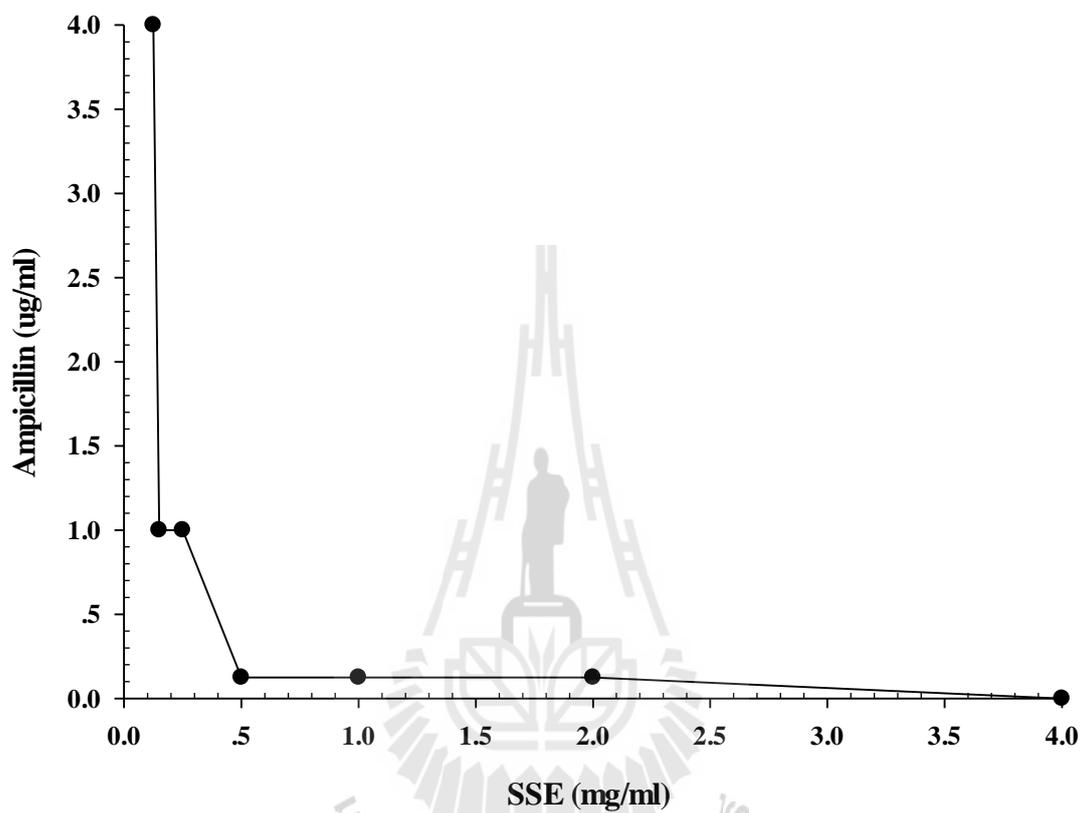
Figure 4.2 Isobologram constructed from checkerboard MIC data showingan antibacterial combinationn of ampicillin plus *S. suberosa* crude extract (SSE) against ARSA 20652.

Ampicillin- resistant *Staphylococcus aureus* DMST 20653 (ARSA)
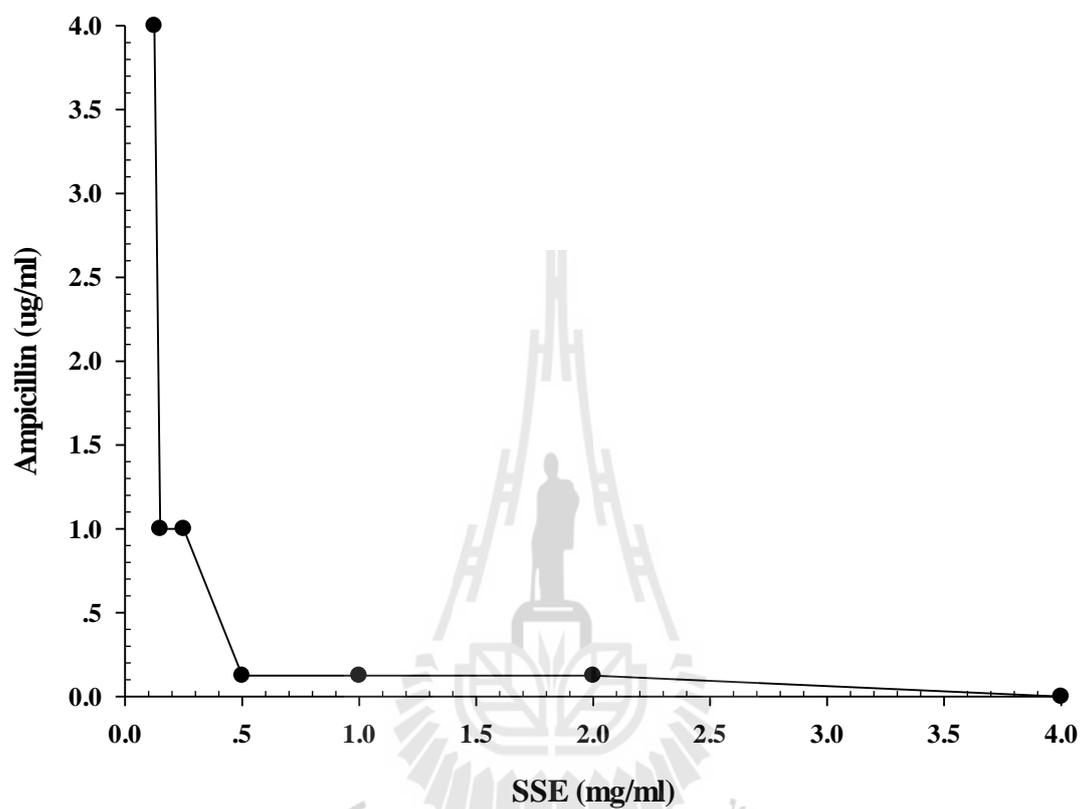
Figure 4.3 Isobologram constructed from checkerboard MIC data showingan antibacterial combinationn of ampicillin plus *S. suberosa* crude extract (SSE) against ARSA 20653.

Staphylococcus epidermidis DMST 15505 (_S. epidermidis_)
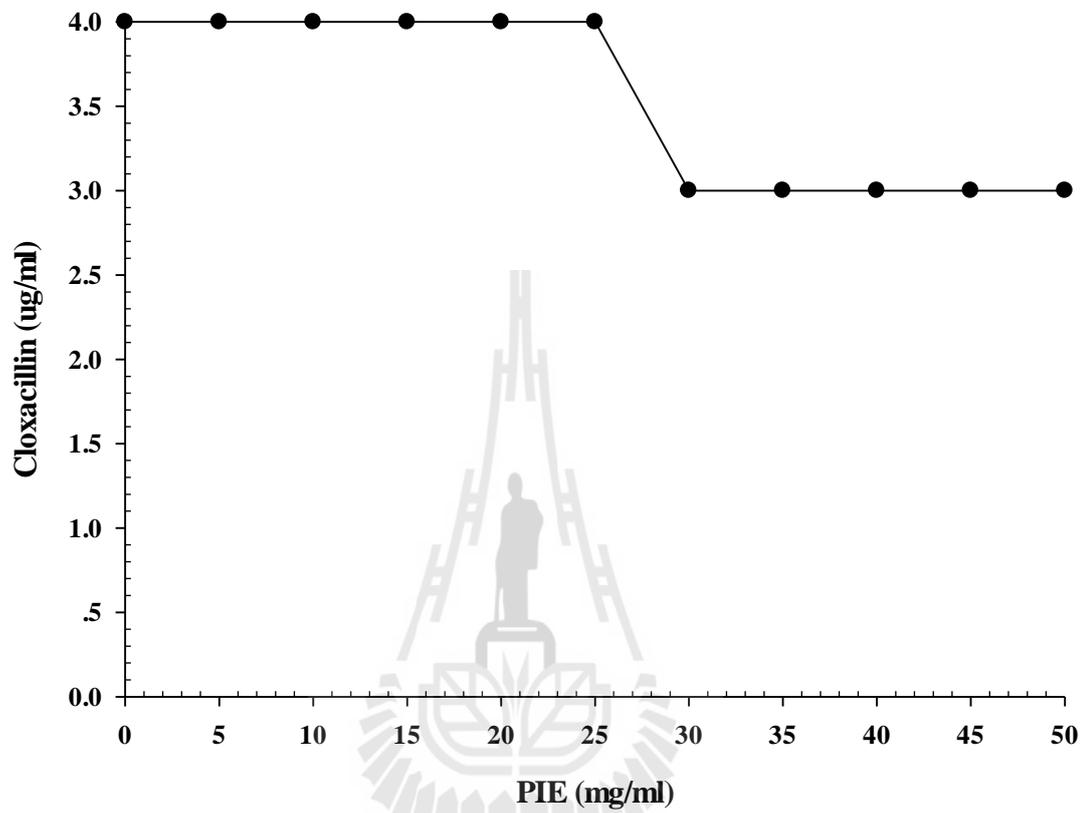
Figure 4.4 Isobologram constructed from checkerboard MIC data showing

antibacterial combination of cloxacillin plus _P. indica_ crude extract (PIE) against

CRSE DMST 15505.

