ฤทธิ์ในการต้านเชื้อของกรดอุสนิคต่อเชื้อสแตปฟิโลคอคคัส ออเรียสและ
สแตปฟิโลคอคคัส เอพิเดอร์มิดิส จากการศึกษาในหลอดทดลองและ
แบบในกายโดยใช้หนอนไหมไทย

นางสาวนุชริยา น้ำคำ

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIMICROBIAL ACTIVITIES OF (+)-USNIC ACID ON *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL

Miss Nutchariya Namkham

A Thesis Submitted in Partial Fulfillment of the Requirements
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(Interdisciplinary Program)
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นุชริยา น้ำคำ : ฤทธิ์ในการต้านเชื้อของกรดอุสนิคต่อเชื้อสแตปฟิโลคอคคัส ออเรียส และ สแตปฟิโลคอคคัส เอพิเดอร์มิดิส จากการศึกษาในหลอดทดลองและ แบบในกาย โดยใช้หนอนไหมไทย. (ANTIMICROBIAL ACTIVITIES OF (+)-USNIC ACID ON *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ภก. ดร. สันทัด จันทร์ประภาพ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ชนิดา พลานุเวช, 68 หน้า.

โรคติดเชื้อที่พบทั้งในชุมชนและในโรงพยาบาลมีสาเหตุมาจากเชื้อสแตปฟิโลคอคคัส ออเรียสและสแตปฟิโลคอคคัส เอพิเดอร์มิดิส ความสามารถในการสร้างไบโอฟิล์มเป็นปัจจัยสำคัญที่ทำให้เชื้อทั้งสองชนิด สามารถรอดชีวิต เพิ่มจำนวน และอาศัยในร่างกายของผู้ป่วยได้ ในงานวิจัยนี้จึงมีความสนใจศึกษาฤทธิ์ต้านจุลชีพ ฤทธิ์ยับยั้งการสร้างไบโอฟิล์ม รวมถึงระยะเวลาในการฆ่าเชื้อสแตปฟิโลคอคคัส ออเรียส และสแตปฟิโลคอคคัส เอพิเดอร์มิดิส ของกรดอุสนิค จากการทดสอบฤทธิ์ต้านจุลชีพโดยวิธีการเจือจางในอาหารเหลว พบว่าความเข้มข้นต่ำสุดของกรดอุสนิคที่สามารถยับยั้งการเจริญของเชื้อสแตปฟิโลคอคคัส ออเรียส และสแตปฟิโลคอคคัส เอพิเดอร์มิดิส มีค่าเท่ากับ 250 และ 62.50 ไมโครกรัม/มิลลิลิตร ตามลำดับ ความเข้มข้นต่ำสุดของกรดอุสนิคที่สามารถฆ่าเชื้อสแตปฟิโลคอคคัส ออเรียส และสแตปฟิโลคอคคัส เอพิเดอร์มิดิส มีค่าเท่ากันทั้งสองเชื้อคือมากกว่า 4.00 มิลลิกรัม/มิลลิลิตร นอกจากนี้การศึกษา time-kill ไม่พบฤทธิ์ของกรดอุสนิคในการยับยั้งการเจริญของเชื้อสแตปฟิโลคอคคัส ออเรียส ในทุกความเข้มข้นที่ใช้ในการทดสอบ (125, 250, 500 and 1000 ไมโครกรัม/มิลลิลิตร) ในขณะที่กรดอุสนิคสามารถยับยั้งการเจริญของเชื้อสแตปฟิโลคอคคัส เอพิเดอร์มิดิส แบบ bacteriostatic ที่ความเข้มข้น 250 ไมโครกรัม/มิลลิลิตร ที่เวลา 12 ชั่วโมงหลังจากทำการบ่ม และพบว่ากรดอุสนิคสามารถยับยั้งการสร้างไบโอฟิล์มของเชื้อทั้งสองชนิดนี้ โดยความสามารถในการยังยั้งการสร้างไบโอฟิล์มขึ้นกับความเข้มข้นของกรดอุสนิค ความเข้มข้นของกรดอุสนิคที่มากกว่าหรือเท่ากับค่าการยังยั้งการเจริญเติบโตของแต่ละเชื้อ สามารถยับยั้งการสร้างไบโอฟิล์มของเชื้อสแตปฟิโลคอคคัส ออเรียส และสแตปฟิโลคอคคัส เอพิเดอร์มิดิส ได้อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อเปรียบเทียบกับ 5% DMSO สำหรับการศึกษาแบบในกายใช้หนอนไหมในการทดสอบประสิทธิภาพการรักษาของกรดอุสนิค พบว่าความเข้มข้นของกรดอุสนิคที่ทำให้หนอนไหมที่ติดเชื้อสแตปฟิโลคอคคัส ออเรียส และสแตปฟิโลคอคคัส เอพิเดอร์มิดิส รอดชีวิตได้ 50% มีค่ามากกว่า 4.00 มิลลิกรัม/มิลลิลิตร (0.57 มิลลิกรัม/กรัม ของหนอนไหม) กรดอุสนิคอาจสูญเสียฤทธิ์ในการรักษาเนื่องมาจากเภสัชจลนศาสตร์ในหนอนไหม นอกจากนั้นจากการศึกษานี้พบว่าหนอนไหมสามารถใช้เป็นโมเดลในการคัดกรองยาสำหรับต้านการติดเชื้อได้ด้วย

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NUTCHARIYA NAMKHAM: ANTIMICROBIAL ACTIVITIES OF (+)-USNIC ACID ON *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIDIS IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL. ADVISOR: SANTAD CHANPRAPARP, Ph.D., CO-ADVISOR: CHANIDA PALANUVEJ, Ph.D., 68 pp.

The common causes of community and hospital-acquired infections are *Staphylococcus aureus* and *Staphylococcus epidermidis*. One of the key factors enables pathogens to survive, colonize and proliferate in body is the ability to form biofilm. In this study investigated the antimicrobial, antibiofilm formation activities and time-kill assay of (+)-usnic acid against *S. aureus* and *S. epidermidis*. Antimicrobial activities were determined by broth microdilution method. The MIC values of (+)-usnic acid for *S. aureus* and *S. epidermidis* were 250 and 62.50 µg/ml, respectively. In addition, MBC values of (+)-usnic acid for both pathogens were more than 4 mg/ml. Furthermore, in time-kill assay showed that (+)-usnic acid has no bacteriostatic activity against *S. aureus* in all concentrations used (125, 250, 500 and 1000 µg/ml) whereas it has bacteriostatic activity against *S. epidermidis* only at 250 µg/ml after 12 h of incubation. (+)-Usnic acid also inhibited biofilm formation against *S. aureus* and *S. epidermidis*. The inhibitory effect on biofilm formation was concentration dependent manner at the concentration equal and greater than MIC values of each microorganism with statistical significance comparing with 5% DMSO ($p < 0.05$). For *in vivo* study, silkworms were utilized for efficacy testing of (+)-usnic acid. ED_{50} of (+)-usnic acid for *S. aureus* and *S. epidermidis* were more than 4.00 mg/ml (0.57mg/g of larva). (+)-Usnic acid may lose its therapeutic effect because of the influence of pharmacokinetic in silkworm model. In addition, silkworm infectious model can also be utilized as a screening tool for drugs discovery.

Field of Study: Pharmacology	Student's Signature ....
Academic Year: 2013	Advisor's Signature ....
	Co-Advisor's Signature ....

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
°C	degree Celcius
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institue
DMSO	dimethyl sulfoxide
eDNA	extracellular DNA
ED_{50}	Median effective concentration
EPS	extracellular polymeric substances
et al	et alibi (and others)
etc.	et cetera (and other things)
h	hour (s)
LD_{50}	Median lethal dose
Log	Decimal logarithm
MBC	Minimal bactericidal concentration
mg	milligram
mg/ml	milligram per milliliter
mg/g of larva	milligram per gram of larva
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration

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min	minute
ml	milliliter
NCCLS	National Committee for Clinical Laboratory Standards
PIA	polysaccharide intercellular adhesion
PSMs	phenol-soluble modulins
QS	quorum sensing
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
µg	microgram
µl	microliter
%	percent

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CHAPTER I

INTRODUCTION

Background and rationale

The genus of *Staphylococcus* is the most important pathogen in human. The pathogenic staphylococci cause a wide spectrum of life-threatening systemic diseases such as infections of the skin, soft tissues, bones, urinary tract and opportunistic infections (Murray, Rosenthal, Kobayashi, & Pfaller, c2002). Staphylococci are gram-positive bacteria that tend to be grape-like clusters. This genus is divided into two major group 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. delphini* and *S. intermidius*. The members of coagulase-negative staphylococcus are *S. epidermidis*, *S. simulans* and *S. xylosus* etc. (Ryan & Ray, c2010). Staphylococci are colonizing on human or animal skin and mucous membrane. The species most commonly associated with human diseases are *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. capitis* and *S. haemolyticus*.

S. aureus is a wide range of serious acute and chronic diseases such as staphylococcal food poisoning, pneumonia, meningitis, empyema, endocarditis, or sepsis with suppuration in any organ. The causes of diseases are the production of toxin or the direct invasion and destruction of tissue. Additionally, *S. epidermidis* is also common causes of human infections especially in patients with medical devices in use such as intravenous catheter infections, shunt infections, prosthetic joint infection and endocarditis etc. This coagulase-negative staphylococcus does not produce α-toxin, exfoliatin, or any of the StaphSAg toxins but they have been shown to have surface adhesions and the ability to produce extracellular polysaccharide biofilms. Moreover, *S. aureus* can also produce biofilms, and although a less frequent colonizer of medical devices, it is likely to produce a more aggressive course and metastatic infections (Murray *et al*., c2002). The ability to form biofilm is one of the key factors enables pathogens to survive, colonize and proliferate in the body. Biofilms can grow on medical devices or range of host tissues. The microorganisms within biofilm are protected against attacks from immune system and antibiotic

agent. Thus, it is resistant to host defense mechanism and antibiotic agent. *S. aureus* and *S. epidermidis* are in attention as the dominant cause of biofilms associated infections (Boles & Horswill, 2011). Therefore, the development of alternative treatment to inhibit bacterial growth and biofilm formation is an important demand.

The previous study of the extract from lichen species such as *Cladonia* (Cladoniaceace) and *Usnea* (Usneaceae) etc. showed antimicrobial potential (Ingo´ lfsdo´ ttir, 2002). *Usnea siamensis* Wainio is Thai herbal drug that has been used to treat diseases in folk medicine. The bioactive compound from this plant is (+)-usnic acid (Kamkaen, 1993). Both *Usnea*-extract and (+)-usnic acid inhibited the growth of aerobic gram-positive bacteria including *Streptooccae*, *Corynebacteria* and methicillin-resistant *S. aureus* (MRSA) (Weckesser *et al*., 2007). Interestingly, the qualitative study by TLC-bioautographic method showed the inhibition zone of (+)-usnic acid against *Bacillus cereus*, *Bacillus subtilis*, *S. aureus* (ATCC 6538P), *S. epidermidis* (Isolated), *Micrococcus luteus* and *Candida albicans* was studied in Thailand (Chaowuttikul, 2013). This study indicates the qualitative information, and then the next step, the quantitative of (+)-usnic acid against *S. aureus* ATCC 6538P and *S. epidermidis* Isolated was studied. Moreover, (+)-usnic acid have not been studied *in vitro* and *in vivo* on efficacy and safety yet.

In vitro screening of drug candidates is insufficient due to problems of toxicities, pharmacokinetics and pharmacodynamics in humans. Preclinical tests in animal models are essential for evaluating the therapeutic effects of drug candidates for further development. The mammals are usually used for drug screening models such as mice, rat and rabbit etc. The use of a large number of mammals brings about a number of problems. They are expensive and highly problematic with regard to ethical issues. To overcome these problems, the development of invertebrate animals as drug screening models is desirable. Invertebrates have been used as an animal model of infectious disease such as *Caenorhabditis elegans* with *Pseudomonas aeruginosa*, and the fruit fly, *Drosophila melanogaster* with *Escherichia coli* (Hamamoto *et al*., 2004; Hamamoto, Tonoike, Narushima, Horie, & Sekimizu, 2009). Interestingly, silkworm also has been used as an animal model of infectious disease. It can be infected with *S. aureus*, *Pseudomonas aeruginosa*, *Vibrio*

cholera, *Stenotrophomonas maltophilia*, *Candida albicans* or *Candida tropicalis*, which pathogenic to human. Moreover, it was previously reported that infection of silkworm larvae was cured by antibiotics, which clinically used in human (Hamamoto *et al*., 2004; Kaito, Akimitsu, Watanabe, & Sekimizu, 2002). The injections into silkworm are available in two routes. First, silkworm is injected into intra-hemolymph, which is similar to the intravenous injection in mouse. Second, silkworm is injected into intra-midgut, which is similar to the oral administration in mouse. Silkworm larvae has a number of advantages: lower cost, smaller space required for the maintenance of silkworm compared to mice and silkworm without the same ethical concerns involved in the use of mammals. Another advantage of this model is that very small amounts of sample can be tested. This is especially important when one is working with limited amounts of samples (Hamamoto *et al*., 2004; Matsumoto, Sumiya, Sugita, & Sekimizu, 2011). A major concern of using invertebrates as an animal model is the toxicity and metabolism of compound (Hamamoto *et al*., 2009). Thus, silkworm is used for preliminary evaluation of antibiotics against human pathogens prior to examination in mammalians.

In this research, we investigated (+)-usnic acid, the bioactive compound of *Usnea siamensis* Wainio, for its antimicrobial activity on *S. aureus* and *S. epidermidis* both *in vitro* and *in vivo* studies. *In vitro* studies, the minimum inhibition concentration (MIC) test (the broth serial dilution) and the minimal bactericidal concentration (MBC) test were used for determining the ability of (+)-usnic acid to kill the microorganism. The time-kill assay was used for examining the rate at which concentrations of an antimicrobial agent kill bacterial isolate. In addition, the microtiter plate biofilm assay was a useful method for assessing bacterial attachment by measuring the staining of the adherent biomass. Moreover, we considered the applicability of the silkworm as an infection model animal for quantitative measurement of the therapeutic effects of (+)-usnic acid against *S. aureus* and *S. epidermidis* infection.

