ฤทธิ์ต้านจุลชีพของสารสกัดหยาบจากกระถินทุ่งและคริซาซินต่อเชื้อสแตปฟิโลคอกคัส ออเรียส และ สแตปฟิโลคอกคัส เอพิเดอร์มิดิส จากการศึกษาแบบนอกกายและแบบในกายโดยใช้หนอนไหมไทย

นางสาววริษฐา สุขพานิช

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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ANTIMICROBIAL ACTIVITIES OF CRUDE EXTRACT OF *XYRIS INDICA* AND CHRYSAZIN ON *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS* IN *IN VITRO* AND *IN VIVO* THAI SILKWORM MODEL

Miss Warista Sukpanich

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	ANTIMICROBIAL ACTIVITIES OF CRUDE EXTRACT OF *XYRIS INDICA* AND CHRYSAZIN ON *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS* IN *IN VITRO* AND *IN VIVO* THAI SILKWORM MODEL
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วริษฐา สุขพานิช : ฤทธิ์ต้านจุลชีพของสารสกัดหยาบจากกระถินทุ่งและคริซาซินต่อเชื้อสแตปฟิโลคอกคัส ออเรียส และ สแตปฟิโลคอกคัส เอพิเดอร์มิดิส จากการศึกษาแบบนอกกายและแบบในกายโดยใช้หนอนไหมไทย (ANTIMICROBIAL ACTIVITIES OF CRUDE EXTRACT OF *XYRIS INDICA* AND CHRYSAZIN ON *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS* IN *IN VITRO* AND *IN VIVO* THAI SILKWORM MODEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : อ. ภก. ดร.สันทัด จันทร์ประภาพ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : อ. ดร.ชนิดา พลานุเวช, 103 หน้า.

S. aureus และ *S. epidermidis* สามารถก่อโรคได้ทั้งในชุมชนและในโรงพยาบาล โดยปัจจัยที่ทำให้เชื้อทั้งสองชนิดนี้สามารถรอดชีวิต เพิ่มจำนวนและอาศัยอยู่ในร่างกายของผู้ป่วยได้คือความสามารถในการสร้างไบโอฟิล์ม งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านจุลชีพ ฤทธิ์ยับยั้งการสร้างไบโอฟิล์ม รวมทั้งระยะเวลาในการฆ่าเชื้อ *S. aureus* และ *S. epidermidis* ของสารสกัดกระถินทุ่งและคริซาซิน จากการทดสอบฤทธิ์ต้านจุลชีพโดยวิธี broth microdilution พบว่าความเข้มข้นต่ำสุดของสารสกัดเอทานอลที่สามารถยับยั้งการเจริญของเชื้อ *S. aureus* และ *S. epidermidis* ได้ มีค่าเท่ากับ 0.5 และ 1 มก./มล. ส่วนความเข้มข้นต่ำสุดของคริซาซินที่สามารถยับยั้งเชื้อ *S. aureus* และ *S. epidermidis* ได้ มีค่าเท่ากับ 1 และ 2 มก./มล. ส่วนการศึกษา time kill พบว่าสารสกัดเอทนอลสามารถออกฤทธิ์ฆ่าเชื้อ *S. aureus* และ *S. epidermidis* ได้ในชั่วโมงที่ 8 และ 10 นอกจากนี้สารสกัดเอทานอลและคริซาซินยังมีประสิทธิภาพในการยับยั้งการสร้างไบโอฟิล์มซึ่งความสามารถในการยับยั้งการสร้างไบโอฟิล์มขึ้นกับความเข้มข้นของสารสกัดและคริซาซิน โดยสารสกัดเอทานอลและคริซาซินที่ความเข้มข้นตั้งแต่ MIC ขึ้นไป สามารถยับยั้งการสร้างไบโอฟิล์มของเชื้อ *S. aureus* และ *S. epidermidis* ได้อย่างมีนัยสำคัญทางสถิติ ($p < 0.01$) เมื่อเปรียบเทียบกับกลุ่มควบคุม ส่วนการศึกษาในสัตว์ทดลองการศึกษานี้ได้นำหนอนไหมมาใช้เพื่อทดสอบประสิทธิภาพการต้านเชื้อของสารสกัดและคริซาซิน พบว่าความเข้มข้นของสารสกัดเอทานอลและคริซาซินที่ทำให้หนอนไหมรอดชีวิตจากการติดเชื้อ *S. aureus* ได้ 50% มีค่าเท่ากับ 3.08 และ >5.00 มก./มล. ส่วนสารสกัดและคริซาซินที่ทำให้หนอนไหมที่ติดเชื้อ *S. epidermidis* รอดชีวิต 50% มีค่าเท่ากับ 5.33 และ >5.00 มก./มล. ดังนั้นจากการศึกษาสรุปได้ว่าสารสกัดเอทานอลของกระถินทุ่งและคริซาซินมีฤทธิ์ในการต้านเชื้อ *S. aureus* และ *S. epidermidis* ทั้งในหลอดทดลองและในสัตว์ทดลองโดยมีกลไกการต้านเชื้อส่วนหนึ่งผ่านทางการยับยั้งการสร้างไบโอฟิล์ม

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชวิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5476240633 : MAJOR PHARMACOLOGY

KEYWORDS : *STAPHYLOCOCCUS AUREUS* / *STAPHYLOCOCCUS EPIDERMIDIS* / *XYRIS INDICA* / CHRYSAZIN / SILKWORM

WARISTA SUKPANICH : ANTIMICROBIAL ACTIVITIES OF CRUDE EXTRACT OF *XYRIS INDICA* AND CHRYSAZIN ON *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS* IN *IN VITRO* AND *IN VIVO* THAI SILKWORM MODEL. ADVISOR : SANTAD CHANPRAPAPH, Ph.D., CO-ADVISOR : CHANIDA PALANUVEJ, Ph.D., 103 pp.

S. aureus and *S. epidermidis* are the common causes of both community and hospital-acquired infections. One of the key factors enable pathogens to survive, colonise and proliferate in body is the ability to form biofilm. The purpose of this study was to evaluate the antimicrobial activities, inhibitory effect of biofilm formation including time of killing *S. aureus* and *S. epidermidis* of *X. indica* and chrysazin, the major bioactive compound found in this plant. Antimicrobial activities were determined by broth microdilution method. MIC of the ethanol extract for *S. aureus* and *S. epidermidis* were 0.5 and 1 mg/ml, whereas MIC of chrysazin for *S. aureus* and *S. epidermidis* were 1 and 2 mg/ml. Time kill studies showed that the ethanol extract exerted bactericidal activity against *S. aureus* and *S. epidermidis* at 8 and 10 h. Furthermore, the ethanol extract and chrysazin also inhibited biofilm formation of *S. aureus* and *S. epidermidis*. The extract and chrysazin showed inhibitory effect on biofilm formation in a concentration dependent manner. At concentration equal and greater than MIC of the ethanol extract and chrysazin showed inhibitory effect on biofilm formation of bacterial pathogen with statistical significance ($p < 0.01$). *In vivo* study, silkworms were utilized for efficacy testing of compounds. ED_{50} of the ethanol extract and chrysazin were 3.08 and >5.00 mg/ml for *S. aureus*, whereas ED_{50} of the ethanol extract and chrysazin for *S. epidermidis* were 5.33 and >5.00 mg/ml. Therefore, these findings showed that the ethanol extract and chrysazin had antimicrobial activities on *S. aureus* and *S. epidermidis* both in *in vitro* and *in vivo*. Moreover, inhibition of biofilm formation may play an important role for the mechanism of action for antimicrobial activity shown.

Department : Pharmacology and Physiology Student's Signature .....

Field of Study : Pharmacology Advisor's Signature .....

Academic Year : 2013 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
°C	degree Celcius
C. elegans	*Caenorhabditis elegans*
C. albicans	*Candida albicans*
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
C. tropicalis	*Candida tropicalis*
C. neoformans	*Cryptococcus neoformans*
D. discoideum	*Dictyostelium discoideum*
DMSO	dimethyl sulfoxide
ED_{50}	Median effective concentration
EPS	extracellular polymer substance
et al.	et alibi (and others)
etc.	et cetera (and other things)
E. coli	*Escherichia coli*
h	hour (s)
L. pneumophila	*Legionella pneumophila*
LD_{50}	Median lethal dose
Log	Decimal logarithm
MBC	Minimum bactericidal concentration
mg	milligram
mg/ml	milligram per milliliter
mg/g larva	milligram per gram larva
MHA	Mueller-Hinton agar
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
min	minute

ml	milliliter
mm	millimetres
NaCl	sodium chloride
NAG, GlcNAc	N-acetylglucosamine
NAM, MurNAc	N-acetylmuramic acid
NCCLS	National Committee for Clinical Laboratory Stardard
nm	nanometer
P. aeruginosa	*Pseudomonas aeruginosa*
pH	log hydrogen ion concentration
R_f value	Retardation factor
S. aureus	*Staphylococcus aureus*
S. capitis	*Staphylococcus capitis*
S. epidermidis	*Staphylococcus epidermidis*
SD	Standard deviation
S. saprophyticus	*Staphylococcus saprophyticus*
S. simulans	*Staphylococcus simulans*
S. xylosus	*Staphylococcus xylosus*
TI	Therapeutic index
V. cholera	*Vibrio cholera*
v/v	volume by volume (ml/100ml)
w/v	weight by volume (g/100ml)
X. indica	*Xyris indica*
µg	Microgram
µl	microlitter
µm	micrometer
%	percent

CHAPTER I

INTRODUCTION

Background and rationale

The genus staphylococcus is gram positive bacteria, which colonize on human or animal skin and mucous membrane. This genus is composes of 32 species and is divided into two major groups namely coagulase-positive staphylococcus and coagulase-negative staphylococcus owing to their ability to produce the enzyme coagulase. The members of coagulase-positive staphylococcus are *Staphylococcus aureus* (*S. aureus*), *Staphylococcus delphini* (*S. delphini*) and *Staphylococcus intermedius* (*S. intermedius*). The members of coagulase-negative staphylococcus are *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus simulans* (*S. simulans*), *Staphylococcus capitis* (*S. capitis*), *Staphylococcus saprophyticus* (*S. saprophyticus*) and *Staphylococcus xylosus* (*S. xylosus*) etc. (Ryan and Ray, 2010). Among these species, *S. aureus* and *S. epidermidis* are the most frequently isolated member of the group of staphylococcus. In addition, *S. aureus* and *S. epidermidis* are always found associated with pathogenic potential in both immunocompetence and immunocompromise patients (Emanuel and Lorrence, 2009). They are the common causes of both community and hospital-acquired infections. *S. aureus* has a wide variety of pathogenicity, ranging from superficial and wound infections to deep and systemic infection, such as scalded skin syndrome, carbuncles, impetigo, wound infection, food poisoning, osteomyelitis, endocarditis and bacteremia etc. At present, *S. epidermidis* is becoming increasingly common causes of human infections especially in patients with medical devices in use, such as intravenous catheter infections, shunt infections, prosthetic joint infections and endocarditis etc. (Murray and Ken, 2002; Gillespie and Hawkey, 2006; Casey *et al*., 2007). Moreover, a critical virulence factors enable *S. aureus* and *S. epidermidis* to survive, colonise and proliferate in human body is the ability to form biofilms. Biofilms are complex microbial communities typically growing adherent to a biotic, such as collagen, fibrinogen and elastin etc.

or abiotic surface, such as shunt, catheter, prosthetic valve and endotracheal tube etc. (Stephen *et al*., 2011). The microorganisms within biofilm are protected against attacks from immune system and antibiotic treatment resulting in *S. aureus* and *S. epidermidis* infections difficult to eradicate and leading to recalcitrant infections later (Bryers, 2008). The ability of *S. aureus* and *S. epidermidis* to form biofilms presents a significant concern for the therapeutic treatment of these infections. Therefore, the development of alternative treatment to inhibit bacterial growth and biofilm formation is important need.

At present, researchers have increasing interest in the search of alternative antimicrobial agents and antibiofilm agents from medicinal plants. Since they have been used in folk medicine for a long time in treatment of infectious disease. Thus, they are safe and efficacious in human use. In previous study, some plants were reported to be able to inhibit the growth and biofilm formation of some pathogens such as *Melaleuca alternifolia* (tea tree oil) extract and *Apis mellifera* (propolis) extract. They could inhibit growth and biofilm formation of *S. aureus* (Hammer *et al*., 2005; Scazzocchio *et al*., 2006). Moreover, thymoquinone, an active principle of *Nigella sativa* had an effective antimicrobial and antibiofilm activities on *S. aureus* and *S. epidermidis* (Chaieb *et al*., 2011). *Xyris indica* (*X. indica*) was used in folk medicine for scabies, ringworm, leprosy and ulcer. Many active compounds have been isolated from *X. indica* including chrysazin, the major bioactive compound found in this plant (Ruangrungsi *et al*., 1993; Sukrong, 1994). Previous *in vitro* study demonstrated that *X. indica* had an antimicrobial activity against *S. aureus* and *S. epidermidis* (Chansukh, 2011). However, *X. indica* extract and chrysazin have not been studied *in vitro* and *in vivo* on efficacy and safety yet.

For *in vivo* studied, vertebrate animal model are utilized for further study about safety and efficacy of candidate compounds. Such animal are mice, rat, rabbit, guinea pig and monkey etc. However, all vertebrate animal models in the laboratory research are often involved with several limitations. One of such limitations is cost. It is not easy to examine therapeutic effect of a large number of candidate compounds at early stages of drug development because of a huge

amount of financial cost for taking care of mammals in laboratory facilities. Another important issue related to the use of large number of mammals for drug development is the ethical issue regulated by laws in each country (Kaito and Sekimizu, 2007). Therefore, in order to solve these problems, several research topics have been developed to use invertebrate animal as alternative infection model for candidate compounds screening. In the research, invertebrate animal models were used for screening about safety and efficacy of candidate compounds and the result obtained from invertebrate models could bring to the further study in vertebrate animal models.

Invertebrates have been used as an animal model for infectious diseases such as *Drosophila melanogaster* (*D. melanogaster*) have been reported to use for *S. epidermidis* and *Escherichia coli* (*E. coli*) infection model (Bernal and Kimbrell, 2000) and *Caenorhabditis elegans* (*C. elegans*) have been used as an animal model for studying *Pseudomonas aeruginosa* (*P. aeruginosa*) infection etc. (Mahajan *et al*., 1999). However, these animal models have shown some limitations on small body size. Therefore, injection experiments must be performed under a microscope. Recently, the researchers from the University of Tokyo, Japan have established silkworm (*Bombyx mori*) as an alternative animal infection model for efficacy testing because of microorganisms that are pathogenic to humans, such as *S. aureus*, *P. aeruginosa*, *Candida albicans* (*C. albican*), *Candida tropicalis* (*C. tropicalis*), *Cryptococcus neoformans* (*C. neoformans*), *Legionella pneumophila* (*L. pneumophila*) and *Vibrio cholera* (*V. cholera*) etc. have lethal effects in silkworms when injected into their hemolymph (mimics mouse intravenous administration). Antibiotics clinically used in humans to treat these microorganisms also have therapeutic effects in silkworms (Kaito *et al*., 2002; Hamamoto *et al*., 2004; Barman *et al*., 2008; Matsumoto *et al*., 2011). Interestingly, silkworms have already been reported as an animal infectious model to evaluate the therapeutic effects of the antiviral agent from traditional medicines and to study antimicrobial effects of chromogenic agents as well (Orihara *et al*., 2008; Fujiyuki *et al*. 2010). Furthermore, silkworms have several advantages in using as an animal infection model for studying microbial pathogenicity and the therapeutic effects of candidate

compounds such as low cost, less of ethical problems associated with the use of mammals, and a body size large enough to handle and inject sample solution into the hemolymph etc.

Objectives of the study

This study was to evaluate:

1. The antimicrobial activities of *X. indica* and chrysazin against *S. aureus* and *S. epidermidis*.

2. Time of killing of *S. aureus* and *S. epidermidis* of *X. indica* and chrysazin

3. The inhibitory effect of biofilm formation of *X. indica* and chrysazin on *S. aur*eus and *S. epidermidis*

Hypothesis to be tested

X. indica and chrysazin can inhibit growth and biofilm formation of *S. aureus* and *S. epider*midis.

Research Design

Experimental research

Scope of the study

This study was performed both *in vitro* and *in vivo* animal model. For *in vitro* study, present study was aimed to evaluate antimicrobial activity, inhibitory effect of biofilm formation and time of killing of *X. indica* and chrysazin against *S. aureus* and *S. epidermidis*. For *in vivo* study, silkworms were used as an animal infection model for studying *S. aureus* and *S. epidermidis* pathogenicity and the therapeutic effects of *X. indica* and chrysazin against these microorganisms.

Benefits from the study

Benefit of the study is to obtain the information of the crude extracts of *X. indica* and chrysazin on antimicrobial and antibiofilm activities against *S. aureus* and *S. epidermidis* in *in vitro* and *in vivo* Thai silkworm model. This information may lead to further step for development of antimicrobial drugs in vertebrate models such as mouse, rat and rabbit etc.

CHAPTER II

LITERATURE REVIEWS

2.1 Description of the genera and morphology

Staphylococcus is gram-positive coccus in the family Micrococcaceae. The cells on gram's stain of gram-positive coccus grow in a pattern resembling a cluster of grapes. However, these microorganisms in clinical material may also appear as a single cell, pairs or short chains. Most staphylococcus is 0.5 to 1 μm in diameter, non-spore forming and facultative anaerobic (able to grow both aerobically and anaerobically on solid media). They are capable of prolonged survival on environmental surfaces in varying conditions. The microorganisms are presented on the skin and mucous membranes of humans and animal. Staphylococcus is an important pathogen in humans both community and hospital-acquired infections. The species of staphylococcus most commonly associated with human disease are *S. aureus* and *S. epidermidis*. They are the cause of widely infections both in immunocompetence and immunocompromised patients such as systemic infection, skin and soft tissues infection, bones infection and gastrointestinal tract infection etc. (สมเกียรติ แลละคณะ, 2547; Murray and Ken, 2002; Harris and Richards, 2006)

2.2 Epidemiology

Sources of infection

Healthy carriers

Staphylococcus is ubiquitous and a part of the normal human flora. All healthy persons have staphylococcus on their skin and transient colonization. The anterior nares are the most frequent site of human colonization, including, the skin (especially when damaged), nasal, axilla, perineum, and oropharynx have also colonized. These colonization sites serve as a reservoir of staphylococcus strains for future infections (Greenwood *et al*., 2007).

Infected lesions

Large numbers of staphylococcus are disseminated in pus and dried exudate discharged from large infected wounds, burns and in sputum coughed from the lung of patients with bronchopneumonia. Direct contact is the most important mode of spread, but airborne dissemination may also occur (Greenwood *et al*., 2007).

Animals

Animals may disseminate staphylococcus and cause human infection, for example milk from a dairy cow with mastitis is causing staphylococcal food poisoning (Greenwood *et al*., 2007).

Thus, acquisition of staphylococcal infection may be exogenous (from external source) or endogenous (from lesion elsewhere in the patient's own body). In addition, staphylococcus can survive on dry surfaces for long periods. This microorganism can be transferred to susceptible person either through direct contact or contact with fomites (such as contaminated clothing, bed linens and medical devices etc.). Therefore, medical personnels must have hygiene to prevent the transfer of staphylococcus from themselves to patients or among patients (Murray and Ken, 2002).

2.3 Physiology and structure

Cell wall is an essential structure to the survival of many bacteria. In staphylococcus (gram-positive bacteria), cell wall or also called murein sacculus is thick approximately 60 to 80 nm and composed of several components (Figure 1) as follows.

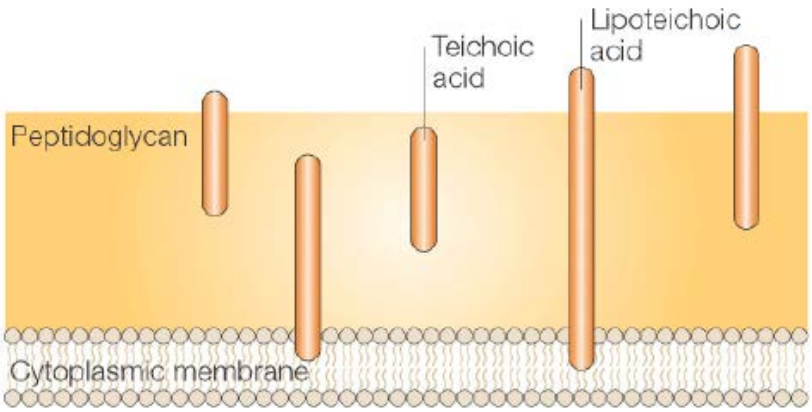


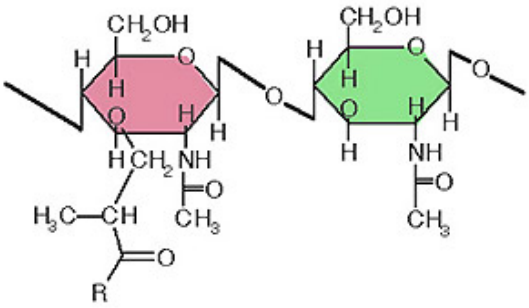
Figure 1 Structure and major components of staphylococcal cell wall including a thick and multilayered peptidoglycan sheath outside of the cytoplasmic membrane. Teichoic acids are linked to and embedded in the peptidoglycan, and lipoteichoic acids extend into the cytoplasmic membrane (Cabeen and Jacobs-Wagner, 2005).

Peptidoglycan

The cell wall consists of several layers of peptidoglycan that forms a mesh-like layer outside the cytoplasmic membrane of bacteria. Running perpendicular to the peptidoglycan sheets is a group of molecules called teichoic acids which are unique to the gram-positive cell wall. Peptidoglycan composed of an overlapping lattice of two sugar residues of β-(1,4) linked N-acetyl glucosamine (NAG or GlcNAc) and N-acetyl muramic acid (NAM or MurNAc) (Figure 2A). Each NAM is attached to a peptide side chains substituent of alternating L- and D-amino acid, for example the glycan chains in *S. aureus* are cross-linked with peptide side chains that composed of the amino acids L-alanine, D-glutamine, DAA (DAA represents a diamino acid such as L-lysine or m-diaminopimelic acid), and two D-alanines, whereas the peptide side chains of *S. epidermidis* consists of the amino acids L-alanine, D-glutamine, DAA, and D-alanine. Peptide side chains are then cross-linked with peptide bridges. This peptide bridges links the terminal COOH of D-alanine of one peptide to an NH_2 group of DAA on a neighboring glycan chain (Figure 2B). Cross-linking between amino acids in different linear amino sugar chains occurs

with the help of the enzyme transpeptidase and results in a 3-dimensional structure that is strong and rigid (Vollmer *et al*., 2008; Murray and Ken, 2002).

(A)



(B)

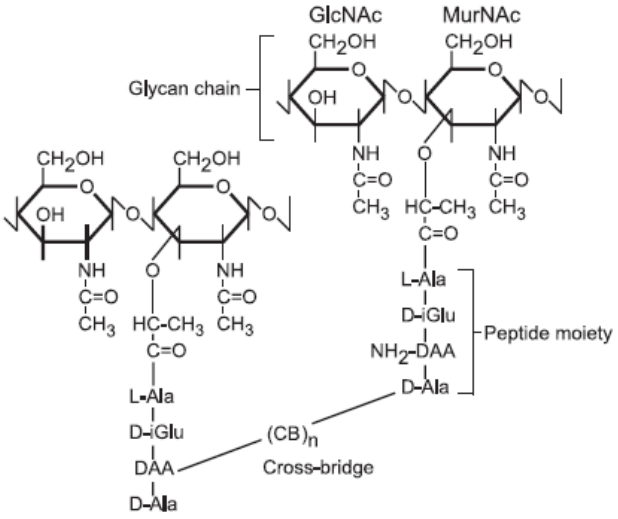


Figure 2 Structure of the peptidoglycan: (A) represents structure of NAM (MurNAc) and NAG (GlcNAc), a backbone of glycan chain, (B) shows a cross-linked between diamino acid (DAA) to the D-alanine (Hung *et al*., 2004)

The main function of peptidoglycan is to preserve cell integrity by withstanding the turgor pressure inside the cell. Inhibition of peptidoglycan biosynthesis of some antibiotics and degradation enzyme are cause of cell wall weaken and resulting in cell lysis. Furthermore, Peptidoglycan contributes to the maintenance of a defined cell shape and serves as a scaffold for anchoring other cell envelope components such as proteins and teichoic acids etc.

