MECHANISM OF ACTION UNDERLYING THE ANTIBACTERIAL ACTIVITY OF LUPINIFOLIN, A FLAVONOID EXTRACTED FROM THE STEM OF *DERRIS RETICULATA* CRAIB., AGAINST *STAPHYLOCOCCUS AUREUS*

Kamol Yusook

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

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กลไกการออกฤทธิ์ต้านแบคทีเรียของลูปินิฟอลินที่สกัดได้จากลำต้นชะเอมเหนือต่อเชื้อสแตปฟิโลคอคคัส ออเรียส

นายกมล อยู่สุข

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีวเวชศาสตร์
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MECHANISM OF ACTION UNDERLYING THE ANTIBACTERIAL ACTIVITY OF LUPINIFOLIN, A FLAVONOID EXTRACTED FROM THE STEM OF *DERRIS RETICULATA* CRAIB., AGAINST *STAPHYLOCOCCUS AUREUS*

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

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กมล อยู่สุข : กลไกการออกฤทธิ์ต้านแบคทีเรียของลูปินิฟอลินที่สกัดได้จากลำต้นชะเอมเหนือต่อเชื้อสแตปฟิโลคอคคัส ออเรียส (MECHANISM OF ACTION UNDERLYING THE ANTIBACTERIAL ACTIVITY OF LUPINIFOLIN, A FLAVONOID EXTRACTED FROM THE STEM OF *DERRIS RETICULATA* CRAIB., AGAINST *STAPHYLOCOCCUS AUREUS*). อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.นวลน้อย จูฑะพงษ์, 80 หน้า.

ในการศึกษาวิจัยนี้ ได้ทำการสกัดสารลูปินิฟอลิน ซึ่งเป็นสารที่มีโครงสร้างในกลุ่ม prenylated flavonoid จากลำต้นชะเอมเหนือ (*Derris reticulata*) โดยได้ทำการตรวจความถูกต้องจาก NMR สเปกตรัมและตรวจยืนยันด้วยการวิเคราะห์มวลสาร (Mass spectrometry) สารละลายลูปินิฟอลินถูกเตรียมด้วยการละลายใน 0.1 N NaOH และเจือจางต่อทันทีในอาหารเลี้ยงเชื้อ Müller-Hinton เพื่อใช้ในการทดสอบฤทธิ์ต้านแบคทีเรีย จากผลการทดสอบที่ได้พบว่า แบคทีเรียแกรมบวกมีความไวต่อสารลูปินิฟอลินมากกว่าแบคทีเรียแกรมลบ จากเชื้อแบคทีเรียแกรมบวกจำนวน 4 สายพันธุ์ พบว่า *Staphylococcus aureus* มีความไวมากที่สุด และจากการทดสอบด้วยวิธี two-fold microdilution พบว่า สารลูปินิฟอลินออกฤทธิ์ยับยั้งการเจริญของแบคทีเรีย *S. aureus* ด้วยค่าความเข้มข้นต่ำสุดในการยับยั้งเชื้อแบคทีเรีย (MIC) และค่าความเข้มข้นต่ำสุดในการฆ่าเชื้อแบคทีเรีย (MBC) เท่ากับ 8 และ 16 ไมโครกรัม/มล. ตามลำดับ ซึ่งฤทธิ์ดังกล่าวมีความแรงน้อยกว่า เมื่อเทียบกับ ampicillin อย่างไรก็ตาม จากกราฟความสัมพันธ์ระหว่างเวลากับผลในการออกฤทธิ์ แสดงให้เห็นว่า ลูปินิฟอลินออกฤทธิ์ได้เร็วกว่า ampicillin โดยฤทธิ์ที่เร็วกว่าของลูปินิฟอลินนี้ สามารถยืนยันได้โดยภาพถ่ายจากกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด และเพื่อตรวจหากลไกการออกฤทธิ์ของลูปินิฟอลิน กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านถูกนำมาใช้เพื่อสังเกตโครงสร้างขนาดเล็กภายในเซลล์แบคทีเรีย *S. aureus* ภาพถ่ายที่ได้แสดงให้เห็นว่า ลูปินิฟอลินทำให้เกิดการฉีกขาดของเยื่อหุ้มเซลล์และผนังเซลล์ เนื่องจากการออกฤทธิ์ที่รวดเร็วดังกล่าวนี้ชี้แนะว่า ลูปินิฟอลินน่าจะออกฤทธิ์ทำลายเยื่อหุ้มเซลล์โดยตรง สมมติฐานนี้ได้รับการพิสูจน์ยืนยันด้วยวิธี Flow cytometry โดยใช้สี $DiOC_2$ เป็นสารบ่งชี้ในการทดสอบ ผลการศึกษาพบว่า อัตราส่วนของสีแดง/สีเขียว ซึ่งบ่งชี้ให้เห็นความสมบูรณ์ของเยื่อหุ้มเซลล์ของแบคทีเรียลดลงอย่างมีนัยสำคัญ เช่นเดียวกับสาร carbonyl cyanide 3-chlorophenylhydrazone ซึ่งเป็นที่ทราบกันทั่วไปว่ามีฤทธิ์เป็น protonophore ที่มีฤทธิ์ทำลายโดยตรงต่อเยื่อหุ้มเซลล์ จากผลการทดลองนี้สรุปได้ว่า ลูปินิฟอลินมีฤทธิ์ยับยั้งการเจริญของแบคทีเรีย *S. aureus* โดยการทำลายเยื่อหุ้มเซลล์

นอกจากนี้ ยังได้ทำการทดสอบความเป็นพิษของลูปินิฟอลินในเม็ดเลือดแดงและเซลล์เพาะเลี้ยง HepG2 พบว่า ลูปินิฟอลินที่ค่าความเข้มข้นเท่ากับ MIC และ MBC ไม่แสดงความเป็นพิษต่อเซลล์เม็ดเลือดแดงและเซลล์ HepG2 อย่างมีนัยสำคัญ ($p < 0.05$) ผลการทดลองที่ได้การศึกษานี้ แสดงให้เห็นว่า ลูปินิฟอลินมีศักยภาพที่จะนำไปใช้เป็นสารต้านเชื้อแบคทีเรียได้

สาขาวิชาปรีคลินิก
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____

KAMOL YUSOOK : MECHANISM OF ACTION UNDERLYING THE ANTIBACTERIAL ACTIVITY OF LUPINIFOLIN, A FLAVONOID EXTRACTED FROM THE STEM OF *DERRIS RETICULATA* CRAIB., AGAINST *STAPHYLOCOCCUS AUREUS*. THESIS ADVISOR : ASSOC. PROF. NUANNOI CHUDAPONGSE, Ph.D. 80 PP.

DERRIS RETICULATA CRAIB./*LUPINIFOLIN*/*STAPHYLOCOCCUS AUREUS*/ANTIMICROBIAL/CELL MEMBRANE DISRUPTION

In this study, lupinifolin, a prenylated flavonoid, was isolated from *Derris reticulata* stem, identified by NMR spectra and confirmed with mass spectrometry. Lupinifolin was freshly prepared by solubilizing in 0.1 N NaOH and immediately diluted in Müller-Hinton broth for testing antibacterial activity. The data showed that Gram-positive bacteria were more susceptible to lupinifolin than Gram-negative bacteria. Of four strains of Gram-positive bacteria tested, *Staphylococcus aureus* was the most susceptible strain. Using the two-fold microdilution method, it was found that lupinifolin possessed antimicrobial activity against *S. aureus* with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 8 and 16 μg/ml, respectively, which was less potent than ampicillin. However, from the time-effect relationship, it was shown that lupinifolin had faster onset than ampicillin. The faster onset of lupinifolin was confirmed by scanning electron microscopy. To investigate the mechanism of action of lupinifolin, transmission electron microscopy (TEM) was performed to observe the ultrastructure of *S. aureus*. The TEM images showed that lupinifolin ruptured the bacterial cell membrane and cell wall. Due to its

fast onset, it is suggested that the action of lupinifolin is likely to be the direct disruption of the cell membrane. This hypothesis was substantiated by the data from flow cytometry using $DiOC_2$ as an indicator. The result showed that the red/green ratio which indicated bacterial membrane integrity was significantly decreased, similar to the known protonophore carbonyl cyanide 3-chlorophenylhydrazone. It is concluded that lupinifolin inhibits the growth of *S. aureus* by damaging the bacterial cytoplasmic membrane.

In addition, cytotoxicity of lupinifolin was evaluated in red blood cells (RBCs) and HepG2 cells. It was found that at MIC and MBC of lupinifolin did not produce significant toxicity on RBCs and HepG2 cell viability ($p < 0.05$). The data obtained from this study suggested that lupinifolin may have the potential to be used as antibacterial agent.

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LIST OF ABBREVIATIONS

ATCC	=	American type culture collection
CCCP	=	Carbonyl cyanide 3-chlorophenylhydrazone
CDC	=	Center for disease control and prevention
$CDCl_3$	=	Deuterated chloroform
CFU	=	Colony forming unit
CLSI	=	Clinical and laboratory standard institute
$DiOC_2$	=	3,3′-diethyloxacarbocyanine iodide
ESI	=	Electrospay ionization
HAI	=	Hospital-acquired infections
MBC	=	Minimum bactericidal concentration
MHB	=	Mueller Hinton broth
MIC	=	Minimum inhibitory concentration
MRSA	=	Methicillin-resistant *Staphylococcus aureus*
MS	=	Mass spectrometry
MTT	=	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	=	Nuclear magnetic resonance
PBS	=	Phosphate buffer solution
SEM	=	Scanning electron microscopy
TLC	=	Thin layer chromatography
TMS	=	Tetramethylsilane

LIST OF ABBREVIATIONS (Continued)

TEM	=	Transmission electron microscopy
TISTR	=	Thailand institute of scientific and technological research

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER I

INTRODUCTION

It is widely known that the incidence of hospital-acquired infections (HAI) is continuously increasing, and that they are responsible for morbidity and mortality in hospitalized patients (Yokoe and Classen, 2008). Recently, the Centers for Disease Control and Prevention (CDC) have reported that in 2014. HAIs were found in central line-associated bloodstream infections, catheter-associated urinary tract infections, certain surgical site infections and hospital-onset *Clostridium difficile* infections (CDC, 2016). Similar to other species, many strains of *Staphylococcus aureus*, such as methicillin-resistant *S. aureus* (MRSA) (Magiorakos et al., 2012). *S. aureus* is a major problem in nosocomial infection diseases such as pneumonia, operative wound infections and bloodstream infections (Lowy, 1998). Infections caused by *S. aureus* include skin lesions such as boils, furuncles and more serious infections, for example, phlebitis, meningitis, endocarditis and urinary tract infections. The mortality rate for nosocomial endocarditis is found higher than that for urinary tract infection when the pathogen is *S. aureus* (Fernandez-Guerrero, Verdejo, Azofra and, de Gorgolas, 1995). The hallmark of staphylococcal infection is the abscess, which consists of a fibrin wall surrounded by inflamed tissues enclosing a central core of pus containing organisms and leukocytes. The organisms may be disseminated hematogenously, even from the smallest abscess. *S. aureus* has a tendency to spread to particular sites, including the bones, joints, kidneys, and lungs.

This may result to virulent sepsis. The symtoms of staphylococcal sepsis are similar to that of Gram-negative sepsis, with fever, hypotension, tachycardia, and tachypnea. Severe cases progress to multiorgan dysfunction, lactic acidosis and death.

Similar to other species, many strains of *S. aureus*, such as methicillin-resistant *S. aureus*, are developing resistance to the available antibacterial agents, creating a serious problem in public health. The organism may acquire genes encoding enzymes, for example β-lactamase that destroys the antibacterial agent before it can have an effect. Due to the increasing prevalence of failures in the treatment of infectious diseases, the identification and development of novel antibacterial compounds are urgently required. Flavonoids derived from natural plants have been proved to have the potential to be new leads for antibacterial drug discovery (Cushnie and Lamb, 2003; Cushnie and Lamb, 2011).

Flavonoids are well known and interesting sources for new antibacterial agents. More than 6000 flavonoid compounds have been purified and identified (Liu, 2011). They are ubiquitous in cells and are commonly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. These compounds have been used in traditional herbal medicine as the principal physiologically active constituents to treat human diseases for centuries. In addition, this class of natural products is becoming the subject of antimicrobial research. Many groups of flavonoids possessing antiviral, antifungal or antibacterial activities have been isolated and identified for the structure (Cushnie and Lamb, 2005).

Derris plants have been received much interest from phytochemical researchers because of their plentiful bioactive compounds of flavonoids. Many of *Derris* flavonoids exhibit wide varieties of biological activities. For example, *D. reticulata* has

been reported to possess anti-diabetic action (Kumkrai, Kamonwannasit, and Chudapongse, 2014) and anti-inflammatory activity (Vongnam, 2013).

Lupinifolin is a prenylated flavonoid isolated from several medicinal plants, such as *Myriopteron extensum* (Soonthornchareonnon, Ubonopas, Kaewsuwan, and Wuttiudomlert, 2004), *Eriosema chinense* (Prasad, Laloo, Kumar, and Hemalatha, 2013), *Albizia myriophylla* (Joycharat et al., 2013) and *Erythrina fusca* (Khaomek et al., 2008). It is also reported to be a major compound of *Derris reticulata* (Chivapat, Chavalittumrong, Attawish, and Soonthornchareonnon, 2009). There are several lines of evidence demonstrating that lupinifolin exerts antimicrobial activities, such as antiviral activity against herpes simplex virus type 1 (Soonthornchareonnon, Ubonopas, Kaewsuwan, and Wuttiudomlert, 2004), antimycobacterial activity against *Mycobacterium tuberculosis* (Sutthivaiyakit et al., 2009) and antibacterial activity against *Bacillus cereus, Corynebacterium diphtheria* and *S. epidermidis* (Soonthornchareonnon, Ubonopas, Kaewsuwan, and Wuttiudomlert, 2004; Sutthivaiyakit et al., 2009). Lupinifolin possesses very strong activity against *Streptococcus mutans* with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 1 and 2 µg/ml, respectively (Joycharat et al., 2013). It has been demonstrated to exhibit antidiarrheal activity on castor oil-induced intestinal fluid accumulation with a significant recovery from Na^+, K^+ loss (Prasad, Laloo, Kumar, and Hemalatha, 2013). The same report showed that antibacterial activity of lupinifolin against bacterial strains mainly implicated in diarrhea such as *B. cereus*.

Several studies have demonstrated the mechanisms of action underlying antimicrobial effects of flavonoids extracted from medicinal plants. Because of a

variety of the structures in this phytochemical class, the mechanism of action previously established by researchers varies dramatically. For example, sophoraflavanone G and catechins alter the fluidity of outer and inner layers of bacterial membranes (Cushnie and Lamb, 2005). Epicatechingallate inhibits cell wall synthesis and increases the quantity of autolysins (Stapleton, Shah, Ehlert, Hara, and Taylor, 2007). 6,8-Diprenyleriodictyol, isobavachalcone, and 4-hydroxylonchocarpin, flavonoids from *Dorstenia* genus, cause damage of cell membrane. In addition, an inhibition of macromolecule synthesis such as DNA, RNA, and proteins have also been found (Dzoyem, Hamamoto, Ngameni, Ngadjui, and Sekimizu, 2013).

Antimicrobial activities of the plant flavonoid lupinifolin has been demonstrated. Therefore, In the present study, lupinifolin was isolated from *D. reticulata* stem and tested for antibacterial activities against four strains of Gram-positive and Gram-negative bacteria. Due to its highest susceptibility to lupinifolin, *S. aureus* was used to investigate the mechanism underlying this antibacterial activity. It is first reported here that lupinifolin purified from *D. reticulata* inhibits growth of *S. aureus* by damaging the bacterial cell membrane. The data obtained from this study will provide scientific evidence to support the use or development of this compound as antimicrobial agent.

CHAPTER II

LITERATURE REVIEW

2.1 *Staphylococcus aureus*

All living organisms can be classified as either eukaryotes or prokaryotes. Eukaryotes are organisms made up of cells that contain a membrane-enclosed nucleus as well as other membrane-enclosed structures outside the nucleus. Prokaryotes are simple cells that lack a nucleus and any other structures enclosed by a membrane. Bacteria are prokaryotic cells whose external structure is composed of cell wall, cytoplasmic membrane, pili, glycocalyx and flagella, whereas bacterial intracellular structure consists of DNA, plasmid, ribosome and cytoskeletons (Lowy, 1998). The major bacterial shapes are spheres (cocci, coccus), rods (bacilli, bacillus), and coccobacilli. Rod-shaped bacteria that have tapered ends are called fusiform bacilli.