***Staphylococcus epidermidis* DMST 15506 (*S. epidermidis*)**
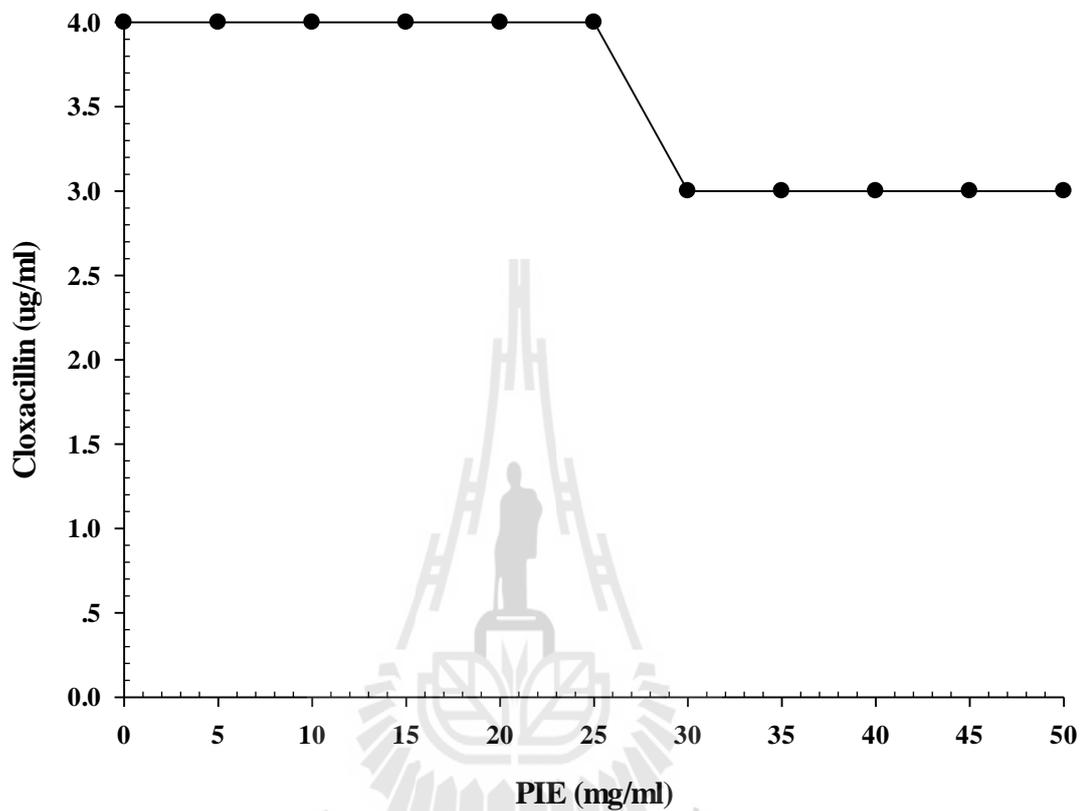
Figure 4.5 Isobologram constructed from checkerboard MIC data showing

antibacterial combination of cloxacillin plus *P. indica* crude extract (PIE) against

CRSE DMST 15506.

***Staphylococcus epidermidis* DMST 15507 (*S. epidermidis*)**
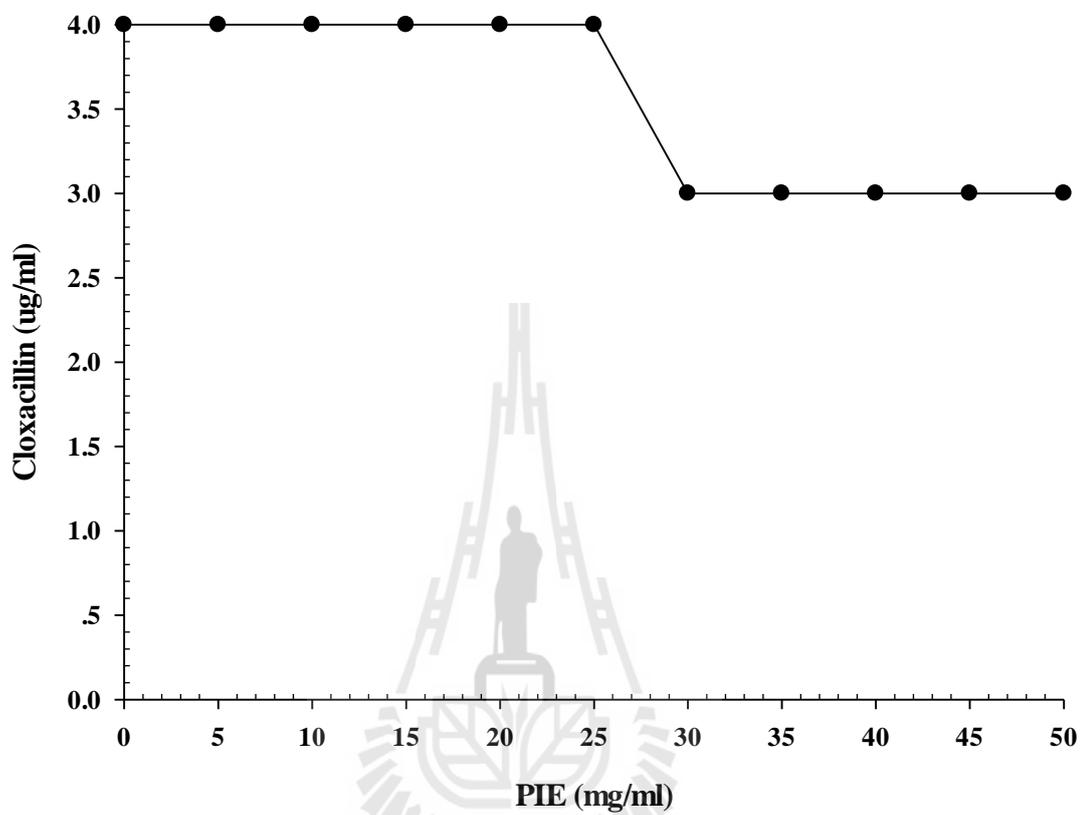
Figure 4.6 Isobologram constructed from checkerboard MIC data showing

antibacterial combination of cloxacillin plus *P. indica* crude extract (PIE) against

CRSE DMST 15507.

4.5 Killing curve determination

The lowest FIC index results from combinations of *S. suberosa* plus ampicillin against ARSA 20651 were chosen for killing curve determination. The determination of killing curve was performed according to CLSI guidelines (CLSI, 2013). The killing curve of ARSA 20651 after exposure to SSE either alone or in combination with ampicillin is presented in Figure 4.7. The control cells revealed no reduction in viable counts and steady increase in log phase viable counts throughout 24 h. Whereas, no significant change was observed in cells treated with the SSE and ampicillin alone. Interestingly, the combination of the SSE plus ampicillin exhibited a steady reduction of $5x10^5$ cfu/mL to 10^3 cfu/mL within 6 h and did not recover within 24 h. These results had also been confirmed antibacterial and synergistic activity of MIC and checkerboard determinations. Synergistic activity was defined as a decrease of equal to or more than 2log10 cfu/mL in comparison to the initial inoculum while the interaction was considered for a bactericidal agent if there was a decrease of equal to or more than 3log10 cfu/mL (Barry et al., 1999; Belley et al., 2008; Mun et al., 2013). Bacteriostatic activity was defined as a reduction of less than 3log10 cfu/mL (Belley et al., 2008). Time killing assay showed synergistic activity of the combination of SSE at 0.5 mg/ml and ampicillin at 0.15 µg/mL against ARSA 20651. Interestingly, this is the first report of the synergistic activity of the ampicillin plus SSE combination against ARSA strains. These results seem consistent with previous findings that galangin, quercetin or baicalein plus ceftazidime exhibited synergistic activity against MRSA result in a large decrease in from 6 to 24 h (Eumkeb et al., 2010). Apart from this, the combinations of baicalin and β-lactam antibiotics showed that the killing of MRSA and beta-lactam-resistant *S. aureus* cells were dramatic