Teichoic acids

Teichoic acids are found within cell wall of staphylococcus and appear to extend to the surface of the peptidoglycan layer. They are phosphate containing polymers that are bound covalently to the peptidoglycan layer. Ribitol teichoic acid with GlcNAc residues (polysaccharide A) is present in *S. aureus* and glycerolteichoic acid with glucosyl residues (polysaccharide B) is present in *S. epidermidis*. Teichoic acids mediate the attachment of staphylococcus to mucosal surfaces through their specific binding to fibronectin. They also assist to provide rigidity to the cell wall (Murray and Ken, 2002; Emanuel and Lorrence, 2009).

Lipoteichoic acid

Structure of lipoteichoic acid contain chains of glycerol phosphate or ribitol and anchored to cell membrane via a diacylglycerol. Lipoteichoic acid has property as antigen. It is released from bacterial cells mainly after bacteriolysis and resulting in stimulation immune response (Talaro, 2009).

Cytoplasmic Membrane

Cytoplasmic membrane is made up of a complex of proteins, lipids and a small amount of carbohydrates. It serves as an osmotic barrier for cell and location for the transport of molecules into bacteria cell (Murray and Ken, 2002).

Coagulase

Coagulase has been identified in *S. aureus* that enables the conversion of fibrinogen to fibrin. Coagulase reacts with prothrombin in blood. The resulting complex is called staphylothrombin, which enables enzyme protease to convert fibrinogen to fibrin and resulting in

blood clot. In laboratory, this property is used to distinguish between different types of coagulase-positive staphylococcus strains and coagulase-negative staphylococcus strains (Murray and Ken, 2002).

Protein A

The surface of *S. aureus* strains (but not *S. epidermidis* strains) is uniformly coated with protein A. This protein is covalently linked to the peptidoglycan layer. It helps inhibit phagocytic engulfment and acts as an immunological disguise (Murray and Ken, 2002).

2.4 Characteristics for identification and subtyping

One of the most important phenotypical features used in the classification of staphylococcus is their ability to produce coagulase, an enzyme that causes blood clot formation. The species are currently recognised as being coagulase-positive staphylococcus, such as *S. aureus*, *S. delphini* and *S. intermedius*, whereas coagulase-negative staphylococcus such as *S. epidermidis*, *S. simulans*, *S. capitis*, *S. saprophyticus* and *S. xylosus* etc. Among other species, *S. aureus* and *S. epidermidis* are important pathogens in humans and the most frequently isolated members of the genus of staphylococcus (Ryan and Ray, 2010; Emanuel and Lorrence, 2009).

S. aureus

S. aureus forms fairly golden-yellow colonies on medium caused by the production of the orange carotenoid staphyloxanthin during their growth. *S. aureus* colonies are circular, 2-3 mm in diameter and shiny surface (Emanuel and Lorrence, 2009). It can colonise on the skin and anterior nares of individuals. A fundamental biological property of this bacterium is its ability to asymptomatically colonize healthy individuals. Thus, it is presumed to be important sources of infections when individuals have healthy weaken (Chambers and Deleo, 2009; Kluytmans *et al*., 1997). In addition, *S. aureus* is a major cause of many serious community and hospital-acquired infections. This pathogen can cause a wide variety of infections, which can be divided into three types (Murray and Ken, 2002).

(A) **(B)**

Figure 3 *S. aureus*: (A) scanning electron microscopy of *S. aureus* cluster, (B) colonies of *S. aureus* on mannitol salt agar (Pillai *et al*., 2012)

1. Superficial and wound infections

Impetigo

Impetigo is a superficial infection affecting mostly young children. It occurs primarily on the face and limbs. Initially, a small macule (flattened red spot) is seen and then a pus-filled vesicle (pustule) on an erythematous base develops. Crusting occurs after the pustule ruptures. Multiple pustule vesicles at different stages of development are common because of pathogen can spread to adjacent skin sites (Murray and Ken, 2002).

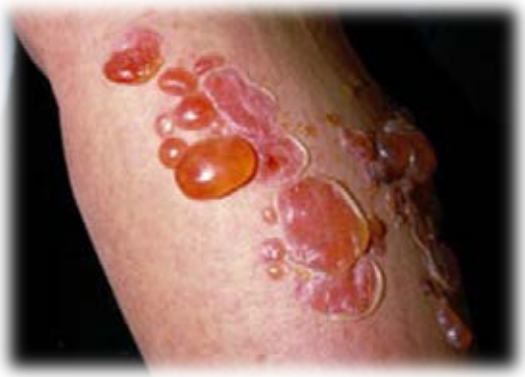


Figure 4 Impetigo caused by *S. aureus* (Oakley, 2013)

Folliculitis

Folliculitis is a pyogenic infection in the hair follicles. The base of the follicle is raised and reddened because of collection of pus beneath the epidermal surface. If this occurs at the base of eyelid, it is called a stye (Murray and Ken, 2002).

Furuncles

The furuncle or boil is a superficial skin infection that typically develops in a sebaceous gland or sweat gland. Blockage of content within the gland duct is causes leading to infection. Infection can spread from a furuncle with the development of one or more abcesses in adjacent subcutaneous tissues. This lesion, known as a carbuncle, is serious infection that may result in bloodstream invasion (Ryan and Ray, 2010).

Carbuncles

Carbuncles occur when furuncles coalesce and extend to the deeper subcutaneous tissue. Unlike patients with folliculitis and furuncles, patients with carbuncles have chills and fevers, indicating the systemic spread of staphylococcus via bacteria to other tissue (Murray and Ken, 2002).

Scalded Skin Syndrome

Scalded skin syndrome results from the production of exfoliative toxin in a staphylococcal lesion. The disease is most common in children. The face, axilla and groin tend to be affected first, but the erythema, bullous formation and subsequent desquamation of epithelial sheets, can spread to all parts of the body. However, the disease occasionally occurs in adults, particularly those who are immunocompromised. Milder version of what is probably the same disease is staphylococcal scarlet fever, in which erythema occurs without desquamation and bullous impetigo (Engleberg *et al*., 2007; Ryan and Ray, 2010).

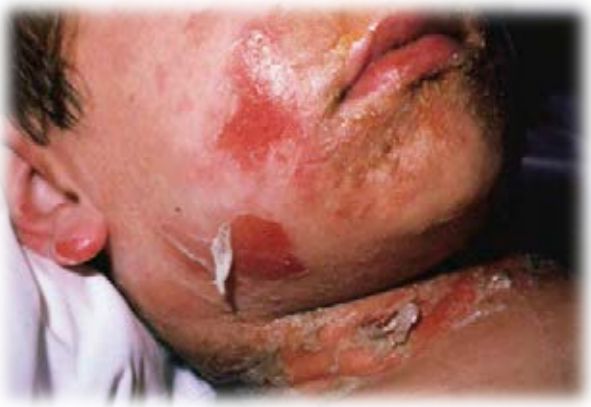


Figure 5 Staphylococcal scalded-skin syndrome (Lowy, 2009)

2. Systemic and life-threatening infections

Bacteremia

S. aureus is a common cause of bacteremia. Although bacteremia caused by most other organisms originate from an identifiable focus of infection, such as an infection of the lungs, urinary tract or gastrointestinal tract etc. but the initial foci of infection in approximately one third of patients with *S. aureus* bacteremia are not known. Most likely, the infection spreads to the blood stream from an innocuous-appearing skin infection. More than 50% of the cases of *S. aureus* bacteremia are acquired in the hospital after a surgical procedure or result from the continued use of a contaminated intravascular catheter. *S. aureus* bacteremia, particularly prolonged episodes, is associated with dissemination to other bodysite, including heart (Murray and Ken, 2002).

Endocarditis

Endocarditis caused by *S. aureus* is a serious disease, with a mortality rate approaching 50%. Although patients with *S. aureus* endocarditis may initially have nonspecific influenza-like symptoms, their condition can deteriorate rapidly with disruption of cardiac output and peripheral evidence of septic embolization (Murray and Ken, 2002).

Pneumonia

S. aureus respiratory disease can develop after the aspiration of oral secretions or from the hematogenous spread of the organism from distant site. Pneumonia is seen primarily in the young aged and patients with cystic fibrosis, influenza, chronic obstructive pulmonary disease and bronchiectasis. Radiographic examination reveals the presence of patchy infiltrates with consolidation or abscesses, the latter consistent with the organism's ability to secrete cytotoxic toxins and enzymes and to form localized abscesses (Murray and Ken, 2002).

Osteomyelitis and Septic Arthritis

S. aureus osteomyelitis can result from the hematogenous dissemination to bone, or it can be a secondary infection resulting from trauma or the extension of disease from an adjacent area. Hematogenous spread generally results from a cutaneous *S. aureus* infection and usually involves the metaphyseal area of long bones, a highly vascularized area of bony growth. This infection is characterized by the sudden onset of localized pain over the involved bone and by high fever. Blood cultures are positive in about 50% of cases.

Furthermore, *S. aureus* is primary cause of septic arthritis in children and in adults who are receiving intraarticular injections or who have mechanically joints. *S. aureus* arthritis is characterized by a painful, erythematous joint, with purulent material obtained on aspiration. Infection is usually demonstrated in the large joints (such as shoulder, knee, hip and elbow). The prognosis in children is excellent, but in adults, it depends on the nature of the underlying disease (Murray and Ken, 2002).

Toxic shock syndrome

The disease is initiated with the localized growth of toxin-producing strains of *S. aureus* in the vagina or a wound and followed by the release of the toxin into the blood stream. Clinical manifestations start abruptly including high fever, vomiting, diarrhea, hypotension and muscle pain. Within 48 h, it may progress to severe shock with evidence of renal and hepatic damage. In

addition, a skin rash may develop, followed by desquamation at deeper level than in scalded skin syndrome (Murray and Ken, 2002; Ryan and Ray, 2010).

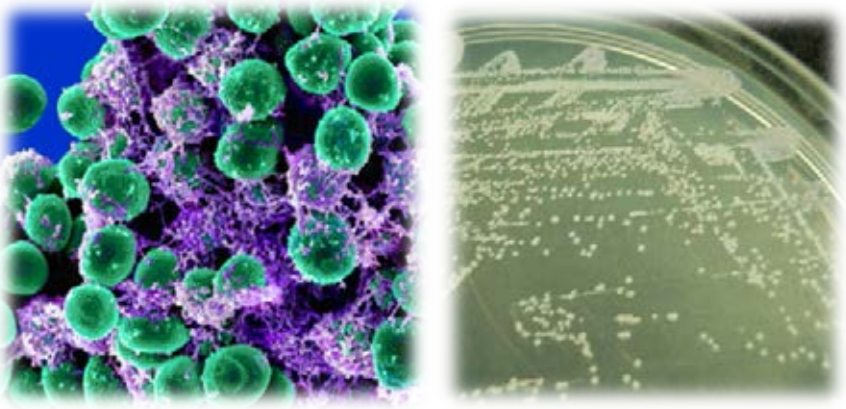
3. Other infection

Food poisoning

Food poisoning is intoxication resulting from the ingestion of food containing performed staphylococcal enterotoxins. Enterotoxins are fairly stable to heat and are not destroyed in food that is cooked at moderate temperatures after the toxin has been formed. After ingestion of contaminated food, the onset of disease is abrupt and rapid, with a mean incubation period of 4 h. Such symptoms as severe nausea, vomiting, diarrhea and abdominal pain. Sweating and headache may occur, but fever is not seen. Recovery generally occurs rapidly except in elderly and in those with another disease (Murray and Ken, 2002).

S. epidermidis

S. epidermidis is currently the most significant member of the coagulase-negative staphylococcus. This pathogen colonizes on the skin and mucous membranes of the human body (such as axillae, head, and nares etc.) and represents the major part of the normal bacterial flora of this habitat. It forms small, white and raised colonies approximately 1-2 mm in diameter after overnight incubation. *S. epidermidis* has gained substantial interest in recent years because it has become the most important cause of hospitals infections, especially in patients with predisposing factors such as indwelling catheters or implanted medical devices. Infecting this microorganism can originate from a number of sources, including the skin at the insertion site, colonisation of the medical device before implant, airborne contamination and microorganisms shed from staff and other healthcare workers. Examples of permanent and semi-permanent medical devices that relate to the infections are prosthetic heart valve, prosthetic joint, central venous catheter and urinary catheter. Currently, Clinical diseases caused by *S. epidermidis* are as follow (Gillespie and Hawkey, 2006; McCann *et al*., 2008).



(A) **(B)**

Figure 6 *S. epidermidis*: (A) scanning electron microscopy of *S. epidermidis* cluster, (B) colonies of *S. epidermidis* on agar plate (http://www.niaid.nih.gov)

Endocarditis

S. epidermidis can infect native and prosthetic heart valves. Infection of native valves is believed to result from the inoculation of organisms onto a damaged heart valve (such as a congenital malformation, damage resulting from rheumatic heart disease).

S. epidermidis is the most common cause of prosthetic valves endocarditis. The mortality rate from prosthetic valves endocarditis approaches 70%. Commonly, prosthetic valves endocarditis occurs within 60 days after implaning the device and is defined as early prosthetic valves endocarditis. It can occur in the first year after surgery, probably caused by inoculation of the organism at the time of surgery. It finally leads to an abscess of the mechanical valve ring. No sign and symptom is consistently diagnosis of prosthetic valves endocarditis, but fever is the most common finding (Gillespie and Hawkey, 2006; Török and Day, 2005).

Prosthetic Joint Infections

S. epidermidis can cause infection of artificial joints, particularly the hip. The patients usually only experience localized pain and mechanical failure of the joint. Systemic signs such as fever and leukocytosis are not prominent. Treatment consists of joint replacement and antimicrobial therapy (Murray and Ken, 2002).

Catheter and Shunt Infections

From 65% of all infections of catheters and shunts are caused by *S. epidermidis*. These infections have become a major medical problem because long-dwelling catheters and shunts are used commonly for the medical management of critically ill patients. *S. epidermidis* are particularly well adapted for causing these infections because they can produce a biofilm (polysaccharide slime) that bonds them to catheters and shunts and protects them from antibiotics and inflammatory cells (Murray and Ken, 2002).

Intravascular line infections

S. epidermidis is the commonest cause of intravascular line infections. Clinical evidence for sepsis includes inflammation, with or without a purulent discharge at the insertion site. Evidence of systemic infection and occasionally metastatic sepsis at other sites may be present (Gillespie and Hawkey, 2006).

2.5 Biofilm

At present, one of the key factors in the pathogenesis and bring about to survive, colonise and proliferate in human body of *S. aureus* and *S. epidermidis* is formation of stable biofilm on surface of body, prosthetic devices or medical devices. Biofilms are complex microbial communities typically growing adherent to a biotic surface of host (such as collagen, fibrinogen and elastin etc.) or abiotic surface (such as shunt, catheter and endotracheal tube etc.). They are generally porous with water channels that allow for the transport of water and nutrients to all cells within the biofilm and for excretion of waste. Biofilms have been implicated as the cause of serious infections that may be fatal to individuals. Although infections are not exclusively the result of biofilm formation, up to 70% of all human infections are caused by biofilm. *S. aureus* and *S. epidermidis* are recognized as the most frequent causes of biofilm-associated infections. In biofilm, *S. aureus* and *S. epidermidis* are protected against attacks from immune system and antibiotic treatment, making *S. aureus* and *S. epidermidis* infections difficult to eradicate and

resulting in recalcitrant infections. The other advantages associated with biofilm formation include protection from the external environment, nutrient availability, metabolic cooperation, and acquisition of new genetic traits (Stephen *et al*., 2011; Bryers, 2008; Otto, 2008).

Stages involved in biofilm development

The conversion from the planktonic (free-floating microorganisms) to biofilm (sessile) form occurs in several stages outlined in Figure 7.

1. Attachment

The first stage of staphylococcal colonization may either proceed as direct attachment of bacteria to uncoated plastic surface or attachment of bacteria to plastic surface coated with host-derived matrix proteins

Direct attachment of bacteria to uncoated plastic surface

The attachment of staphylococcal (the planktonic microorganism) to uncoated plastic material (biomaterials) is dependent on the physico-chemical properties of plastic polymer and bacterial surface for example hydrophobic interactions. As the plastic surface is hydrophobic, and the adhesion of staphylococcal has been shown to be similar on many investigated biomaterials, the main parameter determining bacterial adhesion is the hydrophobicity of the bacterial surface.

Attachment of bacteria to plastic surface coated with host-derived matrix proteins

In human body, the interaction between bacterial pathogen with host-matrix proteins represents an important mechanism of adherence. *S. aureus* and *S. epidermidis* express of so-called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) such as fibrinogen binding protein (Fib), fibronectin binding protein (Fnb) and collagen binding adhesion (Cna) that have the capacity to bind to human matrix proteins such as fibrinogen, fibronectin and often combine binding capacity for several different matrix proteins.

2. Intercellular adhesion

The formation of multicellular cell clusters on top of the monolayer of cells attached to plastic or host cells is the second stage of biofilm formation by *S. aureus* and *S. epidermidis*.

3. Maturation

Maturation of the *S. aureus* and *S. epidermidis* biofilm is characterized by the generation of a slime glycocalyx or extracellular polymeric substance, which encases surface bound organisms. It is able to increase the stability of the biofilm architecture, leading to a more robust structure and making *S. aureus* and *S. epidermidis* colonies difficult to treat by antibiotics and as a barrier to the host immune response. The mature biofilm structure reveals groups of microcolonies, often in mushroom-like forms surrounded by fluid-filled channels that are thought to deliver nutrients and oxygen to all cells in the biofilm and facilitate the removal of metabolic waste.

4. Detachment

In last step, individual bacterial cells, can leave a biofilm and spread from the film surface on the outer side of the mature biofilm to colonise distant sites. Dissemination of bacterial cells and the establishment of additional infecting sites are fundamental importance for the spread of biofilm-associated infection (Vuong and Otto 2002; Stephen *et al*., 2011; Otto, 2008).

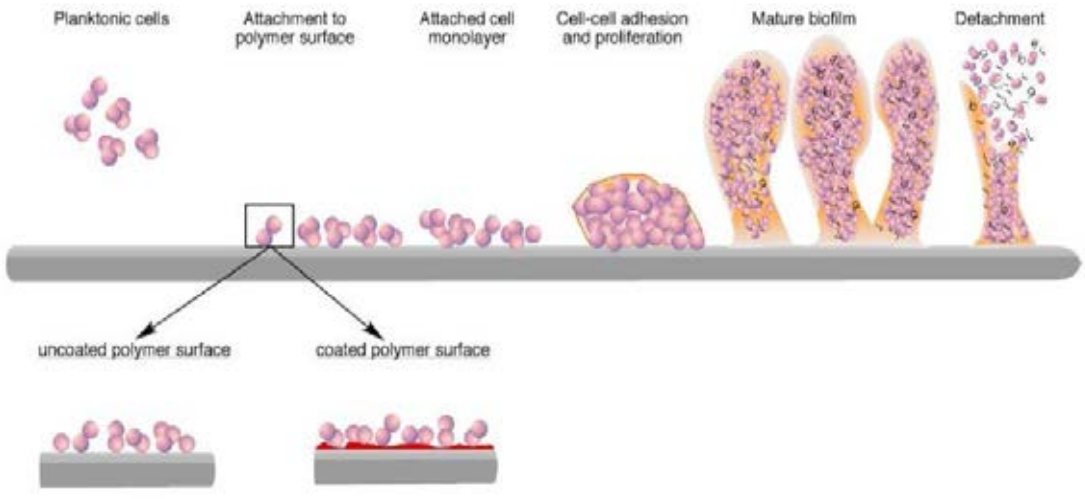


Figure 7 Stages of staphylococcal biofilm formation (Vuong and Otto, 2002)

Roles of biofilm

Biofilm-related with virulence factors and toxin production

In process of infections, bacteria have to orchestrate the expression of a group of molecules that determine pathogenicity, which are collectively known as the virulence factors and toxins. A delicate coordination of these usually species-specific virulence factors and toxins is crucial for the survival of the pathogen and the successful invasion of the host. Hence, pathogens have developed sophisticated regulatory systems to control virulence gene expression in during infection.

Among these regulatory systems, cell-cell communication, also known as quorum sensing system (QS), has gained broad attention in the scientific community. QS system uses small signaling molecules known as auto-inducing peptides (AIPs). At low cell population density, AIPs are low in concentration. When cells reach a certain population density and the AIPs accumulate to a threshold concentration, activation of the QS system occurs and triggers the direct transcription of target gene (*agr*, the accessory gene regulator quorum sensing system). The accessory gene regulator (*agr*) is genus specific. In *S. aureus* and *S. epidermidis* strains, *agr* controls the expression of a series of toxins, other virulence factors and immunoavoidance factors which interact with the innate immune system resulting in *S. aureus* and *S. epidermidis* can survive and proliferate in human body. (Kong *et al*., 2006; McCann *et al*., 2008; Archer *et al*., 2011)

Biofilm resistance to host defenses and antimicrobial drugs

Many of the protective mechanisms of biofilms toward host defenses, in particular those of innate host defense, have been analyzed in *S. aureus* and *S. epidermidis*. Mechanisms of innate host defense allow the human body to respond swiftly to an infection. It is triggered by the recognition of invariant structures on the bacterial surface, such as peptidoglycan, lipopeptides, lipoteichoic acids, or even some species-specific molecules such as the staphylococcal phenol-soluble modulins (PSMs). Innate host defense comprises mainly the activity of phagocytes, the

complement system and antimicrobial peptides (AMPs). However, several studies has been suggested early that the *S. aureus* and *S. epidermidis* "slime" substance, now called extracellular polymer substance (EPS) which enclose bacteria cells, is the main biological purpose to prevent ingestion of bacteria by phagocytes. Phagocytosis, mainly by neutrophils and macrophages, is a major mechanism by which the innate immune system eliminates invading microorganisms. It has been known for a long time that neutrophil and macrophages phagocytosis is impaired against staphylococcus in a biofilm (Fedtke *et al*., 2004; Stephen *et al*., 2011; Otto, 2012). Recent research has given more mechanistic insight, showing that *S. aureus* and *S. epidermidis* biofilms is protected from neutrophil and macrophages-dependent killing by staphylococcal complement inhibitor (SCIN) product resulting in complement inactivation via prevention of the deposition of C3b and immunoglobulin G (IgG). SCIN bound and stabilized C3 convertases, the proteolytic cleavage of C3 into C3a and C3b. C3b (opsonin) is a crucial complement in immunity, because the microbial surface-bound C3b product facilitate the recognition of foreign substances by phagocytic cells. Furthermore, biofilm protect the bacteria by inhibiting the chemotaxis of polymorphonuclear leukocytes, as well as by inhibiting proliferation of mononuclear cells after mitogen exposure. The EPS decreases the activity of antimicrobial peptides (AMPs), likely by hindering them from reaching their predominant target, the cytoplasmic membrane. These mechanisms also facilitate the adherence of bacteria to catheters and other synthetic materials (such as grafts, shunts, prosthetic valves and joints etc.) (Suzan *et al*., 2005).