Objective

To investigate antimicrobial and antibiofilm activities of (+)-usnic acid, the bioactive compound of *Usnea siamensis* Wainio, on *S. aureus* and *S. epidermidis in vitro* and *in vivo* using Thai silkworm model.

Hypothesis

(+)-Usnic acid, the bioactive compound of *Usnea siamensis* Wainio has antimicrobial and antibiofilm activities against *S. aureus* and *S. epidermidis*.

Research design

Experimental Research

Scope of the study

This study was performed both *in vitro* and *in vivo*. For *in vitro* study, the present study was aimed to evaluate antimicrobial activity, inhibitory effect of biofilm formation and time of killing of (+)-usnic acid 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, toxicity of (+)-usnic acid and therapeutic effects of (+)-usnic acid against these microorganisms.

Benefits

Benefit of this study is obtaining information of (+)-usnic acid on antimicrobial and antibiofilm activities against *S. epidermidis* which is superior to *S. aureus* in *in vitro* study. In *in vivo* study, (+)-usnic acid lost its therapeutic effect against *S. aureus* and *S. epidermidis* in silkworm infectious model. This may be due to pharmacokinetic (absorption, distribution, metabolism and excretion) in silkworm. Therefore, further study is required to evaluate antimicrobial and antibiofilm activities of (+)-usnic acid against *S. aureus* and *S. epidermidis* in mammalian model such as rat, mice etc.

CHAPTER II
LITERATURE REVIEWS

2.1 Characteristics and Morphology

Staphylococci are members of family Micrococcaceae. The genus *Staphylococcus* contains at least 30 species. Three main species that responsible for human infections are *S. aureus*, *S. epidermidis* and *S. saprophyticus*. *S. aureus* is the agent of most acute pyogenic and toxic-related staphylococcal infections in human. *S. epidermidis* is a major component of the normal flora of the skin and skin structures, foreign bodies and deep infections in immunocompromised patients (Brooks *et al*., c1995; Gorbach, Bartlett, & Blacklow, 2004). Staphylococci are gram-positive organisms with a diameter between 0.5 to 1.5 µm that occur singly and in pairs, tetrads, short chains and irregular grapelike clusters and ubiquitous colonizers of the skin and mucosa of virtually all animals, including mammals and birds. Staphylococci are non-motile and non-spore forming (Brooks *et al*., c1995; Mandell, Bennett, & Dolin, 2010). The organisms grow at a temperature ranging from 18°C to 40°C and can produce pigments vary from white to deep yellow. *S. aureus* usually forms gray to deep golden yellow colonies as the result of the carotenoid pigments that form during their growth. In contrast to *S. aureus*, *S. epidermidis* usually forms gray to white colonies. Additionally, staphylococci can divide into two major groups owing to their ability to produces the coagulase enzyme; coagulase-positive staphylococcus such as *S. aureus* and *Staphylococcus delphini* and coagulase-negative staphylococcus such as *S. epidermidis* and *Staphylococcus saprophyticus* (Brooks *et al*., c1995; Murray *et al*., c2002).

2.2 Physiology and Structure

Staphylococci contain cell wall structure which is important because the actions of many of the effective classes of antibiotics involve inhibition of cell wall synthesis. Furthermore a number of diagnostic tests that are potentially useful in the management of staphylococcal disease also depend on cell wall characteristics. Cell wall structure consists of peptidoglycan, a polysaccharide polymer containing linked subunits, provides the rigid exoskeleton of the cell wall. Moreover, teichoic acids are

major cell wall component of staphylococci, which are polymer of glycol or ribitol phosphate. *S. aureus* contains ribitol teichoic acid, whereas *S. epidermidis* contains glycerol teichoic acid. Teichoic acids are linked covalently to the peptidoglycan backbone (Gorbach *et al*., 2004). Protein A is covalently linked to the peptidoglycan layer and effectively prevents the antibody-mediated immune clearance of the organism by binding to the Fc receptor of immunoglobulin (Ig)G_1, IgG_2 and IgG_4. Additionally, extracellular protein A can also bind antibodies. *S. aureus* is coated with protein A but not coagulase-negative staphylococci. Capsule formation is variable among staphylococci may be important pathogenically, which inhibit phagocytosis by polymorphonuclear leukocytes unless specific antibodies. This polysaccharide can be released during focal infection (Murray *et al*., c2002).

2.3 Enzymes and Toxins

Staphylococci can produce enzymes and toxins as pathogenic factors. Catalase enzyme can activate potentially bactericidal hydrogen peroxide. This enzyme converts hydrogen peroxide into nontoxic water and oxygen. Other enzyme that may be important in the pathogenicity is hyaluronidase and lipase. Hyaluronidase enzyme hydrolyzes hyaluronic acid, that the part of connective tissue matrix. Lipase enzyme breaks down tissue lipid components. *S. aureus* produces coagulase, an enzyme-like protein that convert fibrinogen to fibrin by binding to prothrombin. In addition, α-toxin is a protein that acts on a wide variety of cell membranes, when injected parenterally, rapid produces and area of necrosis. Moreover, *S. aureus* can produce exfoliatin, enterotoxin and the toxic shock syndrome toxin-1 (TSST-1) that cause human diseases (Brooks *et al*., c1995; Gorbach *et al*., 2004). Exfoliatin is a specific cell membrane ganglioside found only in the stratum granulosum of the keratinized epidermis of young children and rare adults and responsible for the staphylococcal scalded skin syndrome. Enterotoxin is an important cause of food poisoning by stimulates gastrointestinal symptoms in human and animals. TSST-1 is the agent of a potentially lethal multisystem disorder that mimics bacteremic septic shock. In addition, superantigen act by stimulating reflexes in the abdominal viscera, which are transmitted to medullary emetic centers in the brain stem via the vagus nerve (Gorbach *et al*., 2004; Ryan & Ray, c2010).

2.4 Epidemiology

Staphylococci are ubiquitous human parasites. The chief sources of infection are shedding human lesions, fomites contaminated from such lesions and the human respiratory tract and skin. The organisms can be transferred to a susceptible person either through direct contact or through indirect contact such as contaminated clothing. In hospitals, the areas at highest risk for severe staphylococcal infections are the newborn nursery, intensive care units, operating rooms and cancer chemotherapy words. Therefore, medical personnel must use proper hand-washing techniques to prevent the transfer of staphylococci from themselves to patients or among patients (Brooks *et al*., c1995; Murray *et al*., c2002).

2.5 Clinical diseases of *S. aureus*

Staphylococcal Scalded Skin Syndrome

Staphylococcal Scalded Skin Syndrome (SSSS) is a superficial skin disorder that varies from local blistering to impressive generalized scalding. This disease results from the production of exofoliatin in a staphylococcal lesion. The disease is most common in neonates and young children. The face, axilla and groin tend to be affected first, but erythema, bullous formation and subsequent desquamation of epithelial sheets can spread to all parts of the body (Murray *et al*., c2002; Ryan & Ray, c2010).

Toxic shock syndrome

Toxic shock syndrome is characterized by high fever, vomiting, diarrhea, sore throat and muscle pain. Within 48 hours, it may progress to severe shock with evidence of renal and hepatic damage. A skin rash may develop, followed by desquamation at a deeper level than in scalded skin syndrome (Ryan & Ray, c2010).

Staphylococcal food poisoning

Staphylococcal food poisoning result from ingestion of staphylococcal enterotoxin-contaminated food results in acute vomiting and diarrhea within 1 to 5

hours. There is prostration, but usually no fever. Recovery is rapid, except sometimes in the elderly and in those with another disease (Ryan & Ray, c2010).

Cutaneous infections

Impetigo is a superficial infection affecting mostly young children, occurs primarily on the face and limbs. Initially, a small macule is seen, and then a pus-filed vesicle on an erythematous base develops. Folliculitis is a pyogenic infection in the hair follicles. The base of the follicle is raised and reddened, dermal surface. An extension of folliculitis is furuncles. Furuncles are large, painful, raised nodules with an underlying collection of dead and necrotic tissue. Additionally, carbuncles occur when furuncles coalesce and extend to the deeper subcutaneous tissue. Patients with carbuncles have chills and fevers, indicating the systemic spread of staphylococci *via* bacteremia to other tissues. Staphylococcal wound infections can occur in patients after a surgical procedure or after trauma, with organisms colonizing the skin introduced into the wound. Infections are characterized by edema, erythema, pain and an accumulation of purulent material (Murray *et al*., c2002).

Bacteremia and Endocarditis

S. aureus is a common cause of bacteremia. 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. Bacteremias caused by most other organisms originate from an identifiable focus of infection, such as an infection of the lungs, urinary tract, or gastrointestinal tract. Acute 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 and include disruption of cardiac output and peripheral evidence of septic embolization. The initial symptoms may be mild, but fever, chill and pleuritic chest pain caused by pulmonary emboli are generally present. Clinical cure of the endocarditis is the rule, although it is common for complications to occur as the result of secondary spread of the infection to other organs (Murray *et al*., c2002).

Sensitivity and resistance of *S. aureus* to antibiotics

S. aureus is sensitive to penicillin G. Only a very limited percentage of hospital strains and no more than 20 to 30% of community strains do not produce a β-lactamase and are therefore sensitive to penicillin G. Resistance to β-lactam antibiotics is a major clinical problem. *S. aureus* possess a remarkable number of mechanisms for resisting antibacterial action. Thus, depending on the local epidemiologic conditions, 5 to 20% of isolates are resistant to the antibacterial agents commonly used in staphylococcal infections such erythromycin, lincomycin and clindamycin. This percentage seems to be lower for fusidic acid, although clinical experience with this drug is limited. Aminoglycoside-resistant strains have been described with increasing frequency. Rifampin, which is remarkably active against *S. aureus*, cannot be used as a single agent whereas no strain had been described as being fully resistant to vancomycin (Mandell, Bennett, & Dolin, 2000).

2.6 Clinical diseases of *S. epidermidis*

Coagulase-negative staphylococci cause a wide variety of clinical infections, many related to foreign bodies and prosthetic medical devices. They are common cause of nosocomial bacteremia, particularly in areas of the hospital where the use of indwelling vascular catheters is common.

Bacteremia and Endocarditis

Coagulase-negative staphylococci account for approximately 30% of health care-associated bloodstream infections. Most of these infections are caused by involvement of intravascular catheters or other prosthetic medical devices. Immunosuppressed patients, particularly those with severe neutropenia, are at increased risk of coagulase-negative staphylococci bloodstream infection (Gorbach *et al*., 2004).

Catheter and Shunt infections

These infections are a major medical problem because long-dwelling catheters and shunts are used commonly for the medical management of critically ill patients. The coagulase-negative staphylococci are particularly well adapted for causing these infections because they can produce a polysaccharide slime that bonds them to catheters and shunts and protects them from antibiotics and inflammatory cells (Murray *et al*., c2002).

Prosthetic joints infections

Infections of implanted hip and knee prostheses are caused by coagulase-negative staphylococci. The patient usually only experience localized pain and mechanical failure of the joint. Systemic signs such as fever and leukocytosis are not prominent, and blood cultures are usually negative. Treatment of these infections is joint replacement and antimicrobial therapy. The risk of reinfection of the new joint is considerably increased in such patients (Mandell *et al*., 2000; Murray *et al*., c2002).

Antibiotic susceptibility

S. epidermidis is usually resistant to multiple antibiotics, with more than 80% resistant to methicillin. In addition to β-lactams, antimicrobials to which more than 50% of *S. epidermidis* and *S. haemolyticus* nosocomial isolates are resistant include erythromycin, clindamycin, cloramphenicol and tetracycline. Resistance to trimethoprim and gentamicin is high in some hospitals but may be low in others.

Antimicrobials to which most coagulase-negative staphylococci are susceptible *in vitro* include vancomycin, rifampin and ciprofloxacin. The former two agents are the mainstay of the treatment of deep-seated coagulase-negative staphylococcal foreign body infections, although the development of resistance to rifampin during therapy limits the usefulness of this antibiotic. The efficacy of ciprofloxacin and other fluoroquinolones in the treatment of these infections has yet to be adequately determined, but resistance of colonizing coagulase-negative staphylococci also emerges rapidly in patients receiving ciprofloxacin (Mandell *et al*., 2000).

2.7 Biofilm

One of the key factors enables pathogens to survive, colonize and proliferate in the body is the ability to form biofilm. The microorganisms within biofilm are protected against attacks from antimicrobial agents and host defense mechanisms. Both *S. aureus* and *S. epidermidis* are a frequent cause of biofilm-associated infections (Ryan & Ray, c2010). Biofilm can growth on medical devices or host tissues. *S. epidermidis* often cited as being associated with foreign body infections and *S. aureus* with infections on host tissues such as osteomyelitis, septic arthritis and endocarditis (Boles & Horswill, 2011). Bacteria are developing a biofilm in three stages-adherence, maturation and dispersal (Figure 1).

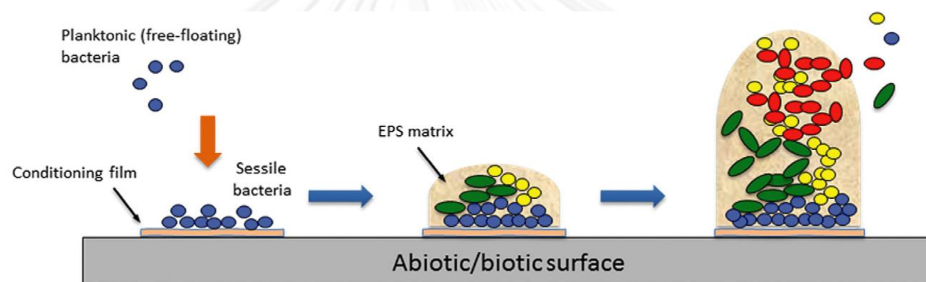

Figure 1 Stage in biofilm formation, initial microorganism attachment to the surface, the biofilm maturation and cell dispersion (Dufour, Leung, & Levesque, 2012)

Adherence

Biofilm form when bacteria adhere to surfaces in aqueous environments and begin to excrete a slimy, glue-like substance that can anchor to variety of materials including metals, plastics, soil particles, medical implant materials and human or animal tissue (Marshall, 2012). Staphylococci adhere to biomaterial by binding to serum matrix proteins coated on the biomaterial and through direct biomaterial interactions (Mandell *et al*., 2010).