reduction by these combinations (Liu et al., 2000). Clearly, the synergistic effect of SSE plus ampicillin against ARSA was observed. For this reason, the elementary mechanism of action such as TEM, CM permeability, and enzyme assay were more investigated.
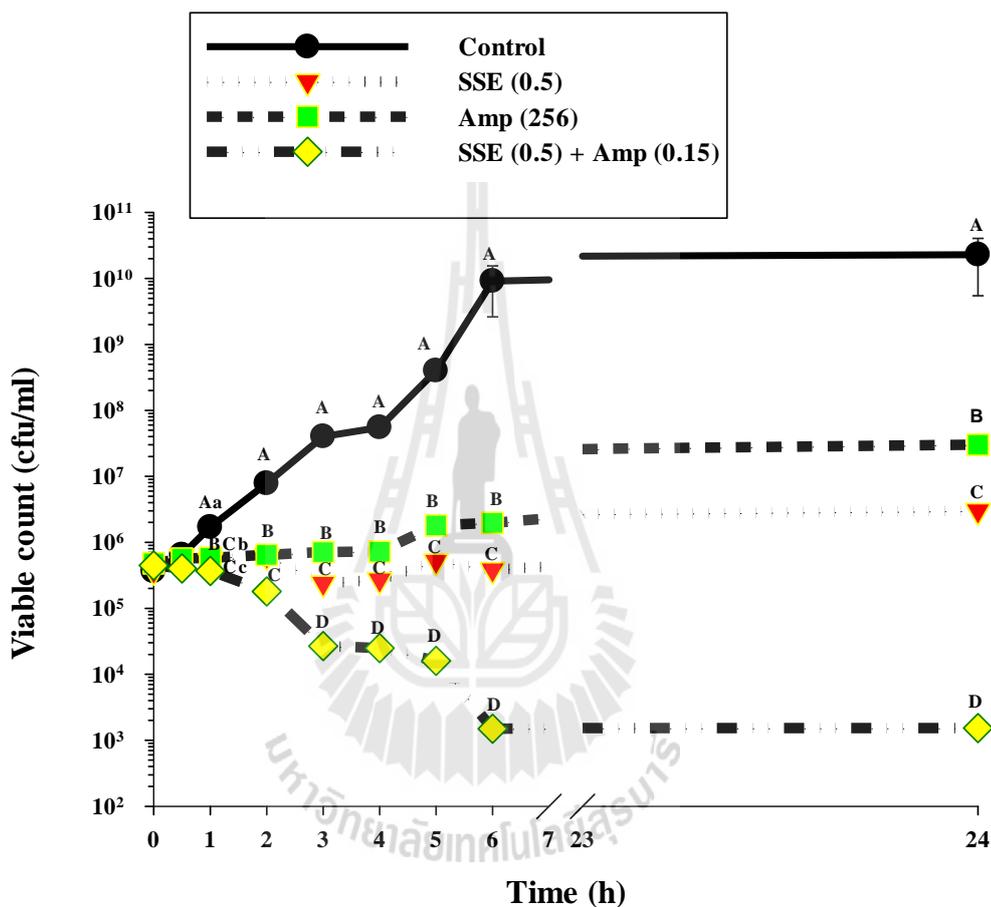
Figure 4.7 Time killing-curves of ARSA 20651. The effect of ampicillin either alone or in combination with SSE on clinical isolates of Methicillin-resistant *Staphylococcus aureus* DMST 20651 (ARSA). The symbol represents: (●) control (antibacterial free); (■) SSE (0.5 mg/mL); (◇) ampicillin (256 µg/mL); (▼) SSE (0.5 mg/mL) + ampicillin (0.15 µg/mL); the values plotted are the means of 4 observations, and the vertical bars indicate the standard errors of the mean. The significant difference between each group at the same time, means sharing the

different superscript letters, was compared using ANOVA and Games-Howell post hoc test at a = $p<0.05$; A = $p<0.01$ are presented.

4.6 Transmission electron microscopy (TEM)

In order to investigate the effect of the *S. suberosa* crude extracts either alone or in combination, on the ultrastructure of ARSA 20651, these treated cells were examined by transmission electron microscopy (TEM). The results of electron microscopic study are shown in Figure 4.8 to 4.11. Also, the comparison of cell area calculated by cell width x cell length (nm) is presented in Figure 4.12.
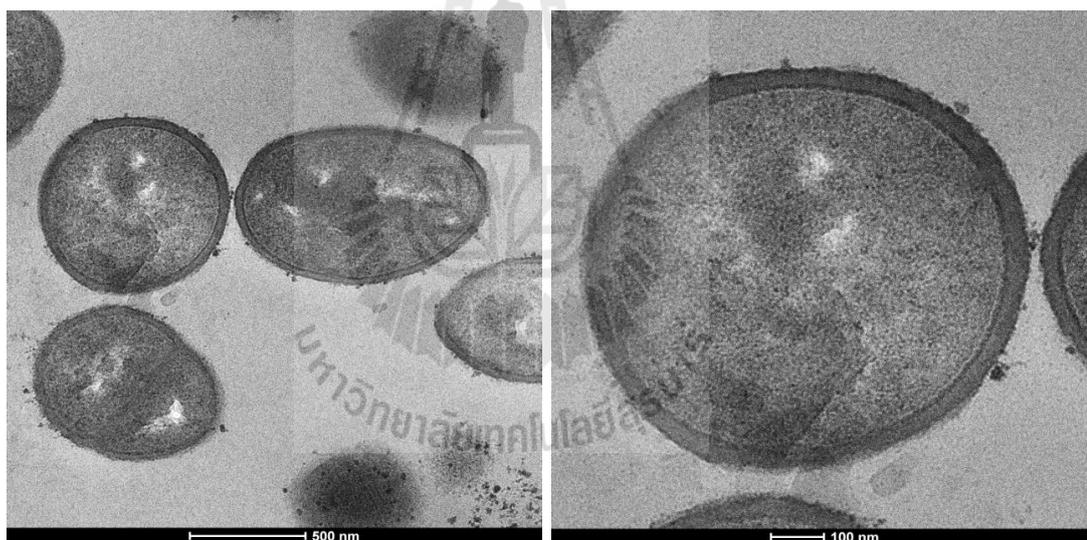
(A)   (B)

Figure 4.8 Ultrathin sections of ARSA 20651 grown for 4 h in CAMHB: (a), (b) control (no antibacterial agent). x19,500, bar = 500 nm (a); x43,000, bar = 100 nm (b).
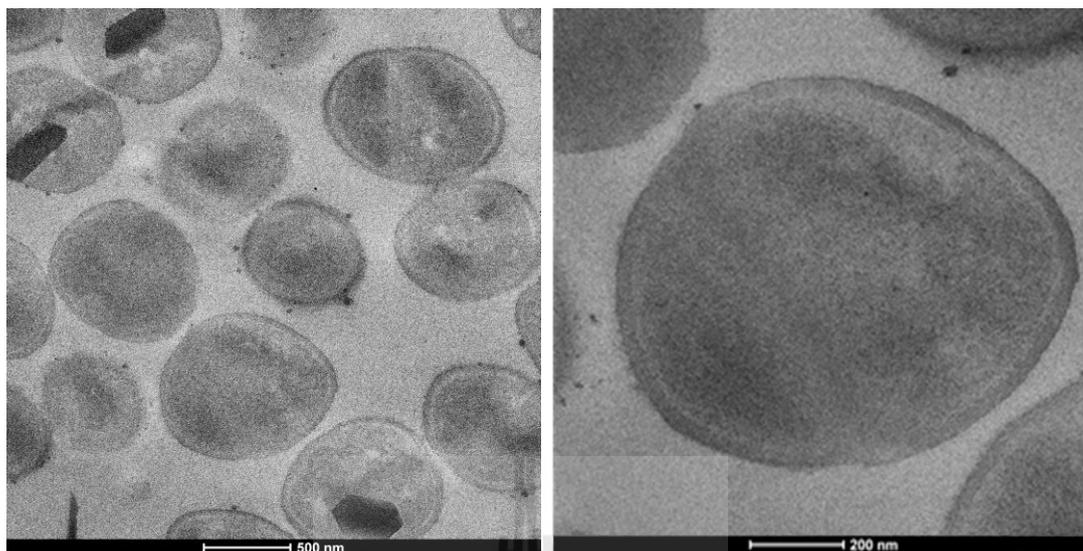
(A)   (B)

Figure 4.9 Ultrathin sections of ARSA 20651 grown for 4 h in CAMHB: (a), (b)

Ampicillin (256 µg/ml). x15,000, bar = 500 nm (a); x38,000, bar = 200 nm (b).
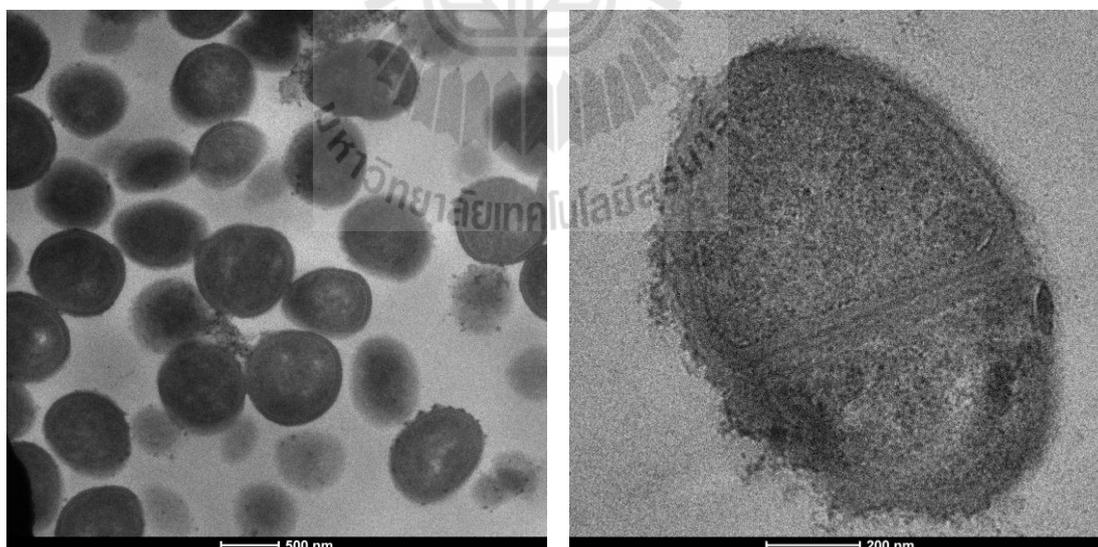
(A)   (B)

Figure 4.10 Ultrathin sections of ARSA 20651 grown for 4 h in CAMHB: (a), (b)