The resistance of bacterial cells in a biofilm system to antimicrobial agents including antibiotics and disinfectants has been widely reported. As a consequence, biofilms that contaminate medical devices, manufacturing surfaces or fluid systems are extremely difficult to eliminate. Three mechanisms of biofilm resistance have been described in Figure 8 and these are believed to work in synergy to bring about reduced susceptibility of bacteria in biofilms. The first mechanism is the possibility of slow or incomplete penetration of the antibiotic into the biofilm. The EPS is a major feature of biofilms and is believed to play a major role in this resistance. The EPS is believed to work as a diffusion barrier, it prevents the complete penetration of antimicrobial drugs and macromolecules into the inner cells. A Second mechanism of antibiotic resistance is subpopulation of microorganisms within biofilm form a unique and differentiates

into a highly protected phenotypic state. Finally, the third mechanism depends on an altered chemical microenvironment within the biofilm. The depletion of a nutrient or accumulation of waste product might cause some bacteria to enter a non-growing state, in which they are protected from killing of antibiotics such as penicillin and vancomycin. Since these antibiotics are only active against growing bacteria cells. Therefore, they have not efficacious in eradicating biofilm infections. (Stewart and Costerton, 2001; Paraje, 2011)

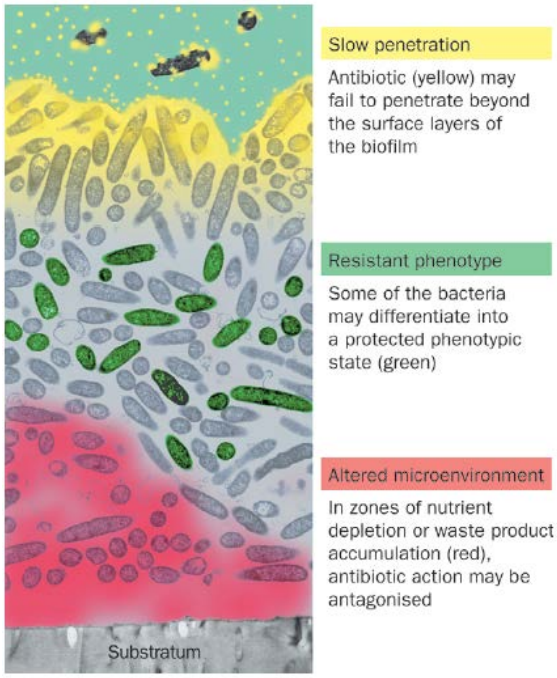


Figure 8 Mechanism of bacteria in biofilm resistance to antimicrobial drugs

(Stewart and Costerton, 2001)

As such, alternative strategies or more effective agents exhibiting activity against biofilm-producing microorganisms are of great interest. Natural agents could represent an interesting approach to limit the emergence and the spread of these organisms, which currently are difficult to treat. Recently, there has been considerable interest in the study of plant materials as sources of new compounds for processing into therapeutic agents.

Medicinal plants

The search for novel antimicrobial agents from natural plants has been of great interest in the last few decades because many infectious diseases have been known to be treated with herbal remedies for a long time. The study of plant extracts and bioactive compounds of plants have shown that many natural plants represent a potential source of alternative anti-infective agents for treatment infectious diseases (Cowan, 1999; Vijay *et al*., 2010). Xyris is a well-known plant in herbal medicine due to the therapeutic efficacy of its different species. One of the most important species of this genus is *Xyris indica* (*X. indica*) which has been used in herbal medicine. Other species of the genus Xyris have also been found to exert antimicrobial activity for example, the ethanol extracts of the aerial parts of *Xyris platystachia* and *Xyris pterygoblephara* had their antimicrobial activities evaluated against standardized strains of *S. aureus* and *Micrococcus luteus* (*M. luteus*) by agar diffusion assay (Ventura *et al*., 2007) and *Xyris pilosa* extracts have been reported against *S. aureus* (Cota *et al*., 2002).

2.6 *Xyris indica* (Kra thin Thung)

X. indica species is the scientific name of a genus of Xyris in the Xyridaceae or Yellow-eyed-grass family. Synonyms of *X. indica* are *X. capito*, *X. paludosa*, *X. robusta* and *X. calocephala*. It distributes in Thailand, Myanmar, India and Malaysia. In Thailand, this plant is found in provincial parts such as Udon thani, Khon Kaen, Chanthaburi and Prachin buri etc. The ecology of this plant is on open, swampy places along ricefields in the lowland up to 100 m altitude include in wet sand or muddy soil. Inflorescences can emerge on October to January (Sukrong, 1994; Swapna *et al*., 2011).

The characteristic features of *X. indica* are described as following: an annually marshy herb, 50-70 cm high; leaf simple, glabrous, radical, elongate, 0.6-0.8 cm broad, bifarious, narrowly loriform, narrowed to an obtuse apex; scape robust, longitudinal ridges, angles acute, spike 1.2-3.1 cm long; bracts many, broader than long, orbicular or cuneately obovoid, tumid,

shining, very coriaceous, margins scarious, lateral bracteoles dorsally winged, wing serrulate; flowers small, regular, yellow, solitary, sessile in the axils of rigid imbricating bracts of a terminal spike, bracteoles deciduous considered by some as sepals; petals 3, yellowish to yellow, long-clawed as long as sepals, limb orbicular, erose; stamens 3, filaments short and broad, inserted on petals, anthers oblong, 2-celled, dorsifixed, dehiscing dorsally, anther cells obtuse, acute at base, arms penicillate, ovary superior, unilocular, style short, arms long, stigmas terminal, truncate, ovules many on 3 parietal placentas, orthotropous; fruit a 1-chambered, loculicidally 3-valved capsule, seeds minute (Guofang and Kral, 2000; Jayaweera,1982).

Figure 9 *X. indica* (Jayaweera, 1982; สุรชัย, 2536)

Phytochemical and pharmacological properties

X. indica has been used in folk medicine as a natural remedy for various diseases such as scabies, ringworm, leprosy and ulcer. Many bioactive compounds have been isolated from this plant including three anthraquinones namely chrysazin, 3-methoxychrysazin and 3-hydroxychrysazin. In addition, isocoumarins and two phytosterols (stigmasterol and α-spinasterol) were isolated from the *X. indica* as well. Among such active compounds, chrysazin has been reported as the major bioactive compound found in this plant (58.90%) (Ruangrungsi *et al*., 1993; Sukrong, 1994). Pharmacological activities were reported for this plant extract such as antimicrobial activities against the growth of *S. aureus* through the inhibition zone (10.33±0.58 mm). Moreover, the extract also showed activities against the growth of *S. epidermidis* (inhibition zone 16.00±1.00 mm) (Chansukh, 2011). In addition, chrysazin, major bioactive compound found in this plant was reported that it could inhibit generating nitric oxide by inhibiting inducible NO synthase (iNOS) and also improve activities of macrophage phagocytosis and natural killer cell in leukemic mice (Lee, 2010; Chen *et al*., 2011).

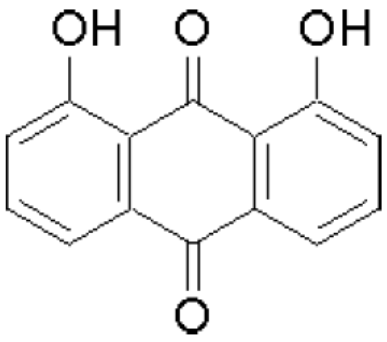


Figure 10 Structures of chrysazin (1,8-dihydroxyanthraquinone) (Lee, 2010)

2.7 Silkworm

Silkworm is the larvae of "*bombyx mori*" moths. Silkworm is domesticated from the wild silkmoth *Bombyx mandarina* which has a range from northern India to northern China, Korea, Japan, Thailand and the eastern regions of Russia. It is an economically important insect because of being a primary producer of silk. When silkworm has become putates, they will secrete a single protein strand as the silk from two gland on the top of the head making the cocoon is cropped for silk. Preferred food of silkworm is white mulberry leaves, but silkworm may also eat leaves of any other mulberry tree. Silkworm belongs to the class Insecta. The domesticated silkworm moth makes up the family Bombycidae and is classified as *Bombyx mori* species. The full genome of the silkworm was published in 2008 by the International Silkworm Genome Consortium. The genome size of the silkworm is approximately 432 Mb (The International Silkworm Genome Consortium, 2008). Recently, silkworm has been the target of scientific study. In several infectious research topics at the University of Tokyo, Japan, silkworms have been being used as an animal model such as infection model including bacterial, fungi and virus infection, natural immunity stimulation model, toxicity model and hyperglycemic model etc.

The life cycle stages of silkworm

Silkworm was born since time immemorial. The duration of the development stages can be controlled completely throughout the life cycle by regulating environmental conditions. Silkworm is holometabolous insect (complete metamorphosis). In time about 6-8 weeks, they complete their life cycle of four different metamorphosing phases consist of egg, larva, pupa and adult (moth). The life cycle of silkworm is presented in Figure 11. The first stage of silkworm starts with a little egg laid by female moth. One female moth lays as many as 300-600 eggs in one night, during gestation (approximately two weeks). Then, the small eggs hatch and grow as the larva, the larva stage is growing through 5 steps consist of first instar to fifth instar larva. The larva just after hatching has many setae on the body surface and the body color is black in normal strains. The young larva is about 10 mm in length. At about two days after hatching, the setae on

the body surface become less conspicuous. After one day the body length reaches about 20 mm and the surface of the skin become glossy. The larva stops eating for 24 h (molting). During this time the larva produces a new cuticle and sheds the old one. This phenomenon of shedding the old skin is called ecdysis. Since molting is repeated 4 times during the larval period, there are 5 feeding periods or instars. The duration of molting is rather constant among different strains. In the 5^{th} instar, larva attains a maximum length of about 50-60 mm and eats voraciously. It is interesting that the fifth instar larva body grows completely and functions actively. This stage has a long period about 20-24 days. When the molting in larva stage reaches to 4 times, the larva fully develop and stop eating. The larva skin becomes transparent and the caterpillar begins to putate. The larva does spinning a cocoon around itself. This process is called mounting. This step often takes more than 3 days. When larva transforms to a pupa, drastic changes occur in various tissue and organs. Several larva tissues and organs are lost and anlages rapidly grow and differentiate into the adult. The last stage starts after two to three weeks in the pupa stage and the adult silkworm so called silkmoth appears from the cocoon. This stage the life span of the silkmoth is about four to six days. When the silkmoth appears from the cocoon, firstly male moth hatches out and moves rapidly around to search for female moth. Female moth hatches 2 days later. Female moth releases the pheromone to attract male moth for mating and stands mating for about one day. After mating, female moth will separate and lay the yellow eggs and dies after laying eggs. In this stage, all organs and tissues are in the final state of differentiation. (Tazima, 1978; ประทีป และคณะ, 2549).

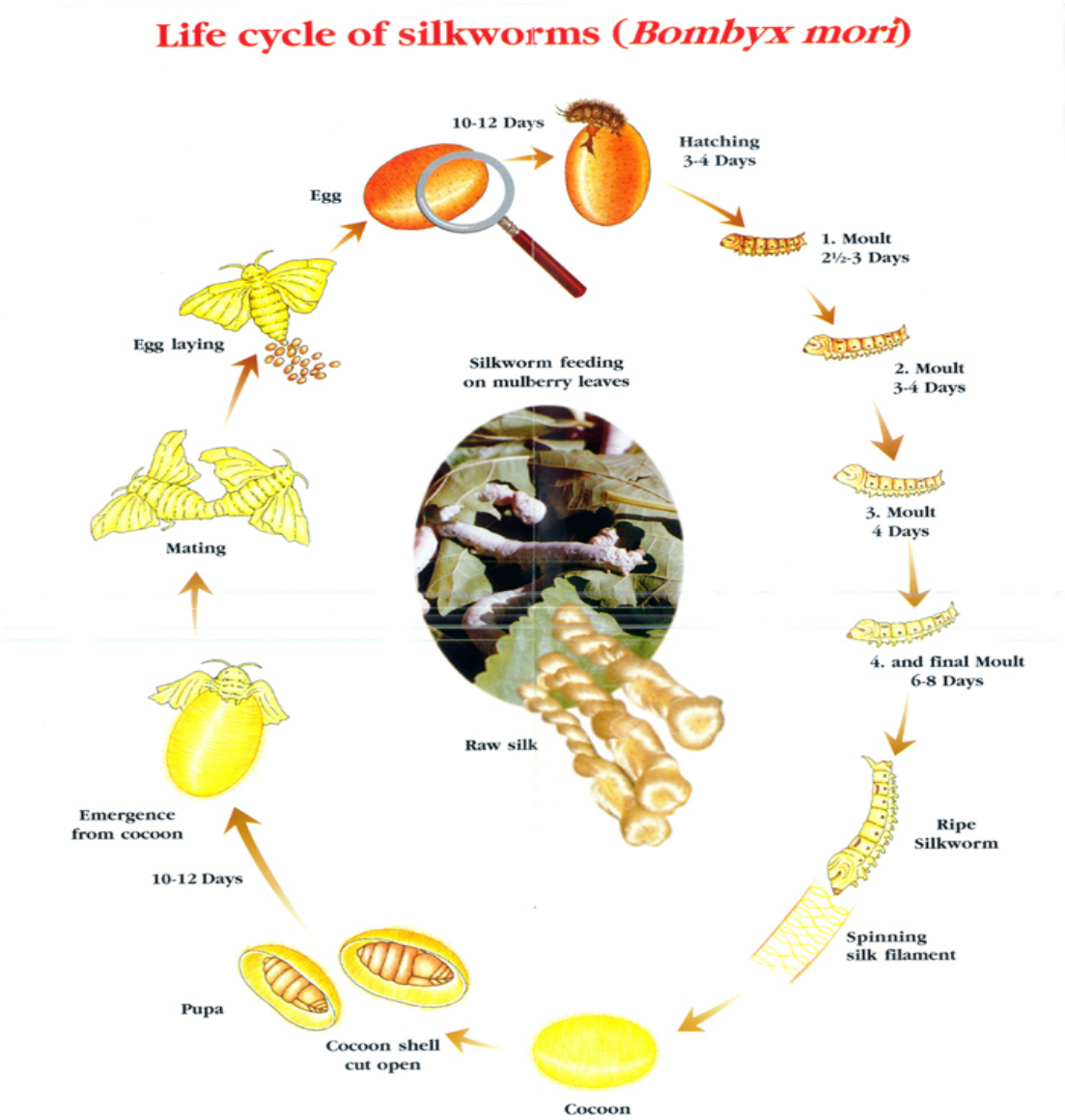


Figure 11 Life cycle of silkworm, *Bombyx mori* composed of 4 major stages including egg, larva, pupa and moth. (Martin, 2010)

Anatomy and physiology

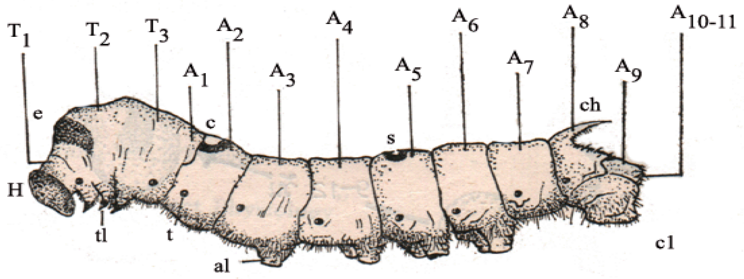
The larva body

Regions of silkworm body are divided into three principle parts, namely, head, thorax and abdomen.

Head has two sets of 6 ocelli which are situated just behind and downward on the left and right face. Antennae are situated a little under the ocelli. The antenna is composed of three short segments. The mouth parts are located downward and in front of the face and are composed of a pair of mandibles and maxillae with labrum and labium. The mandibles are used for mastication and consist of two hard pieces. The maxilla consists of a single maxillary lobe and the maxillary palpi, which is made up of three segments. The labrum hangs down from the frontal portion to form a flap of the mouth. The labium is situated on the ventral region of the head and has a pair of labial palps which form a sensory organ. A spinneret is situated at the proximal position between the two labial palpi.

The thorax comprises three segments with one pair of spiracles and three pairs of thoracic legs. The abdomen is constructed of 11 segments with 8 pairs of spiracles, 4 pairs of abdominal legs, 1 pair of caudal legs and 1 caudal horn (Tazima, 1978)

(A)



T_1 - T_3 ; Thoracic segment , A_1 - A_{11} ; Abdominal segment ,
H ; Head, e; Eye spot , c; Crescents , s ; Star spot,
tl ; Thoracic legs , al ; Abdominal legs, cl ; Caudal legs,
ch ; Caudal horn

(B)

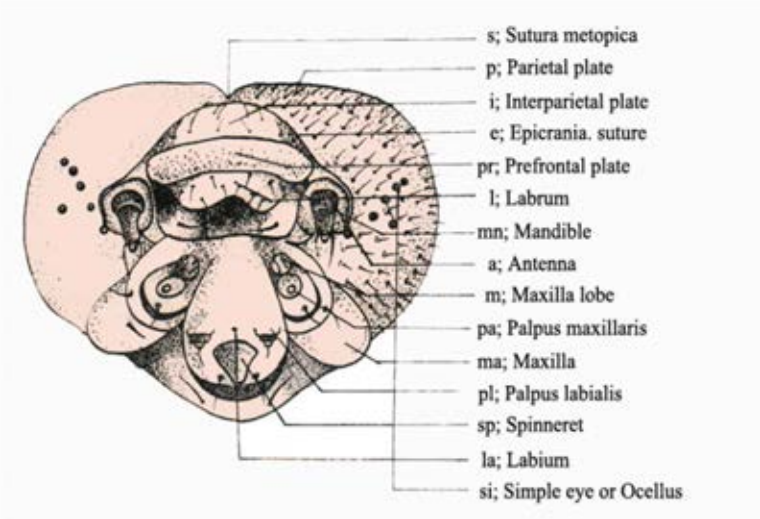


Figure 12 Structure of fifth instar larva just after molting. (A) external region of the silkworm body, (B) header elements of the silkworm (Tazima, 1978)

Sex discrimination at the larval stage

The male has one spot of the Herold imaginal gland in the center line of the ventral side of the 12th segment. The female has four spots of the Ishiwata imaginal gland at ventral side of the 11th and 12th segments (Figure 13). The best time for discrimination between the two sexes is the early 5th instar just after the 4th molt. Discrimination is more or less difficult, depending on the strains (Tazima, 1978).

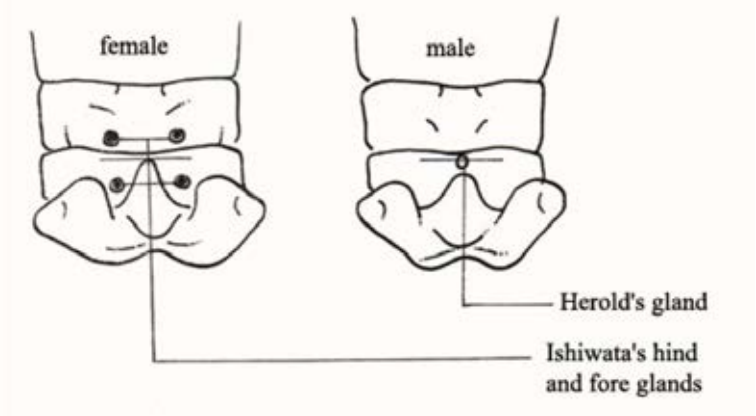


Figure 13 Sex discrimination of silkworm (Tazima, 1978)

Internal anatomy of the larva

The inner organ of a full-grown larva at the 5th instar is shown in Figure 14. Main internal organs are the alimentary canal (fore-gut, mid-gut and hind-gut), trachea, nervous system, dorsal vessel, malpighian tube, silk gland and others (Tazima, 1978).

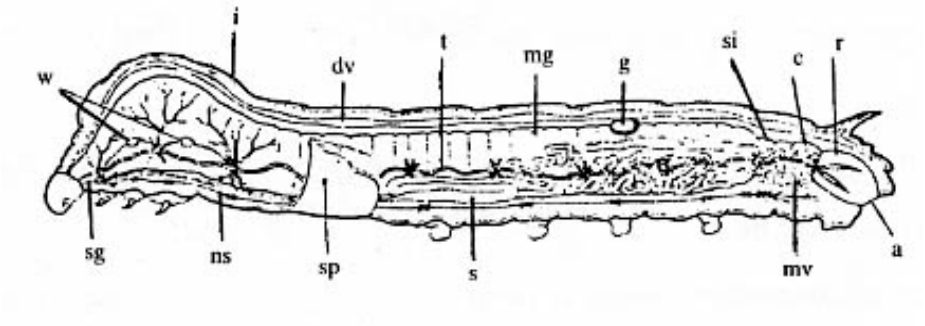


Figure 14 Internal organ of silkworm. a: anus, c: colon, dv: dorsal vessel, g: gonad, i: integument, mg: mid-gut, mv: malpighian vessel, ns: nervous system, r: rectum, si: small intestine, sg: salivary gland, sp: spiracle, t: trachea, w: wing disc (Tazima, 1978)

The alimentary canal

The alimentary canal in silkworms is divided into three main regions include the foregut (stomodaeum), which is ectodermal in origin, the midgut (mesenteron), which is endodermal and the hindgut (protodaeum), which is again ectodermal. In silkworms, these regions are subdivided into various functional parts.

The foregut is composed of pharynx, oesophagus and salivary glands. It is commonly concerned with the storage of food and sometimes helps to fragment the food before it passes to the midgut. The salivary glands can secrete digestive enzymes. These enzymes can hydrolyze protein, starch, sucrose, maltose and trehalose.

The midgut is the largest organ, extending over 8 of the 13 body segments. It is primarily concerned with the production of enzymes and the absorption of the products of digestion. The midgut tissue comprises globlet cells, columnar cells and a basement membrane. The most

characteristic mid gut cells are tall and columnar so called columnar cells with regular microvilli forming a striated border adjacent to lumen. The globlet cells and columnar cells are concerned with enzyme secretion (such as protease, lipase, amylase and maltase etc.) and with the absorption of products of digestion. The basement membranes of cells adjoin to the haemocoel with few opening to hemolymph.

The hindgut is composed of epithelial cells forming numerous projections, each of which is made up of two or three large cuneiform cells. Four regions are recognizable in this part include the small intestine, the colon, the rectum and anus. The hindgut conducts undigested food to the exterior via the anus, but also has other functions. In particular the rectum is involved in salt and water regulation (Tazima, 1978; Wiggle, 1974).

The silk gland

The silk gland is the second largest organ in the silkworm body. It develops from the paired invagination in the labial segments. In the full grown larva it occupies most of the lateral side of the body from the 4^{th} to the 8^{th} segment. It is divided into three distinct parts, anterior, middle and posterior. The number of cells in the silk gland varies according to the strains, but does not differ between the right and the left glands, sexes, or post embryonic developmental stages (Tazima, 1978).

The respiratory system

The respiration is accomplished by the tracheal system, which is constructed of spiracles, tracheae and tracheoles. The respiratory system consists of segmental tracheae which are led to the exterior through 9 pairs of spiracles. The control of respiratory activity is achieved by "diffusion control" due to opening and closing of the spiracles and also by "ventilation control" caused by variations in the frequency and intensity of respiratory movements of the tracheal system. The latter movements include opening or closing, which are influenced by oxygen and carbon dioxide concentrations (Tazima, 1978).

The blood circulation system

The dorsal vessel consists of an abdominal portion called the heart and a thoracic portion known as the aorta. The heart is the pulsating part of the tube with 9 pairs of opening, called the ostia, for admission of the blood. As for the circulatory mechanism of the silkworm larva, the blood flows backwards through the body cavity, enters the ostia at the dorsal vessel of abdominal 6^{th} to 11^{th} segments, and leaves the dorsal vessel at the ostia of 2^{nd} to 5^{th} segments.

The blood or hemolymph circulates round the body cavity between the various organs. It consists of a fluid plasma in which are suspended the blood cells or haemocytes. The content of water in the blood is about 90 to 95% and potential of hydrogen ion (PH) of the blood in 5^{th} instar larva is 6.3 to 6.5. The blood of the silkworm plays a major part in distribution of nutrition to the tissue and carries waste product from them. The blood also has an important role in respiration. The substances dissolved in the blood include proteins, amino acids, carbohydrates and inorganic component. The percentage of the blood based on body weight ranged from 21 to 25% in the larva stage. No significant difference among the values at various development stages and between those of males and females was found.

In general, the density of blood cells (haemocytes) ranges from about 25,000 to 100,000 per mm^3. The blood cell in hemolymph including prohaemocytes, plasmatocytes, granular haemocytes, spherulocytes, cystocyte (coagulocytes). An important function of blood cells is phagocytosis of foreign particles, microorganisms and tissue debris. Various types of blood cells are capable of phagocytosis but the plasmatocytes and granular haemocytes are most important in combating bacteria. The healing of wounds is also effected by the blood or its haemocytes (Chapman, 1982; Murray, 1985).