Maturation

After the initial colonization, the biofilm grows though a combination of cell division and recruitment. Then, maturation of *S. aureus* and *S. epidermidis* biofilm is

generation a slime glycocalyx or extracellular polymeric substance, which encases surface bound organisms. Once bound, the microorganisms produce extracellular polymeric substances (EPS), which compose of polysaccharide intercellular adhesin (PIA) and extracellular DNA (eDNA). eDNA is the major component of both *S. aureus* and *S. epidermidis* biofilms. In addition, biofilm also contains an abundance of PIA. In *S. aureus*, PIA is essential for virulence in murine systemic disease models and is vaccine candidate for both *S. aureus* and *S. epidermidis*. Therefore, PIA is highly advantageous to the organism during the infection process (Mandell *et al*., 2010).

Dispersion

The last stage of biofilm development is dispersal and subsequent spread to other potential sites. Phenol-soluble modulins (PSMs) are regulated by the quorum-sensing global regulator *agr*, act as surfactants, leading to loss of cellular clusters. *S. epidermidis* produce PSMs, which are mediator for detachment of the upper layers of the biofilm. Then, the 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 that are importance for the spread of biofilm-associated infection. PSMs of *S. epidermidis* are pro-inflammatory whereas PSMs of *S. aureus* have been shown to recruit, activate and lyse human neutrophils (Mandell *et al*., 2010).

Biofilms are composed of 80-85% EPS by volume and 15-20% of cells by volume. The EPS can act as diffusion barrier, preventing toxic substances such as antibiotics and disinfectants. One of the mechanisms of resistance within biofilms is that bacteria in such communities regulate gene expression in a coordinated fashion that mediated by bacterial communication. Cell-to-cell communication which called quorum sensing (QS) release signal molecules by bacteria. The signaling molecule of gram-negative bacteria is acyl homoserine lactone (AHL), while gram-positive bacteria use small peptides. The signaling molecule is increase in concentration as a function of bacterial cell density. Bacteria communicate with one another by using autoinducers to regulate their gene expression in response to fluctuation in the cell population density. QS has been linked to biofilm structure and important for the attachment of bacteria to the surface, the maturation of the biofilm or the control of events leading to the dispersion of cells. The microorganisms within biofilm have

reduced susceptibility to antimicrobial agents and host immune defense (Dufour *et al*., 2012).

The mechanisms of biofilm resistance to host defenses are limited penetration of leukocytes and their bactericidal production into the biofilm. The global response regulators and QS activities are increase resistance to leukocytes by decreased ability of leukocytes to engulf biofilm bacteria, genetic switches. Thus bacterial cell in biofilm increase resistance to the immune system and suppression of leukocyte activity through effector regulation (Leid, 2009).

The mechanisms of biofilm resistance to antimicrobial agent have been described in Figure 2. The antibiotics may decrease the amount of bacteria within biofilms, but they dose not completely eradicate the pathogen and thus leads to refractory infections. The first mechanism is incomplete penetration or diffusion of antimicrobial agent into the biofilm. The EPS matrix acts to a major role of resistance. The protection can be due either to physical barrier in antimicrobial diffusion or to direct binding of the antibiotic by the EPS matrix. The second mechanism, antimicrobial agents may be trapped and destroyed by enzymes that produce by bacteria within biofilm. The third mechanism, the growth rate inside the biofilm may be altered. At the bottom layers of the biofilm, nutrients and oxygen are limited, then bacteria enter to a non-grow state (persisters). The persisters are resistant protect from killing by all antimicrobial agent because these antimicrobial agents are only active against growing bacteria cells. The activation of quorum-sensing systems along with different concentration gradients of nutrients, oxygen and metabolic waste products also make important contributions to antimicrobial tolerance and resistant to host immune system. The fourth mechanism, antimicrobial agents are increasing remove from biofilm by multi-drug resistance (MDR) efflux pumps. The origin of these transporters is to remove metabolites and by-products within bacterial cells. Overtime, they evolve to efflux out other harmful molecules such as antimicrobial agents. The fifth mechanism, bacteria within biofilm can express stress-responsive genes and switch to more tolerant phenotypes upon environmental stressors such as starvation, heat and cold shock, cell density, pH and osmolality (Dufour *et al*., 2012; Leid, 2009; Pozo & Patel, 2013).

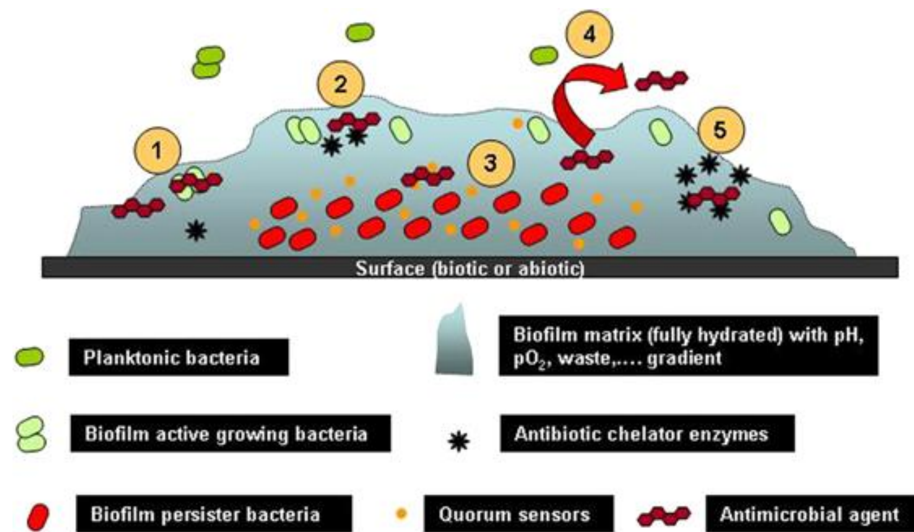

Figure 2 Mechanism of bacteria within biofilm resistance to antimicrobial agents (Pozo & Patel, 2013)

2.8 (+)-Usnic acid

The genus of *Usnea* (Usneaceae) is lichen. *Usnea* has been used as crude drugs in Asia, Africa and Europe for pain relief and fever control. In addition, *Usnea siamensis* Wainio is Thai herbal drug that used to treat diseases in folk medicine (Figure 3). The scientific classification is shown below. The lichens are growing on rocks, tree trunks or other substrate. The chemical classes from lichens are depsides, depsidones and dibenzofuran. Usnic acid is secondary metabolites, which is dibenzofuran derivative. Usnic acid is distributed in species of *Cladonia* (Cladoniaceae), *Usnea* (Usneaceae), *Lecanora* (Lecanoraceae), *Ramalina* (Ramalinaceae) and other (Ingo´ lfsdo´ ttir, 2002).

Kingdom	:	Fungi
Division	:	Ascomycota
Class	:	Lecanorales
Family	:	Parmeliaceae
Genus	:	*Usnea*

Figure 3 *Usnea siamensis* Wainio

The molecular weight of usnic acid [2,6-diacetyl-7,9-dihydroxy-8,9b-dimethyl-1,3(2H,9bH)-dibenzo-flurandione; $C_{18}H_{16}O_7$] is 344.31. Usnic acid is a yellow pigment and exists in two enantiomers forms depending on the methyl group located in position 9b chiral. The configuration of (+)-usnic acid at 9b chiral has been determine by X-ray analysis to be R (Figure 4). The enantiomers have been shown different biological activities. Both of two enantiomers forms of usnic acid are effective against a large variety of gram-positive bacterial strain (Guo et al., 2008; Ingo ́ lfsdo ́ ttir, 2002). Interestingly, the crude extract of *Usnea siamensis* Wainio from Thailand contained of a dibenzofuran derivative: (+)-usnic acid, depside: atranorin and depsidone; isomeric form of stictic acid. (+)-Usnic acid is an active compound from *Usnea siamensis* Wainio. Additionally, (+)-usnic acid is appeared in the sample of Doi intanon, Chaing mai and Pu luang, Loei 4.31% and 3.43% of dry weight, respectivel (Kamkaen, 1993). Antimicrobial activities of (+)-usnic acid against the *S. aureus* and *S. epidermidis* was reported with MIC values of 16 and 4 µg/ml respectively, and MBC value of 64 and 32 respectively (Weckesser *et al*., 2007). The qualitative study by TLC-bioautographic method showed the inhibition zone of (+)-usnic acid against *Bacillus cereus*, *Bacillus subtilis*, *S. aureus* (ATCC 6538P), *S. epidermidis* (Isolated), *Micrococcus luteus* and *Candida albicans* was reported by Chayanon Chaowuttikul, College of Public Health Science Chulalongkorn University 2013 (Chaowuttikul, 2013).

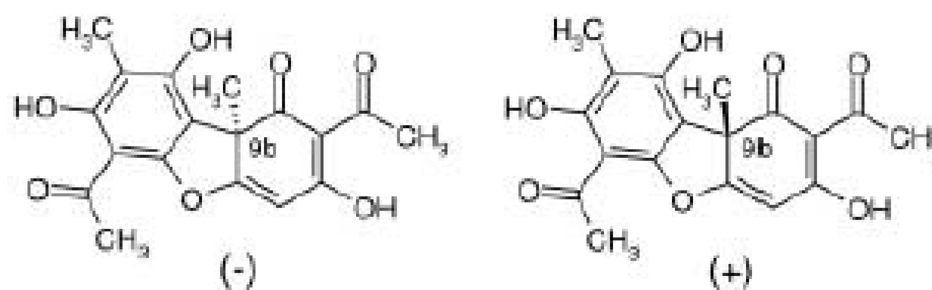

Figure 4 Structure of (-)-usnic acid and (+)-usnic acid (Cocchietto, Skert, Nimis, & Sava, 2002)

2.9 Silkworm

Silkworm is an invertebrate animal. It is the larvae of *Bombyx mori* (Bombycidae) moths. Silkworms undergo complete metamorphosis. Larvae feed on the leave of the mulberry (family Muraceae, genus *Marus*) and other genera in this family. Life cycle of the silkworm composes of four stages are adult, egg, larvae and pupa (Figure 5). The duration of life cycle is six to eight weeks depending upon racial characteristics and climatic condition (Singh).

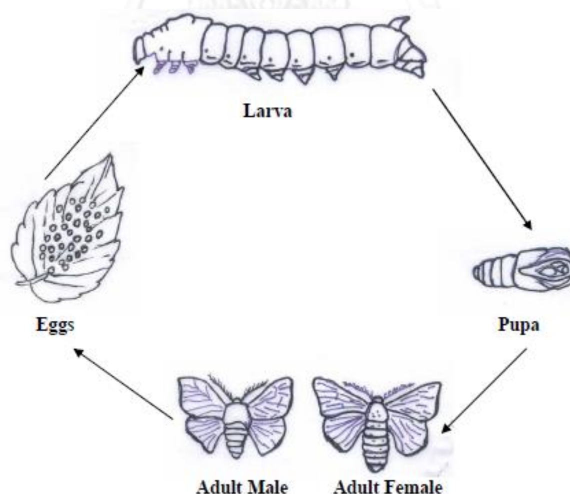

Figure 5 Life cycle of silkworm, *Bombyx mori* composed of 4 major stages including adult, egg, larvae and pupa (Singh)

The first stage of silkworm is egg. The silkworm eggs are round, white and weight around 2000 eggs to gram. They measure 1 to 1.3 mm in length and 0.9 to 1.2 mm in width. Races producing white cocoons lay pale yellow eggs while races producing yellow cocoons lay deep yellow eggs. The second stage of silkworm is

larvae. The eggs hatch into larva after 10 days of incubation. It becomes smoother and lighter in colour due to rapid stretching of the cuticular skin during different instars of the larval stage when the larva grows. The entire body is covered with a thin and elastic chitinous cuticle which is capable of being extended considerably to permit rapid growth of the larva during any instar. The young larva is about 10 mm in length. After 2 days of hatching, the setae on the body become less conspicuous. After 1 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. 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 5th instar, larva attains a maximum length of about 50-60 mm and eats voraciously. The 5th instar larva body grows completely and functions actively. This stage has a long period about 20-24 days.

The larval body composed of head, thorax and abdomen (Figure 6). The head consist of six body segments fused together with a cranium. It carries the appendages: antennae, mandibles, maxillae and labium. There are six pairs of ocelli or larval eyes which are located behind and a little above the base of the antennae. There is a pair of antennae formed of five jointed segments and these are used as sensory organs. The mandibles are well developed and powerful and are adapted for mastication. The maxillary lobe and palpi help in discriminating the taste of food. The prementum is also chitinized, and its distal part carries a median process known as spinneret through which silk is extruded out from the silk gland. The sensory labial palpi are found on both sides of the spinneret.

The thorax has three segments called pro-thorax, meso-thorax and meta-thorax. Each of the thoracic segments carries ventrally one pair of true legs, which are conical in shape and carry sharp distal claws. These claws are not used for crawling but they help in holding the leaves while feeding. The spot on dorsal side of meso-thorax is called eyes.

The abdomen is comprise of eleven body segment, though only nine can be distinguished, as the last three are fused together to form the apparent ninth

segment. Third to sixth and last abdominal segment bear a pair of abdominal legs, which are fleshy, unjointed muscular protuberance. Eighth abdominal segment bears caudal horn on the dorsal side. In females, the sexual marking appear as a pair of milky white spot in each of the eighth and ninth segments and are referred to as Ishiwata's Fore Gland and Ishiwata's Hind Gland respectively. In males a small milky white body known as Herold's Gland appears ventrally in the centre between eighth and ninth segments. There are nine pairs of spiracles placed laterally on either side of the body. They are found on the first thoracic segment and the first eight abdominal segments. These are the breathing or respiration pores.