S. suberosa (2 mg/ml). x17,000, bar = 500 nm (a); x38,000, bar = 200 nm (b).
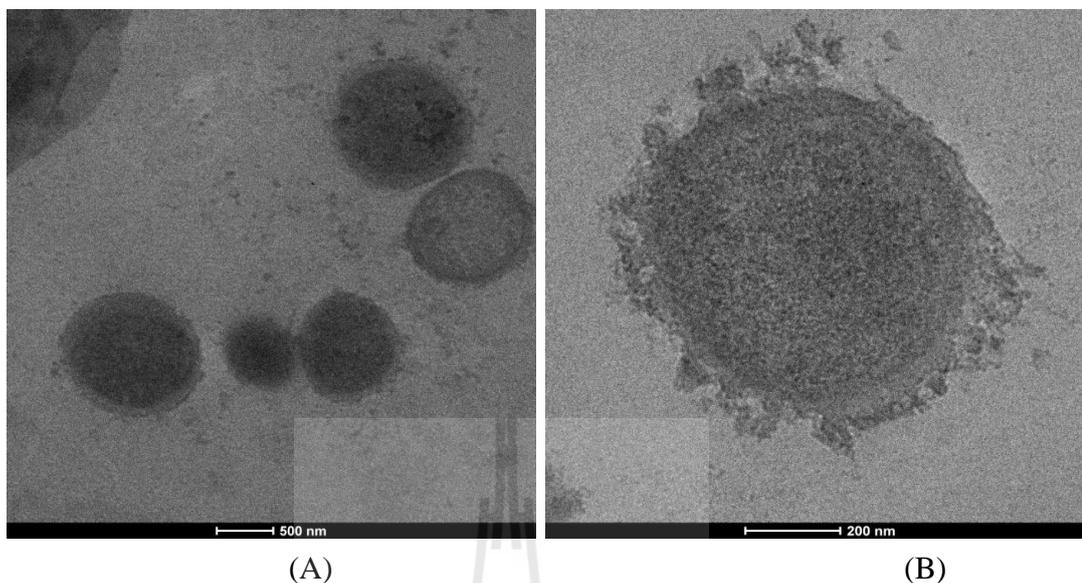
Figure 4.11 Ultrathin sections of ARSA 20651 grown for 4 h in CAMHB: (a), (b) *S. suberosa* (0.40 mg/ml) plus ampicillin (0.11 µg/ml). x8,700, bar = 500 nm (a); x38,000, bar = 200 nm (b).

Electron microscopic investigation clearly exhibited that the cytoplasmic membrane and peptidoglycan of ARSA 20651 grown in the absence of antibacterial agent (control) can be undoubtedly distinguished and no damage to ultrastructure was observed (Figure 4.8A and 4.8B). The ARSA 20651 treated with ampicillin 256 µg/ml alone showed slight peptidoglycan damage to approximately 8-10% of these cells (Figure 4.9A and 4.9B). About 20-23% of these cells treated with SSE 2 mg/ml caused somewhat peptidoglycan damage (Figure 4.10A and 4.10B). Besides, These average cell areas were somewhat smaller than the control and ampicillin groups, but not significant difference ($p > 0.01$) (Figure 4.12). These findings suggest that the SSE treated cells cause rather higher peptidoglycan damage than ampicillin treated cells. Obviously, the synergistic effect was observed in the combination of ampicillin

plus SSE that these cells demonstrated around 70-75% of these cells exhibited marked morphological damage, noticeable peptidoglycan damage (Figure 4.11A and 4.11B). Obviously, These average cell areas were significantly smaller than control and others ($p < 0.01$) (Figure 4.12). So that, TEM results of SSE plus ampicillin treated cells demonstrated that ARSA cells exhibited marked morphological damage, clear peptidoglycan and cytoplasmic membrane damage, and average cell areas significant smaller than control. These results seem consistent with previous findings that the combination of ceftazidime plus galangin caused damage to the ultrastructures of the cells, affected the integrity of the cell walls and led to an increase in cell size of ceftazidime-resistant *S. aureus* (Eumkeb et al., 2010). Similar results have been reported by Jiamboonsri et al. (2011) that the Thai mango seed kernel extract exhibited impaired cell division and ultrastructural changes in cell morphology, including the thickening of the cell walls of MRSA treated with the mango seed kernel extract. Our findings seem consistent with previous reports that the synergistic antibacterial activity of alkaloids alone and in combination with antibiotic against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) are revealed (Zuo et al., 2011). These results can be explained by assuming that SSE may insert synergistic action with ampicillin to inhibit peptidoglycan synthesis leads to marked morphological damage and delay cell division. Also, these results suggest that this crude extract has ability to exert a synergy effect with ampicillin by reversing the resistance to be susceptible strain to its primary antibiotic.

ARSA 20651 cell size (width x length)
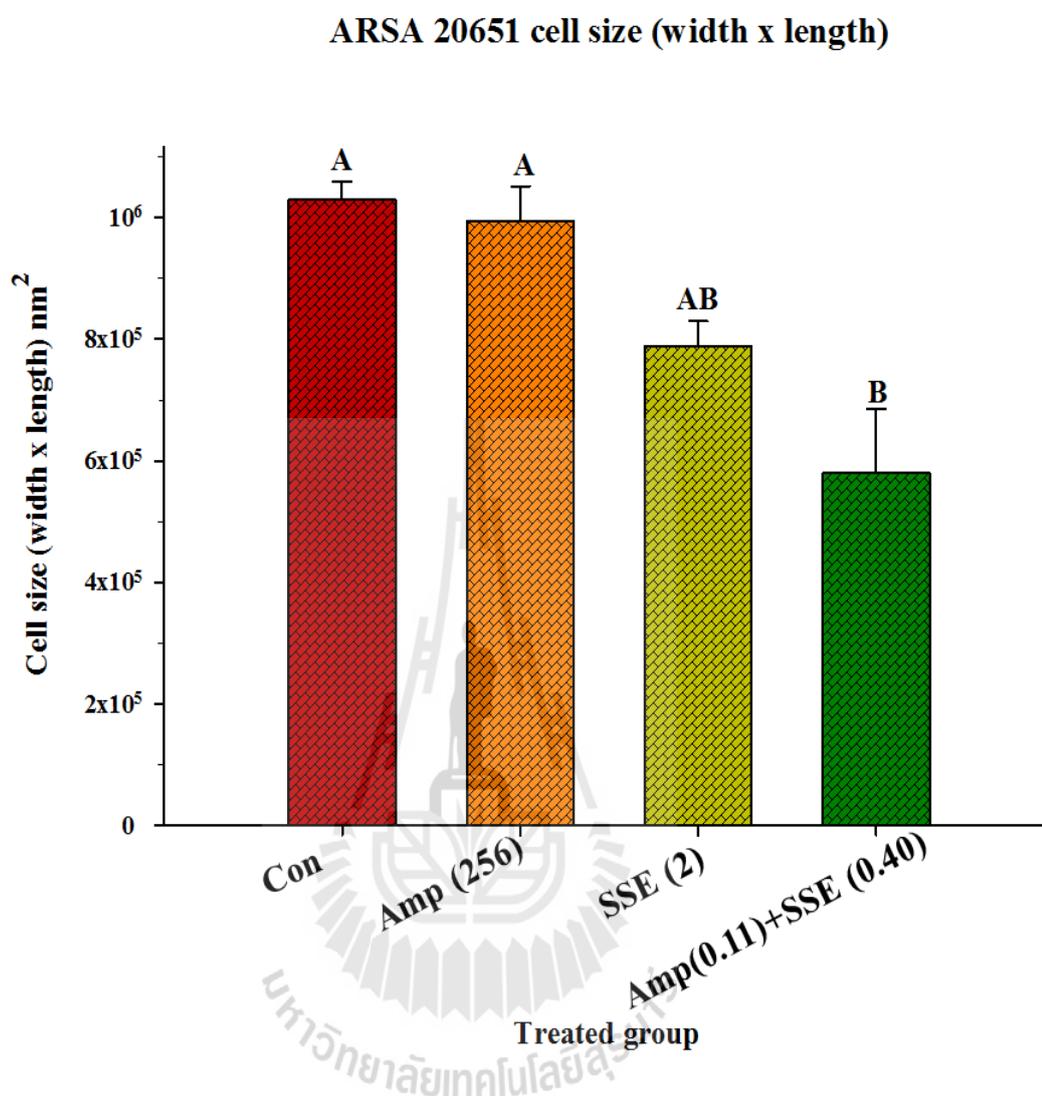
Figure 4.12 The cell area of ARSA 20651 after treatment with SSE, ampicillin either alone or in combination: Con = control (drug free)**;** Amp(256) = ampicillin at 256 µg/ml; SSE(2) = SSE at 2 mg/ml; Amp(0.11) + SSE(0.40) = ampicillin at 0.11 µg/ml plus SSE at 0.40 mg/ml; The graph shows area of cell determined by cell width x cell length (nm^2). The different superscript alphabets are significantly different from each other. Each treated group was compared using one-way ANOVA and Tukey's HSD Post-hoc test, $p<0.01$ are presented.

4.7 CM permeability

The effect of 256 μg/ml ampicillin, 2 mg/ml SSE alone and the combination of 0.11 μg/ml ampicillin plus 0.40 mg/ml SSE on CM permeability determined by cytoplasmic β- galactosidase activity is illustrated in Table 4.4. The result showed that there was no activity of β-galactosidase with increasing time in cells grown without antibacterial agent (control), with ampicillin and SSE alone. Whereas, cells treated with SSE plus ampicillin combination and nisin exhibited β-galactosidase activity (observed yellow) after 1 h exposure time. These results indicated that the combination of SSE plus ampicillin revealed the ability to increase CM permeability of ARSA 20651. These results are approximately similar with previous findings that luteolin either alone or combined with amoxicillin and apigenin alone and in combination with ceftazidime increased CM permeability of amoxicillin-resistant *E. coli* and ceftazidime-resistant *E. cloacae* respectively (Eumkeb and Chukrathok, 2013; Eumkeb et al., 2012). In general, nisin incorporates into the membrane and makes the membrane permeable for ions. So that, both the membrane potential and pH gradient are dissipated (Gao et al., 1991). Apart from this, nisin inhibits peptidoglycan synthesis and forms highly specific pores through interaction with the membrane-bound cell wall precursor lipid II (Wiedemann et al., 2001). The increase in CM permeability may be one of the synergistic action of this combination against ARSA strain. 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. The major proteins in the cell membrane generally have very hydrophobic external surface in the regions of the protein that

make an intimate association with the highest non-polar fatty acid chains (Brock et al., 1994;).