S. aureus, a member of the Micrococcaceae family is a Gram-positive cocci in grape like clusters (Figure 2.1A), which consist of non-spore forming, non-motile, catalase-positive and facultative anaerobic bacteria that can invade the body via broken skin or mucous membrane. The term *Staphylococcus* is derived from the Greek term *staphyle,* meaning “a bunch of grapes”. The cell wall contains peptidoglycan and teichoic acid. The organisms are resistant to temperature as high as 50°C, high salt concentrations, and dry environments. Colonies are usually large (6-8 mm in diameter), smooth, and translucent. The colonies of most strains are pigmented, ranging from cream-yellow to orange (Lowy, 1998). An example of skin

infected by *S. aureus* is shown in Figure 2.1B. This type of microorganism is one of coagulase-positive *staphylococci* which become increasingly recognized as pathogen of nosocomial infection, following osteomyelitis, endocarditis, food poisoning, toxic shock syndrome and more serious skin infections (Ruimy et al., 2010).

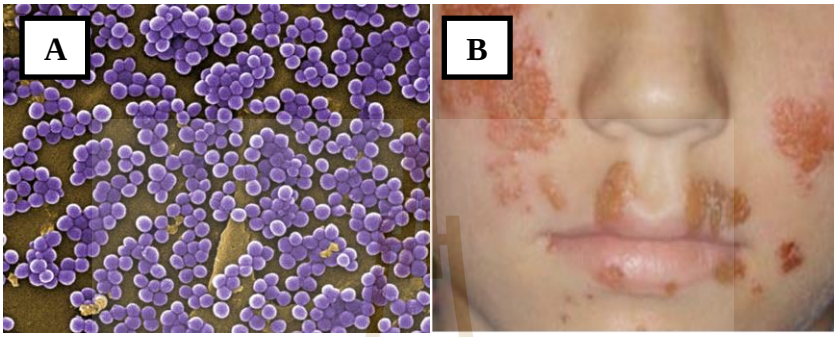


Figure 2.1 Morphology of *S. aureus* (A) and skin lesion of *S. aureus* infection (B) (Carr and Hageman, 2005).

S. aureus can grow at 6 - 46°C, with the optimum temperature at 30 - 37°C. Like most pathogenic bacteria, it best grows in pH 7.2-7.4. *S. aureus* classified as facultative anaerobes are able to grow well in the presence of oxygen and lack growth in the absence of oxygen. *S. aureus* can create toxins, and the enterotoxins are divided into eight types, which are A, B, C1, C2, C3, D, E, and H. Common causes of food poisoning are A and D toxins that have special resistant features. Toxins are not destroyed by heat, even if boiled for half an hour. Common sources of *S. aureus* are bacteria that can be found in the nasal cavity, skin, mucous membranes, respiratory tract, wounds, abscesses, as well as in soil and dust. Food is often contaminated with *S. aureus*, such as meat, poultry, egg products, tuna, chicken, potato salad, macaroni products, pastries, cream pies, chocolate, sandwich and milk products (Lowy, 1998).

S. aureus can cause food poisoning. It is caused by eating food contaminated with the toxin, even in small amounts of less than 1 microgram can cause illness. High volume of *S. aureus* contamination in food of 100,000 cells per gram of food can cause acute infection and acute toxemia of pregnancy. After eating food contaminated with the bacteria within 1-6 hours, a person will start to have symptoms of nausea, vomiting, diarrhea, severe tiredness, abdominal pain and cramps. Most patients with symptomatic severe shock will also see signs of fever. Other severe complications may occur in newborns, the elderly and people with diabetes. However, most will recover within 8-24 hours, depending on the body's resistance and the amount of toxins that get into the body (Carr and Hageman, 2005).

2.2 Antibacterial drugs

Humans have used natural anti-pathogen in the treatment of infectious diseases for over 2500 years. The 19th century marks the beginning of antimicrobials due to the discovery of several antibiotics. They are widely used to prevent and treat infectious diseases. The modern era of antimicrobial therapy started in 1936 with the discovery of sulfonamides and other drugs used in the treatment of infections in patients. During the next decade in 1940, penicillin and streptomycin were used to treat infections in patients. It can be said that the year 1950 was the golden age of antibiotics. Since then, antimicrobial drug discovery has developed into a new type of industry. Increasingly, the synthetic of the drugs for effectiveness and decreasing the side effects on humans is processed. The discovery of antibiotics is the greatest achievement in the medical industry (Hessen and Kaye, 2004).

The use of antimicrobial drugs in the past is a double blade sword. It will be very useful to reduce the rate of morbidity and mortality from infectious diseases. Whenever, if used inappropriately, it will make the cost of healthcare rises because it causes the ability to develop resistant to antimicrobial drugs. To select antimicrobials appropriately, we need to know the clinical features of the syndrome and the infectious diseases, based on information which was obtained from history. Physical examination and appropriate laboratory tests, which are useful in determining the cause of the infection (Colgan and Powers, 2001). We should understand the mechanism of action of drugs, pharmacokinetics, pharmacodynamics and adverse reaction (Hessen and Kaye, 2004). Mechanisms of resistance and sensitivity pattern of bacteria to antimicrobial agents are the factors in treatment the patient. In addition, position of infection, liver function, kidney conditions, immune intolerance, pregnancy and age of the patient are important to follow up for successful therapy (Niederman, 2003).

The basic principles, considered for the treatment of patients with antimicrobial, are to keep in mind that antimicrobials are not antipyretic drugs. Fever caused by different reasons are not necessarily always due to infectious diseases. Some infections do not need treatment with antibiotics such as cold medicine, so patients should be evaluated carefully. All antimicrobials are particularly dangerous when not used properly. In the treatment, drug effectiveness should be appropriately used and adjusted after known the drugs susceptibility testing (Slama et al., 2005). The using of narrow spectrum antimicrobial agents can be prevented drugs resistance microorganisms.

2.2.1 The susceptibility of pathogens to antimicrobial agents

Each pathogen is sensitive to different antibiotics, and even the same type of infection is also sensitive to different drugs. Therefore, it is necessary to perform the culture and the susceptibility testing to the antimicrobial agents, which can be done in several ways by the standards of the Clinical and Laboratory Standards Institute (CLSI) methods. Easy and popular practice commonly disc diffusion susceptibility testing was done by placing a filter paper disc with the antimicrobial at concentrations that are defined as standard on agar plates. After incubation for 16 -18 hours, the antibacterial activity was determined by measuring the diameter of the inhibition zone occurred and the results were demonstrated to resistant, intermediate and susceptible tested. This method will not be able to tell the minimal inhibitory concentration (MIC), which represents to the concentration of the lowest dose that can inhibit the replication of the bacteria. The MIC will focus on the use of antimicrobials, including the size of the dose required to treat certain infections, such as infection of the heart valves and patients infected with *S. pneumoniae* meningitis from the current problem of drug resistance. The MIC can be done in many ways such as broth macrodilution, microdilution, and agar dilution (Niederman, 2005).

The control of microorganisms is critical for the prevention and treatment of diseases. Microbial colonization can lead to disease, disability and death. Thus the control or destruction of microorganisms residing within the bodies of humans is of great importance.

The discovery of the first antibiotic was an accident. Alexander Fleming was working with *S. aureus* in 1928, and while plating this organism he accidentally allowed the fungus *Penicillium* to contaminate his plate. He subsequently observed

that the plate had a uniform growth of *S. aureus* except where the fungi were growing. In this area, Fleming saw a clearly defined region where there was no bacterial growth. This was what eventually became referred to as a zone of inhibition (Strelkauskas, Strelkauskas, and Moszyk-Strelkauskas, 2010).

The growth of microbes are inhibited by a number of diverse molecules. The first group of these molecules that inhibited bacterial growth were natural products isolated from specific microorganisms. As a result of increasing bacterial resistance, these natural molecules have been modified, and several types of semi-synthetic antibiotics have been derived. Penicillin was the original natural molecule used by humans that its activity was restricted to Gram-positive organisms and essentially ineffective against Gram-negative organisms. It had a very narrow spectrum of activity.

2.2.2 Mechanism and site of antibacterial action

Some of antibiotics were obtained naturally. Their structures have been put to chemical modification by removing some chemical groups and adding other in attempts to increase beneficial effects while minimizing the toxic effects. Chemotherapeutic agents can be either cidal or static. The antibacterial drugs are often described as bacteriostatic or bactericidal. Bacteriostatic describes a drug that temporarily inhibits the growth of microorganism whereas bactericidal describes a drug that attaches to its receptor and causes the death of microorganism. Many of the bacteriostatic drugs in higher doses act as bactericidal agents (Purohit, Saluja, and Kakrani, 2006). Base on the mechanism of action, antibacterial agent generally falls within one of the following five categories.

2.2.2.1 Cell wall inhibitors

The bacterial cell wall provides structural integrity to the cell. In prokaryotes, the primary function of the cell wall is to protect the cell from internal turgor pressure caused by the much higher concentrations of proteins and other molecules inside the cell compared to its external environment. It is a necessary component for the survival of the bacteria. While cell wall is found in bacteria, but absent in human cells. Therefore, the most appealing target for antibiotics is the bacterial cell wall. During the construction of the cell wall, synthesis of components are involved to many enzymatic interactions. These processes can be used as targets of antibiotic molecules such as penicillin, cephalosporins, cephamycins, vancomycin and bacitracin. The cell wall is composed of layers of peptidoglycan, which is made up of repeating units of *N*-acetyl glucosamine (NAG) and *N*-acetyl muramic acid (NAM) (Park and Uehara, 2008). The NAG and NAM molecules are cross-linked through the activity of transglycosylase and transpeptidase enzymes. Many antibiotics that target the cell wall act by inhibiting the activity of these two enzymes. The result is that the cell wall is not properly cross-linked leading to weak and unable to endure the environmental pressures (bactericidal). General comparison of Gram- positive and Gram-negative bacterial cell wall structure illustrate in Figure 2.2.

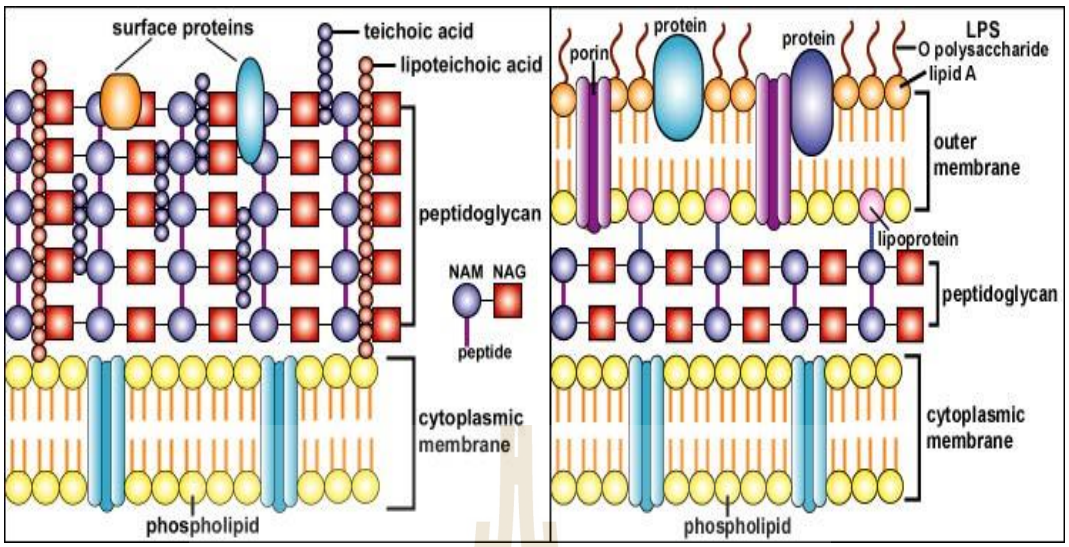


Figure 2.2 Diagrams of Gram-positive (Left) and Gram-negative cell wall (Right) (Strelkauskas, Strelkauskas, and Moszyk-Strelkauskas, 2010).

The cell walls have penicillin-binding proteins (PBPs) in the construction because the β-lactam ring of penicillin binds to these proteins. During active growth of bacteria, new cell wall is continuously being built. It is at this time that the activity of penicillin is most effective, because penicillin prevents the cross-linking of the NAG and NAM units and thereby prevents the formation of an intact cell wall. Consequently, the more rapidly the bacteria are dividing, the more devastating is the effect of penicillin. However, penicillin is effective to against Gram-positive more than Gram-negative that depends on the amounts of peptidoglycan (Strelkauskas, Strelkauskas, and Moszyk-Strelkauskas, 2010).

2.2.2.2 Cell membrane inhibitors

The plasma membrane of a bacterium, also known as the cell membrane or cytoplasmic membrane, is a delicate flexible structure that holds in the internal cellular matrix of cytosol and organelles. The most important features of any

plasma membrane are selective permeability, the production of ATP, and other physiological functions. It is therefore a prime target for antimicrobials such as polymyxin and daptomycin. Because any disruption of this membrane will destroy the ability of bacteria to survive (bactericidal). Gram-negative bacteria have both a plasma membrane and an outer membrane. The outer membrane is rich in lipopolysaccharide and thus increase virulence (Strelkauskas, Strelkauskas, and Moszyk-Strelkauskas, 2010).

2.2.2.3 Protein synthesis inhibitors

Proteins are assembled at a ribosome in combination with messenger RNA, and assembly of a protein begins with the formation of an intact ribosome from two ribosomal subunits. Here amino acids are linked together through peptide bond formation. Because ribosomes are found in both prokaryotic and eukaryotic cells, so the selection of protein synthesis is a target for antibiotic therapy against bacteria. The ribosomes of prokaryotes are not the same as those of eukaryotes. Therefore, antibiotics that target the synthesis of proteins in bacteria do meet the criterion of selective toxicity (Strelkauskas, Strelkauskas, and Moszyk-Strelkauskas, 2010). The bacterial 70S ribosome is composed of two subunits, 30S and 50S. Several targets of antibiotics are located on these subunits. Some mechanisms of antibiotic inactivation involve improper orientation of the mRNA, inability to form peptide bonds, or inhibition of peptide elongation (bacteriostatic). Such as aminoglycosides change 30S subunit shape so mRNA is misread, macrolide binds 50S subunit and prevents mRNA moving through ribosome, tetracycline blocks ribosome docking site of tRNA and chloramphenicol inhibits peptide bond formation (Strelkauskas, Strelkauskas, and Moszyk-Strelkauskas, 2010).

2.2.2.4 Nucleic acid inhibitors

The most obvious target of antibiotic therapy would be the nucleic acids DNA and RNA. Because these molecules have critical roles in the reproduction of bacteria. The disruption in their function will result in the death of the bacteria (bactericidal). The structure of DNA and RNA in bacteria is not different from the structure of these cells in humans so the main difficulty in using nucleic acids as targets is selective toxicity. Currently, over the years a variety of potential antibiotics have been developed, such as rifamycin and quinolone groups. The anti-mycobacterial rifamycins inhibit the growth of most Gram-positive bacteria by binding to the RNA polymerase molecules and disrupt its structure, leading to the polymerase molecule unable to function properly. Similarly, the quinolones have been found to be disrupt the three-dimensional structure of topoisomerase and gyrase (Strelkauskas, Strelkauskas, and Moszyk-Strelkauskas, 2010). These two enzymes are essential for DNA synthesis.

2.2.2.5 Inhibitors of metabolism

The metabolism of folic acid is a molecule using for nucleic acid synthesis. One of the intermediates in the pathway is *para*-amino benzoic acid (PABA). Sulfa drugs competitively inhibit the function of the enzyme that incorporates the PABA molecule into the folic acid metabolic pathway (Strelkauskas, Strelkauskas, and Moszyk-Strelkauskas, 2010). It is referred to as competitive inhibition because the sulfa molecule is remarkably similar in structure to the PABA molecule. The enzyme simply gets fooled into incorporating the sulfa molecule into the folic acid structure instead of the PABA. This stops the pathway and is a cell

death of bacteria (bactericidal) because they cannot survive without folic acid (Strelkauskas, Strelkauskas, and Moszyk-Strelkauskas, 2010).