The third stage of silkworm is pupa. This stage is the inactive resting stage of silkworm. It is a transitional phase during which definite changes take place. During this period of biological activity the larval body and its internal organs undergo a complete change and assume the new form of the adult moth. The pupa is white in colour and soft but gradually turns brown to dark brown and the pupal skin becomes harder. The last stage of silkworm is adult. It is about 2.5 cm in length and pale creamy white. It is incapable of flight because of its feeble wings and heavy body. The body of moth has general plan of insect body organization (Mahesha; Singh; Takeda, 2009).

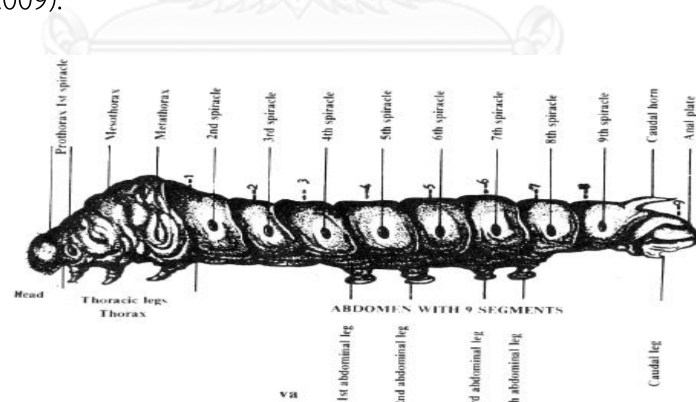

Figure 6 Morphology of silkworm larvae (Mahesha)

Silkworm has been examined as an animal model of human infection with pathogenic bacteria in 2002 by Chikara Kaito and all. They were injected $3x10^7$ cells of *S. aureus*, *Pseudomonas aeruginosa*, *Vibrio cholera* or *Escherichia coli* into intra hemolymph of 5th instar silkworm larvae. The result shown, over 90% of silkworm those were injected with *S. aureus*, *Pseudomonas aeruginosa* or *Vibrio cholera* died

within 2 days (Figure 7). The larvae were injected with these microorganisms had decrease in movement, then they stopped eating, their skin color turned to dark and finally they died. In addition, Chikara Kaito and all also determined the proliferation of microorganisms in larvae body. They observed the number of *S. aureus* in blood and tissues of silkworm after injected *S. aureus* within 2 days (Figure 8). The number of *S. aureus* was increased in both blood and tissues of larvae. The results indicate bacteria were growth in larvae body and were infected in silkworm larvae (Kaito *et al*., 2002).

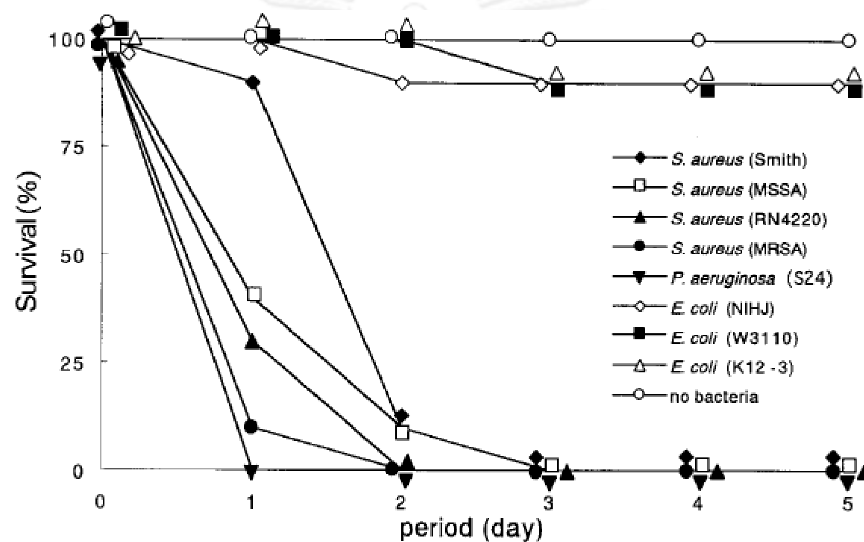

Figure 7 Survival of the silkworm larvae injected with *S. aureus*, *P. aeruginosa*, or *E. coli*. Fifth instar larvae of silkworm (n=10) were injected with $3x10^7$ cells of *S. aureus* (RN4220, Smith, MSSA, MRSA), *P. aeruginosa* (S24) or *E. coli* (K12-3, W3110, NIHJ). The numbers of surviving larvae are shown (Kaito *et al*., 2002)

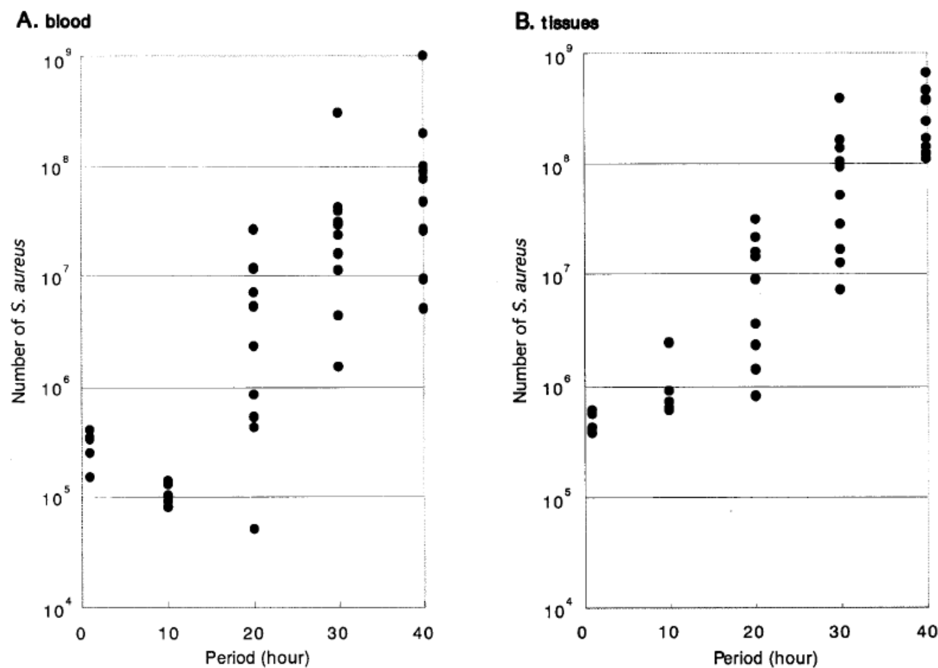

Figure 8 Proliferation of *S. aureus* in blood and tissues of silkworm larvae. Silkworm larvae were injected with $3x10^7$ cells of *S. aureus* MSSA. Animals were sacrificed and blood (A) and tissue (B) were collected. After appropriate dilution, samples were spread on mannit agar plates, incubated at 37ºC overnight, and the number of colonies was counted (Kaito *et al*., 2002)

However, the infectious larvae were cured with antibiotic drugs, which clinically in human. Infection of silkworm larvae by methicillin-sensitive *S. aureus* (MSSA) was cured by ampicillin, oxacillin and vancomycin, in contrast infection by methicillin-resistance *S. aureus* (MRSA) was cured by vancomycin (Kaito *et al*., 2002). The quantitative therapeutic effects of antibiotics drugs in silkworm larvae comparative with those in mice were studied by Hiroshi Hamamoto and all in 2004. The results demonstrated that 50% effective dose (ED_{50}) of antibiotic drugs (vancomycin, minocycline, flomoxet and linezoline) in silkworm are consistent with those in mice, ED_{50}/MIC ratios of those drugs in silkworm are consistent with those drugs in mice too. ED_{50}/MIC ratios are useful for evaluating the pharmacokinetics of antibiotics. The small values indicate that the antibiotics functioned effectively in animal bodies, similar to their functioning in the culture medium in which the MICs were determined. This can achieved if the drug concentrations are maintained in the

body fluid for a long enough period without degradation or modification. They also examined the ED_{50}/MIC ratios of antibiotic drugs in silkworm larvae; the result indicated the ED_{50}/MIC ratios were less than 10 (Table 1) (Hamamoto *et al*., 2004).

Table 1 ED_{50} of antibiotics in a silkworm infection model with *S. aureus* and *S. maltophila* (Hamamoto *et al*., 2004)

Antibacterial agent	Bacteria	ED_{50} in silkworm (μg/g of larva)	MIC (μg/ml)	ED_{50}/MIC ratio in:	
				Silkworm	Mouse
Kanamycin	*S. aureus*	3	8	0.4	
Arbekacin	*S. aureus*	4	8	0.5	
Teicoplanin	*S. aureus*	0.3	0.5	0.6	
Vancomycin	*S. aureus*	0.3	1	0.3	1.1[a]
Tetracycline	*S. aureus*	0.4	0.5	0.8	
Minocycline	*S. aureus*	3.9	0.4	9.8	3.5[b]
Chloramphenicol	*S. aureus*	7	16	0.4	
Flomoxef	*S. aureus*	0.2	0.4	0.5	0.8[b]
Linezolid	*S. aureus*	9	4	2.3	1.5[a]
Minocycline	*S. maltophilia*	7.8	1	7.8	
ST[c]	*S. maltophilia*	57	256	0.2	
IPM/CS[d]	*S. maltophilia*	50	256	0.2	

[a] ED_{50} and MIC were previously reported (4).
[b] ED_{50} and MIC were previously reported (10).
[c] ST, sulfamethoxazole-trimethoprim.
[d] IPM/CS, imipenem-cilastatin.

The absorption system of drugs in the silkworm larva midgut is much simpler than that in mammals. Because of the epithelium of the silkworm larva midgut is composed of a monolayer of cells. Additionally, insects have an open vessel system without a complex blood circulatory system around the midgut. Therefore, compounds that permeate the midgut appear directly in the hemolymph. In mammals drugs absorbed from the intestine are carried by the portal vein and first pass through the liver, but this pathway does not exist in silkworm larvae. The general features of the non-specific transport route are similar between silkworm and mammals. The hydrophilic compounds with a molecular mass of greater than 400 Da did not permeate the silkworm larva midgut, whereas the hydrophobicity of similar-sized compounds had positive effects on the transport rate (Hamamoto *et al*., 2005). Hiroshi Hamamoto and all indicated silkworm infected with pathogen was cured by chloramphenicol and tetracycline both inject into intra-hemolymph and intra-midgut whereas vancomycin and kanamycin was effective when injection into intra hemolymph. Because of vancomycin and kanamycin do not permeate the midgut

thus these antibiotic had not therapeutic effect in the silkworm larva infection model when injection into intra-midgut (Hamamoto *et al*., 2004). Silkworm larvae have a metabolism and excretion pathway. The chemical were metabolized through a pathway common to both mammals and silkworm: reaction with cytochrome P450 then conjugation with hydroxylated compounds and excretion. In insects, fat bodies are suggested to function as a metabolic center, like the liver in mammals. The concentration of the glucose-conjugated form increased in the fat body culture medium in a time-dependent manner (Hamamoto *et al*., 2009).

CHAPTER III
MATERIALS AND METHODS

Materials

1. Silkworms

Thai silkworm 5th instar larvae weighing 0.3 – 0.5 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* isolated from The college of public Health Science, Chulalongkorn University. *S. aureus* ATCC 29213 was used as control test.

3. Chemicals

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

3.2 70% Alcohol

3.3 1% crystal violet

3.4 Dimethyl sulfoxide (RCI labscan, Thailand)

3.5 Glacial acetic acid (BDH Laboratory, England)

3.6 Methanol (RCI Labscan, Thailand)

3.7 Mueller Hinton Agar (BBL, USA)

3.8 Mueller Hinton Broth (BBL, USA)

3.9 0.9% NaCl

3.10 Silkmate

3.11 Sterile distilled water

3.12 (+)-Usnic acid (Sigma, USA)

4. Instruments

4.1 Alcohol lamp stainless

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

4.3 Cylinderes

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4.4 Disposable syringe size 1 milliliter (Nipro, Thailand)

4.5 Hot air oven (WTB binder, Germany)

4.6 Hypodermic needle 27G x 1” (Nipro, Thailand)

4.7 Incubater (Memmert, Germany)

4.8 Larminar airflow cabinet (Astec, Thailand)

4.9 Loop Sterilizer (LabScientific, USA)

4.10 Microcentrifuge tubes (Corning incorporated, Mixico)

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

4.12 Microplate reader (LabSciencetific, USA)

4.13 Multichannel pipette (Biohit, Finland)

4.14 Petric dish (Pyrex, USA)

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

4.16 Shaker bath

4.17 Spreader

4.18 Sterile loop

4.19 T60 Visible spectrophotometer

4.20 96 well microtiter plate (Costar, USA)

Methods

1. Antimicrobial activities testing of (+)-usnic acid (*in vitro* studies)

1.1 Inoculum preparation

Fresh microbial cultures were prepared by streaking bacterial pathogen (*S. aureus* ATCC 6538P, *S. aureus* ATCC 29213 or *S. epidermidis* Isolated) on Mueller-Hinton agar (MHA) plates and incubated at 37ºC for 24 h. After incubation overnight, isolate colonies of each bacterial pathogen were transferred into a tube containing 0.9% NaCl and the turbidity was adjusted to a 0.5 McFarland standard by optical density (OD) 0.08-0.10. The absorbance of suspension was measured at 625 nm (approximately 1.0 x 10^8 CFU/ml) (G. BeŚciak & Surmacz-Górska). This suspension was further diluted 1:100 with MHB (approximately 1.0 x 10^6 CFU/ml). After that, freshly

bacterial suspension was used for each experiment of antimicrobial activities testing (Schwalbe, Steele-Moore, & Goodwin, c2007).