Table 4.3 β-galactosidase activity results of ARSA 20651 after treatment with ampicillin, SSE alone or in combination. Neg, no evidence of activity; Pos, have evidence of activity; Amp (256), ampicillin at 256 μg/ml; SSE (2), *Stephania suberosa* extract at 2 mg/ml; Amp+SSE (0.11+0.40) = Ampicillin 0.11 μg/ml plus SSE 0.40 mg/ml; NIS (8), Nisin at 8 μg/ml was used as a positive control. The experiment was carried out in triplicate observations.

Time	Control (no drug)	Amp (256)	SSE (2)	Amp+SSE (0.11+0.40)	NIS (8) (positive control)
0	Neg	Neg	Neg	Neg	Neg
1	Neg	Neg	Neg	Pos	Pos
2	Neg	Neg	Neg	Pos	Pos
3	Neg	Neg	Neg	Pos	Pos
4	Neg	Neg	Neg	Pos	Pos
5	Neg	Neg	Neg	Pos	Pos

4.8 Enzyme assay

The ability of SSE to inhibit activity of *β*-lactamase type IV isolated from *E. cloacae* was assayed by determining the amount of remaining benzylpenicillin using reverse-phase HPLC. As shown in Figure 4.13, the result displayed that benzylpenicillin treated with SSE was significantly higher than control starting from 5 min ($p < 0.01$). The benzylpenicillin remainder was significantly increased by an

increase in SSE as a concentration-dependent manner. The more SSE concentrations results in more benzylpenicillin remainder of enzyme assay results are in substantial agreement with previous finding that galangin inhibits β-lactamase in a concentration-dependent manner. These results suggest that one activity of SSE against ARSA may involve in β-lactamase inhibition (Eumkeb et al., 2010).
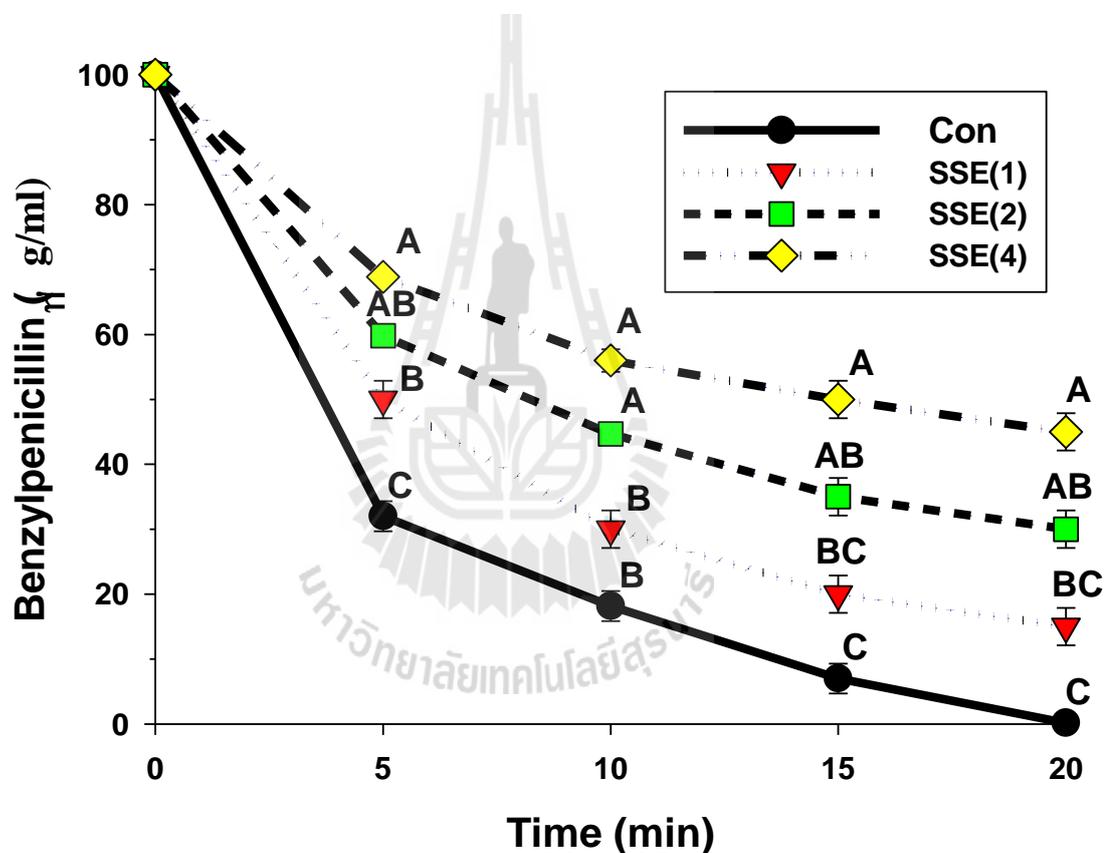
Figure 4.13 The inhibitory activity of SSE against *β*-lactamase type IV from *E. cloacae* in hydrolyzing benzylpenicillin; Con = control (no testing agent); SSE(1) = SSE at 1 mg/ml; SSE(2) = SSE at 2 mg/ml; SSE(4) = SSE at 4 mg/ml. The graph shows the remaining benzylpenicillin at the same time. Means sharing the same superscript are not significantly different from each other (Tukey's HSD, $p < 0.01$).

CHAPTER V

CONCLUSIONS

Antibiotic resistance problem in pathogenic bacteria such as *E. coli* and *S. aureus* has been increasingly emerged worldwide (Zetola et al., 2005). The antibiotic susceptibility of common community-acquired bacteria in Thailand and worldwide has been studied. Community-acquired *Escherichia coli, Klebsiella pneumoniae, Acinetobacter* spp*., Enterobacter* spp and *Staphylococcus aureus* were more susceptible to antimicrobials compared to hospital-acquired strains (Danchaivijitrmd et al., 2005). In developing countries such as Thailand and Malaysia, the high prevalence of community-acquired infections were attributed to poor environmental management, poor personal hygiene and lack of health education (Noor Azian et al., 2007). Therefore, novel antimicrobial agents from plants, which are available in developing countries could in future be used to tackle the increasing number of community-acquired bacterial infections. Furthermore, MRSA quickly spread around the world and are now a major nosocomial and emerging community pathogen. In 2000, 47% of *S. aureus* and 75% of coagulase-negative staphylococcal isolates from intensive care units in the US, and 48% of hospital isolates of *S. aureus* in Portugal were methicillin resistant (Sa-Leao et al., 2000). Until recently, MRSA was mainly a hospital problem, however, there have been reported of community-acquired infection in several countries. The clinical MRSA strains have become an increasingly pressing global problem. Anti-MRSA synergistic effects between plant natural compounds and

conventional antibiotic agents have further been exhibited here as an alternative way of overcoming resistance to current antibiotics (Wagner and Ulrich-Merzenich, 2009). Most dramatically, the proportion of cases of *S. aureus* bacteraemia due to MRSA increased from 1 to 2% in 1990-1998 (Reacher et al., 2000).

Traditionally, the search for novel antimicrobial agents to combat bacterial resistance has focused around products which arise from plant sources. Plant based antimicrobials have enormous therapeutic potential as they are effective in the treatment of infectious diseases while simultaneously reducing many of the side effects that are often associated with synthetic antimicrobials. Natural plant resources are now under investigation as a source of novel agents. However, no work has been done on the effect of *S. suberosa* and *P. indica* on drugs resistant bacteria such as ARSA and CRSE. Therefore, the purpose of this thesis was to examine the antibacterial activity of *S. suberosa* and *P. indica* extracts against these resistant bacterial strains used either singly or in combination with selected β-lactam antibiotics (ampicillin and cloxacillin).

In the present study, the antibacterial activity of *S. suberosa* and *P. indica* against drugs-resistant bacteria of ARSA and CRSE using the broth macrodilution method. These strains showed a high percentage of drug resistance to ampicillin and cloxacillin respectively. The MIC results revealed that these testing *S. aureus* strains were highly resistant to ampicillin alone because of the standard value of the sensitivity of ampicillin against these strains are ≤ 0.25 µg/ml. As well as, SSE demonstrated little bacteriostatic effect against these strains while the reference *S. aureus* strain exhibits susceptible to ampicillin (Clinical, 2012).

The checkerboard determination revealed synergistic effects of ampicillin plus SSE against all of tested ARSA strains with FIC index at <0.13 whereas, no interaction or synergistic effect of PIE plus cloxacillin against all CRSE strains were discovered. As previously documented, drug combination approach by achieving a synergistic effect can eliminate and neutralize the adverse effects (Wagner, 2011). To observed synergistic effects of plant extract and β-lactam could be theoretically the results of the perturbation of the cell membrane coupled with the action of β-lactam antibiotic that inhibits the final stage involved in the synthesis of peptidoglycan of cell wall (transpeptidation reaction), which occurs outside the cell membrane and is mediated by alternative protein binding protein PBP2a encoded by mecA gene (Pinho et al., 2001). This antimicrobial effect has largely been attributed to the alkaloid found in the plant called berberine, which has been shown to intercalate with the DNA of the bacteria preventing basic cell function (Kuo et al., 2004). This compound has been shown to be effective at inhibiting the growth of Gram-positive and Gram-negative bacteria (Villinski et al., 2003). Therefore, a combination of the two could potentially make effective treatment.