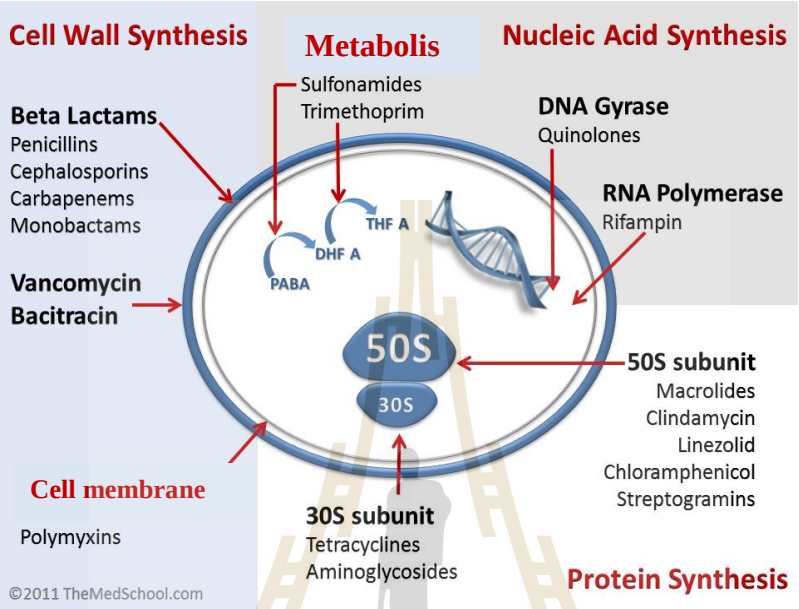


Figure 2.3 The target sites of antibiotics on bacteria (Sahare, Moon, and Shinde, 2013).

2.3 Mechanisms of Antimicrobial Resistance

2.3.1 Intrinsic or natural resistance

Intrinsic or natural resistance may be a result of the drug or active ingredient has no target for the drugs (Tenover, 2006). Microorganisms naturally do not possess target sites for the drugs. In addition, they naturally have low permeability to those agents because of the differences in the chemical nature of the drug and the microbial membrane structures. The Figure 2.4 shows an overview of intrinsic resistance mechanisms. The example shown is of β-lactam antibiotics targeting a penicillin-binding protein (PBP). Antibiotic A can enter the cell via a membrane-

spanning porin protein, reach its target and inhibit peptidoglycan synthesis. Antibiotic B can also enter the cell via a porin, but unlike antibiotic A, it is efficiently removed by efflux. Antibiotic C cannot cross the outer membrane and so is unable to access the target PBP. In this example, it is said that this type of bacteria is naturally resistance to antibiotics B and C.

2.3.2 Acquired resistance

Acquired resistance is often a result from the widespread using of antibiotics and inappropriate in terms of medicine. Bacteria pathogens are trying to adaptation for survival after they are exposed to antimicrobial drugs. Mechanism of resistance is possible in many ways. In summary, mechanisms for acquired resistance consists of the presence of an enzymes that inactivates the antimicrobial agent. A mutation in the antimicrobial agent's target and post-transcriptional or post-translational modification can reduce binding of the antimicrobial agent. The active efflux of bacteria possesses elimination of the drugs. For example, β-lactamase production is the most common mechanism of resistance (Katzung, Masters, and Trevor, 2009). These enzyme is produced by S. *aureus*, *Haemophilus* spp, *P. aeruginosa*, *Enterobacter* spp. and *E. coli*. The reduced affinity of antimicrobial targets lead to reduce the affinity of PBPs in *S. pneumoniae*, *S. aureus* (Ifesan, Joycharat, and Voravuthikunchai, 2009). Some bacteria, such as methicillin-resistant *S. aureus* create a new goal or change the cell wall structure, involving in alteration of target PBP.

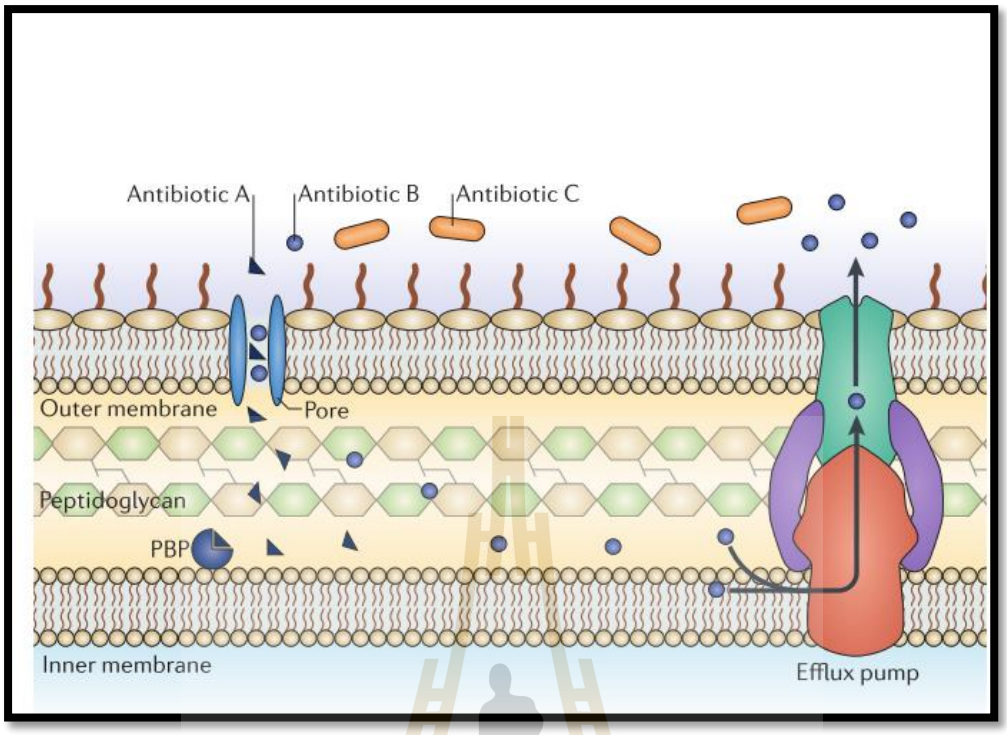


Figure 2.4 Examples of intrinsic mechanisms of resistance (Blair, Webber, Baylay, Ogbolu, and Piddock, 2015).

Table 2.1 Mechanism of resistance to antimicrobial agents (Blair, Webber, Baylay, Ogbolu, and Piddock, 2015).

Antimicrobial agents	**Mode of Action**	**Resistance mechanisms**
β-lactams	Inhibit cell wall synthesis, Cell division	β- lactamases, altered penicillin binding protein, altered GNB outer-membrane porins, active efflux
Glycopeptides (vancomycin, cycloserine)	Inhibit cell wall division	Altered target site
Aminoglycosides	Inhibit protein synthesis (bind to30s ribosome)	Aminoglycoside-modifying enzyme, Decreased membrane permeability, active efflux
Macrolides	Inhibit protein synthesis (bind to 50s ribosome)	Altered target, enzymatic inactivation, active efflux
Tetracycline	Inhibit protein synthesis (bind to 30s ribosome)	Efflux, altered target, enzymatic inactivation, decreased permeability
Chloramphemcol	Inhibit protein synthesis (bind to 50s ribosome)	Chloramphenicol acetyltransferase, active efflux
Rifampin	Inhibits nucleic acid synthesis	Altered target, decreased permeability of membrane
Sulfonamides Trimethoprim	Inhibit folic acid synthesis	Altered target

2.4 *Derris reticulata* Craib.

Derris reticulata Craib., a plant in Leguminosae family, contains flavonoids as its major bioactive compound similar to other plants in genus *Derris*. It is a well-known Thai herbal medicine commonly called as Cha-am-nuea. The picture of this plant is shown in Figure 2.5.

Derris plants are benificial in traditional herbal medicine all over the tropical areas. The biological activities of some species of *Derris* plants have been reported. Some of the significant ethnopharmacology and medical applications of *Derris* plants are shown in Table 2.2.

Plant flavonoids have been demonstrated to possess a variety of biological activities including antibacterial, antiviral, antioxidant, antiulcerogenic, antineoplastic, antihepatotoxic, anti-inflammatory activities and cytotoxicity (Cushnie and Lamb, 2005; Pengelly, 2004). Flavonoids sources are mainly found in fruit, vegetables, nuts, seeds, stems and flowers, tea, wine, propolis and honey. According to biosynthetic origin, flavonoids can be classified into several groups, for example chalcones, catechins, flavanones, isoflavones, anthocyanidins, xanthones, aurones, flavones and flavonols (Figure 2.6). Flavonoids have attracted considerable interest recently because of their potential beneficial effects on human health. They have been reported to possess many useful properties, including antioxidant, anti-inflammatory, antiallergic, vascular, cytotoxic antitumor, oestrogenic, antibacterial activities, and enzyme inhibitory.

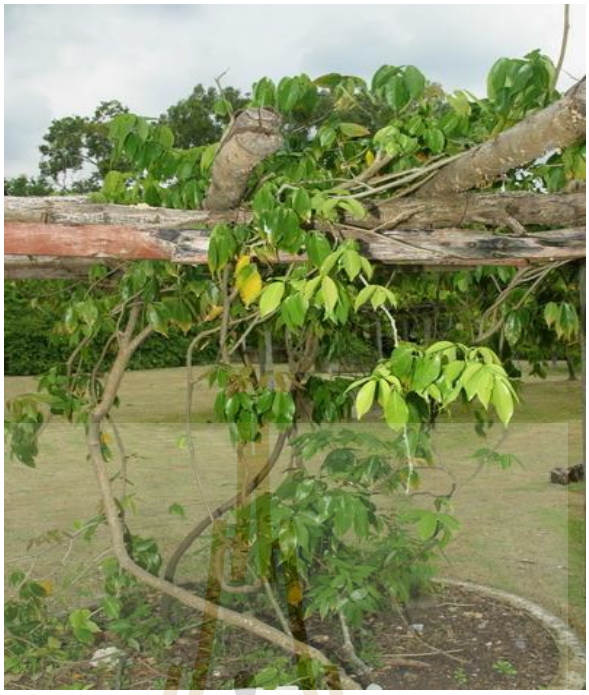


Figure 2.5 *Derris reticulata* (Ruckhachati, 2010).

Table 2.2 Selected ethnomedical applications of *Derris* plants (Kongjinda, 2004).

Sources	Plant part	Ethnomedical activity
D. amazonica	Dried root	Fish poison
D. elliptica	Root	Insecticide
	Dried root	Blood purification
		Fish poison
D. indica	Dried seed	Fish poison
D. malaccensis	Dried root	Fish poison
	Dried root	Leprosy
D. oblonga	Root	Insecticide
D. robusta	Fresh root	Sore throat

Table 2.2 Selected ethnomedical applications of *Derris* plants (Kongjinda, 2004) (Continued).

Sources	Plant part	Ethnomedical activity
D. scandens	Dried stem	Rheumatism
	Dried entire plant	Fish poison
	Dried stem	Analgesic
	Dried stem	Antipyretic
	Dried stem	Arthistic symptoms
	Dried stem	Antidysenteric
	Dried stem	Antidiuretic
D. spruceana	Dried leaf	Fish poison
D. trifoliata	Root	Insecticide
	Dried entire plant	Stimulant
	Dried entire plant	Antipasmodic

Derris plants received much attention from phytochemical researchers because of their plentiful bioactive compounds of flavonoids. Many kinds of *Derris* flavonoids exhibit wide varieties of biological activities. Some flavonoid compounds and their biological activities of certain *Derris* plants are illustrated in Table 2.3.

According to their antimicrobial activities, *D. elliptica, D. indica* and *D. trifoliata* showed varied levels of broad spectrum against 25 pathogens (Khan, Omoloso, and Barewai, 2006). The methanol fractions of leaves and root heart-wood,

as well as petrol, butanol and methanol fractions of the root bark of *D. indica* including ethyl acetate fractions of *D. trifoliate* possessed significant antibacterial activity. *D. scandens* showed a good inhibitory effect on growth of *S. aureus*, *S. epidermidis* and *E. coli* (Sittiwet and Puangpronpitag, 2009). Moreover, it has been used to treat arthritis patients (Laupattarakasem, Houghton, Hoult and Itharat, 2003). Different parts of *D. trifoliate* were used for treatment of wounds, rheumatism, dysmenorrhea and asthma (Jiang et al., 2012).

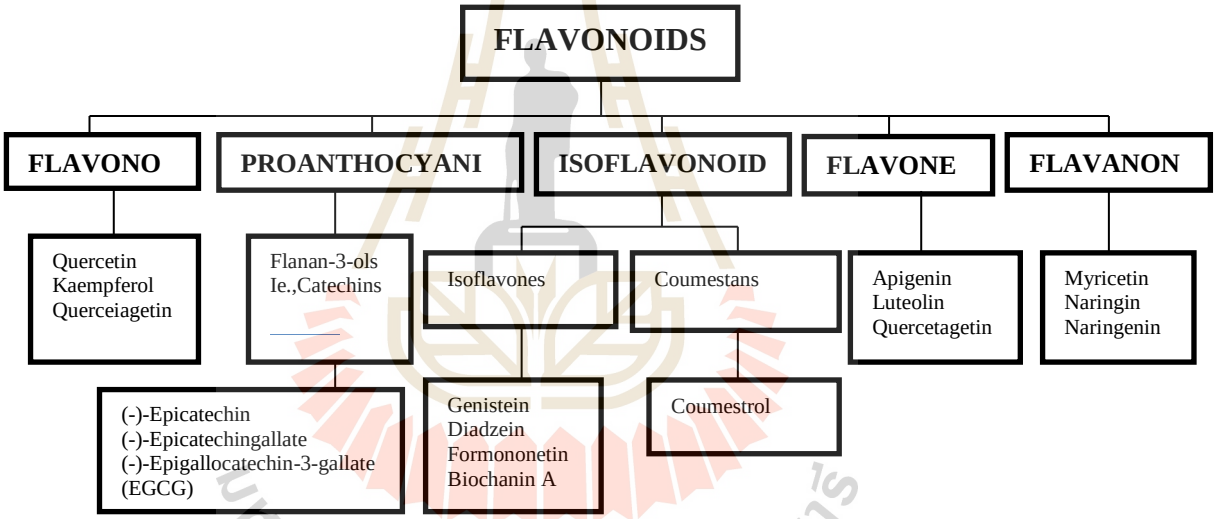


Figure 2.6 Family of major dietary flavonoid groups (Skibola and Smith, 2000).

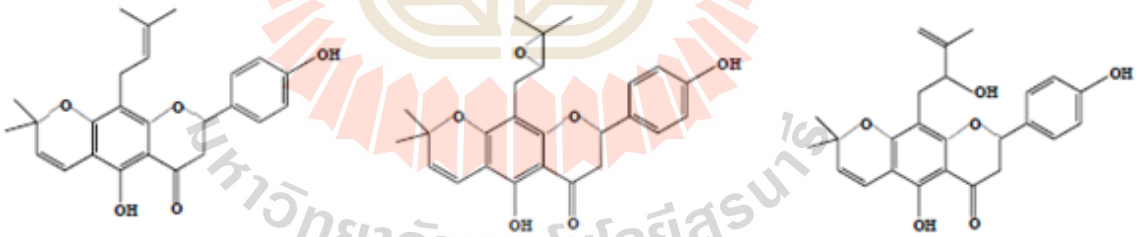
Table 2.3 Active compounds from *Derris* plants and biological activity (Kongjinda, 2004).