1.2 Determination of the minimum inhibitory concentration (MIC) by broth microdilution method

Broth microdilution method was used to obtain MIC values of the (+)-usnic acid against *S. aureus* ATCC 6538P and *S. epidermidis* Isolated. The concentration of (+)-usnic acid was two-fold serial dulution. 100 µl of each concentration of the (+)-usnic acid was dispensed into the wells 1-12 A-C of the 96-well microtiter plate (final concentration ranging from 0.002-4 mg/ml). Then, an equal volume of bacterial pathogen was added and mixed in each well which contained of (+)-usnic acid. Well 1-3 E contained bacterial pathogen with MHB medium which as growth control. While, well 5-7 E was used as negative control, this well contained solvent (5% DMSO) and bacterial pathogen. Similarly, ampicillin preparation (two-fold serial dilution) was included as positive control (final concentration ranging from 0.025-50 µg/ml). The final bacterial concentration in 96-well microtiter plate was 0.5×10^6 CFU/ml. All test plates were covered with lids and incubated at 37°C for 24 h. The lowest concentration of the (+)-usnic acid or ampicillin that inhibited the bacterial growth after incubation was taken as the MIC value of the compound. The experiment was repeated three independent times per microorganism (Schwalbe *et al*., c2007). In addition, *S. aureus* ATCC 29213 was used for quality control, which performed the same experiment as mentioned above.

1.3 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 the (+)-usnic acid or ampicillin that indicated 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*., c2007). In addition, *S. aureus* ATCC 29213 was used for quality control, which performed the same experiment as mentioned above.

1.4 Time kill assay

Microorganisms (*S. aureus* ATCC 6538P or *S. epidermidis* Isolated) were grown overnight at 37°C for 24 h in MHB. The overnight broth was adjusted to a 0.5 McFarland standard in MHB and a further diluted 1:100 with MHB (approximately 1.0 x 10^6 CFU/ml). A stock solution of ampicillin or (+)-usnic acid was diluted in sterile DI water or DMSO respectively to concentrations as exact multiples of the MIC value at 1/2MIC, 1MIC, 2MIC and 4MIC. Later, *S. aureus* suspension was added into sterile test tubes that containing of ampicillin (final concentration 0.05, 0.10, 0.20 and 0.40 µg/ml) or (+)-usnic acid (final concentration 125, 250, 500 and 1000 µg/ml). Similarly, *S. epidermidis* suspension was added into sterile test tubes that containing of ampicillin (final concentration 0.78, 1.56, 3.12 and 6.25 µg/ml) or (+)-usnic acid (final concentration 31.25, 62.50, 125 and 250 µg/ml). All test tubes were incubated with shaking on shaker water bath at 37°C. The final concentration of *S. aureus* or *S. epidermidis* in test tube was 0.5 x 10^6 CFU/ml. Then an aliquot was removed from the test tubes at 0, 2, 4, 6, 8, 10, 12, 24, and 48 h. Then the solution was diluted serially. Finally, 100 µl of the serial dilution sample was spread on MHA plates after that incubated at 37°C for 24 h for the determination of surviving bacteria. The growth control was composed of bacterial pathogen and MHB. These control tube 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 (J. May, C. H. Chana, A. King, Williams, & Frencha, 2000; Soontornpas, Saraya, Chulasiri, Chindavijak, & Mootsikapun, 2005). Bactericidal activity was defined as a reduction of 99.9% (≥ 3 $\log_{10}$ scale) of the total count of CFU/ml in the original inoculums. Bacteriostatic activity was defined as maintenance of or a reduction of less than 99.9% (1-3 $\log_{10}$ scale) of the total of CFU/ml in the original inoculums (Barry *et al*., 1999; Petersen, Jones, & Bradford, 2007). The regrowth was defined as maintenance of the growth of increase ≥1 log scale from nadir (Hanberger, 1992).

1.5 Antibiofilm formation assay

The microorganisms (*S. aureus* ATCC 6538P or *S. epidermidis* Isolated) were grown overnight at 37°C for 24 h in MHB. The overnight broth was adjusted to a 0.5 McFarland standard in MHB and a further diluted 1:100 with 0.25% glucose of MHB (approximately 1.0 x 10^6 CFU/ml). To prevent initial cell attachment, 100 µl of ampicillin (final concentration 0.05, 0.10, 0.20 and 0.40 µg/ml) or (+)-usnic acid (final concentration 125, 250, 500 and 1000 µg/ml) was added into wells of a 96 well microtitre plate that contained of *S. aureus* (final concentration approximately 0.5 x 10^6 CFU/ml). Similarly, *S. epidermidis* (final concentration approximately 0.5 x 10^6 CFU/ml) was added into 96 well microtitre plate that contained of ampicillin (final concentration 0.78, 1.56, 3.12 and 6.25 µg/ml) or (+)-usnic acid (final concentration 31.25, 62.50, 125 and 250 µg/ml). The growth control was contained bacterial pathogen with 0.25% glucose of MHB was served as growth control blank (Stepanovic, Vukovic, Dakic, & Savic, 2000). Sterile DI water or 5% DMSO was used as the negative control. The microtiter plates were covered with lids and incubated at 37°C for 24 h. Following incubation, the biofilm biomass was assayed using the modified crystal violet (CV) staining assay. The contents of each well were removed and washed three times with sterile DI water. Adherent bacteria were fixed with 99.9% methanol for 15 min. All well were then emptied and left to dry. Following drying, the wells were stained with 100 µl of 1% crystal violet and incubated at room temperature for 15 min after which the plates were washed three times with sterile DI water to remove unadsorbed stain. After drying, stain was resolubilised by adding 33.3% glacial acetic acid to each well. Biofilm formation was quantified by measuring the absorbance at 570 nm using a microplate reader; percentage inhibition determined using following equation (Merritt, Kadouri, & O'Toole, 2011; Sandasi, Leonard, Van Vuuren, & Viljoen, 2011).

$$\%\text{inhibition} = \frac{\text{OD negative control} - \text{OD sample}}{\text{OD negative control}} \times 100$$

2. Antimicrobial activities testing of (+)-usnic acid (*in vivo* studies)

2.1 Pathogenicity of bacterial to silkworms

The first day of 5th instar larvae (n=10) were injected with 50 µl of bacterial suspension (*S. aureus* ATCC 6538P or *S. epidermidis* Isolated) ranging from $1.2x10^{1}$ to $1.2x10^{9}$ CFU/ml (ten-fold serial dilution) into hemolymph. The control group (n=10) was also injected with 0.9% NaCl into hemolymph. The survival rate of silkworms was observed at 48 h after the injection. The experiments were performed at three times independently per microorganism. The LD_{50} was determined as the doses of *S. aureus* and *S. epidermidis* that killed half of the silkworms after injection 48 h (Kaito *et al*., 2002). The dose of such microorganisms selected for efficacy study of ampicillin and (+)-usnic acid was the dose of pathogen that killed silkworms 100%.

2.2 Determination of LD_{50} of DMSO

The first day of 5th instar larvae (n=10) were injected with 50 µl of DMSO ranging from 0-100% DMSO into hemolymph. The survival rate of silkworms was observed at 48 h after the injection. The experiments were performed at three times independently. The LD_{50} was determined as the doses of DMSO that killed half of the silkworms after injection 48 h (Kaito *et al*., 2002).

2.2 Determination of LD_{50} of ampicillin or (+)-usnic acid

The first day of 5th instar larvae (n=10) were injected with 50 µl of ampicillin (concentration 5, 10, 20, 40 and 80 mg/ml) or (+)-usnic acid (concentration 0.005, 0.05, 0.5, 2, 4, 5, 10 and 25 mg/ml) into hemolymph. The survival rate of silkworms was observed at 48 h after the injection. The experiments were performed at three times independently. The LD_{50} was determined as the doses of ampicillin or (+)-usnic acid that killed half of the silkworms after injection 48 h (Kaito *et al*., 2002).

2.3 Determination of ED_{50} of ampicillin or (+)-usnic acid

Suspension of bacterial pathogen in 0.9% NaCl (50 µl) that kill all silkworms from the previous experiment were injected into hemolymph of the first day of 5th instar larvae (n=10). 50 µl of 0.25, 0.5, 1, 2, 3 or 4 mg/ml of (+)-usnic acid were

injected into hemolymph immediately after injection of 50 µl of *S. aureus* (1.2 x 10^9 CFU/ml) or of *S. epidermidis* (1.2 x 10^9 CFU/ml). Similarly, 50 µl of 0.01, 0.1, 1, 5 or 10 mg/ml of ampicillin were injected into hemolymph immediately after injection of 50 µl of 1.2 x 10^9 CFU/ml of *S. aureus* or 1.2 x 10^9 CFU/ml of *S. epidermidis*. The control group was injected with 50 µl of 1.2 x 10^9 CFU/ml of *S. aureus* or 1.2 x 10^9 CFU/ml of *S. epidermidis*, into hemolymph of silkworm. The number of surviving silkworms was observed at 48 h after injection. The ED_{50} was determined as the doses of ampicillin or (+)-usnic acid required producing a silkworm survival rate of 50%. The experiments were performed at three times independently (Hamamoto *et al*., 2004; Kaito *et al*., 2002).

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CHAPTER IV

RESULTS

1. Antimicrobial activities testing *in vitro* studies

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

The quantitative analysis of antimicrobial activity was evaluated by broth microdilution method to determine the MIC and MBC values of (+)-usnic acid for *S. aureus* ATCC 6538P and *S. epidermidis*. The results were showed in Table 1. In this experiment, OD_{625} of suspension of *S. aureus* ATCC 6538P and *S. epidermidis* was 0.10 and 0.09 (approximately 1.0×10^8 CFU/ml) respectively. Then, the microorganisms were diluted 100 fold with MHB (approximately 1.0×10^6 CFU/ml) which microorganisms were used to determine MIC by broth microdilution method. Thus the final concentrations of microorganisms were 0.5×10^6 CFU/ml. The antimicrobial activity of (+)-usnic acid was assayed by two-fold serial dilution method to determine MIC value. The broth microdilution method showed the MIC values of (+)-usnic acid for *S. aureus* ATCC 6538P and *S. epidermidis* were 250 and 62.50 µg/ml, respectively. In addition, MBC values of (+)-usnic acid for both *S. aureus* ATCC 6538P and *S. epidermidis* were more than 4 mg/ml. The MBC value is the lowest concentration of the antimicrobial agent indicated that the visible growth of the microorganism cannot be seen on the MHA plate. This results showed that the antimicrobial activities of (+)-usnic acid against *S. epidermidis* was more effective than that against *S. aureus* ATCC 6538P. Ampicillin was used as positive control for these bacterial pathogens. MIC values of ampicillin were 0.10 and 1.56 µg/ml for *S. aureus* ATCC 6538P and *S. epidermidis*, respectively. Moreover, MBC values of ampicillin were 0.39 and 3.12 µg/ml for *S. aureus* ATCC 6538P and *S. epidermidis*, respectively. MIC and MBC values of ampicillin for *S. aureus* ATCC 29213 were the same 0.12 µg/ml.

Table 2 MIC and MBC values of (+)-usnic acid and ampicillin against *S. aureus* and *S. epidermidis*

Substances	*S. aureus* ATCC 6538P		*S. epidermidis* Isolates	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
(+)-Usnic acid	250	> 4,000	62.50	> 4,000
Ampicillin	0.10	0.39	1.56	3.12

1.2 Determination of time kill assay

In time kill assay, the results presented in time kill curve were plotted between $\log_{10}$ of viable colonies (CFU/ml) against time (h). The results were presented in Figure 9-12. In this experiment, OD_{625} of suspension both *S. aureus* and *S. epidermidis* were 0.09 (approximately 1.0 x 10^8 CFU/ml). Then, the microorganisms were diluted 100 fold with MHB (approximately 1.0 x 10^6 CFU/ml). Such microorganisms were used for determining time kill assay. Thus the final concentrations of microorganisms were 0.5 x 10^6 CFU/ml. For *S. aureus*, (+)-usnic acid at 125 µg/ml (1/2MIC) and 250 µg/ml (1MIC) did not inhibit the growth of *S. aureus*. After 2 h of incubation, (+)-usnic acid at 500 µg/ml (2MIC) and 1000 µg/ml (4MIC) inhibited the growth of *S. aureus* with the reduction of bacterial within 1 log scale but did not show bacteriostatic activity. The bacteriostatic activity was the reduction of bacterial 1 to <3 log scale compared with the original inoculum (Petersen *et al*., 2007). (+)-Usnic acid at 500 µg/ml (2MIC), the regrowth was observed at 10 h of incubation and the number of viable colonies of *S. aureus* was more than the original inoculum. (+)-Usnic acid at 1000 µg/ml (4MIC), the regrowth was observed at 12 h of incubation and the number of viable colonies of *S. aureus* was more than the original inoculum. The regrowth was defined as maintenance of the growth of increase ≥1 log scale from nadir (Hanberger, 1992). In addition, after 48 h of incubation, the growth of *S. aureus* in all concentration treated was the same level (Figure 9).