The killing curve determination can confirm MIC and checkerboard determinations that synergistic effect of SSE plus ampicillin caused marked reduction in viable counts of ARSA cells from 6 h and throughout 24 h. Clearly, the synergistic effect of SSE plus ampicillin against ARSA was observed. Moreover, SSE was found to show diverse synergistic effects with β-lactam antibiotics on the susceptibility of ARSA. The killing curve of ARSA cells was also maintained at a low level from 6 to 24 h by ampicillin 0.11 μg/ml in combination with 0.40 mg/ml of SSE. For this

reason, the elementary mechanism of action such as TEM, CM permeability, and enzyme assay were more investigated.

TEM results of SSE plus ampicillin treated cells demonstrated that ARSA cells exhibited marked morphological damage, clear peptidoglycan and cytoplasmic membrane damage, electron-transparent areas in cytoplasm due to lose most of organelles, and average cell areas significant smaller than control. These results can be explained by assuming that SSE may insert synergistic action with ampicillin to inhibit peptidoglycan synthesis leads to marked morphological damage and delay cell division.

The CM permeability revealed that SSE in combination with ampicillin increased cytoplasmic membrane permeability of this strain from 1 h onward (Table 4.4). Accordingly, the increase in CM permeability may be one of the synergistic action of this combination against ARSA strain. Decrease accessibility of antibiotic across bacterial membrane to intracellular target site is one of the most important resistant mechanisms of bacteria via mutation or down regulation of porin genes (Tenover, 2006). Probably, the hyper-permeability of CM caused by SSE plus ampicillin combination may due to leakage of ions and extensive loss of other cell contents, including the intracellular proteins resulting in cell death (Devi et al., 2010). The enzyme assay results of the present study showed that the more SSE concentrations results in a more benzylpenicillin remainder like a concentration-dependent manner. This inhibitory activity may due to formation of SSE and β-lactamase complex results in inactivation of β-lactamase activity. Moreover the β-lactamase activity of *E. cloacae* was also marked inhibited by the combination of ampicillin plus SSE (data not shown). The resistance reversing activity of SSE against

ARSA might also include inhibition of β-lactamase activity. Six new protoberberines and ten known alkaloids were found in *Stephania suberosa* root extracts (Patra et al., 1987). However, the bioactive compounds of *S. suberosa* extract that showed antibacterial effect in this study have not been well characterized. So, further investigation should be focused on active ingredients of SSE that play an important role on antibacterial effect.

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

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APPENDICES

APPENDIX A

CULTURE MEDIA

A.1 Nutrient agar

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

The formula was	g/litre
Yeast extract	4.0
Tryptone	5.0
Glucose	50.0
Potassium dihydrogen phosphate	0.55
Potassium chloride	0.425
Calcium chloride	0.125
Magnesium sulphate	0.125
Ferric chloride	0.005
Manganese sulphate	0.005
Bromocresol green	0.022
Agar	15.0

pH 5.5 ± 0.2

A.2 Cation-adjusted Mueller-Hinton broth (CAMHB)

Oxiod [®] Cation-adjusted Mueller Hinton broth was the medium used for determining the antimicrobial susceptibility testing.

The formula was:

	g/litre
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Ca^{2+}	20 mg/L
Mg^{2+}	10 mg/L
pH 7.3 ± 0.1 @ 25°C	

All culture media were dissolved by water.

A.3 Mueller-Hinton agar

Oxiod [®] Mueller Hinton agar was the medium used for determining the antimicrobial susceptibility testing.

The formula was:

	g/litre
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH 7.3 ± 0.1 @ 25°C	

APPENDIX B

CHEMICALS AND EQUIPMENT

B.1 Chemicals

All chemicals used were laboratory grade otherwise specified.

Ethanol Absolute	Lab grade
Dimethylsulfoxide (DMSO)	AR grade
Sodium chloride	AR grade
Sodium phosphate	Lab grade
Sodium hydroxide	Lab grade
Amonium acetate	AR grade
Acetronitrile	Lab grade
Paraformaldehyde	Lab grade
Glutaraldehyde	Lab grade
Osmiun tetroxide	Lab grade
Methanol	Lab grade
Aradite	Lab grade
Uranyl acetate	Lab grade
Lead acetate	Lab grade
HEPES buffer	Lab grade
Phosphate	Lab grade
PMSF	Lab grade

Ampicillin	AR grade
Cloxacillin	AR grade

B.2 Equipment

Rotary evaporator	Buchi
Heating bath: Büchi heating bath B-490	
Rotavapor: Büchi rotavapor R-200	
Controller: Büchi vacuum controller V-800	
UV-Cabinet II	Camag
Soxhlet apparatus	Buchi
Mixer (Model 5000)	Buchi
Hot air oven (Memmert-600)	
filter paper	
Spectronic 21	Milton Roy
Labofuge	400R Heraeus
Autoclave	Yamato
Laminar air flow	Woerden
Hot air oven	Shellab
Shaking incubator	Heto
Hot plate	VELP scientifica
Refrigerated Incubator	VELP scientifica
Ultramicrotome	JEM
Micropipettors (2-20 μl)	
Witeg	
Micropipettors (100-1000 μl)	Witeg

Cenrtifuge tubes	Pyrex
Spectraphysics	Agilent

B.3 Glassware

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

Pipettes (1, 5, 10 μl)

Measuring cylinder (10, 20 μl)

Petri dishes

Test tubes
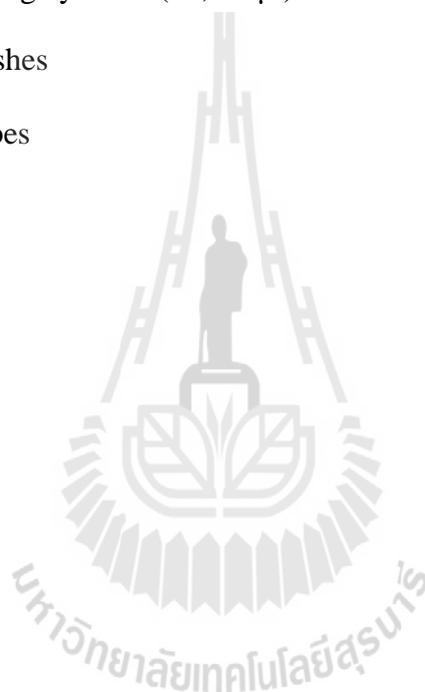
APPENDIX C

BACTERIAL SUSPENSIONS STANDARD CURVES

Figure C.1 to C.7 illustrate that the absorptions of MSSA, ARSA DMST 20651, 20652, 20653, *S. epidermidis* DMST 15505, 15506, 15507 at 500 nm were approximately 1×10^8 CFU/mL at the absorption of 0.10, 0.09, 0.11, 0.12, 0.22, 0.22 and 0.21 respectively.

Standard curve *S. aureus* ATCC 29213
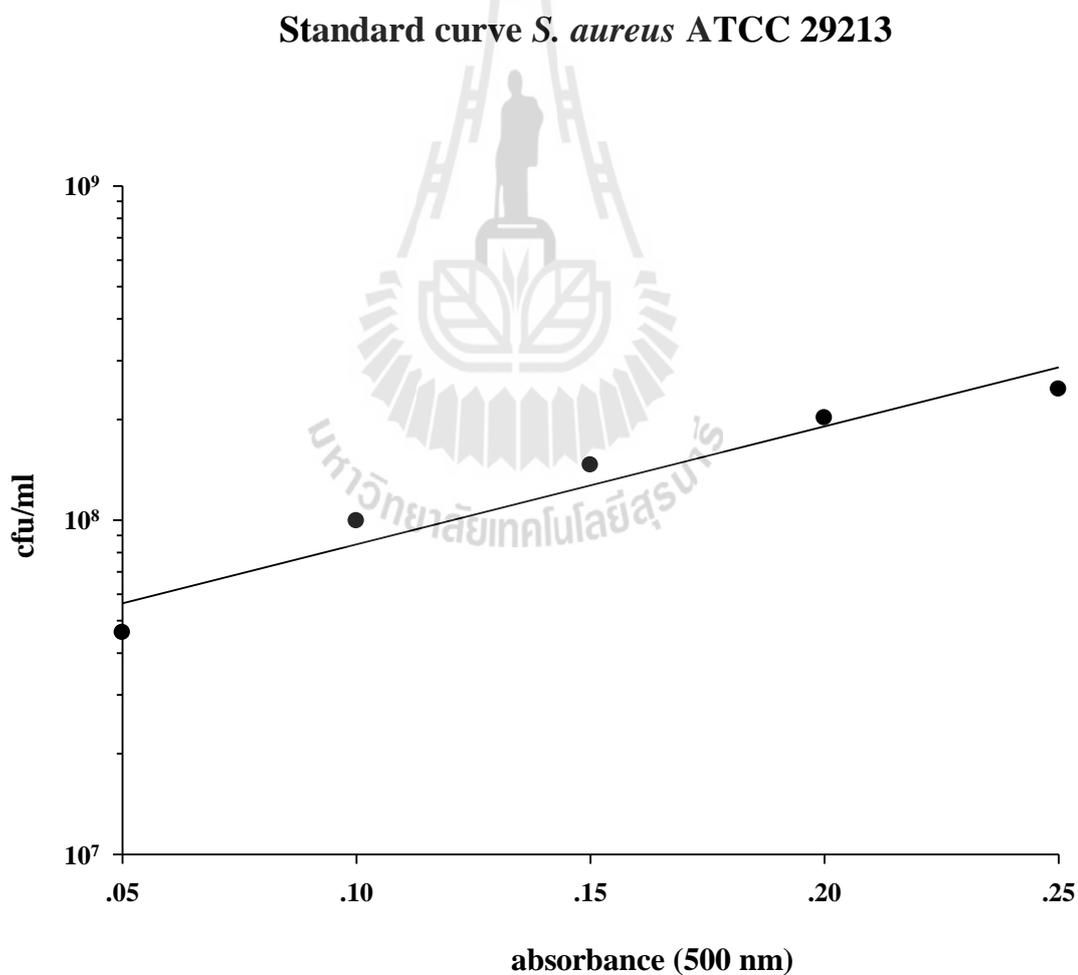
**Figure C.1** Standard curve for suspensions of *Staphylococcus aureus*.ATCC 29213 (MSSA).