The nervous system

The nervous system consists of the brain, the ventral nerve cord and ganglion. Ventral nerve cords are made up of segmented ganglia that are connected by a tract of nerve fibers passing from one side to the other of the nerve cord of the adjacent segments. The silkworm's

brain is six fused ganglia (three pairs) located within the head capsule. Each part of the brain controls a limited spectrum of activities in the body include protocerebrum, the first pair of ganglia are largely associated with vision and they innervate the ocelli. Deutocerebrum, the second pair of ganglia process sensory information collected by the antennae. Tritocerebrum, the third pair of ganglia innervate the labrum and integrate sensory inputs from protocerebrums and deutocerebrums (Chapman, 1982).

Muscle and movement

The muscles composed of skeletal muscles and visceral muscles. The skeletal muscles from bands stretched across the articulations of the body wall, serving to move one segment toward another. The visceral muscles invest the internal organs, such as the alimentary canal (Tazima, 1978).

The malpighin tubules (excretory organ)

In larva, waste products of metabolism are mainly excreated as urine together with feces (dry pellet). The malpighin tubules of silkworm are well-known organs for excretion. The six malpighian tubules open near the border line between small intestinal and colon. The terminal portions of the tubules are intimately associated with the wall of rectum, forming the rectal plexus with an outer layer and inner of the tubules. The terminal portion has an active absorptive function. The terminal portion of malpighin tubules in the body cavity directly absorbs the other substances from the blood and then waste products are excreted as urine (Candy and Kilby, 1975).

Immune system of silkworm

Silkworms have powerful innate immune system against invading pathogens or adulterated thing during their existence. From previous studies it was reported that the innate immune system of silkworms share many fundamental characteristics with the innate immune system of vertebrates (Vilmos and Kurucz, 1998). Innate immunity of silkworms composed of two major types is humoral and cellular immune response. Humoral immune response involves

antimicrobial proteins (AMPs), soluble proteins in the hemolymph. In families of AMPs have main activity for against Gram-positive and Gram- negative bacteria, whereas cellular immune response involve process of phagocytosis which is regulated by hemocytes of silkworms (Tanaka and Yamakawa, 2011).

Utility of silkworm for evaluating the toxicity and efficacy of candidate antimicrobial agents

Once a novel antimicrobial compound is shown to be potent *in vitro*, it is important to establish whether this compound is effective against the target microbe and nontoxic in an animal infection model. All candidate compounds must be tested in animal infection models, and these studies can identify any factors that limit the antimicrobial efficacy of a compound or prevent its potential application in humans. Antimicrobial activity of a compound can be reduced *in vivo* due to degradation by host enzymes, binding to host components, and the effects of other physiological conditions. Therefore, animal infection models provide data on the effectiveness of an antimicrobial agent against an infection *in vivo* and also allow for the assessment of host toxicity. Typically, initial *in vivo* assessments of antimicrobial agent toxicity and efficacy are performed using murine or rodent models. However, mammalian experimentation is costly, time consuming, and requires full ethical consideration. If an initial toxicity and efficacy screen could be achieved in a financially more attractive and ethically more acceptable alternative *in vivo* model, this could reduce the use of mammals and overall costs associated with the early stages of novel antimicrobial discovery and development. As a result, invertebrate animal models of infection have been developed and introduced, including insect and nematode models. Such models provide meaningful data at low cost but do not require the same ethical considerations as required for mammalian studies. Also, the innate immunity, the major pathways of host defence which covered of the response to microbial pathogen, has the similarities between vertebrates and invertebrates (Vilmos and Kurucz, 1998). Therefore, invertebrate animal models are proving to be valuable tools for studying microbial pathogenicity, virulence factor and preclinical assessment of new antimicrobial agents (Garcia-Lara *et al*., 2005; Kaito and Sekimizu, 2007). For example,

Galleria mellonella (Wax Moth Larva) was used as an animal model for studying pathogenicity of microbial pathogen (such as *Aspergillus fumigatus* (*A. fumigatus*), Candida spp., *C. neoformans*, *Acinetobacter baumannii* (*A. baumannii*) and *P. aeruginosa*) and testing candidate antimicrobial agents (Desbois and Coote, 2012). *D. melanogaster* have been reported to use for *S. aureus* and *E. coli* infection model (Bernal and Kimbrell, 2000) and *C. elegans* have been used for *S. aureus* and *P. aeruginosa* infection model (Mahajan *et al*., 1999). However, these invertebrate models were limited on small body size result in it is difficultly to handle for injection experiments. Therefore, they are not suitable for injecting precise volumes of samples into the body fluid, a technique that is essential for quantitative evaluation of bacterial pathogenicity and the therapeutic effects of antimicrobial compounds (Kaito *et al*., 2012). On the contrary, 5^{th} instar larvae of silkworms can be utilized for infection assay because they have several advantages as an animal model for studying bacterial pathogenicity and the therapeutic effect of candidate antimicrobial agents, such as the body size of silkworm 5^{th} instar larvae is big enough to handle, sample solution of pathogens and candidate samples can be injected into the hemolymph (mimics mouse intravenous administration) or midgut (mimics mouse oral administration) of the larvae. Silkworms are easy to raise the number of larvae and they have less of ethical issues compared with the use of mammals. Furthermore, they also ease the rearing and maintenance of silkworms in laboratory. Recently, silkworms were utilized as an alternative animal model for efficacy testing of candidate compounds. In particular, in several bacterial infectious research topics at the University of Tokyo, Japan, silkworms have been being used as an animal model since microorganisms that are pathogenic to humans, such as *S. aureus*, *P. aeruginosa*, *E. coli* (O-157) and *V. cholerae* etc. have lethal effects in silkworms within 2 days when injected into their hemolymph. The larvae injected with these microorganisms had decrease in movement, then they stopped eating and finally their skin color turned dark. Growth of *S. aureus* was observed in larvae blood and tissues. Immunostaining analysis revealed that *S. aureus* proliferated at the epithelial cell surface of the midgut. These results indicate that silkworm larvae

were killed by infection with bacteria pathogens. Infection of silkworm larvae by these pathogens were cured by antibiotics (for example ampicillin and vancomycin etc.), clinically used in humans (Kaito *et al*., 2002). In the next study, the results demonstrated that silkworms were killed by injection of bacteria (*S. aureus*, *Stenotrophomonas maltophilia* (*S. maltophilia*)) and true fungi (*C. albicans*, *C. tropicalis*) that are pathogenic to humans and chloramphenicol, vancomycin and kanamycin, antibiotics clinically used for humans are effective for silkworms. However, injection into intra-midgut of vancomycin and kanamycin is ineffective in silkworms because result from isolation midgut membranes demonstrated that vancomycin and kanamycin were impermeable into the hemolymph. Thus, the ineffectiveness of oral administration of vancomycin and kanamycin to silkworms is due to a lack of intestinal absorption (Hamamoto *et al*., 2004). These results are consistent with results obtained with mice. Silkworms are a promising infection animal model for the identification and evaluation of virulence associated genes. Researcher constructed 100 gene mutants of *S. aureus* by disrupting these genes by targeting using a homologous recombination technique. Gene disruption was confirmed by Southern blotting analysis. Then, 100 strains of disruption mutants were injected into the hemolymph of silkworms. The results indicated that gene-disrupted mutants of three novel genes, named *cvfA*, *cvfB*, and *cvfC* (conserved virulence factor A, B, and C), exhibited decreased lethality in silkworms, whereas the parent strain killed all the population of silkworms within 36 h. These gene-disrupted mutants also showed decreased virulence in mice, indicating that these genes contribute to the virulence of *S. aureus* not only in silkworms but also in mammals (Kaito *et al*., 2005). Silkworm model also was used to study the pathogenic bacterial toxins. The results showed that the injection of culture-supernatants of pathogenic bacteria (for example the culture-supernatants of *S. aureus* and *P. aeruginosa*) or purified bacterial toxins (for example staphylococcal alpha-toxin, beta-toxin, Pseudomonas exotoxin A, and diphtheria toxin) can kill silkworm larvae. Furthermore, a culture-supernatant of a mutant of *agr*, a global virulence regulator of *S. aureus* that is required for exotoxin production, failed to kill silkworm larvae. The results of injection of culture supernatants

derived from disruption mutants of various toxin genes of *S. aureus*, such as beta-toxin gene, alpha-toxin gene and gamma-toxin gene, suggested that beta-toxin is the major factor in the supernatant mediated killing of silkworm. Thus, silkworm could be used as an animal model to study the actions of pathogenic bacterial toxins in animal bodies (Hossain *et al*., 2006). Silkworm larvae were also investigated as an alternative animal model for the toxicity and efficacy testing of novel oxazolidinones and 13 chromogenic agents against *S. aureus* infection. The antimicrobial potential of oxazolidinones and 13 chromogenic agents (such as rifampicin, amidol, pyronin G, coomassie brilliant blue R250/G250, cresyl blue, and safranin etc.) was compared with that of linezolid and vancomycin, antibiotics clinically used in humans to treat bacterial pathogens. These findings indicated that drug screening using the silkworm infection model is useful for evaluating toxicity and potential of candidate antibiotics (Barman *et al*., 2008; Fujiyuki *et al*., 2010). In addition, the study to assess the antiviral effects of the Kampo medicines (Japanese traditional medicines composed of Mao-to, Kakkon-to and Shosaiko-to) using silkworm infection model with baculovirus, *Bombyx mori* nucleopolyhedrovirus has a high similarity when compared with function domains of the herpesvirus and cytomegalovirus DNA polymerases has been conducted as well. The results showed that antiviral drugs used in clinical for humans (such as ganciclovir, foscarnet, vidarabine and ribavirin) showed therapeutic effects in silkworm-baculovirus infection model. The antiviral activity of the Kampo medicines, in particular Mao-to containing cinnamon bark, a component of the traditional Japanese medicine showed a therapeutic effect in silkworm-baculovirus infection model whereas cinnzeylanine, purified compound existing in cinnamon bark, also inhibited the proliferation of herpes simplex virus type I in vero cells. These results suggested that the silkworm-baculovirus infection model is useful for evaluating capability of candidate antiviral agents for apply in treating humans infected with DNA viruses (Orihara *et al*., 2008). Later, the silkworms were developed as an animal model for studying pathogenicity of fungus and evaluating candidate antifungal agents. The serotype A of *C. neoformans*, which is known to have higher mammal pathogenicity than the serotype D, was

also more virulent against the silkworm. Furthermore, the deletion mutants of genes *gpa*1, *pka*1 and *cna*1, which are genes known to be necessary for the pathogenesis in mammals, also showed lower killing activity in silkworms, compared with wild-type of *C. neoformans*. Antifungal drugs such as amphotericin B, flucytosine, fluconazole and ketoconazole showed therapeutic effects in silkworms infected with *C. neoformans*. However, amphotericin B was not therapeutically effective when injected into the midgut of silkworms. This may be due to the fact that amphotericin B is not absorbed from midgut silkworms and this occurs in mammals as well (Matsumoto *et al*., 2011).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Silkworms

Thai silkworm 5th instar larvae weighing 0.6-1.0 g were supplied from The Queen Sirikit Department of Sericulture, Ministry of Agriculture and Cooperatives.

2. Bacterial strains

The bacterial strains used throughout this study were *S. aureus* ATCC 6538P and *S. epidermidis* (Isolate) derived from Department of Microbiology, Faculty of Sciences, Chulalongkorn University.

3. Chemicals

3.1 Amipicillin (T.P. Drug Laboratories Co., Ltd., Thailand)

3.2 70% Alcohol

3.3 Chrysazin (Sigma, USA)

3.4 *X. indica* crude extract (Petroleum ether extract and Ethanol extract) derived from Mr. Kitthisak Chansukh, College of Public Health Sciences, Chulalongkorn University.

3.5 2% (v/v) Crystal violet

3.6 Dimethyl sulfoxide (RCI Labscan, Thailand)

3.7 Ethyl acetate (RCI Labscan, Thailand)

3.8 Glacial acetic acid (BDH Laboratory, England)

3.9 Methanol (RCI Labscan, Thailand)

3.10 Mueller Hinton Agar (BBL, USA)

3.11 Mueller Hinton Broth (BBL, USA)

3.12 NaCl

3.13 Petroleum ether (RCI Labscan, Thailand)

3.14 Silkmate

3.15 Sterile distilled water

4. Instruments

4.1 Alcohol lamp stainless

4.2 Autoclave (ALP Co., Ltd., Japan)

4.3 Cork-borer

4.4 Disposable syringe size 1 milliliter (Nipro, Thailand)

4.5 Glass tank

4.6 Hot air oven (WTB binder, Germany)

4.7 Hypodermic needle 27G x 1" (Nipro, Thailand)

4.8 Incubater (Memmert, Germany)

4.9 Larminar airflow cabinet (Astec, Thailand)

4.10 Loop Sterilizer (LabScientific, USA)

4.11 Mc Farland Densitometer (Gibthai Co., Ltd., Thailand)

4.12 Microcentrifuge tubes (Corning incorporated, Mexico)

4.13 Micropipette size 10, 100 and 1,000 microliter (Gilson, France)

4.14 Microplate reader (LabScientific, USA)

4.15 Multichannel pipette (Biohit, Finland)

4.16 Petri dish (Pyrex, USA)

4.17 Pipette tip size 0.1-10 µl and 100-1,000 µl (Corning incorporated, Mexico)

4.18 Shaker bath

4.19 Spreader

4.20 Sterile loop

4.21 TLC Silica gel 60 F 254 (Merck, Germany)

4.22 96 well microtiter plate (Costar, USA)

Methods

1. Antimicrobial activities testing of *X. indica* extract (*in vitro* studies)

1.1 Inoculum preparation

Fresh microbial cultures were prepared by streaking bacterial pathogen (*S. aureus* or *S. epidermidis*) on Mueller-Hinton agar (MHA) plates and incubated at 37 °C for 24 h. After incubation overnight, isolate colonies of each bacterial pathogen are transferred into a tube containing 0.85% sterile saline and the turbidity was adjusted to a 0.5 McFarland standard by Mc Farland Densitometer (starting inoculum approximately 1.5×10^{8} CFU/ml). After that, freshly bacterial suspension was used for each experiment of antimicrobial activities testing (Schwalbe *et al*., 2007).

1.2 Antimicrobial screening by agar diffusion method

Antimicrobial screening was performed using agar-well diffusion assay. Bacterial suspension from inoculum preparation was mixed with sterile MHA and evenly spread onto the surface of the agar plate. After the media had cooled and solidified, two wells (6 mm in diameter) was punched in the plates with the help of a sterile cork-borer. 20 µl of crude extract (200 mg/ml or 4 mg/well) was added in well. Then, 20% DMSO (a solvent of the ethanol extract) or 100% DMSO (a solvent of the petroleum ether extract) was used as a negative control and was added in another well. Ampicillin (10 mg/ml or 0.2 mg/well) was used as positive control for this study. The MHA plates were then incubated at 37 °C for 24 h. After incubation time, the size of the zone of inhibition was measured and the antimicrobial activity expressed in terms of the average diameter of the zone of inhibition in millimeters (mm). The experiment was repeated three independent times per microorganism (Schwalbe *et al*., 2007).

1.3 Determination of the minimal inhibitory concentration (MIC) by broth microdilution method

Broth microdilution method was used to obtain MIC values of the crude extract against *S. aureus* and *S. epidermidis*. Crude extract was two-fold serially diluted and incorporated with

Mueller Hinton Broth (MHB). 50 µl of each concentration of the crude extract was dispensed into the wells 1-10 of the 96-well microtiter plate. Then, an equal volume of bacterial pathogen was added and mixed in each well which contained crude extract (final concentration ranging from 0.008-4 mg/ml). Wells 11 contained bacterial pathogen with MHB medium and was served as growth control while, well 12 was used as negative control. This well contained solvent of each extract (20% DMSO or 100% DMSO), bacterial pathogen and MHB medium. Similarly, ampicillin preparation (final concentration ranging from 0.049-25 µg/ml) was included as positive control. All test plates were covered with lids and incubated at 37 $^{\circ}$C for 24 h. The lowest concentration of the crude extract that inhibited the bacterial growth after incubation was taken as the MIC value of the crude extract. The experiment was repeated three independent times per microorganism (Schwalbe *et al*., 2007).

1.4 Determination of the minimal bactericidal concentration (MBC)

Contents from each well in the broth microdilution that shown no apparent bacteria growth, were streaked on MHA plate. After streaking, the MHA plate was incubated at 37 $^{\circ}$C for 24 h. The lowest concentration of the crude extract that the visible growth of the microorganism cannot be seen on the MHA plate known as MBC value. The experiment was repeated three independent times per microorganism (Schwalbe *et al*., 2007).

1.5 Determination of time to kill bacteria by time-kill assay

Assay for time to kill bacteria of the ethanol extract was carried out by time kill methodology. The ethanol extract was prepared and incorporated with MHB. Later, *S. aureus* suspension was diluted with MHB and added with the ethanol extract (final concentration ranging from 0.25-4 mg/ml). Similarly, *S. epidermidis* suspension was diluted with MHB and incorporated with the ethanol extract (final concentration ranging from 0.5-4 mg/ml) in sterile test tubes (each tube contains final bacterial concentration approximately $5x10^5$ CFU/ml). The tubes were incubated with shaking on shaker water bath at 37 $^{\circ}$C. 100 µl aliquot was removed from the tubes at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h and was diluted serially. Finally, 100 µl of the serial

diluted samples was spread on MHA plates after that incubated at 37 °C, 24 h for the determination of surviving bacteria. The growth control was composed of bacterial pathogen and MHB. The positive control and negative control (without the ethanol extract) were composed of ampicillin and 20% DMSO, respectively. These control tube were performed the same experiment as mentioned above. Time kill assays were performed at three times independent experiments per microorganism. Viable colonies were calculated to give colony forming unit per milliliter (CFU/ml) and the time kill curve was plotted between $\log_{10}$ of the viable colonies against time (Lynne and Henry, 2010).

1.6 Virulence factor assay: biofilm formation assay

Ethanol extract was tested for its potential to inhibit biofilm formation of *S. aureus* and *S. epidermidis*. The ethanol extract was prepared and incorporated with MHB. 100 µl of the ethanol extract was dispensed into the 96-well microtiter plate. Later, bacterial suspension was diluted with MHB and mixed in the 96-well plate (final bacterial concentration is approximately $5x10^5$ CFU/ml). Final concentrations of the ethanol extract ranging from 0.25-4 mg/ml and 0.5-4 mg/ml for *S. aureus* and *S. epidermidis*, respectively. Each concentration of the ethanol extract with the MHB (without bacterial suspension) as the sample blank. The growth control contained bacterial pathogen with MHB and MHB was served as growth control blank. The positive control was composed of ampicillin (final concentration ranging from 0.049-0.78 µg/ml for *S. aureus* and 0.098-1.56 µg/ml for *S. epidermidis*), bacterial pathogen and MHB, whereas the negative control consisted of 20% DMSO, bacterial pathogen and MHB. The control groups performed the same experiment as mentioned above. The microtiter plates were covered with lids and incubated at 37 °C for 24 h. Thereafter, the contents of each well were removed and washed with 0.85% sterile saline. Adherent bacteria were fixed with 99% methanol for 15 min. All wells were then emptied and left to dry. Each well was stained by 2% crystal violet for 5 min. Excess stain was washed with distilled water and wait for it to dry. After drying, stain was resolubilised by adding 33% glacial acetic acid to each well. Biofilm formation was quantified by measuring the absorbance at

570 nm using a microplate reader (Hammer *et al*., 2005), the percentage of biofilm inhibition was calculated using following equation (Chaieb *et al*., 2011)

$$\frac{[(\text{OD growth control} - \text{OD growth control blank}) - (\text{OD sample} - \text{OD sample blank})]}{(\text{OD growth control} - \text{OD growth control blank})} \times 100$$

2. Qualitative analysis of chrysazin in *X. indica* extract by using thin layer chromatography (TLC)

Mobile phase solvent system was composed of petroleum ether : ethyl acetate having ratio of 1 : 8 (v/v) and was filled in the glass tank. 3 µl aliquots of the crude extract and chrysazin stardard were spoted onto the silica gel plate. The plate was dried in a hood for 5 min before the development. The plate was dipped in the glass tank in such a way that the spots did not touch the solvent directly. The plate was developed until the solvent front moved to about 1 cm below the top edge of the plate. After that, it was removed and dried by dryer. The spots were visualized under UV light at 365 nm and R_f value was calculated using following equation (Sukrong, 1994)

$$R_f \text{ value} = \frac{\text{distance traveled by substance}}{\text{distance traveled by solvent front}}$$

3. Antimicrobial activities testing of chrysazin (*in vitro* studies)

3.1 Antimicrobial screening by agar diffusion method

Antimicrobial screening was performed using agar-well diffusion assay. Bacterial suspension from inoculum preparation was mixed with sterile MHA and evenly spread onto the surface of the agar plate. After the media had cooled and solidified, two wells (6 mm in diameter) was punched in the plates with the help of a sterile cork-borer. 20 µl of chrysazin (10 mg/ml or 0.2 mg/well) was added in well. Then, 100% DMSO (a solvent of chrysazin) was used as a negative control and was added in another well. Ampicillin (10 mg/ml) was used as positive control for this study. The MHA plates were then incubated at 37 $^{\circ}$C for 24 h. After incubation time, the size of the zone of inhibition was measured and the antimicrobial activity expressed in terms of the average diameter

of the zone of inhibition in millimeters (mm). The experiment was repeated three independent times per microorganism (Chansukh, 2011).

3.2 Determination of the minimal inhibitory concentration (MIC) by broth microdilution method

Broth microdilution method was used to obtain MIC values of chrysazin against *S. aureus* and *S. epidermidis*. Chrysazin was two-fold serially diluted and incorporated with Mueller Hinton Broth (MHB). 50 µl of each concentration of chrysazin was dispensed into the wells 1-10 of the 96-well microtiter plate. Then, an equal volume of bacterial pathogen was added and mixed in each well which contained chrysazin (final concentration ranging from 0.008-4 mg/ml). Wells 11 contained bacterial pathogen with MHB medium and was served as growth control while, well 12 was used as negative control. This well contained solvent of chrysazin (100% DMSO), bacterial pathogen and MHB medium. Similarly, ampicillin preparation (final concentration ranging from 0.049-25 µg/ml) was included as positive control. All test plates were covered with lids and incubated at 37 °C for 24 h. The lowest concentration of chrysazin that inhibited the bacterial growth after incubation was taken as the MIC value of chrysazin. The experiment was repeated three independent times per microorganism (Ramli, 2011).

3.3 Determination of the minimal bactericidal concentration (MBC)

Contents from each well in the broth microdilution that shown no apparent bacteria growth, were streaked on MHA plate. After streaking, the MHA plate was incubated at 37 °C for 24 h. The lowest concentration of chrysazin that the visible growth of the microorganism cannot be seen on the MHA plate known as MBC value. The experiment was repeated three independent times per microorganism (Ramli, 2011).

3.4 Determination of time to kill bacteria by time-kill assay

Assay for time to kill bacteria of chrysazin was carried out by time kill methodology. Chrysazin was prepared and incorporated with MHB. Later, *S. aureus* suspension was diluted with MHB and added with chrysazin (final concentration ranging from 0.5-4 mg/ml). Similarly,

S. epidermidis suspension was diluted with MHB and incorporated with the ethanol extract (final concentration ranging from 1-4 mg/ml) in sterile test tubes (each tube contains final bacterial concentration approximately $5x10^5$ CFU/ml). The tubes were incubated with shaking on shaker water bath at 37 °C. 100 µl aliquot was removed from the tubes at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h and was diluted serially. Finally, 100 µl of the serial diluted samples were spread on MHA plates after that incubated at 37 °C, 24 h for the determination of surviving bacteria. The growth control was composed of bacterial pathogen and MHB. The positive control and negative control (without chrysazin) were composed of ampicillin and 100% DMSO, respectively. These control tube were performed the same experiment as mentioned above. Time kill assays were performed at three times independent experiments per microorganism. Viable colonies were calculated to give colony forming unit per milliliter (CFU/ml) and the time kill curve was plotted between $\log_{10}$ of the viable colonies against time (Lynne and Henry, 2010).