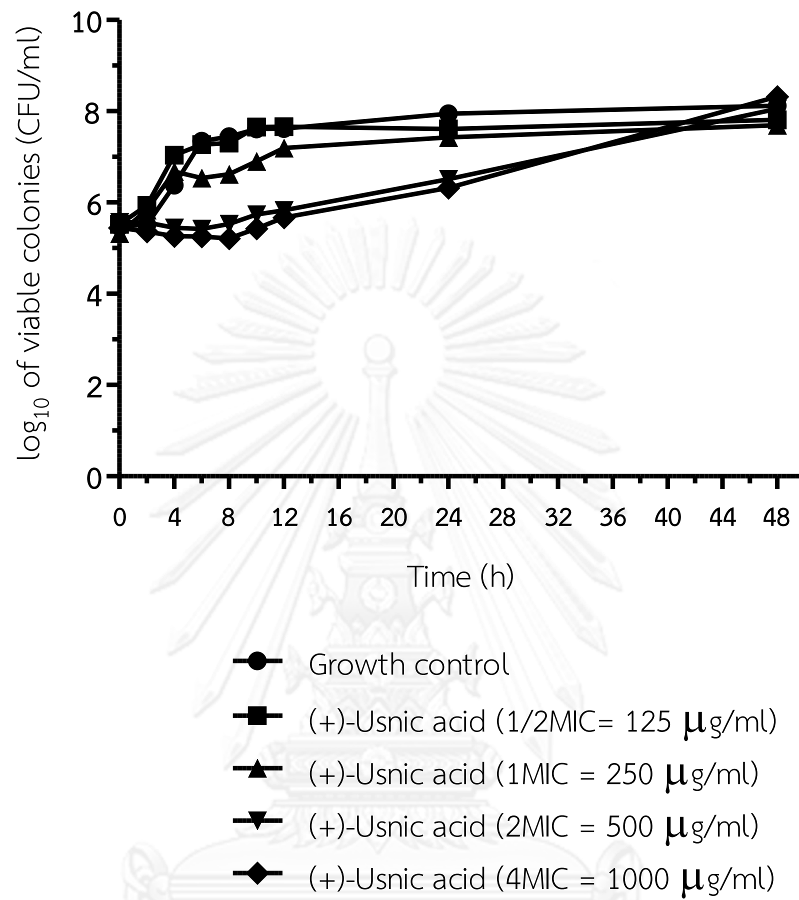

Figure 9 Time kill curve of (+)-usnic acid against *S. aureus*

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For *S. epidermidis*, (+)-usnic acid at 31.25 µg/ml (1/2MIC) and 62.50 µg/ml (1MIC) did not inhibit the growth of *S. epidermidis*. After 8 h of incubation, (+)-usnic acid at 125 µg/ml (2MIC) inhibited the growth of *S. epidermidis* with the reduction of bacterial within 1 log scale but did not show bacteriostatic activity. Moreover, after 12 h of incubation, (+)-usnic acid at 250 µg/ml (4MIC) inhibited the growth of *S. epidermidis* with the reduction of bacterial more than 1 log scale which showed bacteriostatic activity. The bacteriostatic activity was the reduction of bacterial 1 to <3 log scale compared with the original inoculum (Petersen *et al*., 2007).

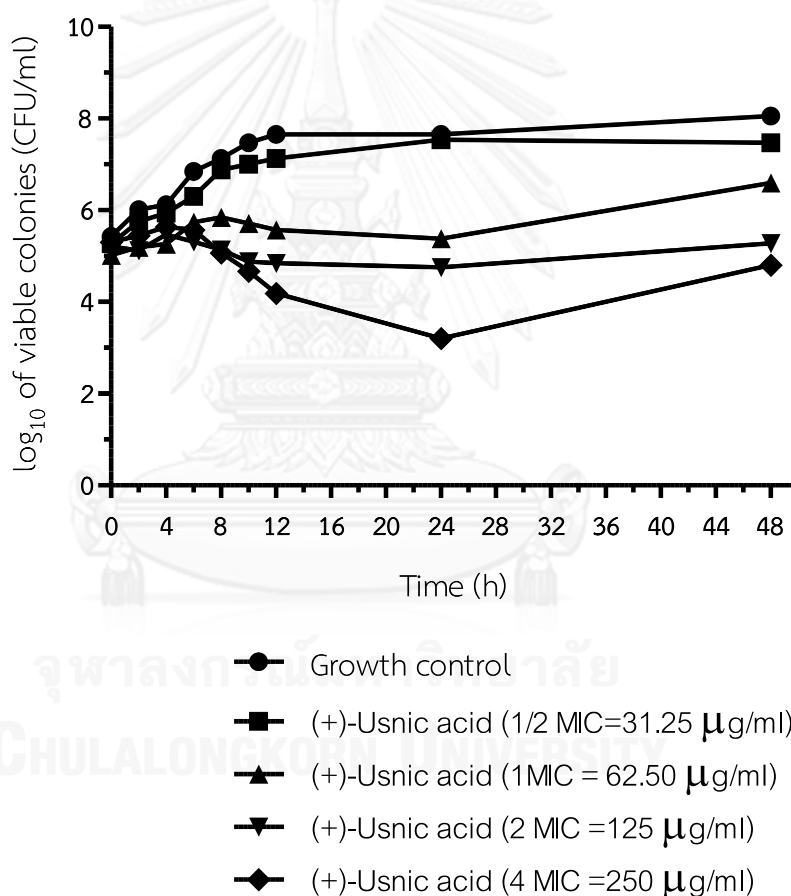

Figure 10 Time kill curve of (+)-usnic acid against *S. epidermidis*

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For *S. aureus*, ampicillin at 0.05 μg/ml (1/2MIC) inhibited the growth of *S. aureus* with the reduction of bacterial more than 1 log scale which showed bacteriostatic activity after 8 h of incubation and the regrowth was observed at 24 h of incubation. Ampicillin at 0.10 μg/ml (1MIC) and 0.20 μg/ml (2MIC) inhibited the growth of *S. aureus* with the reduction of bacterial more than 1 log scale which showed bacteriostatic activity after 4 h of incubation and the regrowth was observed at 48 h of incubation. The regrowth was defined as maintenance of the growth of increase ≥1 log scale from nadir (Hanberger, 1992). The bacteriostatic activity was the reduction of bacterial 1 to <3 log scale compared with the original inoculum (Petersen *et al*., 2007). Ampicillin at 0.40 μg/ml (4MIC) inhibited the growth of *S. aureus* with the reduction of bacterial more than 3 log scale which showed bactericidal activity after 12 h of incubation. After 24 h of incubation with ampicillin at 0.40 μg/ml (4MIC) the viable colonies of *S. aureus* were not seen (Figure 11). The bactericidal activity was the reduction of bacterial equal and more than 3 log scale compared with the original inoculum (Petersen *et al*., 2007).

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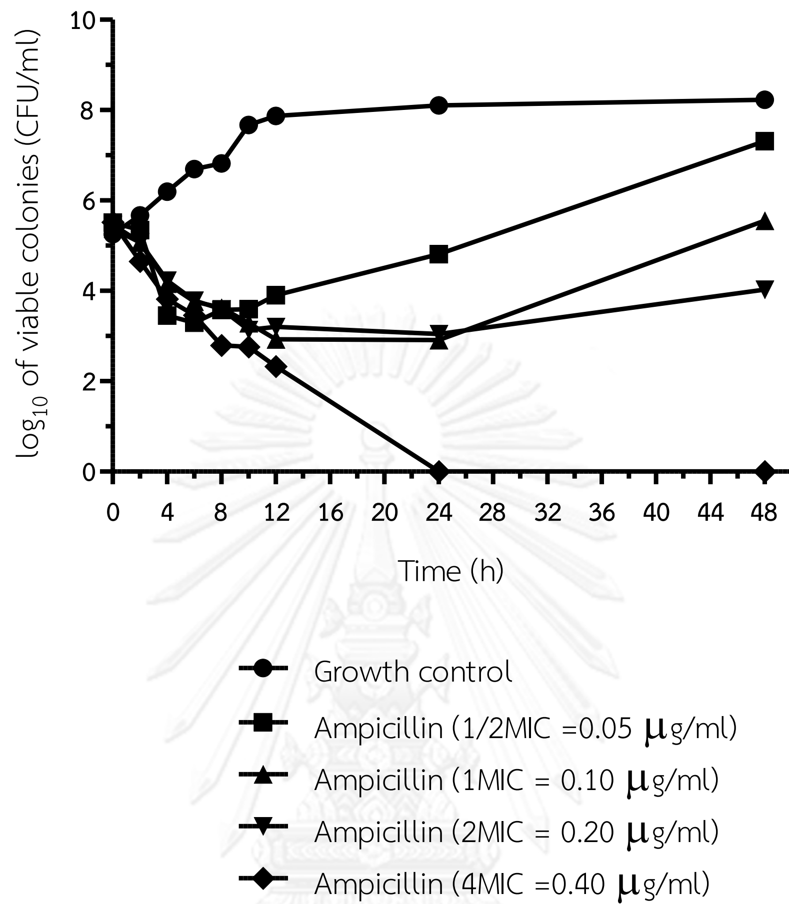

Figure 11 Time kill curve of ampicillin against *S. aureus*

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For *S. epidermidis*, ampicillin at 0.78 µg/ml (1/2MIC) inhibited the growth of *S. epidermidis* with the reduction of bacterial more than 1 log scale which showed bacteriostatic activity after 6 h of incubation and the regrowth was observed at 24 h of incubation. Ampicillin 1.56 µg/ml (1MIC), 3.12 µg/ml (2MIC) and 6.25 µg/ml (4MIC) inhibited the growth of *S. epidermidis* with the reduction of bacterial more than 1 log scale which showed bacteriostatic activity after 4 h of incubation. The bacteriostatic activity was the reduction of bacterial 1 to <3 log scale compared with the original inoculum (Petersen *et al*., 2007). Ampicillin at 1.56 µg/ml (1MIC), the regrowth was observed at 48 h of incubation. The regrowth was defined as maintenance of the growth of increase ≥1 log scale from nadir (Hanberger, 1992). Ampicillin at 1.56 µg/ml (1MIC) inhibited the growth of *S. epidermidis* with the reduction of bacterial more than 3 log scale which showed bactericidal activity after 24 h of incubation. Ampicillin at 3.12 µg/ml (2MIC) and 6.25 µg/ml (4MIC) inhibited the growth of *S. epidermidis* with the reduction of bacterial more than 3 log scale which showed bactericidal activity after 12 h of incubation. Ampicillin at 6.25 µg/ml (4MIC), the viable colonies of *S. aureus* were not seen after 24 h of incubation (Figure 12). The bactericidal activity was the reduction of bacterial equal and more than 3 log scale compared with the original inoculum (Petersen *et al*., 2007).

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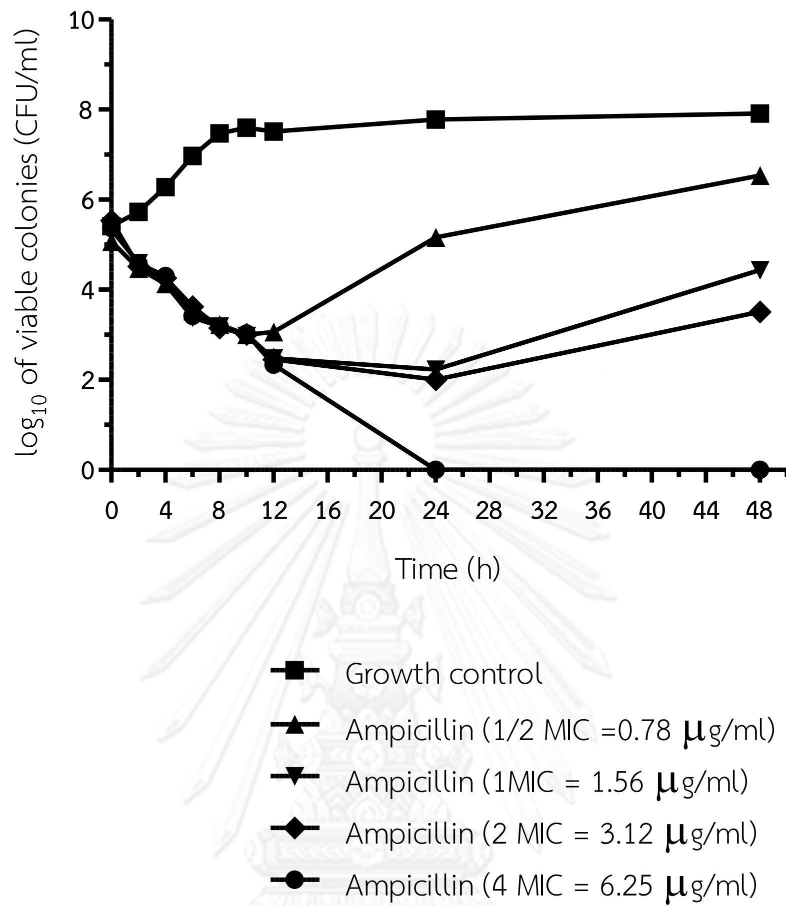

Figure 12 Time kill curve of ampicillin against *S. epidermidis*

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1.3 Inhibition of biofilm formation assay

The result of inhibition of biofilm formation assay was expressed as percentages of inhibition of biofilm formation and compared with 5% DMSO (Figure 13-16). In this experiment, OD_{625} of suspension *S. aureus* and *S. epidermidis* were 0.09 and 0.10, respectively (approximately 1.0×10^{8} CFU/ml). Then, the microorganisms were diluted 100 fold with MHB (approximately 1.0×10^{6} CFU/ml). Such microorganisms were used for determining inhibition of biofilm formation assay. Thus the final concentrations of microorganisms were 0.5×10^{6} CFU/ml. The effect of (+)-usnic acid on inhibition of biofilm formation of *S. aureus* and *S. epidermidis* was concentration dependent manner at concentration equal or greater than MIC value with statistical significance comparing with 5% DMSO ($p < 0.05$). The results also showed that at same concentration (250 µg/ml) (+)-usnic acid exhibited more antibiofilm activity against *S. epidermidis* than *S. aureus* with the percentage of biofilm inhibition of 97.50 and 36.68 respectively. Ampicillin exhibited antibiofilm activity against both *S. aureus* and *S. epidermidis* but not in concentration dependent manner. Interestingly, at concentration 250 µg/ml (4MIC) of (+)-usnic acid the percentage of biofilm inhibition was similar to ampicillin at concentration 6.25 µg/ml (4MIC) for *S. epidermidis*, which was 97.50%.