Standard curve *S. aureus* DMST 20651
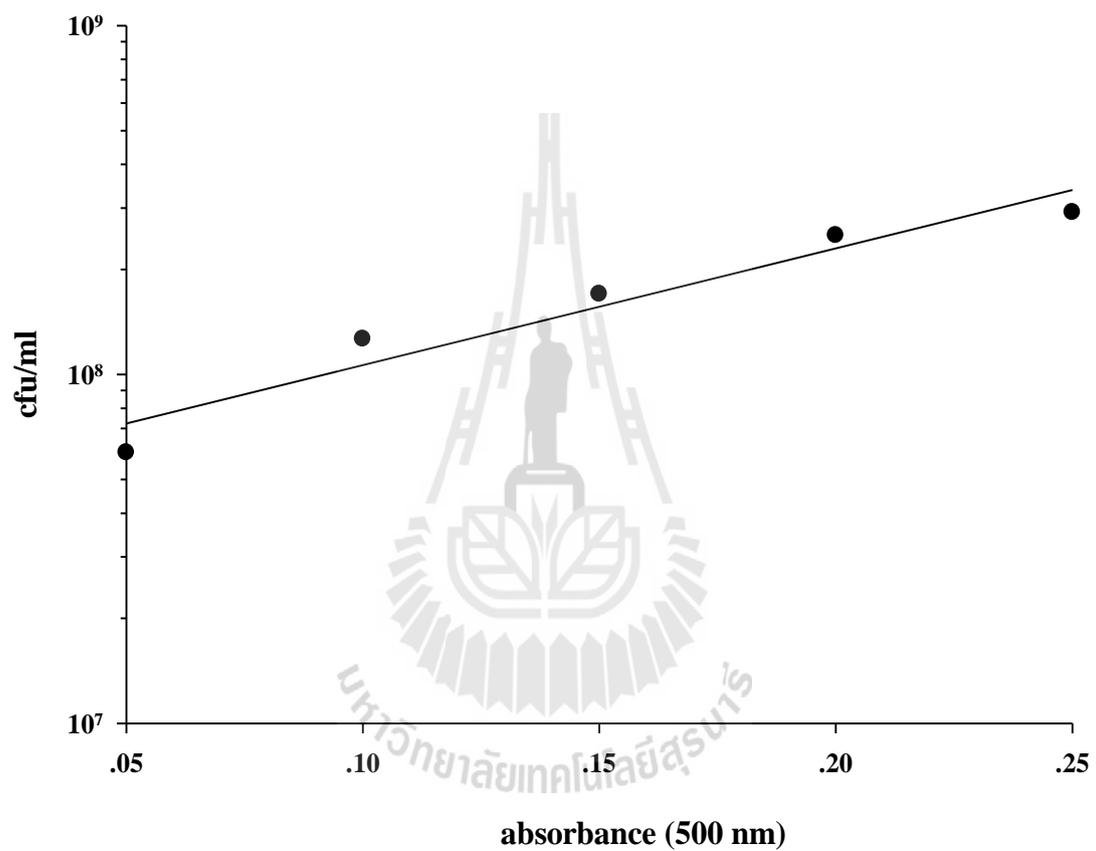
Figure C.2 Standard curve for suspensions of ampicillin-resistant *Staphylococcus*

aureus DMST 20651 (ARSA).

Standard curve *S. aureus* DMST 20652
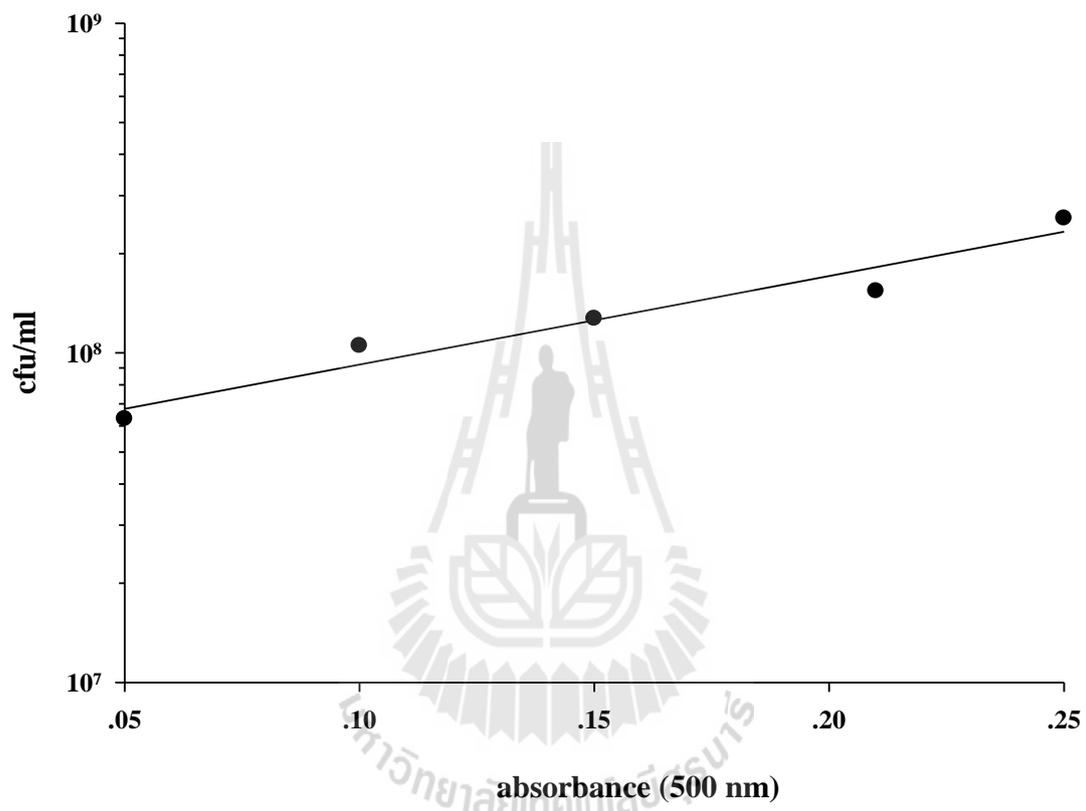
**Figure C.3** Standard curve for suspensions of ampicillin-resistant *Staphylococcus aureus* DMST 20652 (ARSA).

**Standard curve *S. aureus* DMST 20653**
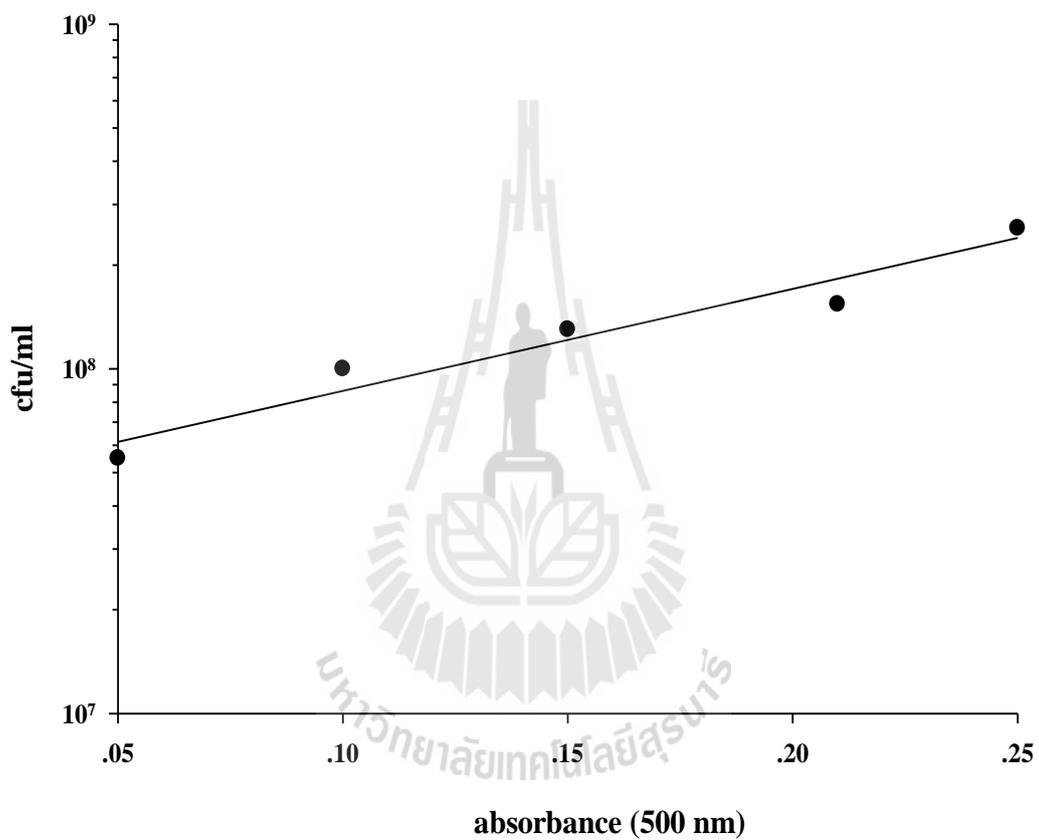
**Figure C.4** Standard curve for suspensions of ampicillin-resistant *Staphylococcus aureus* DMST 20653 (ARSA).