3.5 Virulence factor assay: biofilm formation assay

Chrysazin was tested for its potential to inhibit biofilm formation of *S. aureus* and *S. epidermidis*. Chrysazin was prepared and incorporated with MHB. 100 µl of chrysazin was dispensed into the 96-well microtiter plate. Later, bacterial suspension was diluted with MHB and mixed in the 96-well plate (final bacterial concentration is approximately $5x10^5$ CFU/ml). Final concentrations of chrysazin ranging from 0.5-4 mg/ml and 1-4 mg/ml for *S. aureus* and *S. epidermidis*, respectively. Each concentration of chrysazin with the MHB (without bacterial suspension) as the sample blank. The growth control contained bacterial pathogen with MHB and MHB was served as growth control blank. The positive control was composed of ampicillin (final concentration ranging from 0.049-0.78 µg/ml for *S. aureus* and 0.098-1.56 µg/ml for *S. epidermidis*), bacterial pathogen and MHB, whereas the negative control consisted of 100% DMSO, bacterial pathogen and MHB. The control groups performed the same experiment as mentioned above. The microtiter plates were covered with lids and incubated at 37 °C for 24 h. Thereafter, the contents of each well were removed and washed with 0.85% sterile saline.

Adherent bacteria were fixed with 99% methanol for 15 min. All wells were then emptied and left to dry. Each well was stained by 2% crystal violet for 5 min. Excess stain was washed with distilled water and wait for it to dry. After drying, stain was resolubilised by adding 33% glacial acetic acid to each well. Biofilm formation was quantified by measuring the absorbance at 570 nm using a microplate reader (Hammer *et al*., 2005), the percentage of biofilm inhibition was calculated using following equation (Chaieb *et al*., 2011)

$$\frac{[(\text{OD growth control} - \text{OD growth control blank}) - (\text{OD sample} - \text{OD sample blank})]}{(\text{OD growth control} - \text{OD growth control blank})} \times 100$$

4. Antimicrobial activities testing of *X. indica* extract and chrysazin (*in vivo* studies)

4.1 Pathogenicity of bacterial to silkworms

Silkworms (n = 10) were injected with 50 µl of bacterial suspension ranging from $1.5x10^{1}$ to $1.5x10^{8}$ CFU/ml into hemolymph. Control group (n = 10) was also injected with 0.85% saline into hemolymph. The mortality rate of silkworms was observed at 48 h after the injection. Pathogenicity of bacterial to silkworms was performed at three times independent experiments per microorganism. The LD_{50} was determined as the doses of *S. aureus* and *S. epidermidis* that killed half of the silkworms (Kaito *et al*., 2002).

4.2 Determination of LD_{50} of the ethanol extract and chrysazin

Silkworms (n = 10) were injected with 50 µl of the ethanol extract solution (concentration ranging from 6.25-200 mg/ml) or chrysazin (concentration ranging from 0.5-5 mg/ml) into the hemolymph. The control group (n = 10) was injected via intra-hemolymph of silkworm with 50 µl of 20% DMSO. The mortality rate of silkworms was observed at 48 h after the injection. The LD_{50} of ampicillin were performed the same experiment as mentioned above by used concentration ranging from 7.81-250 mg/ml. The LD_{50} of the ethanol extract, chrysazin and ampicillin was performed at three times independent experiments. The LD_{50} value was determined as the doses of the ethanol extract, chrysazin or ampicillin that killed half of the silkworms (Kaito *et al*., 2002; Fujiyuki *et al*., 2010).

4.3 Determination of ED_{50} of the ethanol extract and chrysazin

Suspensions of bacterial pathogen in 0.85% NaCl (50 µl) that kill all silkworms from the previous experiment were injected into hemolymph. The ethanol extract (concentration ranging from 0.25-16 mg/ml) or chrysazin (concentration ranging from 0.5-5 mg/ml) was injected into hemolymph immediately after injection of *S. aureus* pathogens, whereas the ethanol extract (concentration ranging from 0.5-16 mg/ml) or chrysazin (concentration ranging from 1-5 mg/ml) were injected into hemolymph after injection of *S. epidermidis*. Similarly, the positive control group and the negative control group were injected with 50 µl of ampicillin and 20% DMSO, respectively into intra-hemolymph of silkworm. The number of surviving silkworms was observed at 48 h after the injection. The ED_{50} of the ethanol extract or ampicillin was performed at three times independent experiments. The ED_{50} was determined as the doses of the ethanol extract, chrysazin or ampicillin required producing a silkworm survival rate of 50% (Kaito *et al*., 2002; Fujiyuki *et al*., 2010).

CHAPTER IV

RESULTS

1. Antimicrobial activities testing (*in vitro* studies)

1.1 Antimicrobial activities screening by agar diffusion method

The results of the antimicrobial activity by the agar diffusion method of the crude extract and chrysazin are presented in Table 1. The overall of results represented at three independent experiments per microorganism. From Table 1, the ethanol extract from *X. indica* and chrysazin showed antimicrobial activity against both *S. aureus* and *S. epidermidis* while the petroleum ether extract did not exhibit any activity against these microorganisms (no inhibition zone). The diameters of inhibition zone of the ethanol extract and chrysazin were 19.30±0.60 and 11.30±0.61 mm, respectively for *S. aureus* whereas, the zone of inhibition of the ethanol extract and chrysazin against *S. epidermidis* was 16.67±0.60 and 8.30±0.61 mm, respectively.

Table 1 The mean of inhibition zone of the extract from *X. indica* and chrysazin against *S. aureus* and *S. epidermidis*

Substances	**Inhibition zone (mm)**[a]	
	S. aureus	***S. epidermidis***
***X. indica* extract**		
Petroleum ether extract	NA	NA
Ethanol extract	19.30±0.60	16.67±0.60
Bioactive compound		
Chrysazin	11.30±0.61	8.30±0.61
Positive control		
Ampicillin	48.67±0.62	33.00±0.00
Negative control		
20% DMSO	NA	NA
100% DMSO	NA	NA

a = Inhibition zones are the mean value ± SD, NA = No Activity

1.2 Determination of the minimal inhibitory concentration (MIC) by broth microdilution method and minimum bactericidal concentration (MBC)

The antimicrobial activities of the crude extract and chrysazin against target pathogens were examined quantitatively by broth microdilution method. Results of MIC and MBC values given in Table 2 showed that both the ethanol extract and chrysazin have antimicrobial activities against *S. aureus* and *S. epidermidis*. The MIC values of the ethanol extract showed activity against *S. aureus* and *S. epidermidis* at 0.5 and 1 mg/ml, respectively whereas MIC values of chrysazin against *S. aureus* and *S. epidermidis* were 1 and 2 mg/ml, respectively. However, the petroleum ether extract did not have activity against both *S. aureus* and *S. epidermidis*.

Table 2 MIC and MBC values of the extract from *X. indica* and chrysazin against *S. aureus* and *S. epidermidis*

Substances	***S. aureus***		***S. epidermidis***	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
***X. indica* extract**				
Petroleum ether extract	NA	NA	NA	NA
Ethanol extract	0.5	4	1	4
Bioactive compound				
Chrysazin	1	> 4	2	> 4
Positive control				
Ampicillin	0.098*	0.195*	0.195*	0.39*
Negative control				
20% DMSO	NA	NA	NA	NA
100% DMSO	NA	NA	NA	NA

* μg/ml, NA = No Activity

1.3 Determination of time to kill bacteria by time-kill assay

In time kill assay, the graphical results were presented in Figure 15-20. Time kill studies showed that at concentration equal or greater than MIC of the ethanol extract exhibited bacteriostatic activities with the reduction of bacterial pathogens 1 to < 3 log scale (90% to < 99.9%) compared with the original inoculum. 0.5 mg/ml (MIC), 1 mg/ml (2MIC) and 2 mg/ml (4MIC) of the extract started to inhibit *S. aureus* (about 1 log scale reduction) at 48, 10 and 6 h respectively, whereas 1 mg/ml (MIC) and 2 mg/ml (2MIC) of the extract started to inhibit *S. epidermidis* (about 1 log scale reduction) at 12, and 8 h, respectively. 4 mg/ml (MBC) of this extract was bactericidal activities against *S. aureus* and *S. epidermidis* with the reduction of bacterial pathogens ≥ 3 log scale (≥ 99.9%) compared with the original inoculum at 8 and 10 h, respectively. Chrysazin was considered to be bacteriostatic against *S. aureus* and *S. epidermidis* at concentrations equal or greater than MIC. 1 mg/ml (MIC), 2 mg/ml (2MIC) and 4 mg/ml (4MIC) of chrysazin started to inhibited *S. aureus* (about 1 log scale reduction) at 48, 10 and 6 h respectively, whereas 2 mg/ml (MIC) and 4 mg/ml (2MIC) of chrysazin started to inhibit *S. epidermidis* (about 1 log scale reduction) at 12 and 8 h, respectively. Furthermore, time kill curve showed that ampicillin, standard antibiotic, was bactericidal activities against *S. aureus* and *S. epidermidis* at 12 and 14 h, respectively.

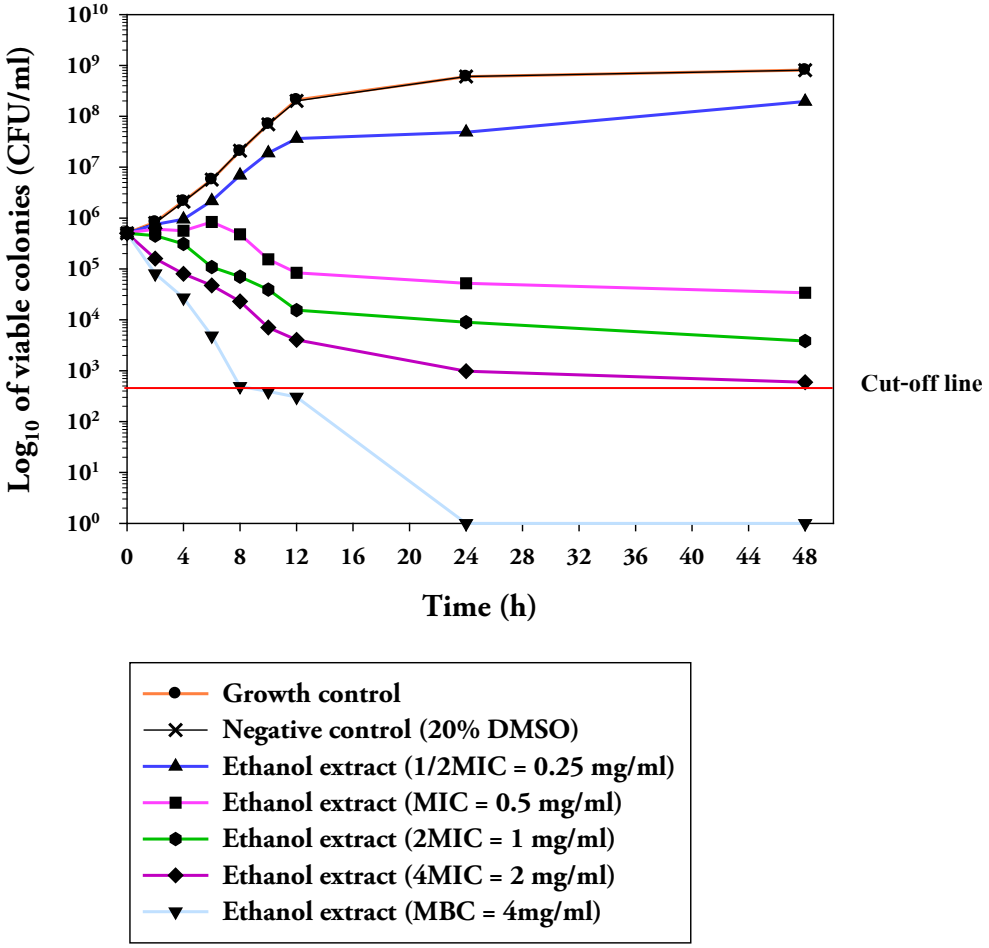


Figure 15 Time-kill curves of ethanol extract against *S. aureus*. Cut-off line represents ≥ 3 log scale reduction compared with the original inoculum (bactericidal activity).

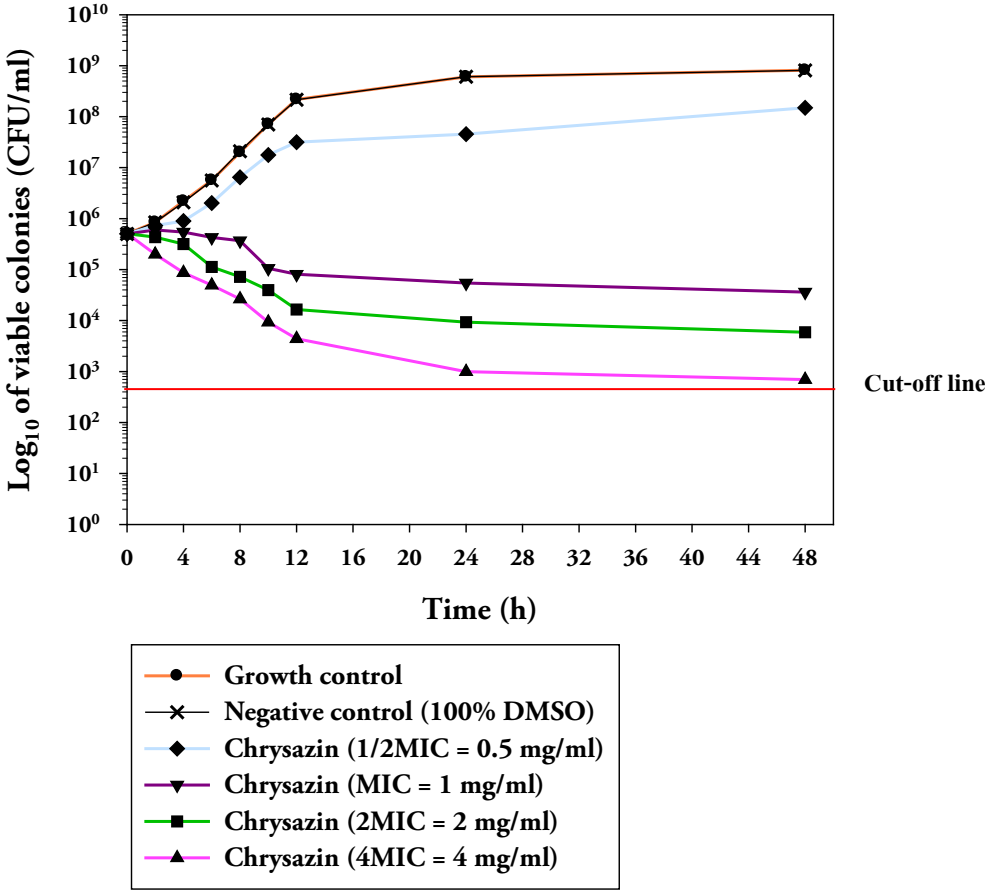


Figure 16 Time-kill curves of chrysazin against *S. aureus*. Cut-off line represents ≥ 3 log scale reduction compared with the original inoculum (bactericidal activity).

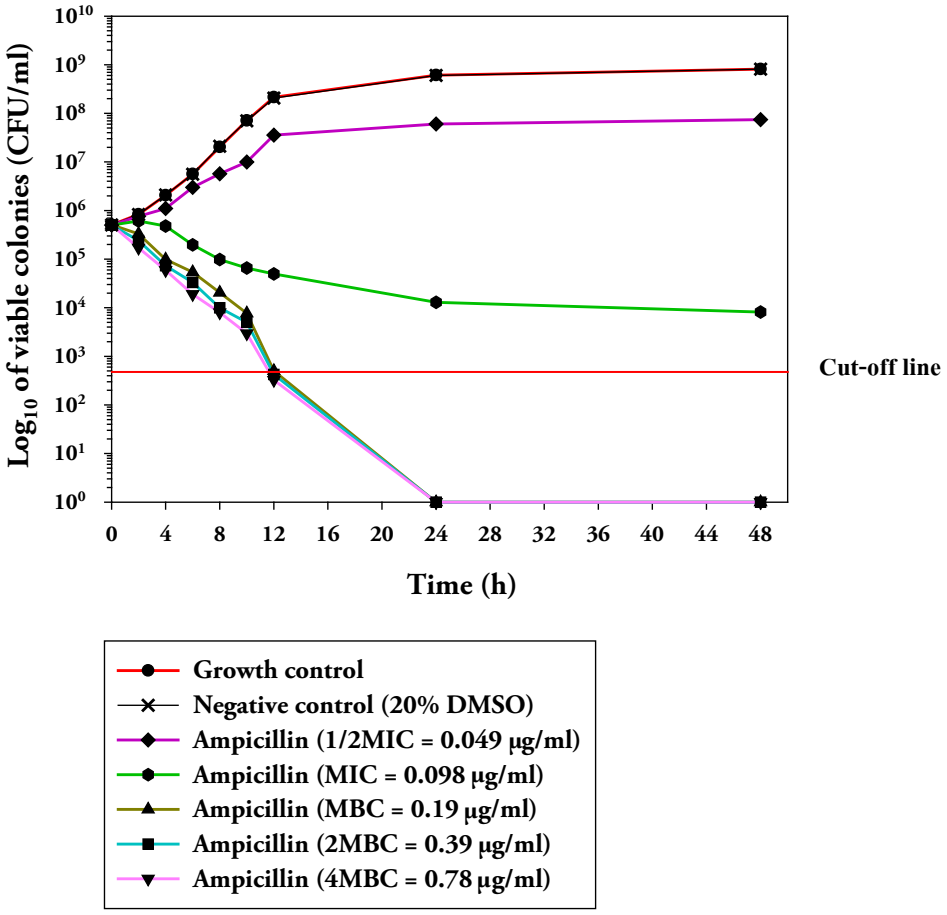


Figure 17 Time-kill curves of ampicillin against *S. aureus* (positive control). Cut-off line represents ≥ 3 log scale reduction compared with the original inoculum (bactericidal activity).

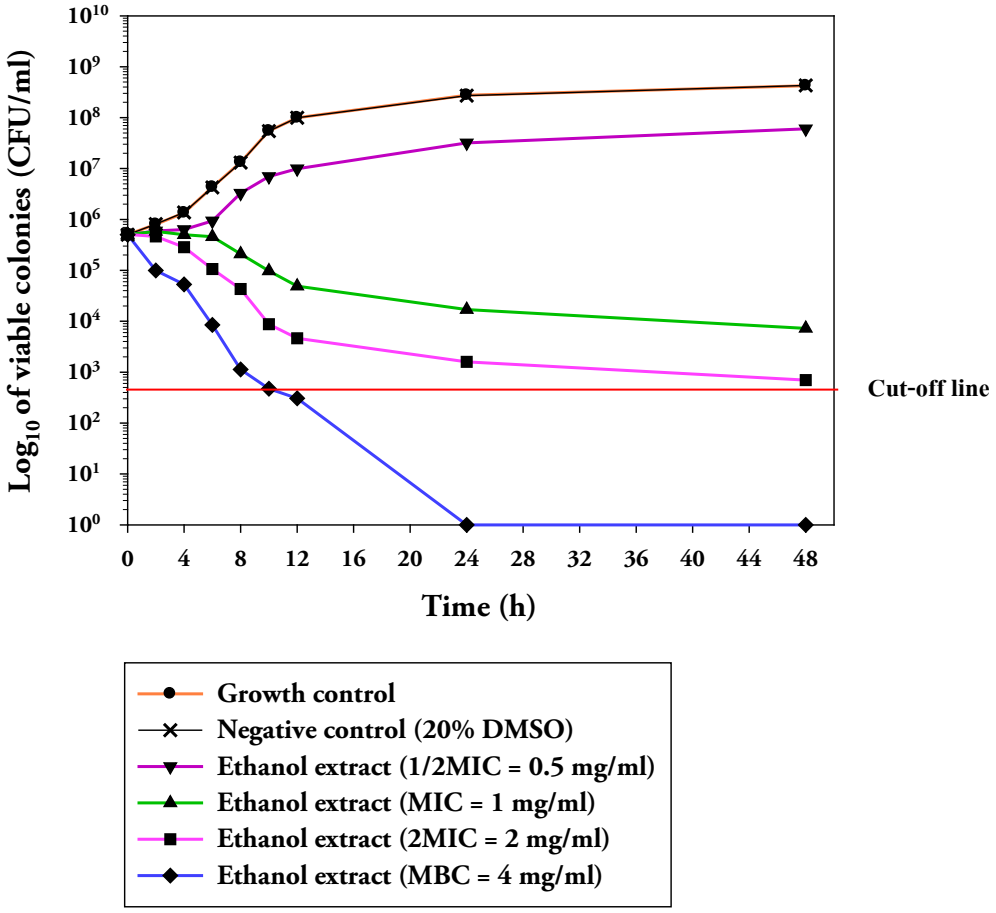


Figure 18 Time-kill curves of ethanol extract against *S. epidermidis*. Cut-off line represents ≥ 3 log scale reduction compared with the original inoculum (bactericidal activity).

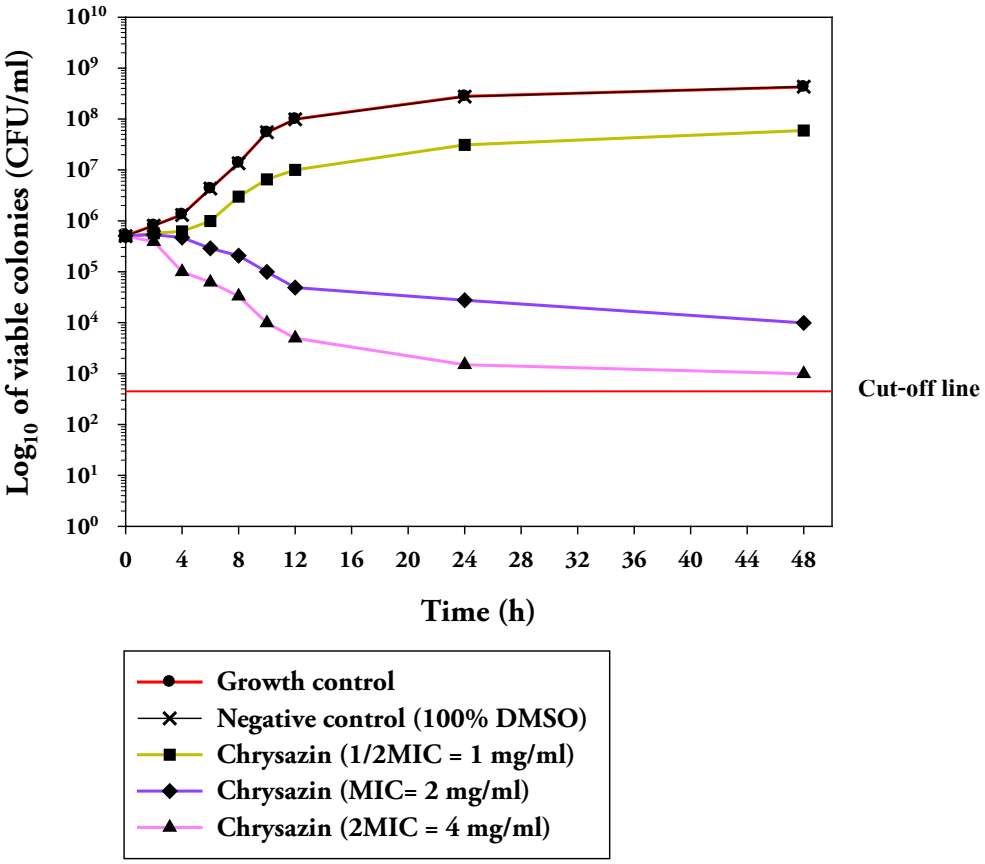


Figure 19 Time-kill curves of chrysazin against *S. epidermidis*. Cut-off line represents ≥ 3 log scale reduction compared with the original inoculum (bactericidal activity).