Compounds	Plant	Biological activity
Deguelin	*D. malaccensis*	antibacterial
Dehydrorotenone	*D. malaccensis*	antibacterial
Dereticulatin	*D. reticulata*	cytotoxic against P-388 Cell line
Derrisisoflavone A	*D. scandens*	anti-dermatophyte
Derrisisoflavone B	*D. scandens*	anti-dermatophyte
Derrisisoflavone C	*D. scandens*	anti-dermatophyte
Derrisisoflavone D	*D. scandens*	anti-dermatophyte
Derrisisoflavone E	*D. scandens*	anti-dermatophyte
Derrisisoflavone F	*D. scandens*	anti-dermatophyte
Elliptone	*D. malaccensis*	antibacterial
Epoxylupinifolin	*D. reticulata*	cytotoxic against P-388 Cell line
Erysenegalensein E	*D. scandens*	anti-dermatophyte
12a-hydroxyrotenone	*D. malaccensis*	antibacterial
Lupalbigenin	*D. scandens*	anti-dermatophyte
Lupinifolin	*D. reticulata*	cytotoxic against P-388 Cell line
Lupinisoflavone G	*D. scandens*	anti-dermatophyte

In addition, the ethanolic extract of *D. reticulata* stem contains anti-inflammatory activity by inhibiting the production of several known inflammatory mediators in LPS-activated macrophages (Vongnam, 2013). *D. reticulata* has been used in traditional medicine for the relief of thirst and as an expectorant (Mahidol,

Prawat, Prachyawarakorn, and Ruchirawat, 2002). Moreover, recently it was demonstrated to possess anti-diabetic activity, resulting from its pancreatic cytoprotective effect and inhibition of intestinal glucose absorbtion (Kumkrai, Kamonwannasit, and Chudapongse, 2014). As contributing to the hypoglycemic action, *D. indica* has been shown to exert intestinal α-glucosidase inhibitory activity (Ranga Rao et al., 2009).

Phytochemicals isolated from some species of *Derris* plants have been reported to possess biological activities. Three pyranoflavanones, lupinifolin, 2''', 3''' - epoxylupinifolin and dereticulatin were identified from the stems of *D. reticulata* Benth. (Mahidol et al., 1997). These compounds shown in Figure 2.7.



Lupinifolin 2‴,3‴-Epoxylupinifolin Dereticulatin

Figure 2.7 Isolated compounds from *Derris reticulata* Benth (Mahidol et al., 1997).

Lupinifolin is the prenylated flavanone that have been isolated from several medicinal plants, such as *Derris reticulate* Benth. (Mahidol et al., 1997), *Myriopteron extensum* (Soonthornchareonnon, Ubonopas, Kaewsuwan, and Wuttiudomlert, 2004), *Eriosema chinense* (Prasad, Laloo, Kumar, and Hemalatha, 2013) and *Albizia myriophylla* (Joycharat et al., 2013). The solubilities of lupinifolin in solvents depend on their existing forms which is less soluble in water, but easily soluble in methanol, ethanol, trichoromethane, and other organic solvents. There are several lines of evidence demonstrating that lupinifolin exerts antimicrobial activities, such as an antiviral activity against herpes simplex virus type 1 (Soonthornchareonnon, Ubonopas, Kaewsuwan, and Wuttiudomlert, 2004), antimycobacterial activity against *Mycobacterium tuberculosis* (Sutthivaiyakit et al., 2009) and antibacterial activity against *B. cereus, Corynebacterium diphtheria, S. epidermidis* (Soonthornchareonnon et al., 2004; Sutthivaiyakit et al., 2009). Lupinifolin possess a strong activity against *Streptococcus mutans* with MIC and MBC ranging from 0.25-8 µg/ml (Joycharat et al., 2013). It has been demonstrated to exhibit antidiarrhoeal activity on castor oil-induced intestinal fluid accumulation with a significant recovery from Na^+, K^+ loss. The same report showed antibacterial activity of lupinifolin against bacterial strains mainly implicated in diarrhea such as *B. cereus* (Prasad, Laloo, Kumar, and Hemalatha, 2013). Acute toxicity study also encourages the therapeutic use of lupinifolin because it did not affect on body weight, food consumption or the animal's health investigated on hematological and biochemistry tests (Chivapat, Chavalittumrong, Attiwist, and Soonthornchareonnon, 2009).

For anti-carcinogenesis, lupinifolin, isolated from plants belonging to *Citrus tamurana*, *C. medica* and *D. trifoliata*, was tested on Epstein-Barr virus (EBV) early antigen activation induced by 12-*O*- tetradecanoylphorbol-13-acetate in Raji cells (Itoigawa et al., 2002). The results showed that lupinifolin possessed inhibitory activity against EBV without exhibiting any cytotoxicity. *In vitro* bioassay of lupinifolin demonstrated that the growth of P-388 cells was inhibited at the concentration of 0.4-0.5 μg/ml (Mahidol et al., 1997). In addition, lupinifolin illustrated a marked inhibitory effect on mouse skin tumor promotion by *in vivo* two-stage carcinogenesis test (Itoigawa et al., 2002). The inhibitory activity was shown to be correlated with the number of prenyl groups in the molecule. The presence of one or more prenyl side-chains is suggested be an important structural characteristic for the inhibitory effect of flavanones. One of the proposed reasons for the enhanced biological activities of prenylated flavonoids is that the prenylation of the flavonoid core increases the lipophilicity and the membrane permeability of the compound (Sasaki, Kashiwada, Shibata, and Takaishi, 2012).

Table 2.4 Flavanones from *Derris* genus (Khan, Omoloso, and Barewai, 2006).

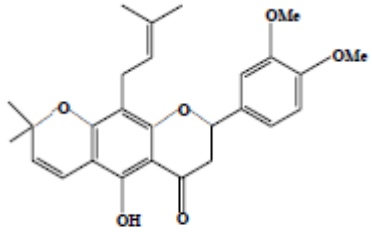
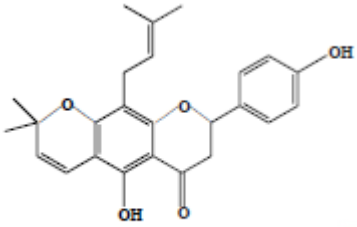
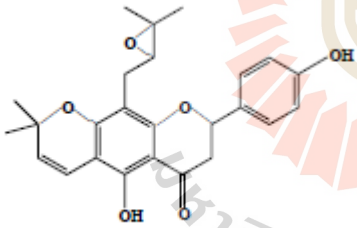
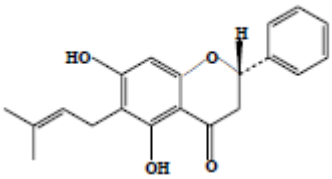
Compounds	Plant organs	Plant sources
 3'-methoxylupinifolin	Root	*D. laxiflora*
 Lupinifolin	Stems	*D. reticulata*
 2''',3'''-Epoxylupinifolin	Stems	*D. reticulata*
 5,7-Dihydroxy-6-prenylflavanone	Root Wood	*D. florobunda* *D. rariflora*

Table 2.4 Flavanones from *Derris* genus (Khan, Omoloso, and Barewai, 2006) (Continued).

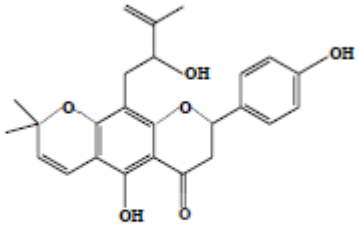
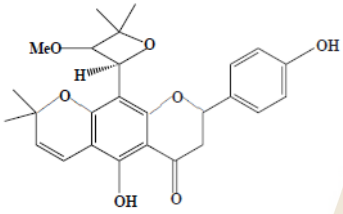
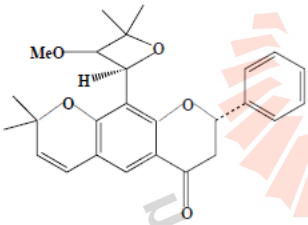
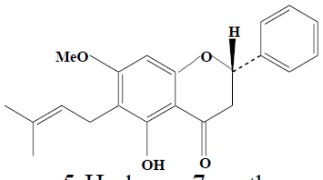
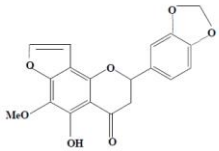
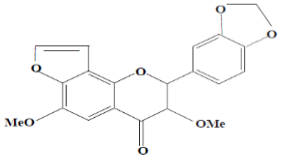
Compounds	Plant organs	Plant sources
 Dereticulatin	Stems	*D. reticulata*
 Derriflavanone	Root	*D. laxiflora*
 Epiderriflavanone	Root	*D. laxiflora*
 5-Hydroxy-7-methoxy-6-prenylflavanone	Wood	*D. rariflora*

Table 2.4 Flavanones from *Derris* genus (Khan, Omoloso, and Barewai, 2006) (Continued).

Compounds	Plant organs	Plant sources
 Root Methylenedioxy-(3',4')-5-hydroxy-6-methoxyfurano-(7,8,2",3")-flavanone	Root	*D. araripensis*
 3,5,6-trimethoxyfurano-(7,8,2",3")-flavanonol	Root	*D. araripensis*

2.5 Antibacterial mechanisms of action of flavonoids

2.5.1 Inhibition of nucleic acid synthesis

Several lines of evidence have suggested that DNA and/or RNA are targets of various flavonoids. For example, using a radioactive precursors, it was found that robinetin, myricetin and (−)-epigallocatechin inhibited the growth of *Proteus vulgaris* by intercalation or hydrogen bonding with the stacking of nucleic acid bases, resulting in the interfering of DNA synthesis (Mori, Nishino, Enoki, and Tawata, 1987).

Topoisomerases, enzymes that alter the supercoiling of double-stranded DNA, are the molecular targets of the antibiotics quinolones. The topoisomerases act by transiently cutting one or both strands of the DNA. Topoisomerase type I cuts one strand whereas topoisomerase type II cuts both strands of the DNA to relax the coil

and extend the DNA molecule. DNA gyrase or topoisomerase type II is an enzyme within the class of topoisomerase that relieves strain while double-stranded DNA is being unwound by helicase. Fourteen flavonoids were selected to investigate for antibacterial mechanisms in a variety strains of bacteria. The data suggested that seven flavonoids, including quercetin and apigenin, from the tested 14 compounds possessed antibacterial activities in part by their inhibition of DNA gyrase (Ohemeng, Schwender, Fu, and Barrett, 1993). From separate study, it was demonstrated that quercetin inhibited *E. coli* DNA gyrase caused by its binding to GyrB subunit and inhibition of GyrB ATPase activity (Plaper, Golob, Hafner, Oblak, Solmajer, and Jerala, 2003). Bernard and co-workers also found that the glycosylated flavonol rutin exhibited antibacterial activity against a permeable *E. coli* strain by inducing *E. coli* topoisomerase IV-dependent DNA cleavage (Bernard et al., 1997). Using a technique known as the SOS chromotest, it was found that the glycosylated flavonol rutin inhibited growth of *E. coli* cells by producing an SOS response. Finally, a study using 4-quinolone resistant strains of *S. aureus* by Cushnie and Lamb (Cushnie and Lamb, 2006) suggested that topoisomerase IV and the relatively homologous gyrase enzyme are involved in the antibacterial mechanism of action of galangin.

2.5.2 Inhibition of cytoplasmic membrane function

The antibacterial actions of sophoraflavanone G have gained increasing attention. The mechanism of action of this flavanone has been reported by several groups of researchers (Sakagami et al., 1998; Tsuchiya et al., 1994). Using liposomal model membranes, the data showed that sophoraflavanone G significantly increase fluorescence polarisation of the liposomes, suggesting that it reduced the fluidity of

outer and inner layers of bacterial membranes. (Tsuchiya and Iinuma, 2000). It has been widely accepted that catechins, a group of flavonoids in green tea, possess antibacterial activity. Using the same cell membrane model, Ikigai and coworkers (Ikigai, Nakae, Hara, and Shimamura, 1993) concluded that epigallocatechin gallate primarily act on and damage bacterial membranes, resulting in leakage of small molecules from the intraliposomal space. Two hypotheses proposed by the author has been (1) directly penetratign and disrupting the barrier function; and (2) causing membrane fusion, resulting in the leakage of intramembranous materials and aggregation. From the same study, catechins were found to have greater activity against Gram-positive than Gram-negative bacteria. Interestingly, it appeared that leakage induced by epigallocatechin gallate was significantly lower when liposome membranes were prepared containing negatively charged lipids. It was therefore suggested that the low catechin susceptibility of Gram-negative bacteria may be depend on the presence of lipopolysaccharide acting as a barrier (Ikigai, Nakae, Hara and Shimamura, 1993). Moreover, another group of investigators studied the mechanism of (−)-epicatechin gallate and 3-O-octanoyl-(+)-catechin in MRSA clinical isolate cells using fluorescent stain propidium iodide as an indicator of cell membrane integrity (Sato, Tsuchiya, Akagiri, Takagi, and Iinuma, 1997). The data from this work was accordance with the result from Ikigai's experiment, substantiating the hypothesis that catechins act on and damage bacterial membranes.

At least two more lines of evidence supporting this hypothesis have been documented. First, the effect of galangin upon cytoplasmic integrity in *S. aureus* has been investigated by measuring loss of internal potassium (Cushnie and Lamb, 2005). The data suggest that galangin induces cytoplasmic membrane damage and potassium

leakage. However, whether galangin damages the membrane directly, or indirectly as a result of autolysis or cell wall damage and osmotic lysis is inconclusive (Cushnie and Lamb, 2011). Second evidence was reported by the investigation of antimicrobial action of propolis by Mirzoeva and colleagues (Mirzoeva, Grishanin, and Calder, 1997). Synergistic effect between propolis and other antibiotics such as tetracycline and ampicillin has been published (Stepanovic, Antic, Dakic, and Svabic-Vlahovic, 2003). It was suggested that the effect of propolis on membrane permeability and membrane potential may contribute to the synergism effect and decrease the resistance of cells to another antibacterial agent.

2.5.3 Inhibition of energy metabolism

The antibacterial mode of action of two retrochalcones (licochalcone A and C) from the roots of *Glycyrrhiza inflata* was investigated (Haraguchi, Tanimoto, Tamura, Mizutani, and Kinoshita, 1998). These flavonoids were demonstrated to have inhibitory activity against *S. aureus* and *Micrococcus luteus* but not against *E. coli*. Since inhibition of macromolecule biosynthesis by these compounds was found, a possible mechanism in interfering with energy metabolism was hypothesized by a group of investigators (Haraguchi, Tanimoto, Tamura, Mizutani, and Kinoshita, 1998). The data showed that the licochalcone A and C inhibited oxygen consumption in the Gram-positive bacteria, *M. luteus* and *S. aureus*, but not in the Gram-negative bacteria *E. coli.* These data were correlated well with the observed spectrum of antibacterial activity. It was further demonstrated that the inhibition site of licochalcones A and C was between CoQ and cytochrome c in the bacterial respiratory electron transport chain.

CHAPTER III

MATERIALS AND METHODS

3.1 Plant material

3.1.1 Plant collection and preparation

Derris reticulata Craib. was collected from Prachinburi province, Thailand by the former Ph.D. student (Dr. Pakarang Kumkrai). Botanical identification was performed by Dr. Paul J. Grote, School of Biology, Suranaree University of Technology (SUT). A voucher specimen (Pharm-Chu-006) was deposited at School of Preclinical Sciences, SUT. The stems were cut into small pieces and dried at 50°C in hot-air oven. The dried stems were stored at room temperature until used for extraction.

3.1.2 Purification of lupinifolin

Sixty grams of dried stems were extracted with 400 ml of hexane using a Soxhlet extractor. After washing twice with deionized water, the extract became turbid due to precipitation of lupinifolin. The hexane layer was collected and heated at 65°C until the extract became clear, and was then left at room temperature overnight for crystallization. The purity of the yellow needle-shaped lupinifolin crystals was first analyzed by TLC. Dichrolomethane:methanol (95:5) was used as the mobile phase and the composition of the extract was detected by UV light at 254 nm. Specific rotation was measured with a Bellingham & Stanley P 20 polarimeter.

Yellow crystallized lupinifolin was dissolved in 10 ml of chloroform (1.10 g/ml) and analyzed with a 20.0 cm polarimeter tube. The calculated specific rotation was -10.0°, which matched the published value (Mahidol et al., 1997). Further identification was carried out with nuclear magnetic resonance (NMR) and mass spectrometry (MS).

3.2 Identification of lupinifolin

3.2.1 Nuclear magnetic resonance (NMR)

The purified lupinifolin was confirmed by NMR spectra on a 500 MHz NMR spectrometer (Bruker AVANE III HD; Fällanden, Switzerland) with a CPP BBO 500 CryoProbe. Deuterated chloroform ($CDCl_3$) was used as solvent and tetramethylsilane (TMS) was used as reference standard. The ^{1}H- and ^{13}C- NMR spectra were collected at frequencies of 500.366 and 125.83, respectively. They were consistent with the previously published data (Mahidol et al., 1997).