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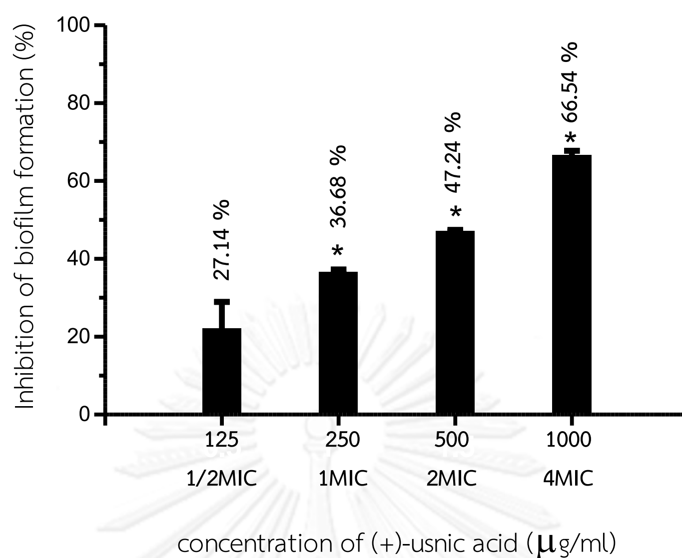

Figure 13 Inhibition of biofilm formation of *S. aureus* by (+)-usnic acid. Error bars represent the mean ± SEM, *p<0.05

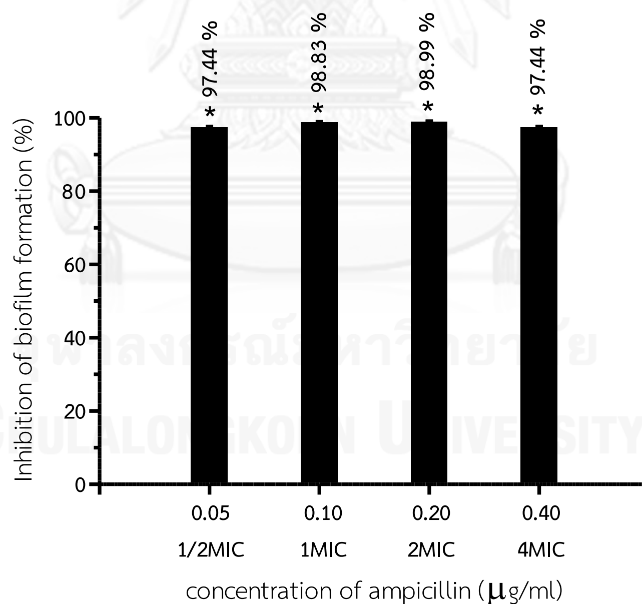

Figure 14 Inhibition of biofilm formation of *S. aureus* by ampicillin. Error bars represent the mean ± SEM, *p<0.05

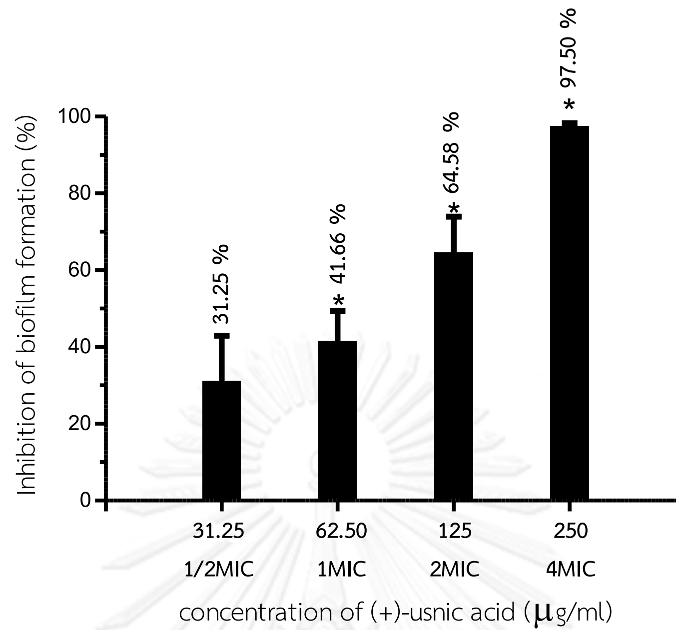

Figure 15 Inhibition of biofilm formation of *S.epidermidis* by (+)-usnic acid. Error bars represent the mean ± SEM, *p<0.05

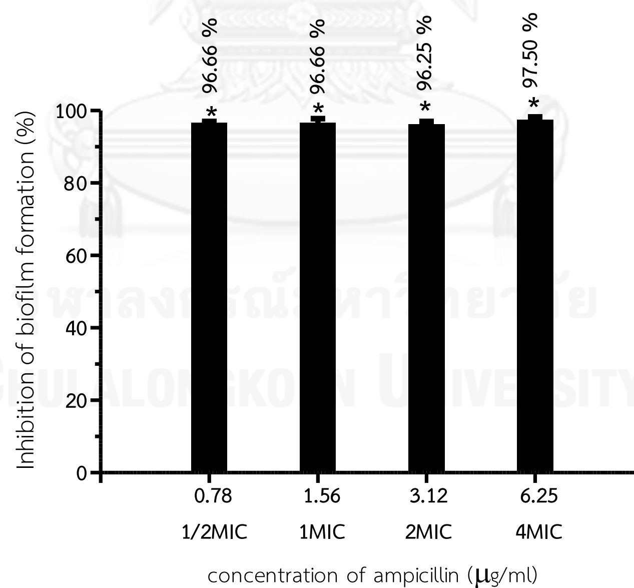

Figure 16 Inhibition of biofilm formation of *S. epidermidis* by ampicillin. Error bars represent the mean ± SEM, *p<0.05

2. Antimicrobial activities testing *in vivo* studies

2.1 Pathogenicity of bacterial to silkworms

This study examined whether injection of *S. aureus* or *S. epidermidis* pathogenic to humans could kill silkworms. Their skin color of the larvae injected with *S. aureus* or *S. epidermidis* turned dark. The medium lethal doses (LD_{50}) of *S. aureus* or *S. epidermidis* were $5.01x10^6$ CFU/ml ($7.50x10^5$ CFU/g of larva) and $7.59x10^6$ CFU/ml ($1.14x10^6$ CFU/g of larva) within 48 h, respectively (Figure 17-18). Additionally, when $1.20x10^9$CFU/ml ($1.80x10^8$CFU/g of larva) of both *S. aureus* and *S. epidermidis* was injected into intra-hemolymph of silkworms, all of silkworms died within 48 h. These concentrations of the microorganisms were used for determination of efficacy effect of (+)-usnic acid. Silkworm larvae survived 48 h when injected with 0.9% NaCl.

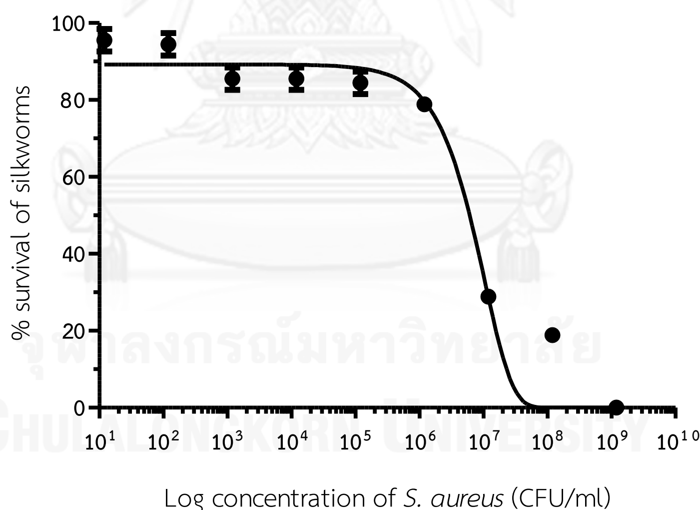

Figure 17 The percentage of survival of silkworm received *S. aureus* at various concentrations

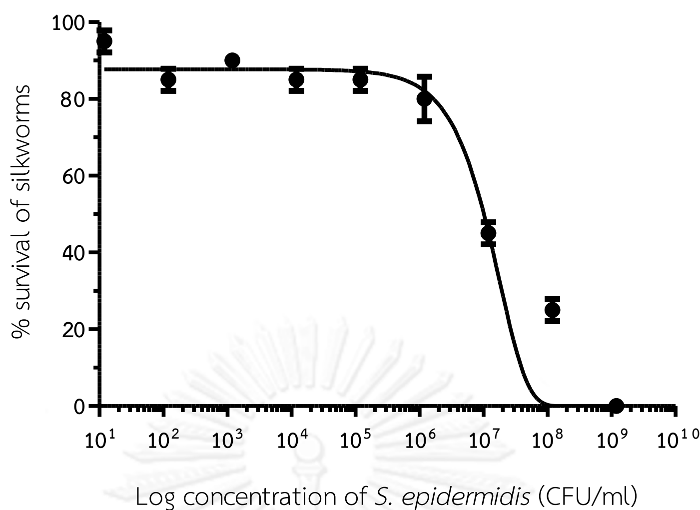

Figure 18 The percentage of survival of silkworm received *S. epidermidis* at various concentrations

2.2 Determination of toxicity of ampicillin, DMSO and (+)-usnic acid

The weight of 5th instar larva was average 0.413 g/larva. Silkworms were injected with 50 µl of ampicillin, DMSO or (+)-usnic acid in various concentrations into intra-hemolymph and the survival rate was observed at 48 h after injection. The LD_{50} value of DMSO was 12.19%. Injection of 50 µl of 5% DMSO into intra-hemolymph of silkworms showed the survival rate was 100% at 48 h after injection (Figure 19). Then this concentration was used as solvent of (+)-usnic acid. In addition, LD_{50} value of ampicillin or (+)-usnic acid was more than 80 mg/ml (more than 9.68 mg/g of larva) and 5.32 mg/ml (0.64 mg/g of larva), respectively (Figure 20). The data was showed in Table 3.

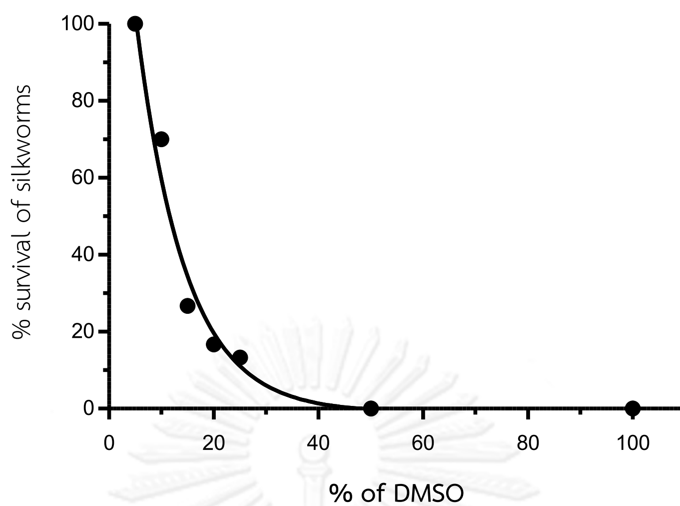

Figure 19 The percentage of survival of silkworm received DMSO at various concentrations

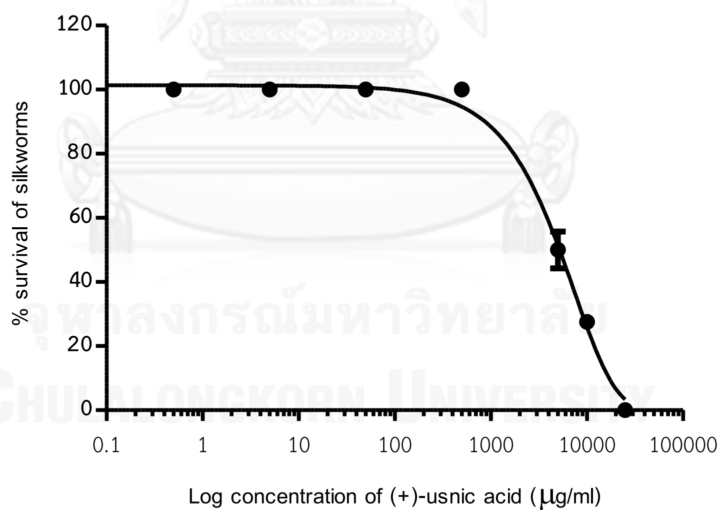

Figure 20 The percentage of survival of silkworm received (+)-usnic acid at various concentrations

2.3 Determination of efficacy of ampicillin and (+)-usnic acid

The results were presented in effective dose (ED_{50}) which the concentration of ampicillin or (+)-usnic acid were showing 50% survival of the silkworm that infection with bacterial pathogen (Figure 21-22). The survival of silkworms was determined 48 h after injection of the bacterial suspension and ampicillin or (+)-usnic acid into intra hemolymph. The weight of 5th instar larva was average 0.353 g/larva. *S. aureus* or *S. epidermidis* concentration 1.20×10^9 CFU/ml (1.80×10^8 CFU/g larva) could kill silkworm 100% after injection into intra-hemolymph within 48 h used for control group. The ED_{50} values of ampicillin against *S. aureus* or *S. epidermidis* was 1.26 mg/ml (0.18 mg/g of larva) and 7.70 mg/ml (1.09 mg/g of larva), respectively whereas the ED_{50} values of (+)-usnic acid against *S. aureus* or *S. epidermidis* was more than 4 mg/ml (0.57 mg/g of larva) (Table 3).