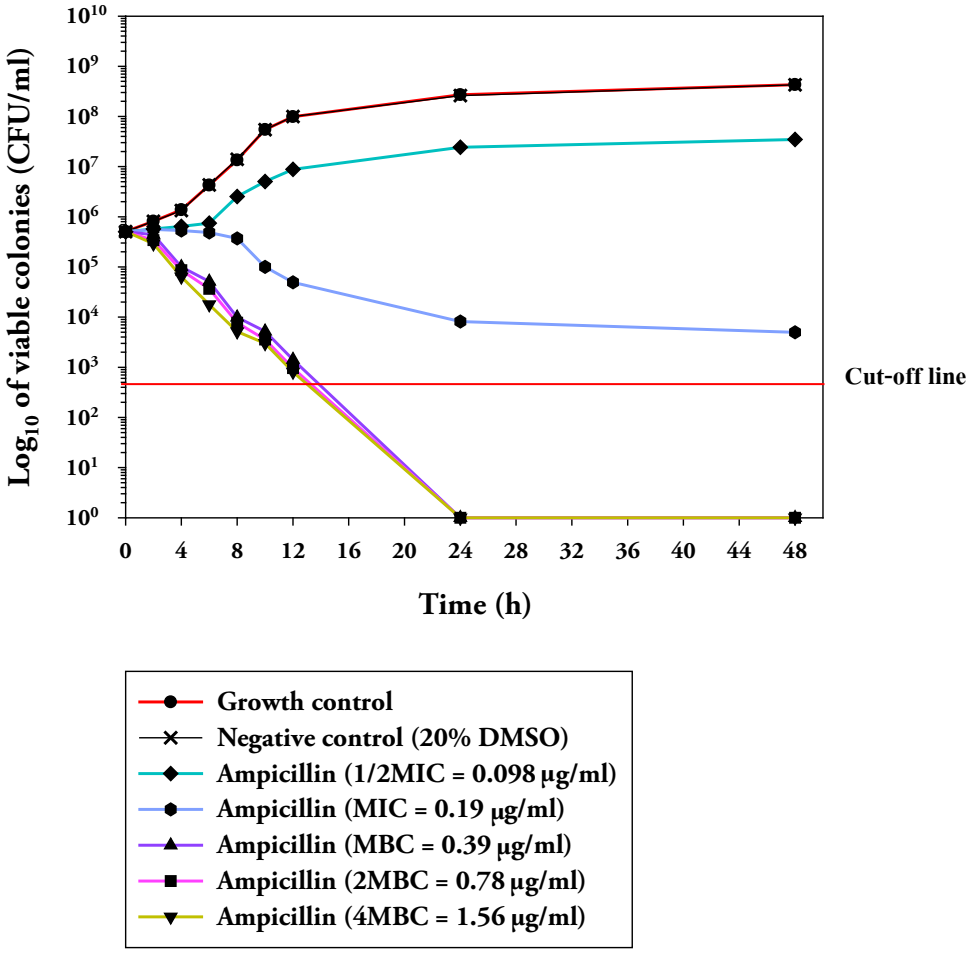


Figure 20 Time-kill curves of ampicillin against *S. epidermidis* (positive control). Cut-off line represents ≥ 3 log scale reduction compared with the original inoculum (bactericidal activity).

1.4 Virulence factor assay: biofilm formation assay

In order to find natural antibiofilm agents able to inhibit bacterial biofilm formation, this study tested the activity of the ethanol extract and chrysazin on bacterial biofilm positive strains. The result expressed as percentages of inhibition of biofilm formation and compared difference statistical significant with negative control group (Figure 21 to 24). Both the ethanol extract and chrysazin showed inhibitory effect on biofilm formation of *S. aureus* and *S. epidermidis* with statistical significance ($p < 0.01$) in almost all of concentrations except in the sub-MIC. Similarly, the result showed that each concentration of ampicillin (positive control) could inhibit biofilm formation of these pathogens with statistical significance ($p < 0.01$) except in the sub-MIC.

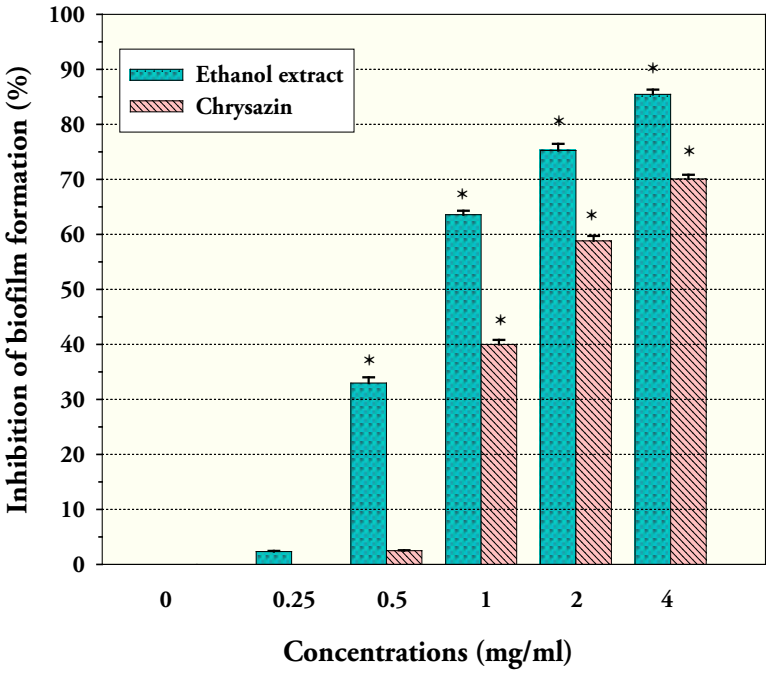


Figure 21 Inhibition of biofilm formation of *S. aureus* by the ethanol extract and chrysazin. Error bars represent the mean ± SD, * $P < 0.01$; ethanol extract or chrysazin groups compare with negative control group (without ethanol extract or chrysazin)

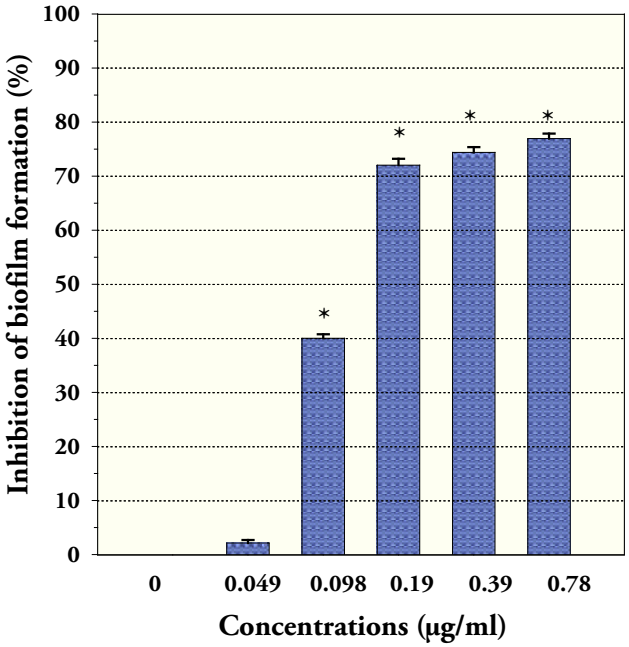


Figure 22 Inhibition of biofilm formation of *S. aureus* by ampicillin (positive control). Error bars represent the mean ± SD, * $P < 0.01$; ampicillin groups compare with negative control group (without ampicillin)

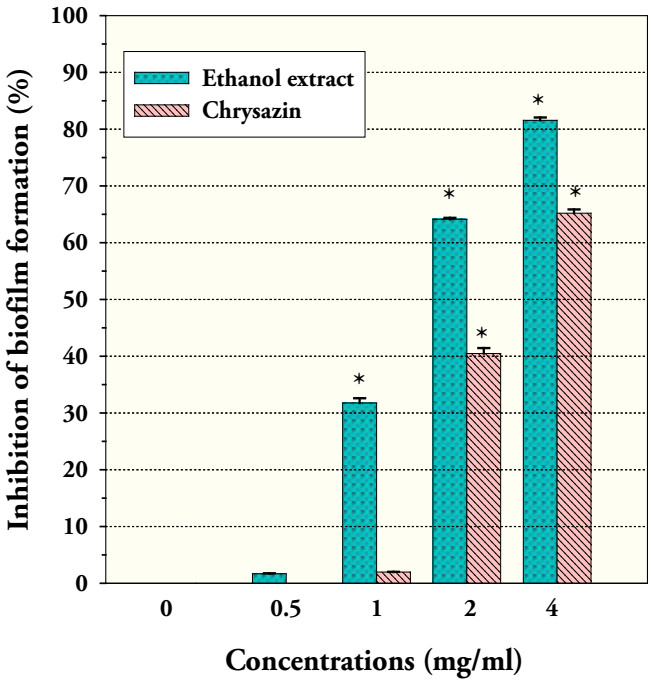


Figure 23 Inhibition of biofilm formation of *S. epidermidis* by the ethanol extract and chrysazin. Error bars represent the mean ± SD, * $P < 0.01$; ethanol extract or chrysazin groups compare with negative control group (without ethanol extract or chrysazin)

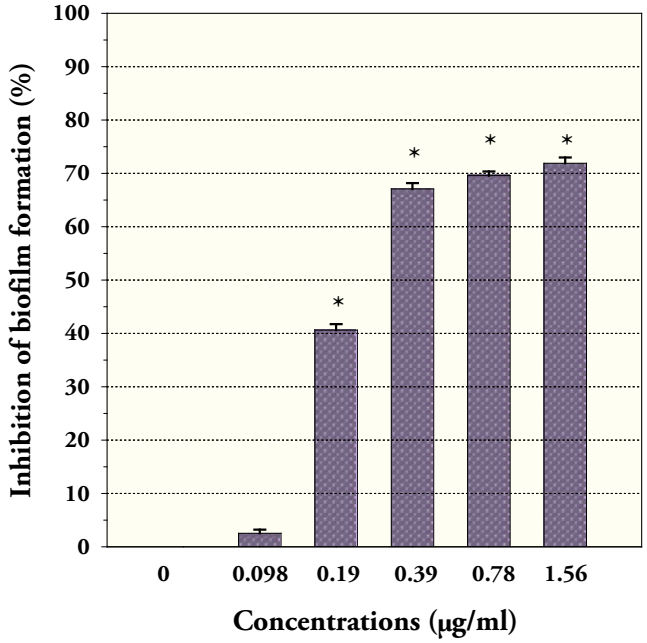


Figure 24 Inhibition of biofilm formation of *S. epidermidis* by ampicillin (positive control). Error bars represent the mean ± SD, * $P < 0.01$; ampicillin groups compare with negative control group (without ampicillin)

2. Qualitative analysis of chrysazin in the crude extract of *X. indica* by using thin layer chromatography (TLC)

For the qualitative identification of chrysazin in the crude extact from *X. indica* by TLC analysis, the result from observation under UV light indicated the presence of chrysazin in the ethanol extract, whereas the petroleum ether extract not presented band of chrysazin on TLC plate. Table 3 presented the R_f value of chrysazin in *X. indica* extract and Figure 25 showed photographs that obtained from the study.

Table 3 R_f value of chrysazin in *X. indica* extract

***X. indica* extract**	**R_f value**
Ethanol extract	0.5
Petroleum ether extract	No R_f value

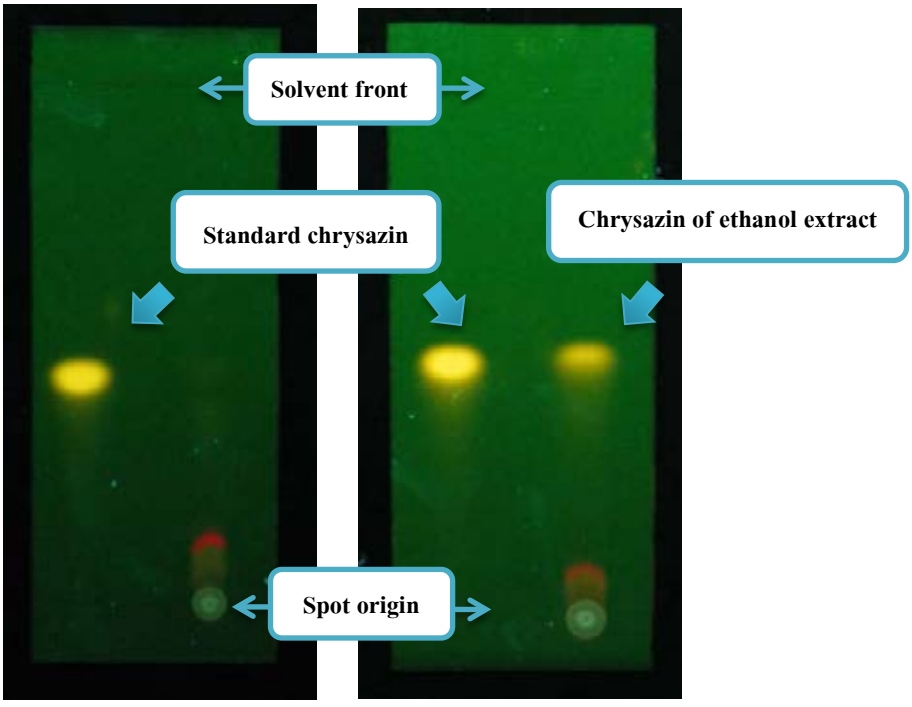


Figure 25 Photographs of TLC plate of the petroleum ether extract (A) and the ethanol extract (B) from *X. indica*

3. Antimicrobial activities testing (*in vivo* studies)

3.1 Pathogenicity of bacterial to silkworms

This study was performed in order to examine whether injection of bacteria that are pathogenic to humans could kill silkworms. The result showed that silkworms injected with *S. aureus* or *S. epidermidis* into hemolymph were normal for several hours, later activity of the silkworms decreased and finally their skin color turned dark. The median lethal doses (LD_{50}) of *S. aureus* or *S. epidermidis* were $3.13x10^3$ CFU/ml ($1.93x10^2$ CFU/g larva) and $3.99x10^3$ CFU/ml ($2.46x10^2$ CFU/g larva), respectively (Figure 26 and 27). Furthermore, when 1.5×10^6 CFU/ml ($9.26x10^4$ CFU/g larva) of *S. aureus* or *S. epidermidis* was injected into silkworm hemolymph, all silkworms died within 2 days.

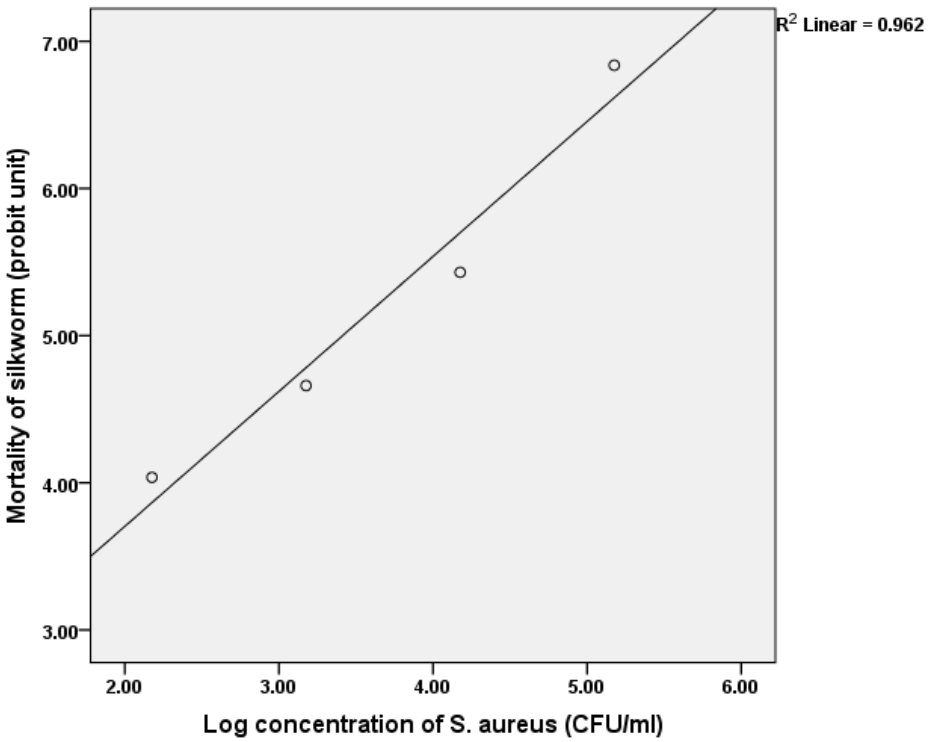


Figure 26 Median lethal dose (LD_{50}) of *S. aureus* in silkworm

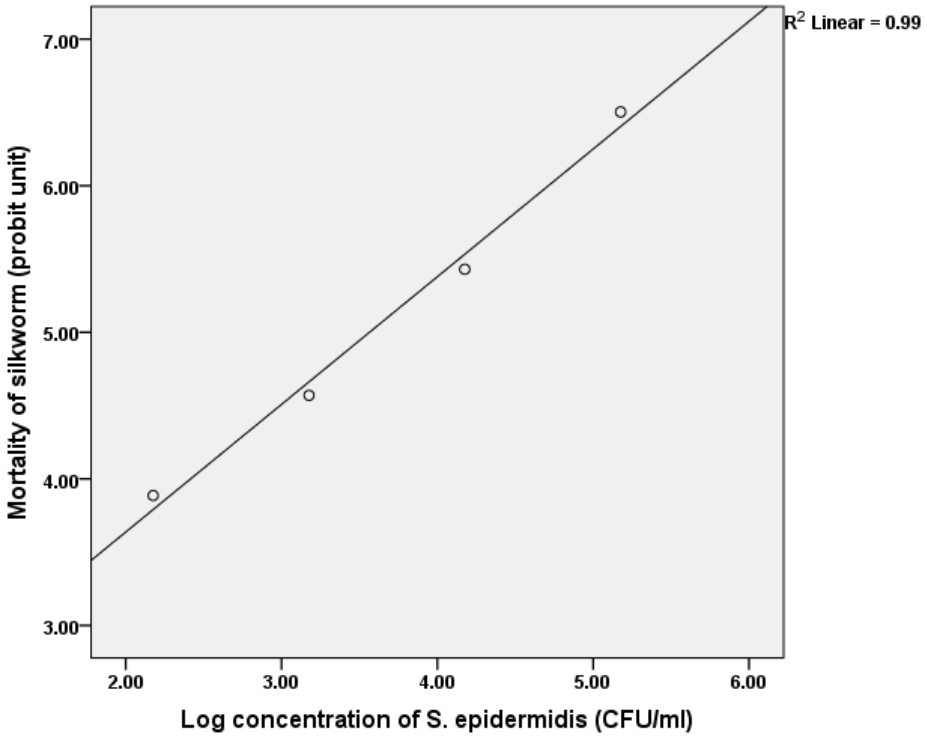


Figure 27 Median lethal dose (LD_{50}) of *S. epidermidis* in silkworm

3.2 Determination of LD_{50} of the ethanol extract and chrysazin

In order to investigate the toxicity of the ethanol extract and chrysazin to silkworms, they were injected with 50 µl of the ethanol extract or chrysazin in various concentrations into hemolymph and the mortality rate of silkworms was observed at 48 h after injection. The result showed that the LD_{50} values of the ethanol extract and chrysazin in silkworm was 84.19 mg/ml (4.68 mg/g larva) (Figure 28) and >5.00 mg/ml (>0.28 mg/g larva), respectively. The toxicity study of ampicillin was present in Figure 29. The LD_{50} value of ampicillin that killed half of the larvae was 102.76 mg/ml (5.71 mg/g larva).

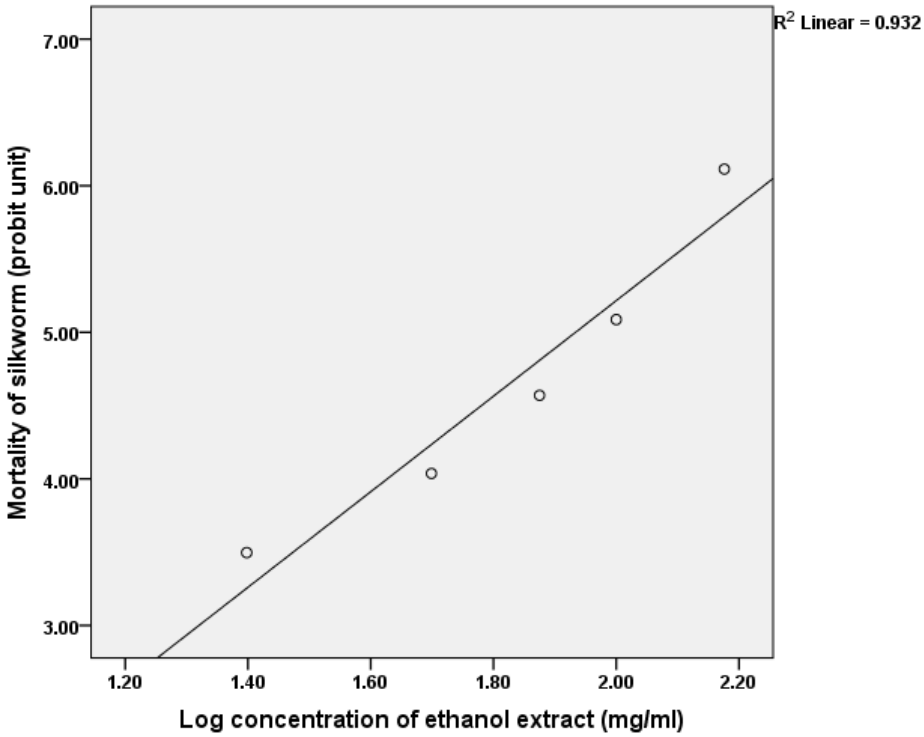


Figure 28 Median lethal dose (LD_{50}) of ethanol extract in silkworm

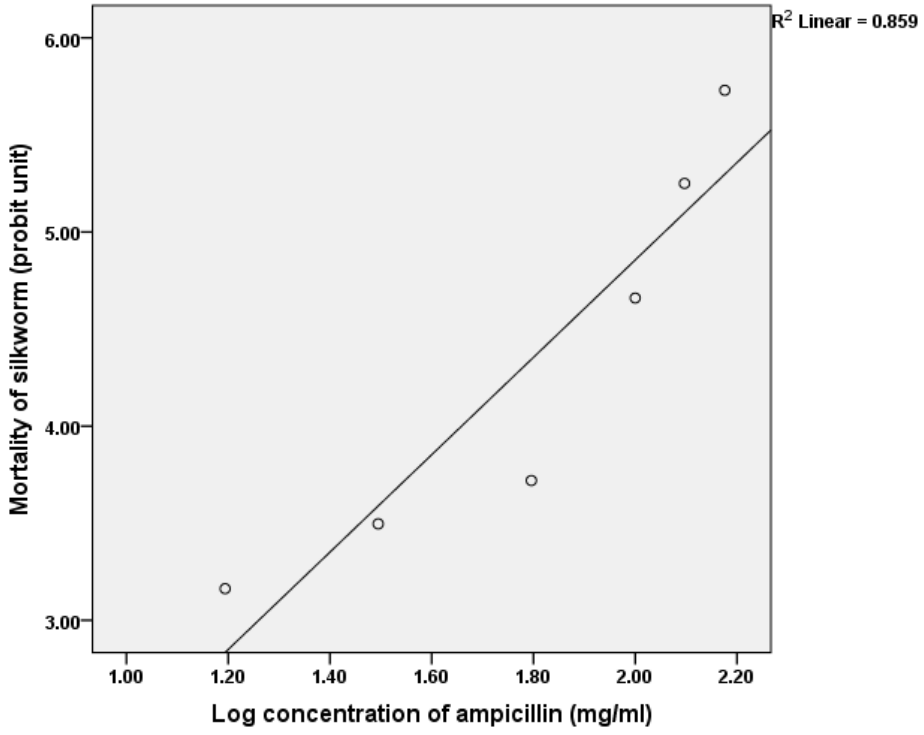


Figure 29 Median lethal dose (LD_{50}) of ampicillin in silkworm

3.3 Determination of ED_{50} of the ethanol extract and chrysazin

This study was to investigate whether the ethanol extract and chrysazin were effective in silkworms infected with *S. aureus* or *S. epidermidis*. The survival of silkworms was determined 2 days after injection of the bacterial suspension and the ethanol extract, chrysazin or ampicillin into hemolymph. Concentrations of *S. aureus* and *S. epidermidis* that could kill all of silkworms were used in control group. The ED_{50} values, concentration that showing 50% survival of the silkworms, of the ethanol extract and ampicillin against *S. aureus* and *S. epidermidis* were presented in Figure 30-31 and Figure 32-33, respectively. The ED_{50} values obtained from the ethanol extract, chrysazin and ampicillin against *S. aureus* were 3.08 mg/ml (0.18 mg/g larva), >5.0 mg/ml (>0.29 mg/g larva) and 0.22 mg/ml (0.013 mg/g larva), respectively whereas the ED_{50} values of the ethanol extract, chrysazin and ampicillin against *S. epidermidis* were 5.33 mg/ml (0.33 mg/g larva), >5.0 mg/ml (>0.31 mg/g larva) and 0.44 mg/ml (0.028 mg/g larva), respectively.