3.2.2 Mass spectrometry (MS)

The structure of lupinifolin was also confirmed by its mass spectrum. The yellow lupinifolin crystals were dissolved in methanol (containing 0.1% formic acid) and injected directly to the electrospay ionization (ESI) source of a Bruker micro-TOF-Q mass spectrometer (Bremen, German). The ESI source was used in positive mode, and the scan range of the mass detector was 50-1500 *m/z*. The expected valued for detection of $[M+H]^+$at *m/z* is 407.1853 ($C_{25}H_{27}O_5$).

3.3 Antibacterial assays

3.3.1 Disc diffusion

Bacteria used in this study were obtained from Thailand Institute of Scientific and Technological Research (TISTR). The antibacterial activities of lupinifolin were evaluated with Gram-positive bacteria *S. aureus* (TISTR 1466), *S. epidermidis* (TISTR 518), *B. subtilis* (TISTR 008) and *B. cereus* (TISTR 687), and Gram-negative bacteria *E. coli* (TISTR 780), *Pseudomonas aeruginosa* (TISTR 781), *Enterococcus aerogenes* (TISTR 1540), *Salmonella typhi* (TISTR 292) and *Proteus mirabilis* (TISTR 100). The screening of the antibacterial activity was done by the disc diffusion method (Humeera et al., 2013). Bacterial suspensions were prepared by inoculating one loopful of a pure colony into Mueller-Hinton Broth (MHB), incubated overnight and diluted in 0.9% NaCl. Cell suspensions, which adjusted turbidity equivalent to that of a 0.5 McFarland standard (~10^8 cfu/ml), were inoculated on Mueller-Hinton Agar (MHA) plates by swabbing over the entire agar surface. Lupinifolin (25, 50, 75 µg/disc) was impregnated on filter paper discs (Whatman No.1, 6 mm diameter) and then placed on the previously inoculated agar plate. After 24 h of incubation at 37°C, the antibacterial activity was determined by measuring the diameter of the inhibition zones formed around the disc. Ampicillin (10 µg) and 0.1 N NaOH (10 µl) were used as positive and vehicle controls, respectively.

3.3.2 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A modified broth microdilution method according to Clinical and Laboratory Standard Institute Guidelines (CLSI, 2012) was used to determine the MIC and MBC of lupinifolin (Joycharat et al., 2013). Two-fold serial dilutions of lupinifolin

were made in MHB using 96-well flat-bottom microtiter plates. A suspension of mid-logarithmic growth phase bacteria in MHB adjusted to 5×10^5 cfu/ml was added to each well. The final concentrations of lupinifolin ranged from 0.25-32 µg/ml. Ampicillin and 0.1 N NaOH (at the same volume as for lupinifolin) were used as positive and vehicle controls, respectively. The MIC was considered to be the lowest concentration of the agents showing no visible growth of microorganism after incubation at 37°C for 24 h. The MBC determination was carried out by sub-culturing 20 µl from the broth with no growth onto MHA plates after 24 h incubation at 37°C. All tests were performed in triplicate independent experiments.

3.3.3 Time-course of inhibitory effect

Staphylococcus aureus cells at mid-logarithmic growth phase (1.8×10^8 cfu/ml: 100 ml) were incubated with lupinifolin at MIC in 250-ml flasks. The optical density was measured to compare the onset of inhibitory activity of lupinifolin to ampicillin for 24 h at 37°C.

3.4 Determination of the mechanism of action

3.4.1 Scanning electron microscopy (SEM)

Staphylococcus aureus cells at mid-logarithmic growth phase (1.8×10^8 cfu/ml) were treated with either 8 µg/ml lupinifolin or 0.25 µg/ml ampicillin for 1, 3, 6 h. After incubation in 37°C shaking incubator (200 rpm), the cells were spun down and MHB medium were removed. The cell pellets were spread on 0.1% gelatin-coated slides and air-dried for 15 min, and then fixed with 4% paraformaldehyde at 4°C for 1 h. After fixation, the specimens were washed with phosphate buffer solution (PBS)

twice and post-fixed with 1% Osmium at 4°C for 30 min. The samples were then washed twice with PBS at 4°C for 10 min and dehydrated twice with serial graded concentrations of ethanol (50, 70, 80, 90 and 95%) at 4°C for 7 min, followed by 100% ethanol. The samples were then dried to the critical point under CO_2 with Leica EM CPD300 dryer (Vienna, Austria) and stained with gold ions in a pressure metallic chamber. Microscopy was performed with a JEOL JSM-6010LV scanning electron microscope (Tokyo, Japan).

3.4.2 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was used to visualize the change in morphology at the membrane and cell wall ultrastructure of *S. aureus* after treatment with lupinifolin. TEM preparations were made in accordance with the previously reported method with slight modifications (Ghosh, Indukuri, Bondalapati, Saikia, and Rangan, 2013).

The bacterial samples were prepared similar to the SEM method. After lupinifolin treatment for 12 h, cells were gently washed with 0.1 M PBS (pH 7.2), fixed with 2.5% glutaraldehyde in PBS and rinsed with PBS. Post-fixation was then carried out with 1% osmium tetroxide (Electron Microscopy Sciences: Hatfield, PA, USA) in 0.1 M PBS for 2 h at room temperature. After washing in the buffer, the samples were dehydrated using sequential exposure for acetone concentrations ranging from 20 to 100%. Subsequently, infiltration and embedding were performed using Spurr's resin (EMS). Finally, the samples were sectioned using an ultramicrotome with a diamond knife and were mounted on copper grids. They were stained with 2% uranyl acetate and lead citrate. The samples were viewed with a JEM-1230 electron microscope (Tokyo,

Japan). The morphology of bacterial cells was observed and compared to ampicillin-treated cells as positive control.

3.4.3 Flow cytometry analysis

In this study, flow cytometry was used to measure bacterial cell membrane integrity. Bacterial membrane potential was determined by using the carbocyanine dye (3,3′-diethyloxacarbocyanine iodide; $DiOC_2$) according to the method previously described (Eun et al., 2012). Bacterial cells at mid-logarithmic growth phase (1×10^6 cells/ml) were resuspended in PBS and treated with lupinifolin (8 µg/ml) or ampicillin (0.25 µg/ml). Then, 10 µl of 3 mM $DiOC_2$ was added to each tube and mixed. The samples were incubated at room temperature for 15 min and then the signal was examined. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 5 µg/ml) was used to produce a positive depolarized control. The analysis of the cells was performed using a flow cytometer (FACScan; BD Biosciences, San Jose, CA, U.S.A.) equipped with CellQuest software (BD Biosciences).

3.4.4 DNA laddering assay

S. aureus cells (5×10^5 cfu/ml) were incubated with lupinifolin at MIC and MBC for 8 h. Then, genomic DNA of *S. aureus* cells were extracted by using the NucleoSpin Tissue kit (Macherey-Nagel, Germany). The DNA was electrophoresed in 0.7% agarose gel and visualized by MaestroSafe nucleic acid gel stain reagent (Maestrogen, USA). The gel was photographed under ultraviolet light. Autolysis cells by 0.05% Triton-X and untreated cells were used as positive and negative controls, respectively.

3.5 *In vitro* cytotoxicity assay

To compare the antibacterial activity and toxicity to mammalial cell, *in vitro* cytotoxicity tests of lupinifolin were performed as follows.

3.5.1 Hemolysis of rabbit red blood cells

To test the direct toxicity of lupinifolin on mammalian cell membranes, a hemolysis test was conducted using rabbit red blood cells (RBCs). RBCs (50% in PBS) were treated with lupinifolin at doses similar to those in the cytotoxicity test for 24 h in 96-well plates. After incubation, optical density at 600 nm was measured using a spectrophotometric microplate reader (Bio-Rad; Hercules, CA, USA).

3.5.2 Preparation of HepG2 cell culture

HepG2 cells obtained from American Type Culture Collection (ATCC, HB-8065) were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C, with 5% CO_2, and 95% relative humidity. The cells with good proliferation were digested and passaged with 0.25% trypsin-EDTA solution at 37°C for 5 min.

3.5.3 MTT assay

HepG2 cells were used to determine the cytotoxicity of lupinifolin by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cui, Zhang, Wang, Chen, Zhang, and Tong, 2014). The cells (2×10^5 cells/well) were seeded in triplicate into 96-well culture plates overnight. The medium was removed and replaced with fresh medium containing different concentrations of lupinifolin ranging from 5 to 100 µg/ml. After 24 h incubation, the media were discarded and 20 µl of MTT solution (5 mg/ml in PBS) were added to each well followed by incubation for 4 h at

37°C with 5% CO_2. The MTT solution was then carefully removed. The insoluble purple formazan products formed in living cells were dissolved by 100 µl of dimethyl sulfoxide (DMSO). Absorbance was read at 570 nm using a microplate reader (Bio-Rad). Cell viability was expressed as a percentage after comparison with the control group which was assumed to have 100% viability.

3.5.4 Trypan blue exclusion assay

HepG2 cells (2×10^5 cells/well) were prepared similar to the experiment for MTT assay. After treatment with various concentration of lupinifolin ranging from 0-100 µg/ml for 24 h, cells were harvested by digestion with 0.25% trypsin-EDTA solution at 37°C for 5 min. The cell suspension was mixed with an equal volume of 0.4% (w/v) trypan blue. The number of viable (unstained) and dead (stained) cells were counted by hemacytometer under a light microscope. The results were calculated and expressed as a percentage of live cells compared to control.

3.6 Statistical analysis

Data were expressed as means ± SD and the comparisons between different groups were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test, unless stated otherwise. A *p* value less than 0.05 was considered to show a statistically significant difference.

CHAPTER IV

RESULTS

4.1 Purification of lupinifolin

Lupinifolin extracted from the stem of *D. reticulate* (Figure 4.1A) in the present study is shown in Figure 4.1B. The active compound appeared to be needle yellow-shaped crystals. The crystals obtained were dissolved in ethanol and submitted to TLC. Figure 4.2 shows that the extracted lupinifolin was relatively pure, suggesting that it could be used for further analysis by NMR.

Figure 4.1 *D. reticulata* Craib. stem (A) and yellow needle-shaped crystals of the extracted lupinifolin (B)

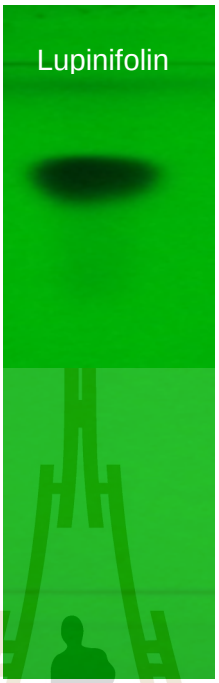


Figure 4.2 TLC chromatogram of the extracted compound detected by UV light at 254 nm.

4.2 Identification of lupinifolin

The purified of lupinifolin was identified by ^{1}H and ^{13}C NMR spectroscopic data as well as comparison with previously reported data (Mahidol et al., 1997). The spectra (appendix A and B) of the extracted lupinifolin was consistent with the published data as shown in Table 4.1.

Chemical structure of lupinifolin isolated from *D. reticulata* stem was illustrated in Figure 4.3. Its formula was confirmed by mass spectrometry. In the positive mode, $[M+H]^{+}$ at m/z 407.1850 (Figure 4.4) which was in accordance with the monoisotopic mass of lupinifolin (406.1780) was recorded. The purity of lupinifolin obtained from this study was more than 95% based on the NMR spectrum.

Table 4.1 Comparison of ^{1}H and ^{13}C NMR spectra of the extracted compound and lupinifolin.

Position	Yellow needle-shaped compound[a]		Lupinifolin[b]	
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
4	196.68		196.84	
7	159.75		160.13	
8a	159.53		159.44	
5	157.50		156.48	
4′	156.56		156.09	
3‴	130.99		131.11	
1′	129.62		130.60	
2′/6′	127.56	7.32 (d, 8.4)	127.66	7.31 (d, 8.4)
3″	125.91	5.50 (d, 10.0)	126.02	5.52 (d, 10.1)
2‴	122.56	5.14 (dd, 7.2,7.2)	122.40	5.16 (dd, 7.2,7.2)
4″	115.67	6.64 (d, 10.0)	115.53	6.64 (d, 10.1)
3′/5′	115.67	6.87 (d, 8.4)	115.53	6.89 (d, 8.4)
8	108.59		108.73	
6	102.70		102.79	
4a	102.69		102.61	
2	78.80	5.34 (dd, 12.8,2.8)	78.47	5.33(dd, 12.6,3.0)
2″	78.05		78.20	
$CDCl_3$	77.37			
$CDCl_3$	77.11			

Table 4.1 Comparison of ^{1}H and ^{13}C NMR spectra of the extracted compound and lupinifolin (Continued).

Position	Yellow needle-shaped compound[a]		Lupinifolin[b]	
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
$CDCl_3$	76.86			
3	43.23	3.04 (dd,17.6,12.8)	42.97	3.06(dd,17.1,12.6)
		2.80 (dd,17.6,3.0)		2.81 (dd,17.1,3.0)
6″	28.39	1.45 (s)	28.25	1.46 (s)
5″	28.29	1.44 (s)	28.33	1.45 (s)
4‴	25.80	1.65 (s)	25.78	1.66 (s)
1‴	21.47	3.21 (d, 7.2)	21.42	3.22 (d, 7.2)
5‴	25.80	1.65 (s)	25.78	1.66 (s)
5-OH		12.24 (s)		12.24 (s)

[a] Recorded in $CDCl_3$ at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR

[b] Recorded in $CDCl_3$ at 300 MHz for ^{1}H-NMR and 75.6 MHz for ^{13}C-NMR, cited in (Mahidol et al., 1997)

4.3 Antibacterial activity of lupinifolin

In order to investigate the antibacterial activity of lupinifolin, susceptibility of bacteria pathogens was evaluated by using the disc diffusion assay. It was found that lupinifolin, at 25, 50 and 75 μg/disc, inhibited growth of all Gram-positive bacteria tested, but not that of Gram-negative bacteria. The diameters of inhibition zone caused by lupinifolin compared to ampicillin as positive control are shown in Table 4.2.

Lupinifolin evidently possessed antibacterial activities against Gram-positive bacteria, therefore, they were further used to evaluate the MIC and MBC values by using the microdilution method. The MIC and MBC of lupinifolin against *S. aureus*, *B. subtilis* and *B. cereus* obtained from this study were found at 8 and 16 µg/ml, respectively. While the MIC and MBC of lupinifolin against *S. epidermidis* were at 16 and 32 µg/ml, respectively. The assay was carried out in triplicate. As found to be more often responsible for a nosocomial infection and higher incidence in drug resistance than the other pathogens tested, *S. aureus* was selected for further investigation.

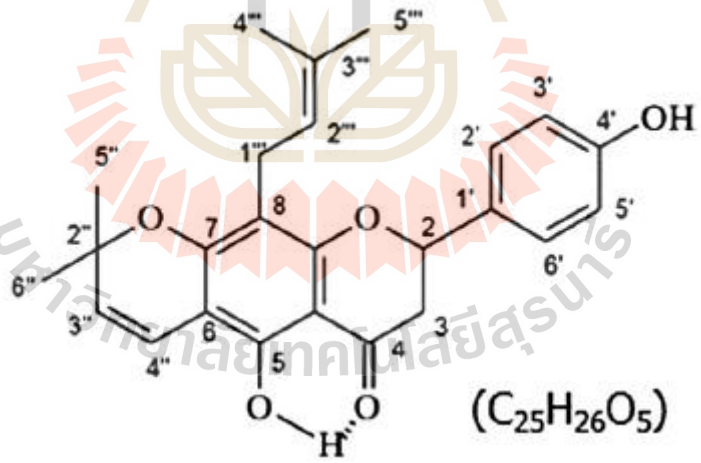


Figure 4.3 Chemical structure of lupinifolin (Soonthornchareonnon, Ubonpas, Kaewsuwan, and Wuttiudomlert, 2004).