Standard curve *S. epidermidis* DMST 15505
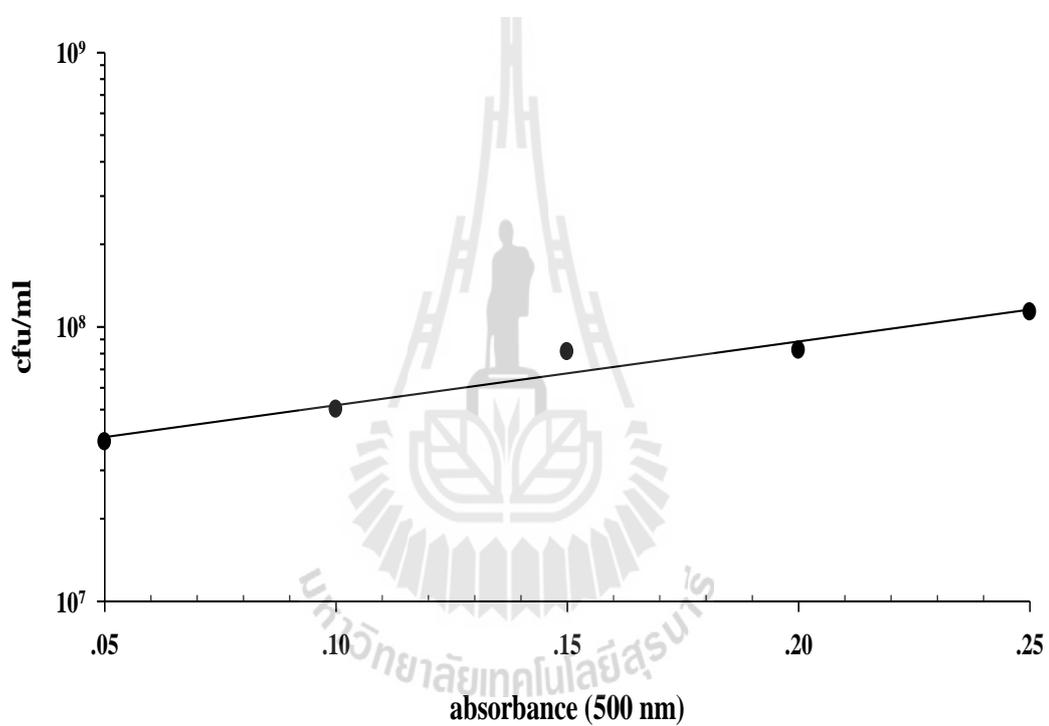
**Figure C.5** Standard curve for suspensions of Cloxacillin resistant *S. epidermidis* DMST15505 (CRSE).

Standard curve *S. epidermidis* DMST 15506
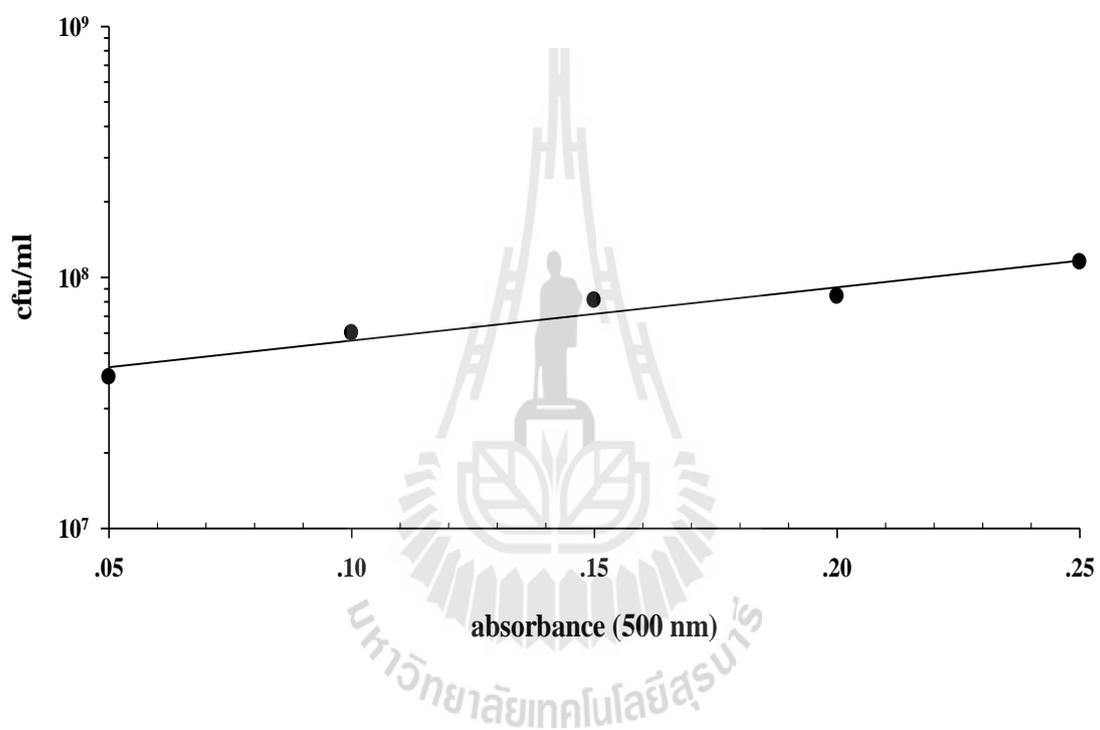
Figure C.6 Standard curve for suspensions of Cloxacillin resistant *S. epidermidis* DMST15506 (CRSE).

Standard curve *S. epidermidis* DMST 15507
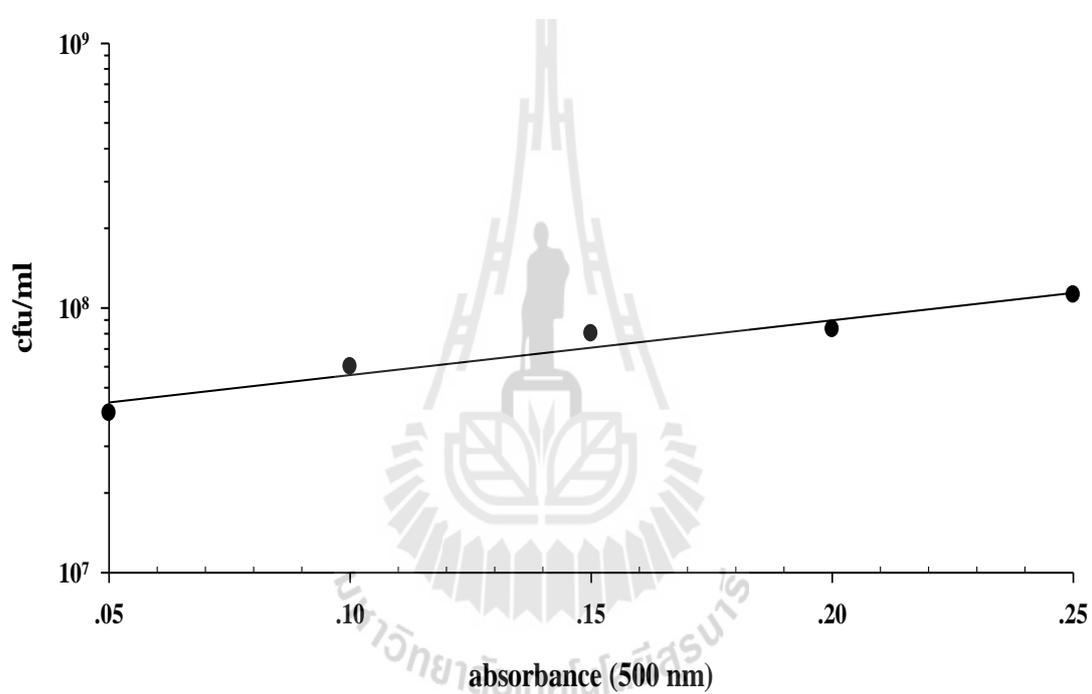
Figure C.7 Standard curve for suspensions of Cloxacillin resistant *S. epidermidis* DMST15507 (CRSE).

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2. Teethaisong, Y., Autarkool, N., and Eumkeb, G. (2014). Synergistic antibacterial activity of *Boesenbergia rotunda* extract and β-lactam antibiotic combination against multidrug-resistant bacteria. Proceeding of the 5th International Conference on Natural Products for Health and Beauty (NATPRO 5). Prince of Songkla University Hat Yai, Songkhla, Thailand.

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