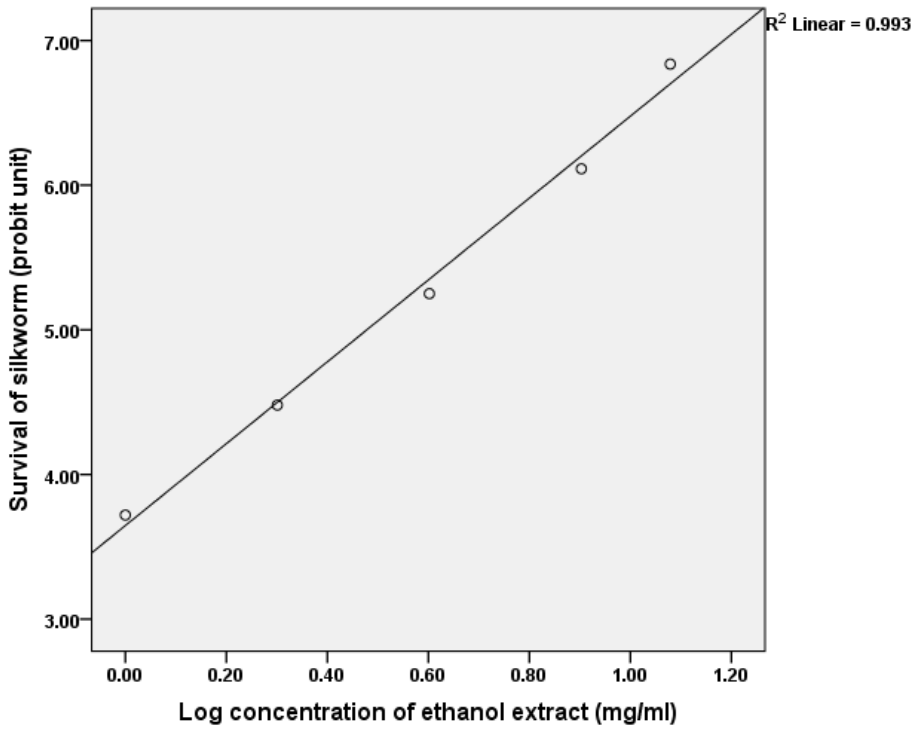


Figure 30 Median effective dose (ED_{50}) of ethanol extract in silkworm infected *S. aureus*

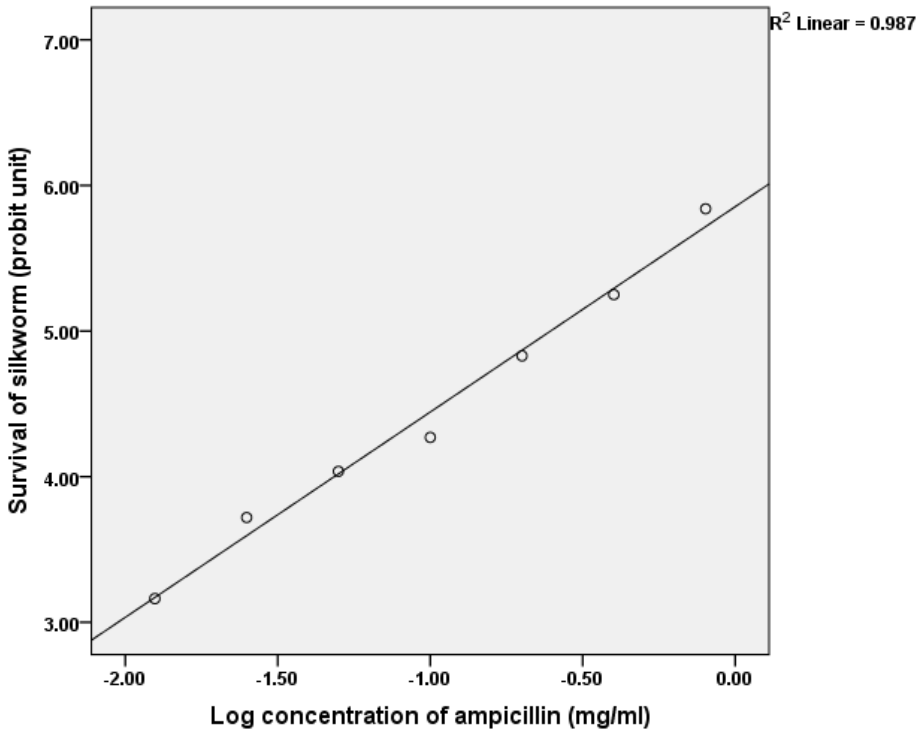


Figure 31 Median effective dose (ED_{50}) of ampicillin in silkworm infected *S. aureus*

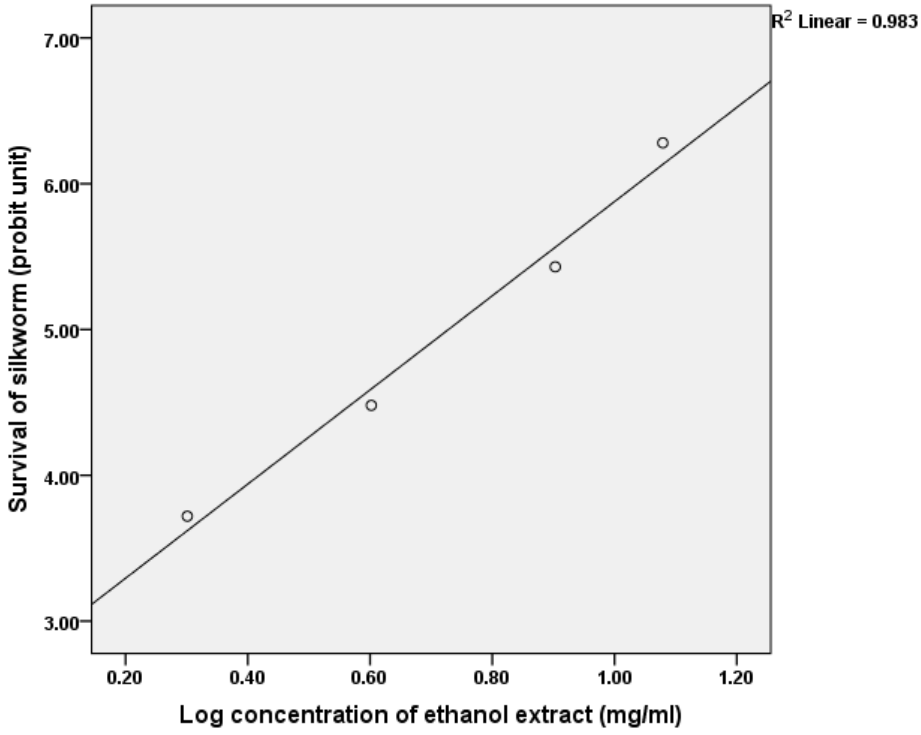


Figure 32 Median effective dose (ED_{50}) of ethanol extract in silkworm infected *S. epidermidis*

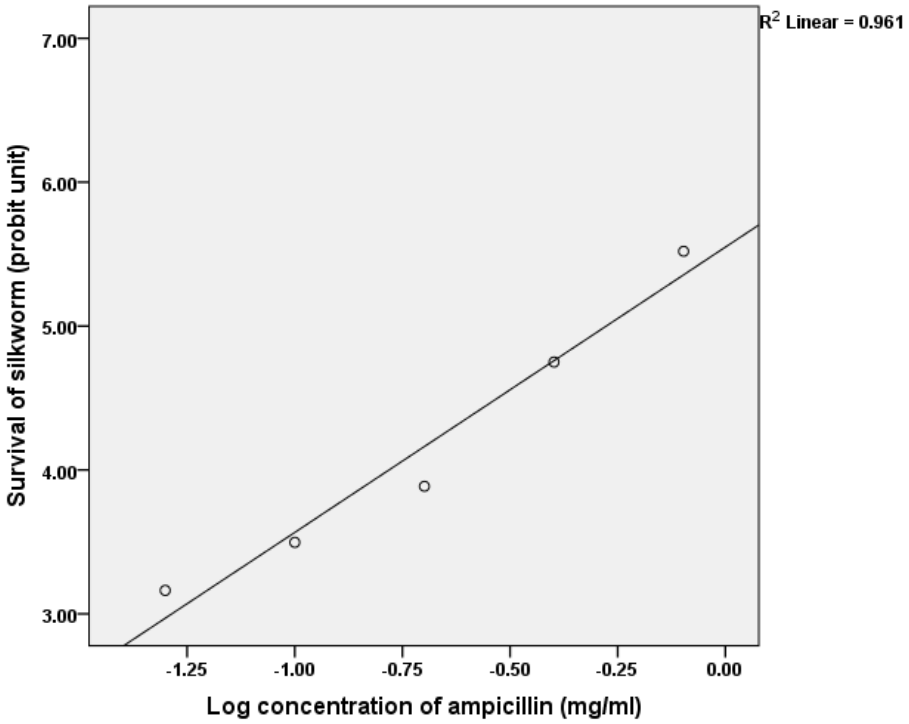


Figure 33 Median effective dose (ED_{50}) of ampicillin in silkworm infected *S. epidermidis*

CHAPTER V

DISCUSSION AND CONCLUSIONS

S. aureus and *S. epidermidis* are the most common causes of both community and hospital-acquired infections. They are able to infect every human organ and cause pathogenesis ranging from superficial skin infections to life-threatening infections. One of the key factors enables *S. aureus* and *S. epidermidis* to survive, colonise and proliferate until difficult to eradicate and cause harm in human body is the ability to form biofilm. At present, several research topics have studied antimicrobial and antibiofilm activities of medicinal plants for alternative treatment. In this study, the screening antimicrobial activity of the ethanol extract of *X. indica* and chrysazin against bacterial pathogens was evaluated by measuring the inhibition zone. The quantitative analysis of the antimicrobial activity was evaluated by broth microdilution method to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of ethanol extract and chrysazin while the bactericidal and bacteriostatic activity including time of killing microorganisms was assessed further by time kill assay. From the study, the results of the experiments both *in vitro* and *in vivo* were consistent. Ethanol extract and chrysazin had antimicrobial and antibiofilm activities against both *S. aureus* and *S. epidermidis*.

Firstly, preliminary study by agar diffusion assay indicated that the bacterial pathogens were inhibited by ethanol extract and chrysazin. The inhibition zones of ethanol extract equal to 19.30±0.60 mm and 16.67±0.60 mm for *S. aureus* and *S. epidermidis*, respectively, whereas chrysazin had the inhibition zones against *S. aureus* and *S. epidermidis* equal to 11.30±0.61 mm and 8.30±0.61 mm, respectively. However, petroleum ether extract did not exhibit antimicrobial activities against *S. aureus* and *S. epidermidis*. The possibility may be due to the fact that (i) petroleum ether extract had no bioactive compound (chrysazin) to inhibit these pathogens or (ii) petroleum ether extract composed of non-polar compounds which are difficult to diffuse in agar medium (Valgas *et al*., 2007; Pimentel *et al*., 2013). Therefore, other experiment should be tested

again to confirm the result such as broth microdilution method. Such method allows direct contact between the extract with bacterial pathogens. In addition, 20% DMSO and 100% DMSO, the negative control of the extract and chrysazin, did not exhibit inhibition against the tested bacterial whereas, ampicillin, standard antimicrobial drug, inhibited both *S. aureus* and *S. epidermidis* with inhibition zone larger than ethanol extract and chrysazin.

Extract and chrysazin were further estimated quantitatively by broth microdilution method. The results showed that ethanol extract exhibited antimicrobial activities against the bacterial pathogens at the MIC values equal to 0.5 mg/ml for *S. aureus* and 1 mg/ml for *S. epidermidis*, whereas MIC values of chrysazin for *S. aureus* and *S. epidermidis* were 1 and 2 mg/ml, respectively. The MBC values of ethanol extract required to kill *S. aureus* and *S. epidermidis* were found to be 4 mg/ml. While the MBC values of chrysazin against *S. aureus* and *S. epidermidis* were > 4 mg/ml. In this study, petroleum ether extract did not exhibit antimicrobial activities against both *S. aureus* and *S. epidermidis* the same as in agar diffusion experiment. Therefore, the active compound that contributes to this result may be chrysazin. In addition, ampicillin was used as a positive control for bacterial pathogens. MIC values of ampicillin against *S. aureus* and *S. epidermidis* were 0.098 µg/ml and 0.195 µg/ml, respectively. MIC values of ampicillin against *S. aureus* and *S. epidermidis* lower than breakpoint values which specified by Clinical and Laboratory Standards Institute (CLSI). CLSI reported breakpoint of MIC value of ampicillin against susceptible staphylococcus strains ≤ 0.25 µg/ml (CLSI, 2012). Hence, from obtained result suggested that strains of *S. aureus* and *S. epidermidis* in this study were susceptible strains to ampicillin.

In time kill assay, *S. aureus* and *S. epidermidis* were examined and samples were collected at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h, respectively. Viable colony count was determined by 10 fold-serial dilution on MHA plate. The results are presented in terms of the $\log_{10}$ CFU/ml of viable colonies. Bacteriostatic activity was defined as 1 to <3 $\log_{10}$ scale reduction (90% to < 99.9%) compared with original inoculum, whereas bactericidal activity was defined as a 3 $\log_{10}$

CFU/ml or greater reduction (≥ 99.9%) compared with original inoculum (NCCLS, 1999; Petersen *et al*., 2007). At concentration equal or greater than MIC of the ethanol extract were classified as bacteriostatic against *S. aureus* and *S. epidermidis*. 0.5 mg/ml (MIC), 1 mg/ml (2MIC) and 2 mg/ml (4MIC) of extract started to exhibit bacteriostatic activity (about 1 log scale reduction) against *S. aureus* at 48, 10 and 6 h, whereas 1 mg/ml (MIC) and 2 mg/ml (2MIC) of extract started to exhibit bacteriostatic activity against *S. epidermidis* (about 1 log scale reduction) at 12, and 8 h, respectively. Ethanol extract at concentration 4 mg/ml (MBC) showed bactericidal activity against *S. aureus* or *S. epidermidis* with a 3 $\log_{10}$ scale reduction observed at 8 and 10 h, respectively. Bacterial regrowth of *S. aureus* or *S. epidermidis* could not be observed after incubating for 24 h and longer. Time kill studies showed that chrysazin was bacteriostatic against *S. aureus* and *S. epidermidis* at concentrations equal or greater than MIC. 1 mg/ml (MIC), 2 mg/ml (2MIC) and 4 mg/ml (4MIC) of chrysazin showed bacteriostatic activity with 1 log scale reduction in colonies count for *S. aureus* at 48, 10 and 6 h respectively, whereas 2 mg/ml (MIC) and 4 mg/ml (2MIC) of chrysazin showed bacteriostatic activity with 1 log scale reduction in colonies count for *S. epidermidis* at 12 and 8 h, respectively. In addition, ampicillin (positive control) showed bactericidal activities as time dependent manner. At concentration greater than MIC of ampicillin, 3 $\log_{10}$ scale reduction in viable colony count for *S. aureus* and *S. epidermidis* was observed at 12 and 14 h, respectively.

In biofilm assay, the effect of ethanol extract and chrysazin on biofilm formation of *S. aureus* and *S. epidermidis* was determined since these pathogens are well known for their biofilm-producing. In biofilm, *S. aureus* and *S. epidermidis* are protected from external harm. Furthermore, biofilm formation correlates with the observation that microorganisms within biofilm are less susceptible to antimicrobial drugs and innate immune response (McCann *et al*., 2008; Archer *et al*., 2011). Hence, development of antibiofilm forming agents appears to be an interesting approach in the reduction of severity of bacterial infection. Based on the present results, both ethanol extract and chrysazin inhibited biofilm formation of *S. aureus* and *S. epidermidis*. The results showed that at MIC and above, both ethanol extract and chrysazin could inhibit biofilm formation of *S. aureus* and *S. epidermidis* with statistical significance ($p < 0.01$).

From previous antimicrobial activities testing (agar diffusion assay, broth microdilution method and time kill assay) showed that ethanol extract and chrysazin were more pronounced on *S. aureus* than *S. epidermidis*. These results are consistent with the results obtained from biofilm formation assay, where the ethanol extract and chrysazin showed inhibitory effect of biofilm formation on *S. aureus* superior to *S. epidermidis*. The decline of bacterial biofilm formation may be due to the fact that ethanol extract and chrysazin inhibited planktonic cell (free-living bacteria) or step of attachment of bacteria to polymer surface that contributing to the formation of biofilm. This inhibition of biofilm formation resulted in cell-cell signaling or quorum sensing system less than threshold level. This signal is dependent on the level of autoinducer peptides (AIPs), small molecule produced by bacterial cells, and causes the expression of accessory gene regulator (*agr*). This gene plays a major role in control pathogenesis, toxin production and many virulence factors such as hemolysin, leucosidin and hyaluronidase etc. of these bacterial pathogens. Furthermore, inhibitory effects of biofilm formation of ethanol extract and chrysazin are concentration dependent manner (Figure 21 and 24). The highest concentrations of ethanol extract and chrysazin (4 mg/ml) could inhibit biofilm formation up to 85.46% and 70.07%, respectively for *S. aureus* and up to 81.57% and 65.18%, respectively for *S. epidermidis*. However, at concentration 4 mg/ml (MBC value) of ethanol extract, the inhibition of biofilm formation could not reach 100%. These may be correlated with time course of biofilm formation of *S. aureus* and *S. epidermidis*. The ability to form biofilm of these pathogens has been reported to start after 2-4 h of incubation (Sun *et al*., 2012; Hola *et al*., 2006). However, the result obtained from time kill assay demonstrated that ethanol extract exhibited bactericidal activity (≥3 log scale reduction) against *S. aureus* and *S. epidermidis* at 8 and 10 h, respectively and these pathogens were completely eliminated after 24 h, respectively. Thus, bacterial pathogens may still form some biofilms before ethanol extract killed them.

In *in vivo* study, it was performed in order to examine whether injection of bacteria that are pathogenic to humans could kill silkworms or not. The result demonstrated that silkworms

were killed by injection of *S. aureus* and *S. epidermidis* suspension within 2 days. This results support previous study reported that *S. aureus* could infect in silkworms due to influence of *cvfA*, *cvfB*, and *cvfC* gene which conserved in staphylococcus species and required for pathogenesis of *S. aureus* in silkworms and mice (Kaito *et al*., 2005). Later, the toxicity study was performed to evaluate the LD_{50} of ethanol extract, chrysazin and ampicillin in order to select dose range that are save for using in the efficacy study in silkworm infection model. LD_{50} value of ethanol extract and chrysazin were 84.19 mg/ml (4.68 mg/g larva) and >5 mg/ml (>0.28 mg/g larva), respectively whereas LD_{50} value of ampicillin were 102.76 mg/ml (5.71 mg/g larva). In the last experiment, ethanol extract and chrysazin were examined for the therapeutic effect (ED_{50}) against *S. aureus* and *S. epidermidis* by using silkworm infection model. A highest concentration of ethanol extract, chrysazin and ampicillin for testing therapeutic effect should be lower than LD_{50} value approximately 5-10 fold except for chrysazin which LD_{50} could not be determined exactly (LD_{50}> 5 mg/ml). Therefore, the highest concentration of chrysazin used in efficacy testing is equal to the highest concentration used in toxicity testing (5 mg/ml). From the study, the results demonstrated that ethanol extract and chrysazin that showed antimicrobial activities in *in vitro* also had therapeutic effects on silkworm infected with these pathogens. ED_{50} values of ethanol extract against *S. aureus* and *S. epidermidis* were 3.08 mg/ml (0.18 mg/g larva) and 5.33 mg/ml (0.33 mg/g larva), respectively. Silkworm could survive from infection completely (100%) after receiving ethanol extract at concentration up to 16 mg/ml (0.94 mg/g larva). Although, silkworm received ethanol extract via intra-hemolymph (mimics mouse intravenous administration) resulted in bioavailability of ethanol extract was 100%. However, concentrations used in this study which allow silkworm to survive completely (100% survival) were more than MIC value that obtain from the study in *in vitro* (32 fold for *S. aureus* and 16 fold for *S. epidermidis*). Such results may be due to the fact that ethanol extract may lose its therapeutic effect because of the influence of other pharmacokinetic parameters for example plasma protein binding, metabolism and excretion. Ethanol extract may lose its antimicrobial activity in the presence of silkworm plasma by binding to silkworm plasma proteins and leading to the lower concentrations of free compounds available in hemolymph, then resulting in lower therapeutic effects in silkworm infection model. Therefore the higher concentration of ethanol extract was needed as seen in the aforementioned results. In addition, therapeutic efficacy of ethanol extract may decrease also because of the metabolism by

enzymes in silkworm or because of the excretion via urine or feces of silkworm. ED_{50} value of chrysazin against these bacterial pathogens was >5 mg/ml. 100% survival of silkworm could not be observed because of the limitation in term of solubility of chrysazin. Therefore the highest concentration used in testing for therapeutic effect of chrysazin could not be titrated higher than 5 mg/ml. However, some survival of silkworm could also be observed at some concentrations of chrysazin used in the experiment. This may be due to the fact that chrysazin showed bacteriostatic activity against *S. aureus* and *S. epidermidis* in time kill assay. Thus, it may inhibit bacterial growth and allow immune system in silkworm getting rid of bacterial pathogens later. In addition, ampicillin, antibiotic clinically used for human, was also effective in silkworms. ED_{50} values of ampicillin against *S. aureus* and *S. epidermidis* were 0.22 mg/ml (0.013 mg/g larva) and 0.44 mg/ml (0.028 mg/g larva), respectively. Again ampicillin may act synergistically to kill bacterial pathogens in conjunction with immune system in silkworm (Ahlstedt, 1981). Regarding, LD_{50} values and ED_{50} values obtained from the experiment, therapeutic index (TI) can be calculated. This of drug value is the ratio of dose that produces toxicity to dose that produces a clinically desired or effective response ($TI = LD_{50}/ED_{50}$). The larger the therapeutic index value, the saver the compound. If the calculated TI value is small, the compound should be monitored closely and carefully for any sign of toxicity. In this study, ethanol extract has calculated therapeutic index 27.33, whereas ampicillin, positive control used in the experiment, has calculated therapeutic index 467.09. Such results suggested that ampicillin, antibiotic clinically used for human, is safer than ethanol extract.

All results obtained from *in vitro* studies, in terms of antimicrobial activity of ethanol extract and chrysazin against *S. aureus* and *S. epidermidis*, are consistent with results obtained from the *in vivo* studies. However, the results indicated that ethanol extract of *X. indica* has greater potency than chrysazin, a major bioactive compound found approximately 58.90% in this plant and in is study also confirmed this phytochemical compound by TLC analysis. Furthermore, *X. indica* extract contains many bioactive compounds namely, 3-methoxychrysazin, 3-hydroxychrysazin, isocoumarins, stigmasterol and α-spinasterol in amount equal to 6.08%, 4.48%, 11.43%, 5.93% and 13.18%, respectively. Among such active compounds, 3-hydroxy-

chrysazin and isocoumarins have been reported antimicrobial activities against *S. aureus* and *S. epidermidis* (Ruangrungsi *et al*., 1993; Devienne *et al*., 2005; Qadeer, 2008). Therefore, additive or synergistic effect between chrysazin and such bioactive compounds of ethanol extract may contribute to the greater antimicrobial activity of ethanol extract of *X. indica* than chrysazin, a single major bioactive compound shown.

These findings demonstrated that ethanol extract of *X. indica* and chrysazin had antimicrobial activities against *S. aureus* and *S. epidermidis* both in *in vitro* and *in vivo* Thai silkworm infection model. Moreover, inhibition of biofilm formation may play an important role for the mechanism of action for antimicrobial activity shown. This study also suggested that both the ethanol extract and chrysazin may be useful as a natural antimicrobial agent and an antibiofilm agent in the future. However, further study is required to evaluate antimicrobial and antibiofilm activities of this compound against *S. aureus* and *S. epidermidis* in mammalian model.