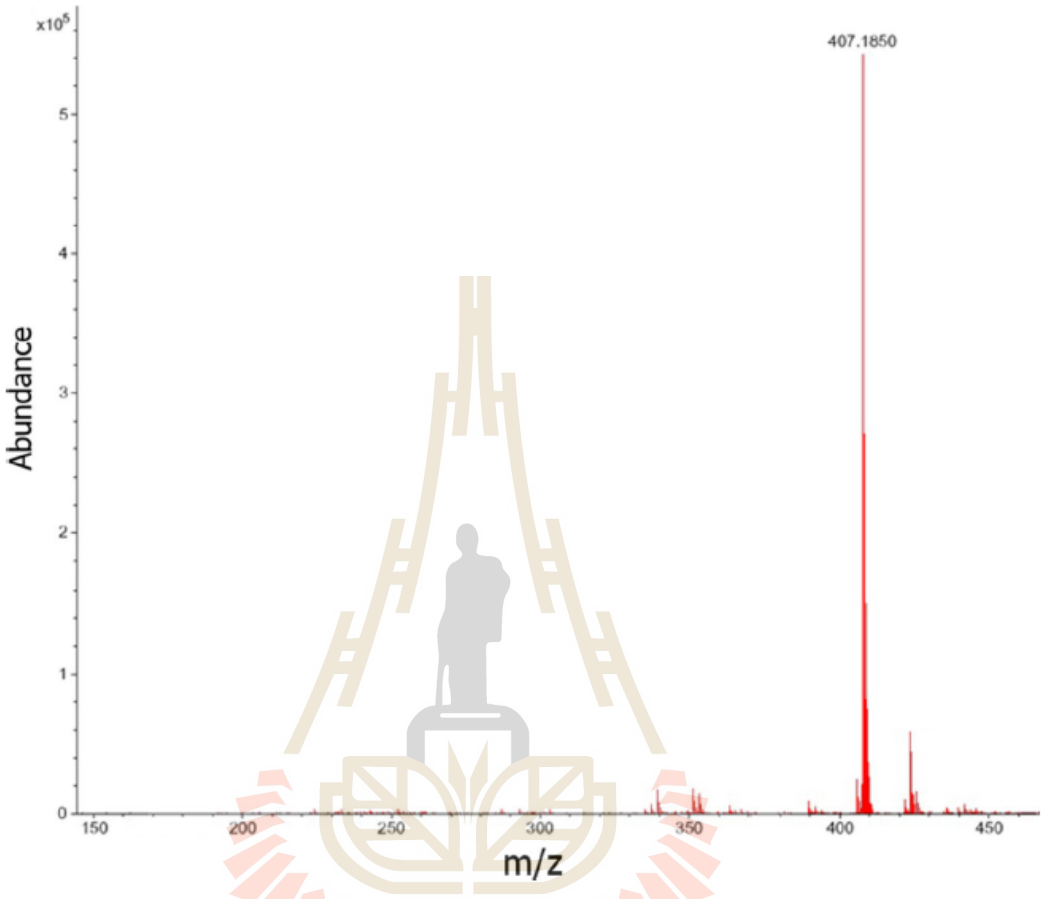


Figure 4.4 Mass spectrum of lupinifolin.

Table 4.2 Antibacterial activity of lupinifolin.

	Diameter of inhibitions zone (mm)			
	Lupinifolin (µg)			Ampicillin (µg)
Microorganism	25	50	75	10
Gram-positive				
Staphylococcus aureus	11 ± 0.6	15 ± 0.6	16 ± 0.7	37 ± 1.7
Staphylococcus epidermidis	14 ± 0.5	18 ± 0.6	21 ± 0.6	52 ± 1.1
Bacillus cereus	10 ± 1.1	13 ± 0.6	25 ± 0.6	13 ± 0.6
Bacillus subtilis	8 ± 0.1	11 ± 0.2	14 ± 0.5	28 ± 1.7
Gram-negative				
Escherichia coli	n.i.	n.i.	n.i.	20 ± 0.6
Enterobacter aerogenes	n.i.	n.i.	n.i.	n.i.
Salmonella typhi	n.i.	n.i.	n.i.	30 ± 0.6
Pseudomonas aeruginosa	n.i.	n.i.	n.i.	n.i.
Preteus mirabilis	n.i.	n.i.	n.i.	31 ± 1.1

n.i.; no inhibition zone, Data are means ± SD (*n*=3).

Table 4.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of lupinifolin from *D. reticulata* against Gram-positive bacteria compared with ampicillin.

Microorganisms	**Lupinifolin**		**Ampicillin**	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
S. aureus	8	16	0.25	1
S. epidermidis	16	32	0.25	0.25
B. subtilis	8	16	16	32
B. cereus	8	16	8	16

4.4 Time-course of inhibitory effect

Time-course effects curves was observed with lupinifolin and ampicillin. Lupinifolin evidently had faster onset than ampicillin. It inhibited the growth of *S. aureus* within the first hour of incubation, whereas ampicillin was seen to affect bacterial growth later at three hours of incubation (Figure 4.5).

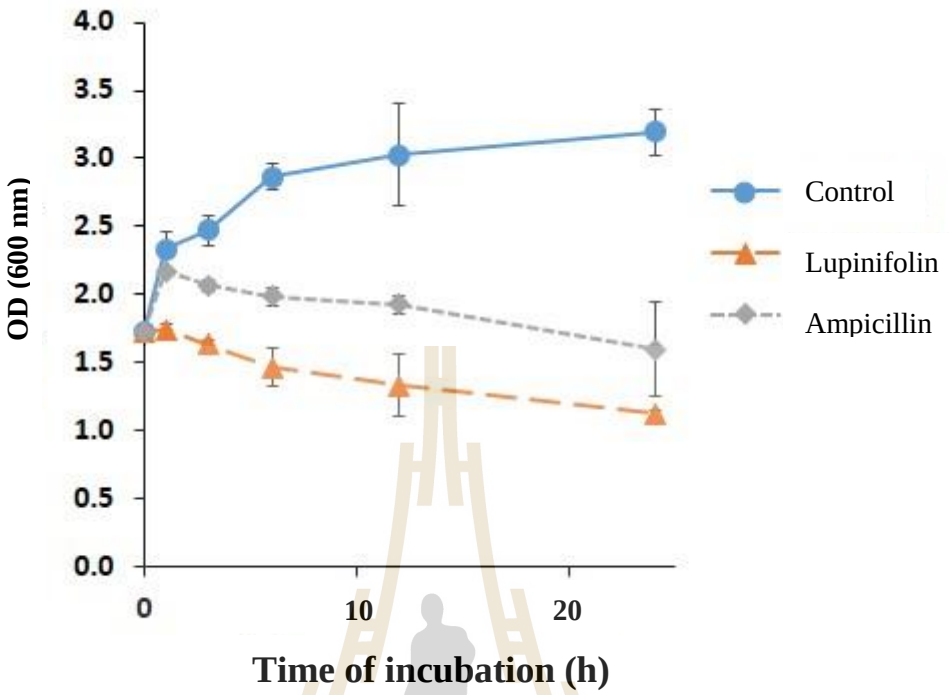


Figure 4.5 Time-course effect of lupinifolin. *S. aureus* bacteria cells were treated with lupinifolin (8 µg/ml) and ampicillin (0.25 µg/ml) as described in Materials and methods. The growth of bacteria was subsequently determined using spectrometry (600 nm) at 0, 1, 3, 6, 12 and 24 h of incubation. Values are expressed as mean ± SD (*n*=3).

4.5 Morphological changes of *S. aureus* treated with lupinifolin

S. aureus was treated with the lupinifolin at MIC (8 µg/ml) and incubated at 37°C for 6 h. SEM analyses were performed and compared to untreated and ampicillin-treated groups. Control bacteria in the absence of the extract showed regular morphology (Figure 4.6A, D, G), whereas cells treated with lupinifolin (Figure 4.6 B, E, H) and ampicillin (Figure 4.6C, F, I) appeared swollen and distorted after 3 h of incubation.

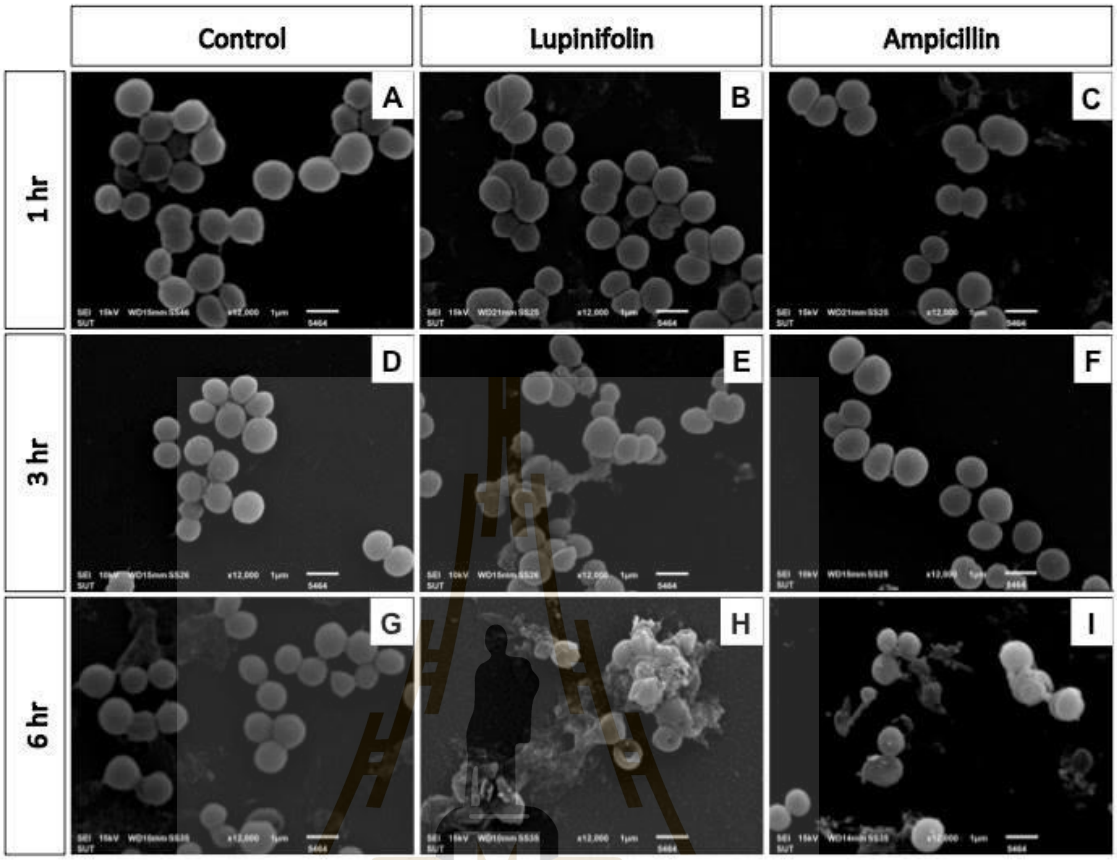


Figure 4.6 Scanning electron micrographs of *S. aureus* treated with lupinifolin. Cells were treated as described in ‘‘Materials and methods’’. The action of lupinifolin (8 µg/ml) was faster than that of ampicillin (0.25 µg/ml). At 1 h, abnormal morphology could not be seen in any treated cells (B, C) compared to control (A). The damaged cells were observed after treatment for 3 h only by lupinifolin (E), but not ampicillin (F). At 6 h of incubation, cells treated with lupinifolin and ampicillin were destroyed, as shown in (H) and (I), respectively, compared to regular shape of control (G). Enlargement: *bar* = 1 µm, 12,000×

TEM analysis was conducted and the data showed that after incubation with MIC for 12 h, lupinifolin, as well as ampicillin, obviously ruptured bacterial cell membrane and/or cell wall (Figure 4.7). Cell death and irregular shape of bacterial cells were seen in the treated groups, lupinifolin and ampicillin. Damage of cell wall and cell membrane of dividing cells were observed after 12 h of incubation with lupinifolin and ampicillin, compared with control.

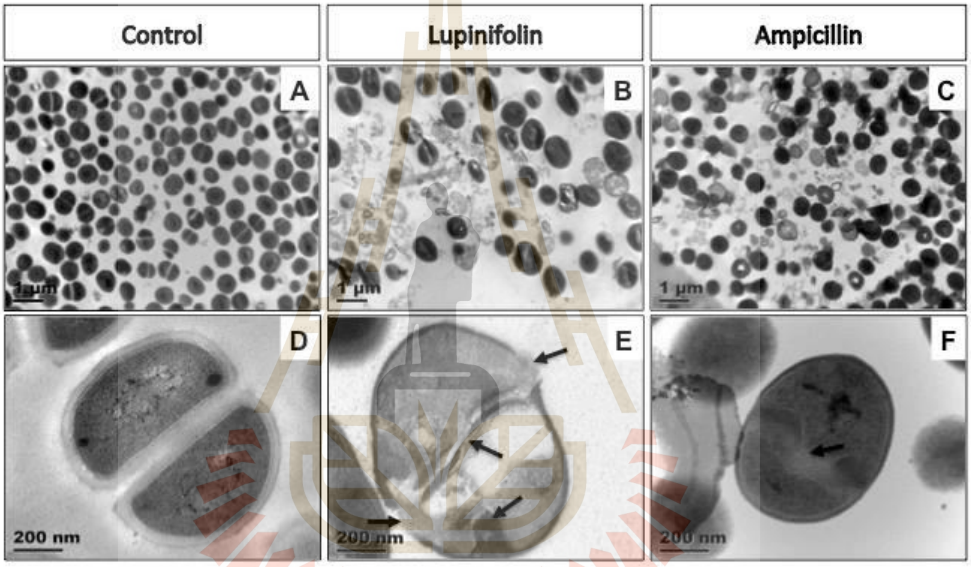


Figure 4.7 Transmission electron micrographs of *S. aureus* treated with lupinifolin. A-C overview of control and cells treated with lupinifolin (8 µg/ml) and ampicillin (0.25 µg/ml), respectively. Cell death and irregular shape of bacterial cells were seen in the treated groups, lupinifolin (B) and ampicillin (C). Damage to cell wall and cell membrane of dividing cells (indicated by *arrows*) were observed after 12 h of incubation with lupinifolin (E) and ampicillin (F), compared with control (D). Enlargement: *bar* = 1 µm, 10,000×; *bar* = 0.2 µm, 50,000×

4.6 Effect of lupinifolin on bacterial cell membrane

The fluorescent probe $DiOC_2$ was used to measure bacterial membrane potentials. Red/green fluorescence ratio of bacterial cells treated with CCCP and lupinifolin, but not ampicillin, dropped dramatically (Figure 4.8). Figure 4.8B showed a significant decrease in red/green ratio after only 15 min of treatment with CCCP and lupinifolin ($p < 0.05$) compared to control which indicated that membrane potential dissipated rapidly.

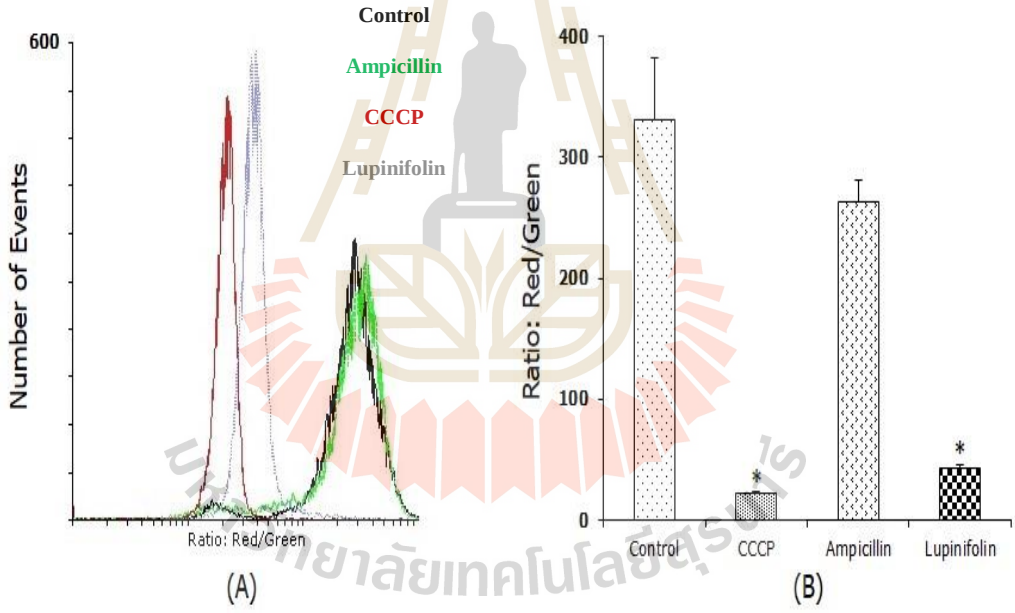


Figure 4.8 Effect of lupinifolin on membrane potential. (A) After incubation of 30 µM $DiOC_2$ in the presence of 8 µg/ml of lupinifolin for 15 min, red/green ratiometric histogram was shift to the left similar to CCCP (5 µg/ml), a known protonophore. (B) Red/green ratios were calculated using population mean fluorescence intensities. It was found that lupinifolin and CCCP, but not ampicillin (0.25 µg/ml), significantly reduced

the red/green ratio. *p < 0.05; statistically significant difference compared to control. Values are expressed as mean ± SD (n=3).

4.7 Results from DNA laddering assay

In this study, lupinifolin was found to have no effect on bacterial nucleic acid. The results shown in Figure 4.9 revealed that bacteria treated with lupinifolin at the concentration of MIC had no DNA fragmentation similar to untreated and ampicillin-treated cells. Whereas, bacteria treated 0.05% Triton X-100 gave smear band as DNA degradation occurred from autolysis.

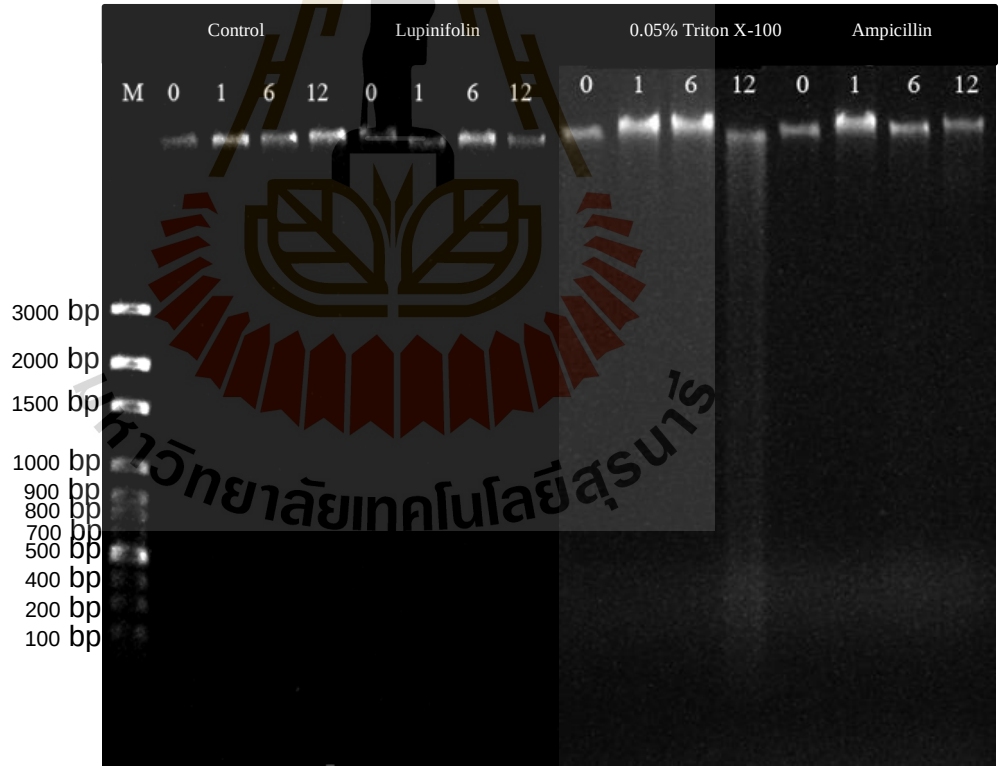


Figure 4.9 Results of DNA laddering assay. Bacterial DNA, islolated from *S. aureus*, were treated with lupinifolin (8 µg/ml), 0.05% Triton X-100 (positive control) and ampicillin (0.25 µg/ml) for 0, 1, 6 and 12 h, and then visualized on a 0.7% agarose gel

stained with MaestroSafe nucleic acid gel stain reagent. As seen in the figure, after 12 h of incubation, only 0.05% Triton X-100 caused DNA fragmentation. Lane M is marker.

4.8 Cytotoxicity of lupinifolin

4.8.1 Hemolytic effect on rabbit red blood cells

It was found that in the concentration up to 40 µg/ml, lupinifolin did not significantly disrupt RBC membranes after 24 hr of exposure (Figure 4.10), However, at the concentration ≥ 80 µg/ml, lupinifolin produced strong hemolysis effect at the first hour of incubation.

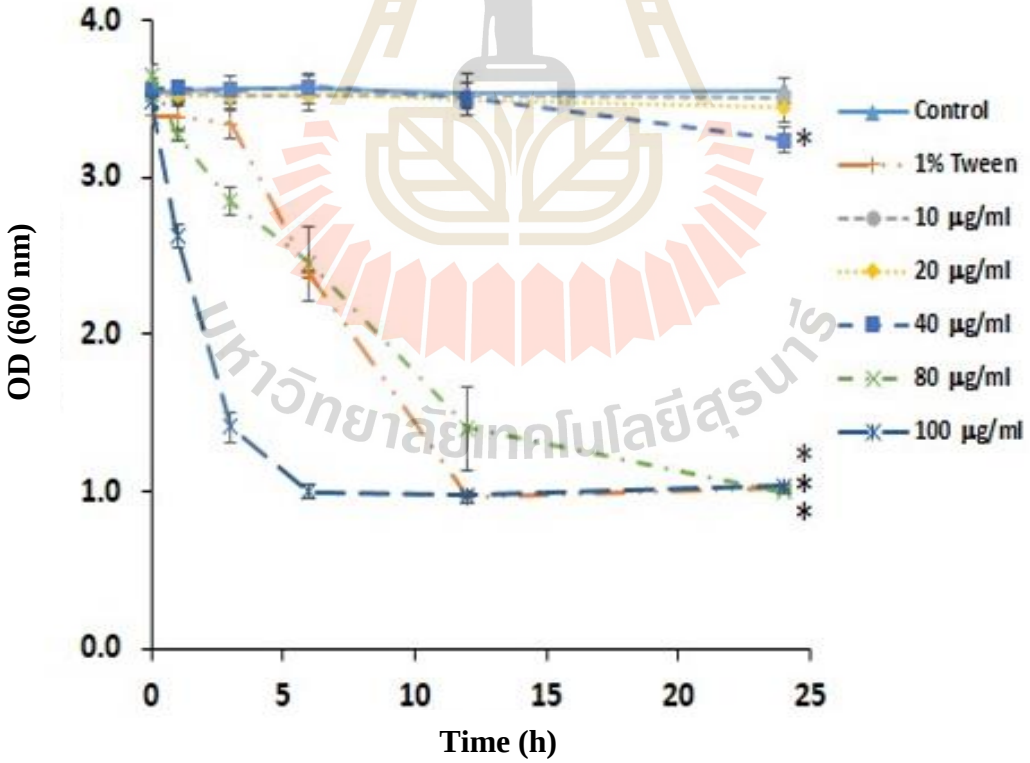


Figure 4.10 Toxic effect of lupinifolin on red blood cells. Hemolytic effect of lupinifolin on rabbit red blood cells (RBCs). Two-way ANOVA followed by Student-

Newman-Keuls test was used to analyse the data. It was found that lupinifolin at concentration of ≤ 40 µg/ml did not significantly affect the RBC after 24 h of incubation, whereas at concentrations of 80 µg/ml and higher caused RBC lysis in the first hour of incubation. Values are expressed as mean ± S.D. (n = 3).

* $p < 0.05$ statistically significant difference compared to control.

4.8.2 Cytotoxicity of lupinifolin on HepG2 cells

It was found that lupinifolin at MIC and MBC did not affect HepG2 cell viability (Figure 4.11). The IC_{50} of lupinifolin on cell viability measured by MTT and trypan blue exclusion assays were 78.3 ± 5.6 and 66.7 ± 13.3 µg/ml, respectively.

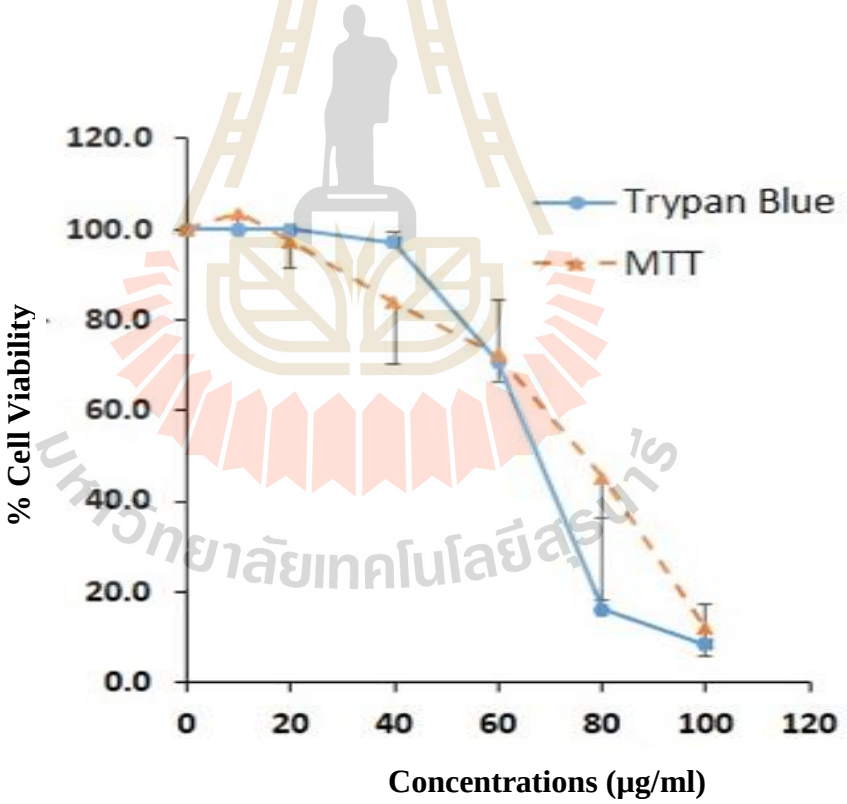


Figure 4.11 Effect of lupinifolin on cell viability of HepG2 cells measured by MTT and trypan blue assays. The calculated IC_{50}s were 78.3 ± 5.6 and 66.7 ± 13.3 µg/ml, respectively. Values are expressed as mean ± S.D. (n = 3).

* $p < 0.05$ statistically significant difference compared to control.

CHAPTER V

DISCUSSION AND CONCLUSION

In addition to the plant species mentioned earlier in Chapter I (page 3), lupinifolin has been found to be a constituent in at least ten more species, including *Citrus medica* (Chan, Li, Shen, and Wu, 2010), *C. limonia* (Chang, 1990), *Dorstenia mannii* (Ngadjui, Kouam, Dongo, Kapche, and Abegaz, 2000), *Euchresta formosana* (Matsuura, Iinuma, Tanaka, and Mizuno, 1995), *Tephrosia pumila* (Pethakamsetty, Seru and Kandula, 2010) and *Lonchocarpus guatamalensis* (Ingham, Tahara, and Dziedzic, 1988). It also occurs in plants of the genus *Derris*, such as *D. trifoliate* (Ntie-Kang, Onguene, Lifongo, Ndom, Sippl, and Mbaze, 2014), *D. scandens* (Ganapaty, Sumitra, and Steve, 2006) and *D. laxiflora* (Lin, Chen, and Kuo, 1991). This indicates that sources of lupinifolin are readily available in nature. Classical extraction techniques can be used to extract bioactive compounds from medicinal plants. Most of these techniques are based on the extracting power of different solvents, heating or mixing. In order to obtain bioactive compounds from plants, soxhlet extraction has widely been used for extracting valuable bioactive compounds from various natural sources (Azmir et al., 2013). This method is usually more efficient than simple refluxing and produces a higher yield of extract with less volume of solvent. In the present study, hexane was used to extract lupinifolin from *D. reticulata* stem by soxhlet extraction method. Crystallization method was used to further purify lupinifolin from the hexane extract. In the crystallization process, lupinifolin in hexane was concentrated to be a saturated

solution by heating. The saturated solution was then kept at room temperature with a cover, but not completely sealed to allow lupinifolin to gradually crystallize while the impurities and the other compounds remain in the solvent. The compound crystal was separated from solution by careful filtration. The purified lupinifolin was obtained as yellow needle-shaped crystal (Figure 4.1B).

In principle, TLC can be used to optimize conditions of column chromatography, including selective the mobile and solid phases, identify compounds isolated from column, as well as confirm purity of the isolates (Liu, 2011). The result from TLC (Figure 4.2) showed relatively high purity of the isolated lupinifolin.

The structure of lupinifolin, depicted in Figure 4.3, was verified by comparison of the NMR spectroscopic data in Table 4.1 with values in the previous report (Mahidol et al., 1997) which are perfect match. MS, an analytical technique that measures the molecular masses of chemicals and atoms precisely by converting them into charged ions, is one of the most powerful tools to study the structure of organic compounds (Liu, 2011). In this study, structure and formula of the obtained lupinifolin was confirmed by MS.

Because of its nonpolar structure, lupinifolin is very soluble in organic solvents, but sparingly soluble in water. Estimated from Kow (Octanol-Water Partition Coefficient), the water solubility of lupinifolin at 25°C is 0.009 mg/L (http://www.chemspider.com/Chemical-Structure.10305920.html). When dissolved in alcohol or dimethyl sulfoxide, it precipitates after dilution in aqueous buffer. This problem was similar to that encountered by a group of researchers who studied the effect of curcumin on 4-hydroxy-2-nonenal protein (Kurien and Scofield, 2007). To

avoid precipitation in aqueous media, nonpolar chemicals can be dissolved in acidic or basic solutions before dilution. In the present study, lupinifolin was freshly prepared by solubilizing in 0.1 N NaOH and immediately diluted in Müller-Hinton broth (MHB) for antibacterial test. With this method, lupinifolin remained soluble at all dilutions. The same volume of 0.1 N NaOH used in each experiment was also tested and found not to significantly affect the growth of bacterial cells compared to control.

It is widely known that phenolic compounds in plants comprise several groups of phytochemicals such as tannin, terpenoids and flavonoids. Over the years, the antibacterial activities of flavonoids have been increasingly documented. Plants in *Derris* genus have been reported to produce numerous flavones, isoflavones and flavanones. Therefore, not surprisingly, *Derris* plants such as *D. heyneana* Benth. and *D.trifoliata* Lour. have been found to possess significant antimicrobial, larvicidal, pesticidal, and anti-fungal activities (Ganapaty, Sumitra, and Steve, 2006; Jiang et al., 2012). Moreover, the extract from *D. scandens* stem has been found to possess antibacterial activities against *S. aureus*, *S. epidermidis* and *E. coli* (Sittiwet and Puangpronpitag, 2009). Previously, a group of Thai researchers has reported antibacterial activities of the crude hexane extract of *Myriopteron extensum* stem against Gram-positive bacteria, such as *S. aureus*, *S. epidermidis*, *B. cereus* and *B. subtilis* (Soonthornchareonnon, Ubonopas, Kaewsuwan, and Wuttiudomlert, 2004). It is suggested that lupinifolin may be an active compound because it possesses very strong antimicrobial activity against Gram-positive bacteria (Joycharat et al., 2013).

In the present study, antibacterial activities of lupinifolin were screened in eight different bacterial species by the disc diffusion method. In agreement with the previous

reports (Khaomek et al., 2008; Soonthornchareonnon et al., 2004), it appeared that only Gram-positive bacteria were susceptible to lupinifolin (Table 4.2). Of four species of Gram-positive bacteria tested, *Staphylococcus* seemed to be the most sensitive, as revealed by the inhibition zone. Because *S. aureus* is the most highly opportunistic Gram-positive bacteria tested, the MIC and MBC of lupinifolin against this microbe was further evaluated using the microdilution method, and were found to be 8 and 16 μg/ml, respectively (Table 4.3).