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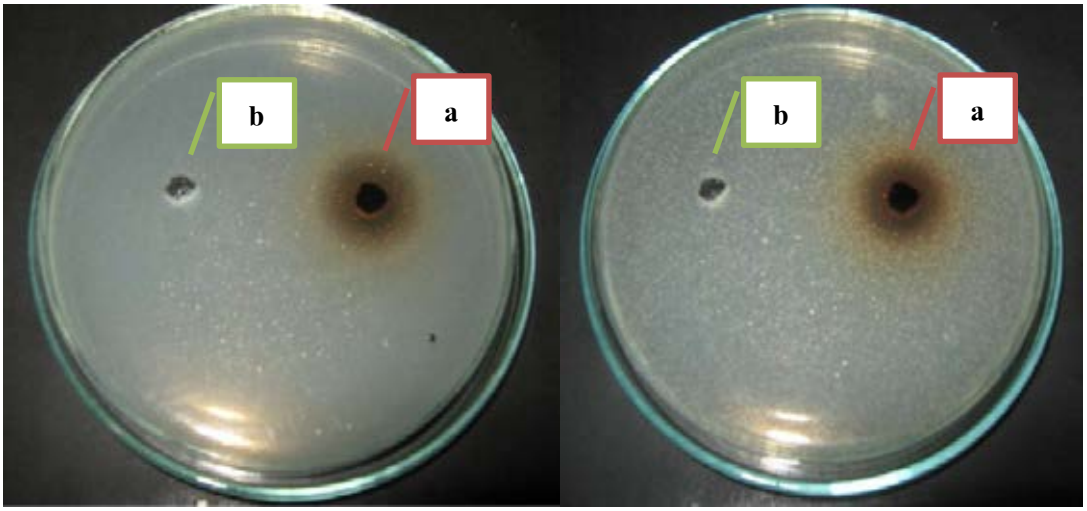
APPENDICES

Appendix A

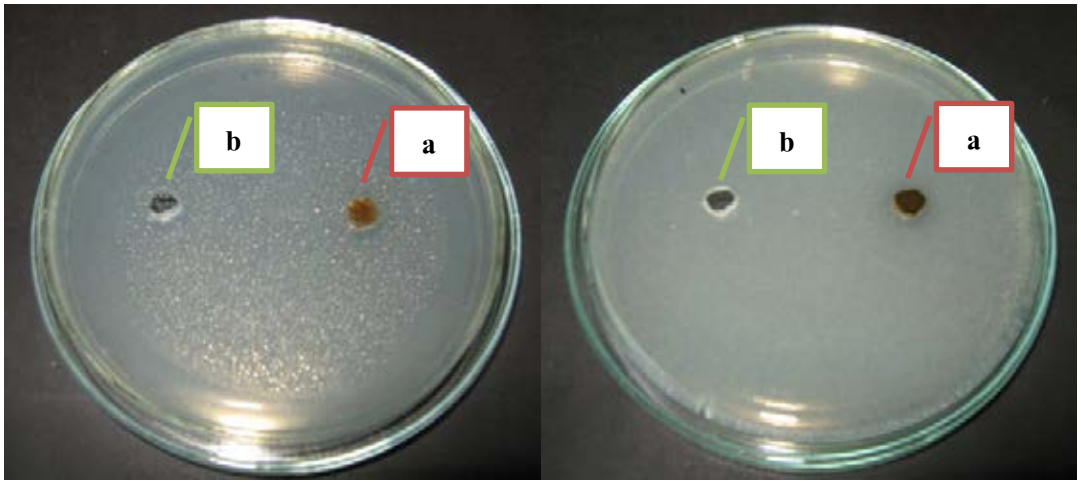
Data of Antimicrobial Activities Testing

(*in vitro* studies)



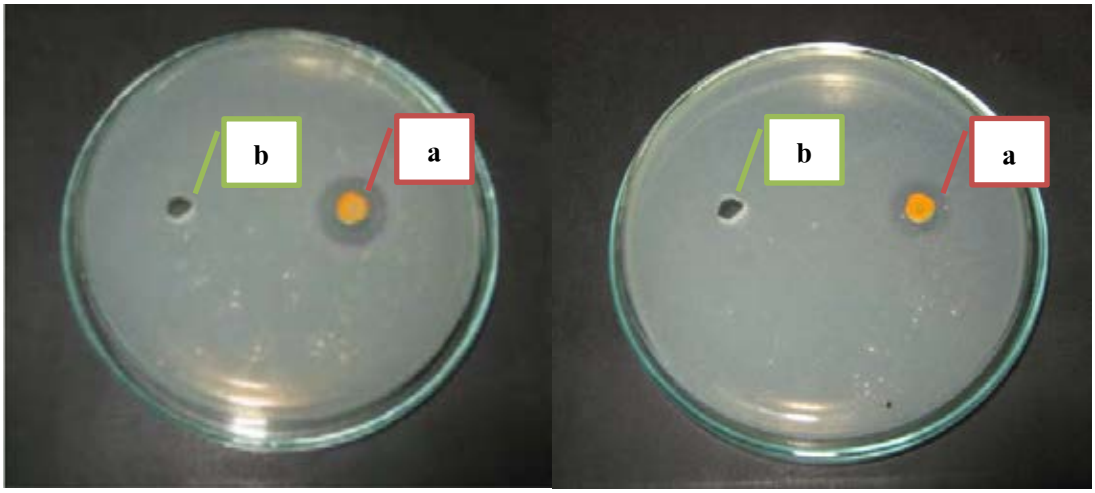
A B

Figure 34 Inhibition zone of the ethanol extract from *X. indica* against *S. aureus* (A) and *S. epidermidis* (B): a. the ethanol extract, b. 20% DMSO



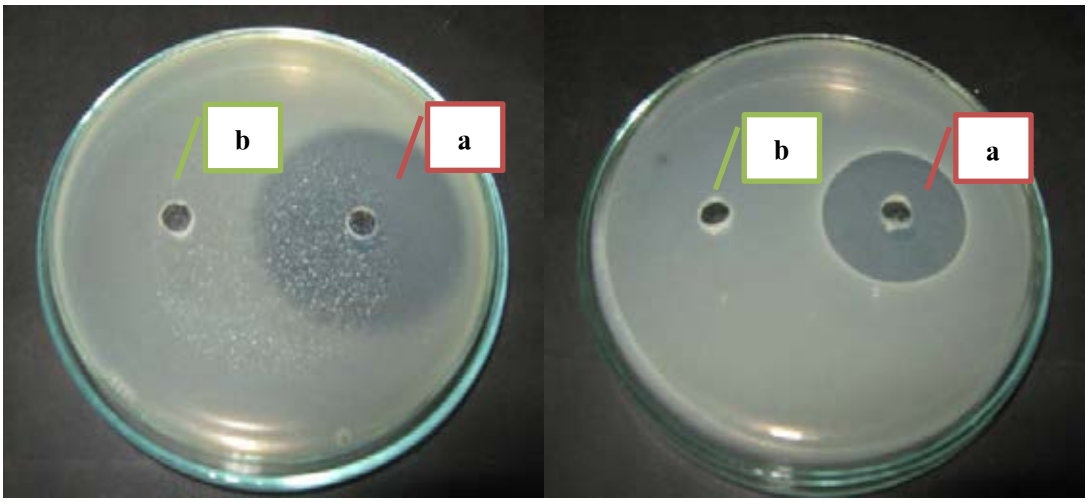
A B

Figure 35 Inhibition zone of the petroleum ether extract from *X. indica* against *S. aureus* (A) and *S. epidermidis* (B): a. the petroleum ether extract, b. 100% DMSO



A B

Figure 36 Inhibition zone of chrysazin against *S. aureus* (A) and *S. epidermidis* (B): a. chrysazin, b. 100% DMSO



A B

Figure 37 Inhibition zone of ampicillin (positive control) against *S. aureus* (A) and *S. epidermidis* (B): a. ampicillin, b. 20% DMSO

Table 4 Survival colonies of *S. aureus* received the ethanol extract in Time kill assay

Hours	Survival colonies (CFU/ml)						
	Growth control	Negative control	Extract 0.25 mg/ml	Extract 0.5 mg/ml	Extract 1 mg/ml	Extract 2 mg/ml	Extract 4 mg/ml
0	$5.08x10^5$	$5.00x10^5$	$5.04x10^5$	$5.10x10^5$	$5.02x10^5$	$5.02x10^5$	$5.00x10^5$
2	$8.30x10^5$	$8.00x10^5$	$7.49x10^5$	$6.05x10^5$	$4.53x10^5$	$1.60x10^5$	$8.06x10^4$
4	$2.15x10^6$	$2.09x10^6$	$9.46x10^5$	$5.64x10^5$	$3.10x10^5$	$8.00x10^4$	$2.70x10^4$
6	$5.68x10^6$	$5.70x10^6$	$2.18x10^6$	$8.38x10^5$	$1.09x10^5$	$4.75x10^4$	$4.83x10^3$
8	$2.06x10^7$	$2.10x10^7$	$6.90x10^6$	$4.79x10^5$	$7.05x10^4$	$2.30x10^4$	$4.90x10^2$
10	$7.00x10^7$	$6.97x10^7$	$1.90x10^7$	$1.55x10^5$	$3.93x10^4$	$7.07x10^3$	$3.90x10^2$
12	$2.10x10^8$	$2.00x10^8$	$3.65x10^7$	$8.37x10^4$	$1.55x10^4$	$4.03x10^3$	$3.03x10^2$
24	$6.01x10^8$	$6.04x10^8$	$4.85x10^7$	$5.23x10^4$	$8.97x10^3$	$9.77x10^2$	0.00
48	$8.13x10^8$	$8.07x10^8$	$1.95x10^8$	$3.39x10^4$	$3.83x10^3$	$5.93x10^2$	0.00

Table 5 Survival colonies of *S. aureus* received chrysazin in Time kill assay

Hours	Survival colonies (CFU/ml)					
	Growth control	Negative control	Chrysazin 0.5 mg/ml	Chrysazin 1 mg/ml	Chrysazin 2 mg/ml	Chrysazin 4 mg/ml
0	$5.06x10^5$	$5.02x10^5$	$5.03x10^5$	$5.02x10^5$	$5.09x10^5$	$5.11x10^5$
2	$8.50x10^5$	$8.46x10^5$	$7.32x10^5$	$6.00x10^5$	$4.33x10^5$	$2.00x10^5$
4	$2.20x10^6$	$2.10x10^6$	$9.00x10^5$	$5.46x10^5$	$3.17x10^5$	$8.70x10^4$
6	$5.65x10^6$	$5.61x10^6$	$2.03x10^6$	$4.30x10^5$	$1.13x10^5$	$4.96x10^4$
8	$2.00x10^7$	$2.10x10^7$	$6.48x10^6$	$3.70x10^5$	$7.21x10^4$	$2.66x10^4$
10	$7.10x10^7$	$7.03x10^7$	$1.78x10^7$	$1.06x10^5$	$3.97x10^4$	$9.33x10^3$
12	$2.18x10^8$	$2.15x10^8$	$3.17x10^7$	$8.13x10^4$	$1.65x10^4$	$4.43x10^3$
24	$6.05x10^8$	$6.10x10^8$	$4.55x10^7$	$5.45x10^4$	$9.30x10^3$	$9.97x10^2$
48	$8.17x10^8$	$8.13x10^8$	$1.50x10^8$	$3.63x10^4$	$5.90x10^3$	$6.97x10^2$

Table 6 Survival colonies of *S. aureus* received ampicillin in Time kill assay

Hours	Survival colonies (CFU/ml)						
	Growth control	Negative control	Extract 0.049 µg/ml	Extract 0.098 µg/ml	Extract 0.19 µg/ml	Extract 0.39 µg/ml	Extract 0.78 µg/ml
0	$5.08x10^5$	$5.04x10^5$	$5.02x10^5$	$5.02x10^5$	$5.09x10^5$	$5.00x10^5$	$5.06x10^5$
2	$8.30x10^5$	$8.25x10^5$	$7.62x10^5$	$6.11x10^5$	$3.30x10^5$	$2.40x10^5$	$1.70x10^5$
4	$2.05x10^6$	$2.10x10^6$	$1.10x10^6$	$4.80x10^5$	$9.87x10^4$	$7.33x10^4$	$5.90x10^4$
6	$5.63x10^6$	$5.60x10^6$	$3.00x10^6$	$1.96x10^5$	$5.40x10^4$	$3.31x10^4$	$1.90x10^4$
8	$2.02x10^7$	$2.11x10^7$	$5.68x10^6$	$9.83x10^4$	$2.05x10^4$	$9.97x10^3$	$7.97x10^3$
10	$7.10x10^7$	$7.00x10^7$	$9.97x10^6$	$6.54x10^4$	$7.67x10^3$	$5.00x10^3$	$2.96x10^3$
12	$2.14x10^8$	$2.06x10^8$	$3.56x10^7$	$4.97x10^4$	$5.00x10^2$	$4.23x10^2$	$3.23x10^2$
24	$6.09x10^8$	$6.00x10^8$	$6.04x10^7$	$1.29x10^4$	0.00	0.00	0.00
48	$8.13x10^8$	$8.10x10^8$	$7.40x10^7$	$8.13x10^3$	0.00	0.00	0.00

Table 7 Survival colonies of *S. epidermidis* received the ethanol extract in Time kill assay

Hours	Survival colonies (CFU/ml)					
	Growth control	Negative control	Extract 0.5 mg/ml	Extract 1 mg/ml	Extract 2 mg/ml	Extract 4 mg/ml
0	$5.04x10^5$	$5.00x10^5$	$5.02x10^5$	$5.03x10^5$	$5.09x10^5$	$5.02x10^5$
2	$8.00x10^5$	$8.09x10^5$	$5.95x10^5$	$5.82x10^5$	$4.64x10^5$	$9.96x10^4$
4	$1.35x10^6$	$1.37x10^6$	$6.33x10^5$	$5.02x10^5$	$2.85x10^5$	$5.29x10^4$
6	$4.30x10^6$	$4.25x10^6$	$9.33x10^5$	$4.57x10^5$	$1.06x10^5$	$8.46x10^3$
8	$1.33x10^7$	$1.31x10^7$	$3.27x10^6$	$2.10x10^5$	$4.30x10^4$	$1.13x10^3$
10	$5.46x10^7$	$5.43x10^7$	$6.97x10^6$	$9.76x10^4$	$8.76x10^3$	$4.73x10^2$
12	$9.97x10^7$	$9.93x10^7$	$9.90x10^6$	$4.88x10^4$	$4.63x10^3$	$3.06x10^2$
24	$2.76x10^8$	$2.70x10^8$	$3.19x10^7$	$1.70x10^4$	$1.59x10^3$	0.00
48	$4.23x10^8$	$4.30x10^8$	$6.03x10^7$	$7.23x10^3$	$6.97x10^2$	0.00

Table 8 Survival colonies of *S. epidermidis* received chrysazin in Time kill assay

Hours	Survival colonies (CFU/ml)				
	Growth control	Negative control	Chrysazin 1 mg/ml	Chrysazin 2 mg/ml	Chrysazin 4 mg/ml
0	$5.10x10^5$	$5.00x10^5$	$5.02x10^5$	$5.08x10^5$	$5.00x10^5$
2	$8.10x10^5$	$8.04x10^5$	$5.70x10^5$	$5.36x10^5$	$3.91x10^5$
4	$1.33x10^6$	$1.30x10^6$	$6.23x10^5$	$4.70x10^5$	$1.00x10^5$
6	$4.27x10^6$	$4.30x10^6$	$9.90x10^5$	$2.89x10^5$	$6.23x10^4$
8	$1.37x10^7$	$1.35x10^7$	$3.00x10^6$	$2.07x10^5$	$3.32x10^4$
10	$5.43x10^7$	$5.50x10^7$	$6.51x10^6$	$9.96x10^4$	$9.86x10^3$
12	$9.93x10^7$	$9.90x10^7$	$1.00x10^7$	$4.89x10^4$	$4.96x10^3$
24	$2.79x10^8$	$2.76x10^8$	$3.10x10^7$	$2.77x10^4$	$1.49x10^3$
48	$4.26x10^8$	$4.33x10^8$	$5.96x10^7$	$9.90x10^3$	$9.93x10^2$

Table 9 Survival colonies of *S. epidermidis* received ampicillin in Time kill assay

Hours	Survival colonies (CFU/ml)						
	Growth control	Negative control	Extract 0.098 µg/ml	Extract 0.19 µg/ml	Extract 0.39 µg/ml	Extract 0.78 µg/ml	Extract 1.56 µg/ml
0	$5.09x10^5$	$5.04x10^5$	$5.00x10^5$	$5.00x10^5$	$5.02x10^5$	$5.00x10^5$	$5.08x10^5$
2	$8.13x10^5$	$8.10x10^5$	$5.72x10^5$	$5.60x10^5$	$4.40x10^5$	$3.41x10^5$	$2.90x10^5$
4	$1.37x10^6$	$1.33x10^6$	$6.40x10^5$	$5.31x10^5$	$9.97x10^4$	$8.60x10^4$	$6.36x10^4$
6	$4.26x10^6$	$4.30x10^6$	$7.47x10^5$	$4.86x10^5$	$5.12x10^4$	$3.66x10^4$	$1.77x10^4$
8	$1.35x10^7$	$1.40x10^7$	$2.53x10^6$	$3.69x10^5$	$9.87x10^3$	$7.73x10^3$	$5.13x10^3$
10	$5.47x10^7$	$5.43x10^7$	$5.04x10^6$	$1.00x10^5$	$5.20x10^3$	$3.57x10^3$	$3.00x10^3$
12	$9.90x10^7$	$9.96x10^7$	$8.83x10^6$	$4.93x10^4$	$1.42x10^3$	$9.60x10^2$	$8.07x10^2$
24	$2.70x10^8$	$2.60x10^8$	$2.43x10^7$	$8.13x10^3$	0.00	0.00	0.00
48	$4.30x10^8$	$4.27x10^8$	$3.46x10^7$	$4.97x10^3$	0.00	0.00	0.00

Table 10 Percentage of inhibition of biofilm formation of *S. aureus* received the ethanol extract

Ethanol extract (mg/ml)	**Percentage of inhibition of biofilm formation (Mean ± SD)**
0.00	0.00
0.25	2.34±0.67
0.50	32.97±1.05
1.00	63.57±0.73
2.00	75.25±1.20
4.00	85.46±0.86

Table 11 Percentage of inhibition of biofilm formation of *S. aureus* received chrysazin

Chrysazin (mg/ml)	**Percentage of inhibition of biofilm formation (Mean ± SD)**
0.00	0.00
0.50	2.49±0.82
1.00	40.00±0.81
2.00	58.81±0.90
4.00	70.07±0.76

Table 12 Percentage of inhibition of biofilm formation of *S. aureus* received ampicillin

Ampicillin (µg/ml)	**Percentage of inhibition of biofilm formation (Mean ± SD)**
0.00	0.00
0.049	2.19±0.56
0.098	40.08±0.74
0.19	72.09±1.19
0.39	74.46±0.98
0.78	77.02±0.92

Table 13 Percentage of inhibition of biofilm formation of *S. epidermidis* received the ethanol extract

Ethanol extract (mg/ml)	**Percentage of inhibition of biofilm formation (Mean ± SD)**
0.00	0.00
0.50	1.67±0.51
1.00	31.75±0.85
2.00	64.11±0.80
4.00	81.57±0.85

Table 14 Percentage of inhibition of biofilm formation of *S. epidermidis* received chrysazin

Chrysazin (mg/ml)	**Percentage of inhibition of biofilm formation (Mean ± SD)**
0.00	0.00
1.00	1.97±0.70
2.00	40.48±0.95
4.00	65.18±0.67

Table 15 Percentage of inhibition of biofilm formation of *S. epidermidis* received ampicillin

Ampicillin (µg/ml)	**Percentage of inhibition of biofilm formation (Mean ± SD)**
0.00	0.00
0.098	2.54±0.67
0.19	40.62±1.11
0.39	67.07±1.10
0.78	69.58±0.77
1.56	71.89±1.09

Appendix B

Data of Antimicrobial Activities Testing

(*in vivo* studies)

Table 16 Mortality rate of silkworm received *S. aureus* at various concentrations

***S. aureus* (CFU/ml)**	**Mean of number of silkworm death (% mortality)**
$1.5x10^1$	0.00
$1.5x10^2$	16.67
$1.5x10^3$	36.67
$1.5x10^4$	66.67
$1.5x10^5$	96.67
$1.5x10^6$	100.00
$1.5x10^7$	100.00
$1.5x10^8$	100.00

Table 17 Mortality rate of silkworm received *S. epidermidis* at various concentrations

***S. epidermidis* (CFU/ml)**	**Mean of number of silkworm death (% mortality)**
$1.5x10^1$	0.00
$1.5x10^2$	13.33
$1.5x10^3$	33.33
$1.5x10^4$	66.67
$1.5x10^5$	93.33
$1.5x10^6$	100.00
$1.5x10^7$	100.00
$1.5x10^8$	100.00

Table 18 Mortality rate of silkworm received the ethanol extract of *X. indica* at various concentrations

Ethanol extract (mg/ml)	**Mean of number of silkworm death (% mortality)**
6.25	0.00
12.50	0.00
25.00	6.67
50.00	16.67
75.00	33.33
100.00	53.33
150.00	86.67
200.00	100.00

Table 19 Mortality rate of silkworm received chrysazin at various concentrations

Chrysazin (mg/ml)	**Mean of number of silkworm death (% mortality)**
0.50	0.00
1.00	0.00
2.00	0.00
3.00	0.00
4.00	0.00
5.00	0.00

Table 20 Mortality rate of silkworm received ampicillin at various concentrations

Ampicillin (mg/ml)	**Mean of number of silkworm death (% mortality)**
7.81	0.00
15.63	3.33
31.25	6.67
62.50	10.00
100.00	36.67
125.00	60.00
150.00	76.67
250.00	100.00

Table 21 Survival rate of silkworm infected with *S. aureus* received the ethanol extract at various concentrations

Ethanol extract (mg/ml)	**Mean of number of silkworm survival (% survival)**
0.25	0.00
0.50	0.00
1.00	10.00
2.00	30.00
4.00	60.00
8.00	86.67
12.00	96.67
16.00	100.00

Table 22 Survival rate of silkworm infected with *S. aureus* received chrysazin at various concentrations

Chrysazin (mg/ml)	**Mean of number of silkworm survival (% survival)**
0.50	0.00
1.00	0.00
2.00	0.00
3.00	10.00
4.00	30.00
5.00	43.33

Table 23 Survival rate of silkworm infected with *S. aureus* received ampicillin at various concentrations

Ampicillin (mg/ml)	**Mean of number of silkworm survival (% survival)**
0.00625	0.0
0.0125	3.33
0.025	10.00
0.05	16.67
0.10	23.33
0.20	43.33
0.40	60.00
0.80	80.00
1.60	100.00

Table 24 Survival rate of silkworm infected with *S. epidermidis* received the ethanol extract at various concentrations

Ethanol extract (mg/ml)	**Mean of number of silkworm survival (% survival)**
0.50	0.00
1.00	0.00
2.00	10.00
4.00	30.00
8.00	66.67
12.00	90.00
16.00	100.00

Table 25 Survival rate of silkworm infected with *S. epidermidis* received chrysazin at various concentrations

Chrysazin (mg/ml)	**Mean of number of silkworm survival (% survival)**
1.00	0.00
2.00	0.00
3.00	0.00
4.00	10.00
5.00	36.67

Table 26 Survival rate of silkworm infected with *S. epidermidis* received ampicillin at various concentrations

Ampicillin (mg/ml)	**Mean of number of silkworm survival (% survival)**
0.0125	0.00
0.025	0.00
0.05	3.33
0.10	6.67
0.20	13.33
0.40	40.00
0.80	70.00
1.60	100.00

VITAE

Miss Warista Sukpanich was born on October 30, 1985 in Bangkok, Thailand. She graduated with Bachelor Degree of Pharmacy from Faculty of Pharmaceutical Sciences, Mahidol University. Consequently, she had started her work at Faculty of Medicine Vajira Hospital, Bangkok. She had enrolled for Master's Degree in Pharmacology at the department of Pharmacology and Physiology, faculty of Pharmaceutical science Chulalongkorn University since June, 2011.