It has been suggested that there are three principal direct mechanisms of action underlying the antibacterial activities of plant flavonoids (Cushnie and Lamb, 2005) : (1) inhibition of nucleic synthesis, (2) inhibition of cytoplasmic membrane function, and (3) inhibition of energy metabolism. As shown by time-course effect curves (Figure 4.4), lupinifolin evidently had a faster onset than ampicillin. It inhibited the growth of *S. aureus* within the first hour of incubation, whereas ampicillin was seen to affect bacterial growth later, at 3 hours.This observation was confirmed by the data from SEM (Figure 4.6). SEM images showed some damages on bacterial morphology caused by lupinifolin and this effect occurred sooner than with ampicillin. The change in morphology of *S. aureus* was similar to that caused by ampicillin, suggesting that one of the targets of lupinifolin is the bacterial cell membrane or cell wall. To test our hypothesis, TEM analysis was conducted. The data showed that after incubation at MIC concentrations for 12 h, the morphology of bacteria treated with lupinifolin, as well as ampicillin, was changed compared to control (Figure 4.7A-C and with higher magnification Figure 4.7D-F) ; ruptured bacterial cell membrane and/or cell walls were observed. During cell division, cell wall synthesis is located between the daughter cells.

As indicated by an arrow in Figure 4.7F, the effect of ampicillin, an inhibitor of cell wall synthesis, was observed there, whereas the cell wall damages caused by lupinifolin was seen around the cell, as indicated by arrows in Figure 4.7E. In accordance with the fast onset of action, it is likely that the target of action of lupinifolin may be through disrupting the cell membrane, not interfering with cell wall synthesis as ampicillin does. This is because the inhibitory effect on cell wall synthesis needs more time than the direct interference effect on cell membrane structure. After damaging the cell membrane, which acts as a barrier for most molecules, bacteria degrade the cell ' s permeability control, resulting in an increase in intracellular pressure and subsequently destruction of the cell wall.

Flow cytometry is a laser-based technology for rapidly analyzing large numbers of cells or particles individually using light-scattering, fluorescence, and absorbance measurements. Flow cytometric assays have been developed to determine both cellular properties such as cell size, granularity of cytoplasm, cell viability, and membrane potential, and the characteristics of cellular components such as, DNA, RNA, total protein, lipid, enzyme activity, surface receptors, and intracellular calcium. This technique has been applied to characterize distinct physiological conditions in bacteria including responses to antibiotics, medicinal plants, pathogen-host interactions, cell differentiation during biofilm formation, and the mechanisms governing development pathways (Ambriz-Avina, Contreras-Garduno and Pedraza-Reyes, 2014). Many applications of flow cytometry are based on fluorescence monitoring. The cellular parameters can be measured using either intrinsic or extrinsic fluorescence. Different extrinsic fluorescent dyes have been used for analyzing different specific parameters in

flow cytometry. For example, 2′,7′-bis-carboxyethyl-5,6-carboxyfluorescein (BCECF-AM), 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), fluorescein isothiocyanate (FITC) and propidium iodide are for used monitoring intracellular pH, membrane potential, nucleic acid and protein, respectively.

To corroborate the postulated mechanism that lupinifolin directly acts on bacterial cell membrane, the fluorescent probe $DiOC_2$ was used to measure bacterial membrane potentials of *S. aureus*. When exposed to bacterial cells, molecules of $DiOC_2$ enter cells and reside either in the membrane or the cytoplasm. In normal cells, $DiOC_2$ emits green fluorescence at 530 nm, but the fluorescence shifts toward red at 576 nm as the dye molecules self-associate at the higher cytosolic concentrations caused by large membrane potentials. With higher cytosolic concentration, the ratio of fluorescent light emitted at $\lambda_{576}/\lambda_{530}$ (red/green ratio) increases. Conversely, when the bacterial membrane potential is dissipated by eliminating the proton gradient with proton ionophores such as CCCP, $DiOC_2$ cannot accumulate inside the cell and the red/green ratio consequently decreases. For several Gram-positive bacteria, including *S. aureus*, the $DiOC_2$ red/green ratio has been shown to vary with the magnitude of proton gradient (Probes, Revised: 13–May–2004). In the present study, the red/green fluorescence ratio of bacterial cells treated with CCCP and lupinifolin, but not ampicillin, dropped dramatically (Figure 4.8A). Figure 4.8B shows a significant decrease in red/green ratio after only 15 min of treatment with CCCP and lupinifolin ($p < 0.05$) compared to control, which indicated that the membrane potential dissipated rapidly. Antibiotics, including ampicillin, that do not target the bacterial membrane have been shown to decrease the potential over a longer period of exposure (Eun et al., 2012). The rapid

action of lupinifolin strongly suggests that the dissipation of membrane potential is due to its direct effect on the bacterial cell membrane.

CCCP is widely known as a protonophore whose structure is an aromatic compound with a negative charge. It collapses cell membrane potential by transporting protons across the membrane when it attaches to the molecule. Several flavonoids have aromatic structures with hydroxyl groups which are able to dissociate and produce negatively-charged molecules similar to CCCP. It is possible that the flavonoid lupinifolin, which also has an aromatic structure with a side-chain hydroxyl group, acts as an ionophore that moves protons and/or positive-charged molecules across lipid bilayers similar to CCCP.

The results found from this study appeared similar to curcumin I which was demonstrated to inhibit the growth of *S. aureus* (Tyagi, Singh, Kumari, and Mukhopadhyay, 2015). Curcumin is an important natural component of the rhizome *Curcuma longa* or turmeric. Propidium iodide uptake and calcein leakage assays were investigated by using flow cytometry technique. It is suggested that its mechanism of action of curcumin I is related to the damaging of bacterial cell membrane, thus impairing the permeabilization of bacterial membranes.

Programmed cell death is genetically regulated from apoptosis that involved in the development and viability of multicellular organisms. Extrinsic and intrinsic stimuli can trigger apoptosis such as radiation, oxidative stress and genotoxic chemical. Chromosome condensation, DNA fragmentation and phosphatidylserine exposure are demonstrated characteristic markers of apoptosis. This effect leads to membrane dissipation and loss of structural integrity. Therefore, bacterial cell death can be induced

by antibiotics that exhibit physiological and biochemical hallmarks of apoptosis (Dwyer, Camacho, Kohanski, Callura, and Collins, 2012). To examine whether the action of lupinifolin on cell membrane is not a consequence of DNA fragmentation caused by apoptosis, DNA laddering assay was performed. As shown in Figure 4.9, unlike 0.05% Triton-X 100, lupinifolin did not show any smear of bacterial DNA fragmentation after 12 h exposure.

To test the toxicity of lupinifolin against mammalian cell membranes, the hemolysis of rabbit red blood cells (RBCs) was examined by spectrophotometric microplate reader. As shown in Figure 4.10, lupinifolin at the concentrations up to 40 µg/ml did not significantly affect RBC membranes after 24 h of treatment. However, at concentration $\geq$ 80 µg/ml, lupinifolin produced a strong hemolysis effect in the first hour of incubation. The toxicity of lupinifolin in mammalian cells was further studied using HepG2 cells. Similar to the effect on RBCs, it is found that at MIC and MBC of lupinifolin did not affect HepG2 cell viability (Figure 4.11). The IC_{50}s of lupinifolin on cell viability measured by MTT and trypan blue exclusion assays were found approximately at 80 µg/ml which was ten times of MIC.

From the current *in vitro* experimental data, the margin of safety of lupinifolin seemed to be narrow, however, *in vivo* safety data of lupinifolin extracted from the same plant, *D. reticulata*, have been reported (Chivapat, Chavalittumrong, Attawish, and Soonthornchareonnon, 2009). Oral administration of lupinifolin in mice at high dose (5 g/kg body weight) showed no acute toxicity. No animal died after 14 days of drug administration. In the same study, a subacute toxicity study was performed in

Wistar rats for 28 days. The results showed that lupinifolin did not affect body weight, food consumption or animals' health.

Conclusion

In the current study, lupinifolin from *D. reticulata* Craib. stem was purified and characterized by spectroscopic techniques, NMR and MS. Its antibacterial properties were evaluated. Time course inhibitory effects and the data from SEM showed that lupinifolin produced reletively fast onset, suggesting the direct action on cell membrane rather than inhibition of cell wall synthesis. The TEM images clearly revealed that lupinifolin caused damages to the cell membranes of *S. aureus*. The compromised state of the bacterial membranes was further evaluated by flow cytometry analysis which confirmed the strong bactericidal action of lupinifolin. DNA fragmentation assay suggested that antibacterial activity of lupinifolin did not involve with bacterial DNA.

In conclusion, the mechanism of action underlying the antibacterial activity of lupinifolin against Gram-positive bacterial of lupinifolin is first reported here. The results obtained from this study provide direct evidence to support the hypothesis that lupinifolin inhibits bacterial growth by damaging the cytoplasmic membrane. The data suggested that lupinifolin may have the potential to be used as antibacterial agent. However, its *in vivo* efficacy needs further investigation.

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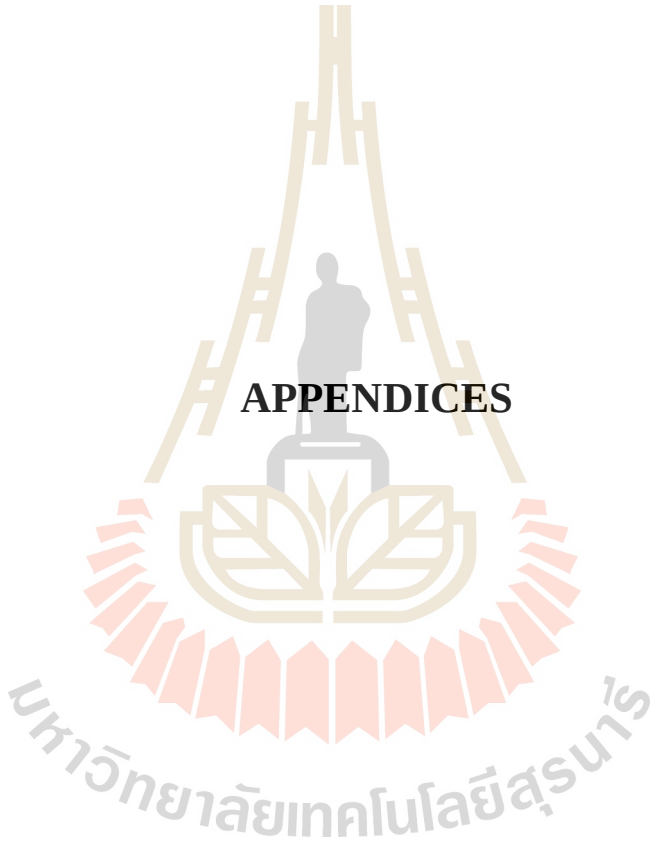
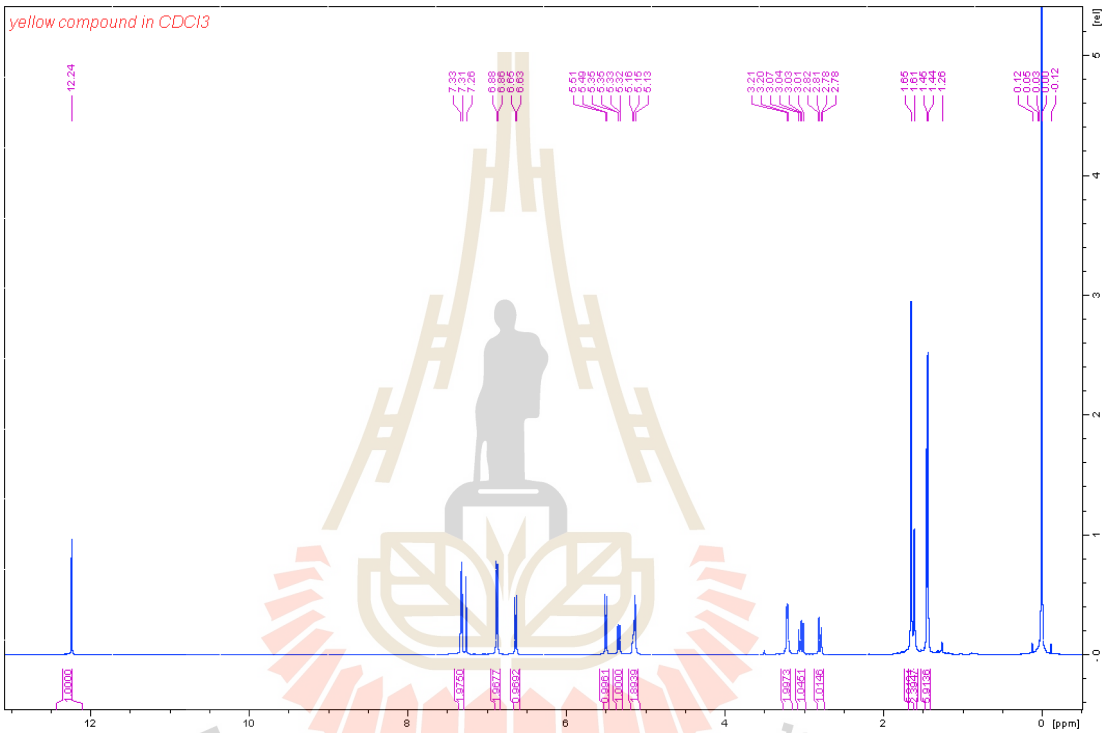
APPENDICES



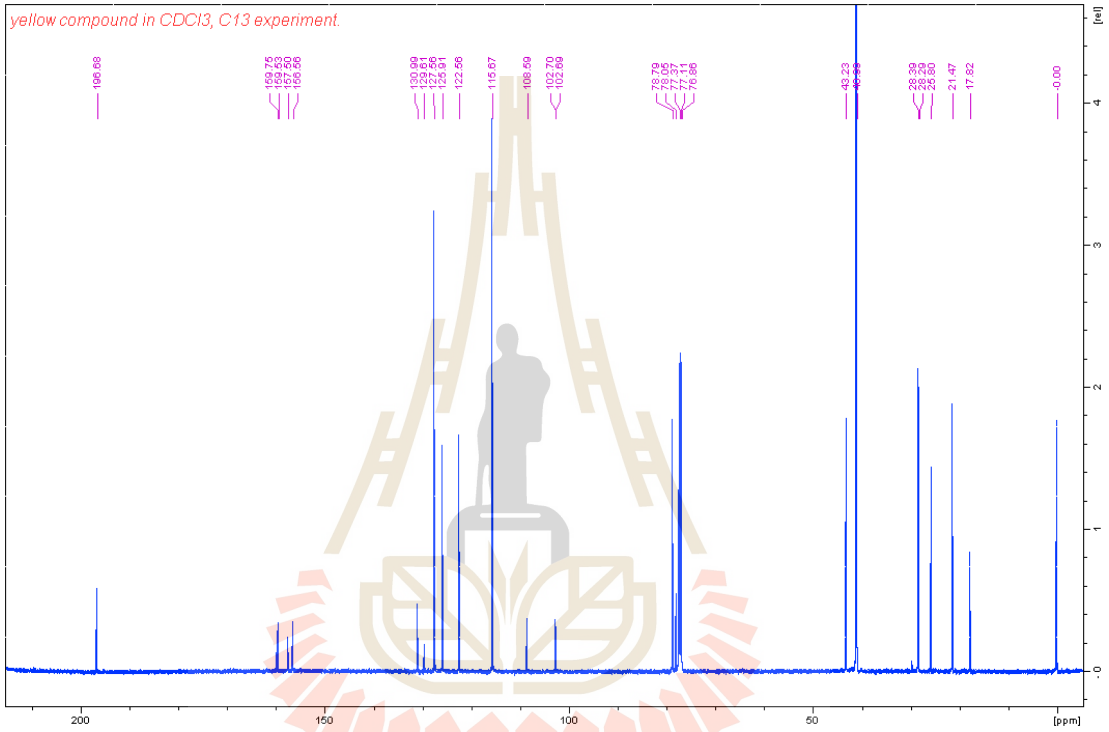
APPENDIX A

^{1}H NMR spectra of lupinifolin.



APPENDIX B

^{13}C NMR spectra of lupinifolin.



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