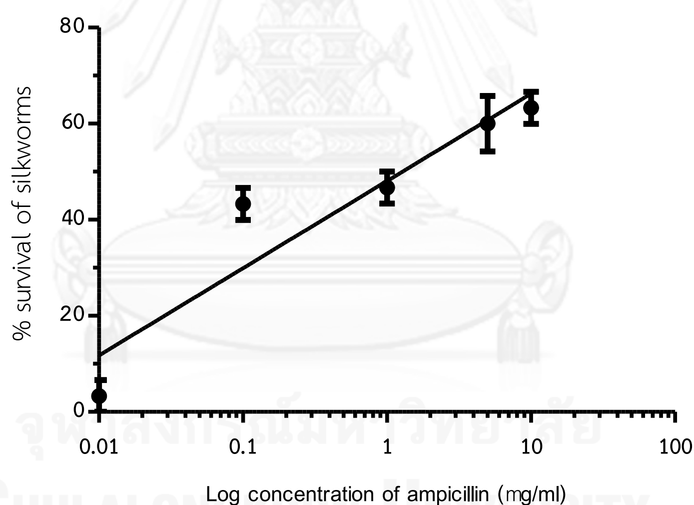

Figure 21 The percentage of survival of silkworm infected with *S. aureus* received ampicillin at various concentrations

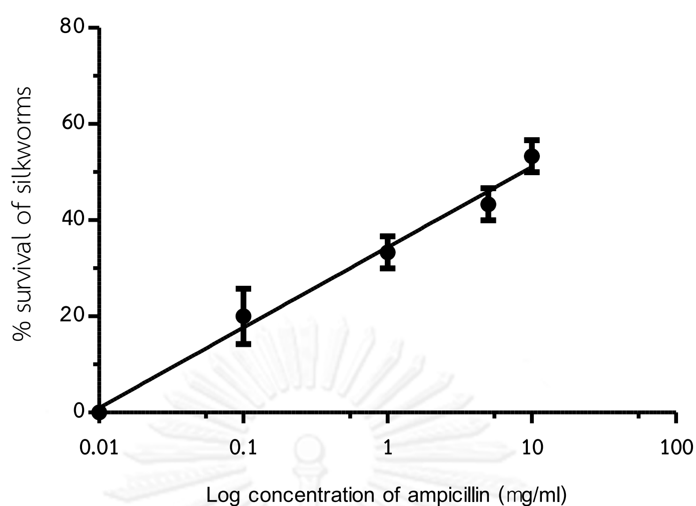

Figure 22 The percentage of survival of silkworm infected with *S. epidermidis* received ampicillin at various concentrations

Table 3 LD_{50}; the concentrations of ampicillin, (+)-usnic acid and DMSO which 50% of the injected larvae were killed 48h later. ED_{50}; the concentration of ampicillin or (+)-usnic acid which 50% of the injected silkworm larvae survived 48h later.

Substances	Larva LD_{50} (mg/ml)	ED_{50} (mg/ml)	
		S. aureus	***S. epidermidis***
Ampicillin	>80	1.26	7.70
(+)-Usnic acid	5.32	>4.00	>4.00
DMSO	12.13*	N.D.	N.D.

* %

N.D. = not determined

CHAPTER V
DISCUSSION AND CONCLUSION

In this study, the antimicrobial activity of (+)-usnic acid was demonstrated quantitatively by broth microdilution method. The results showed that (+)-usnic acid exhibited antimicrobial activity against the bacterial pathogens at the MIC values of 250 and 62.50 µg/ml for *S. aureus* ATCC 6538P and *S. epidermidis* Isolated, respectively, whereas the MBC values of (+)-usnic acid required to kill both *S. aureus* ATCC 6538P and *S. epidermidis* Isolated were more than 4000 µg/ml. Therefore the results indicated that antimicrobial activities of (+)-usnic acid against *S. epidermidis* Isolated was more effective than that against *S. aureus* ATCC 6538P. This result is consistent with the result from the previous study of (+)-usnic acid and *Usnea*-extract. They exhibited antimicrobial activity against *S. aureus* and *S. epidermidis* Isolated (Weckesser *et al*., 2007). Ampicillin was used as positive control for these bacterial pathogens. MIC values of ampicillin were 0.10 and 1.56 µg/ml for *S. aureus* ATCC 6538P and *S. epidermidis* Isolated, respectively. MBC values of ampicillin were 0.39 and 3.12 µg/ml for *S. aureus* ATCC 6538P and *S. epidermidis* Isolated, respectively. MIC value of ampicillin for *S. aureus* ATCC 29213, quality control strain, was 0.12 µg/ml. This result indicated that MIC value of ampicillin against *S. aureus* ATCC 29213 was a little bit lower than MIC value reported by Clinical and Laboratory Standards Institute (CLSI) (*CLSI M100-S23*, 2013).

Time kill assay was used for determination rate of (+)-usnic acid kills microorganisms. The samples were collected at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h, respectively. Viable colony count was determined by 100 µl of serial dilution on MHA. The results were presented in time kill curve that plot between $\log_{10}$ of viable colonies against time (J. May *et al*., 2000; Soontornpas *et al*., 2005). In this study, (+)-usnic acid has no bacteriostatic activity against *S. aureus* in all concentrations used (125, 250, 500 and 1000 µg/ml) whereas it has bacteriostatic activity against *S. epidermidis* only at 250 µg/ml after 12 h of incubation. Regrowth of *S. aureus* occurred with (+)-usnic acid at 500 and 1000 µg/ml with the incubation times at 10 and 12 h respectively. In addition, ampicillin has bacteriostatic activity against *S. aureus* in the following concentrations: 0.50, 0.10 and 0.20 µg/ml with the incubation

time of 8, 4 and 4 h respectively whereas ampicillin also has bactericidal activity against *S. aureus* at concentration 0.40 µg/ml with the incubation time of 12 h. The positive control, ampicillin, has bacteriostatic activity against *S. epidermidis* in the following concentrations: 0.78 and 1.56 µg/ml with the incubation time of 6 and 4 h respectively whereas ampicillin also has bactericidal activity against *S. epidermidis* in the following concentrations: 3.12 and 6.25 µg/ml with the incubation time of 12 h.

Result from the antibiofilm formation assay showed that (+)-usnic acid at concentration equal or greater than MIC of (+)-usnic acid could inhibit biofilm formation of both *S. aureus* and *S. epidermidis* with statistical significance ($p < 0.05$) in concentration dependent manner. This result support previous study reporting that (+)-usnic acid could inhibit biofilm formation of *S. aureus* by kill the attached cells (Francolini, Norris, Piozzi, Donelli, & Stoodley, 2004). This study showed that (+)-usnic acid exhibited more antibiofilm activity against *S. epidermidis* than *S. aureus* with the percentage of biofilm inhibition of 97.50 and 36.68 respectively at same concentration (250 µg/ml). Interestingly, at concentration 250 µg/ml of (+)-usnic acid the percentage of biofilm inhibition was quite similar to ampicillin for *S. epidermidis*, whereas the percentage of biofilm inhibition at concentration 250 µg/ml of (+)-usnic acid was lower comparing with ampicillin for *S. aureus*. This result indicated the efficacy of (+)-usnic acid against *S. epidermidis* was similar to ampicillin against *S. epidermidis*.

The ability to form biofilm has been reported to starting after 4-12 h of incubation (Sun, Zhang, Chen, Chen, & Han, 2012). The action of (+)-usnic acid may protect the attached of bacterial cells because (+)-usnic acid influence to QS. QS has been reported that a signaling molecule for linked to biofilm structure and important for the attachment of bacteria to the surface, the maturation of the biofilm or the control of events leading to dispersion of cells (Dufour *et al*., 2012). Other mechanism of action which may contribute to antimicrobial and antibiofilm formation activities of (+)-usnic acid is the inhibition of expression of regulator gene (*agr*) that plays a major role in control pathogenesis, toxin production and many virulence factors such as hemolysin, leucosidin and hyaluronidase etc. of these bacterial pathogens (Dufour *et al*., 2012; Francolini *et al*., 2004).

For all results presented above, it is indicated that (+)-usnic acid has more antimicrobial and antibiofilm activities against *S. epidermid* than *S. aureus*. The results from time kill assay were consistent with the antimicrobial activity by broth microdilution method and antibiofilm formation assay.

In vivo study, silkworm model was used as an animal model to investigate toxicity and efficacy of (+)-usnic acid. First, we determined the toxicity of (+)-usnic acid, DMSO and ampicillin in silkworm model. The LD_{50} of (+)-usnic acid, DMSO and ampicillin are as followings: 5.32 mg/ml (0.64 mg/g of larva), 12.15% and more than 80 mg/ml (more than 9.68 mg/g of larva) respectively. We also demonstrated the pathogenicity study in silkworm model. The result showed that LD_{50} of *S. aureus* and *S. epidermidis* were 5.01×10^{6} CFU/ml (7.50×10^{5} CFU/g of larva) and 7.59×10^{6} CFU/ml (1.14×10^{6} CFU/g of larva) respectively. This result is consistent with the result from the previous study that *S. aureus* and *S. epidermidis* killed silkworm within 2 days (Kaito *et al*., 2002; Sukpanich, 2013). We therefore selected the inoculum concentration of both *S. aureus* and *S. epidermidis* at 1.2×10^{9} CFU/ml for further study in efficacy of (+)-usnic acid in silkworm model in order to be sure that silkworm larva must be killed 100% by the inoculum used.

For efficacy study, the results showed that the ED_{50} values of (+)-usnic acid for *S. aureus* or *S. epidermidis* were more than 4 mg/ml (more than 0.57mg/g of larva) whereas the ED_{50} of ampicillin, antibiotic clinically used for human, for *S. aureus* and *S. epidermidis* were 1.26 mg/ml (0.18 mg/g of larva) and 7.70 mg/ml (1.09 mg/g of larva), respectively. The ED_{50}/MIC ratio of (+)-usnic acid were more than 16 and more than 64 for *S. aureus* and *S. epidermidis* respectively. Theoretically, the higher the ratio of ED_{50}/MIC, the more impact the pharmacokinetic in silkworm larvae body involved (Hamamoto et al., 2004). Such pharmacokinetics may include absorption, distribution, metabolism and excretion. Based on the result from efficacy study, (+)-usnic acid significantly lost its therapeutic effect comparing to the result from *in vitro* study. This indicated that the influence of pharmacokinetic parameters for example plasma protein binding, metabolism and excretion play an important roles in silkworm model. Moreover, therapeutic index, ratio of LD_{50}/ED_{50}, of (+)-usnic acid was less than 1.33 for both *S. aureus* and *S. epidermidis* which is a narrow

therapeutic index. Thus, (+)-usnic acid must be dosed carefully. This is a limitation of (+)-usnic acid when applied to the clinical setting in terms of safety. For ampicillin therapeutic index, ratio of LD_{50}/ED_{50}, of (+)-usnic acid was more than 63.49 and more than 10.39 for *S. aureus* and *S. epidermidis* respectively which is broader therapeutic index comparing to the one of (+)-usnic acid.

In conclusion (+)-usnic acid had antimicrobial activities and antibiofilm formation against *S. aureus* and *S. epidermidis* in all *in vitro* models, however it antimicrobial activities in *in vivo* model (silkworm model) are lessen against both *S. aureus* and *S. epidermidis*. This may be due to pharmacokinetic parameter in silkworm model as mentioned above. Therefore, further study is required to evaluate antimicrobial and antibiofilm formation activities of (+)-usnic acid against *S. aureus* and *S. epidermidis* in mammalian model such as rat, mice etc and silkworm infectious model can also be used as a screening tool for drugs discovery.

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APPENDICES

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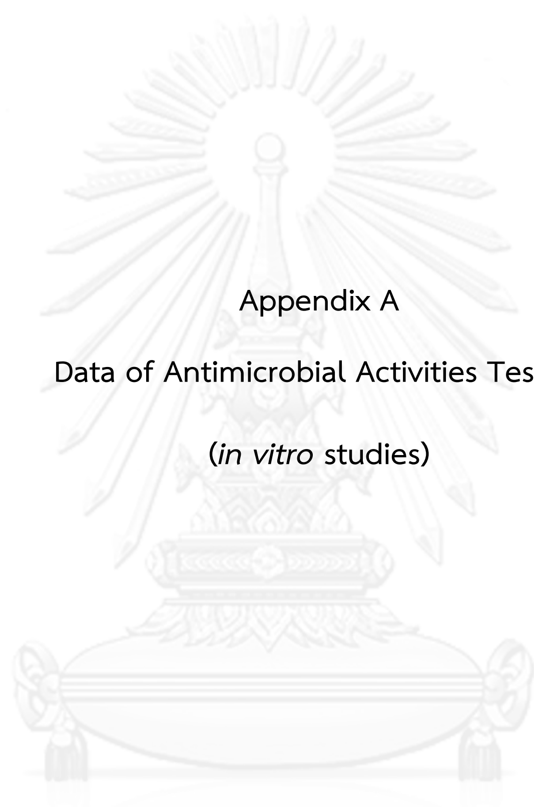

Appendix A

Data of Antimicrobial Activities Testing

(*in vitro* studies)

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Table 4 Survival colonies of *S. aureus* received ampicillin

Hours	Survival colonies (CFU/ml)				
	Growth control	Ampicillin 0.05 μg/ml (1/2MIC)	Ampicillin 0.10μg/ml (1MIC)	Ampicillin 0.20 μg/ml (2MIC)	Ampicillin 0.40 μg/ml (4MIC)
0	1.76×10^{5}	3.27×10^{5}	2.36×10^{5}	2.92×10^{5}	3.37×10^{5}
2	4.70×10^{5}	2.26×10^{5}	1.18×10^{5}	1.20×10^{5}	4.42×10^{4}
4	1.56×10^{6}	2.93×10^{5}	1.45×10^{4}	1.70×10^{4}	6.53×10^{3}
6	5.00×10^{6}	2.02×10^{5}	5.75×10^{3}	6.20×10^{3}	2.94×10^{3}
8	6.62×10^{6}	3.80×10^{3}	4.28×10^{3}	3.95×10^{3}	6.33×10^{2}
10	4.70×10^{7}	4.00×10^{3}	1.93×10^{3}	1.38×10^{3}	5.75×10^{2}
12	7.43×10^{7}	8.00×10^{3}	8.50×10^{2}	1.26×10^{3}	2.15×10^{2}
24	1.56×10^{8}	6.52×10^{4}	8.20×10^{2}	1.15×10^{3}	0.00
48	1.69×10^{8}	2.07×10^{7}	3.57×10^{5}	1.06×10^{5}	0.00

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Table 5 Survival colonies of *S. aureus* received (+)-usnic acid

Hours	Survival colonies (CFU/ml)				
	Growth control	(+)-Usnic acid 125 µg/ml (1/2MIC)	(+)-Usnic acid 250 µg/ml (1MIC)	(+)-Usnic acid 500 µg/ml (2MIC)	(+)-Usnic acid 1,000 µg/ml (4MIC)
0	2.99×10^{5}	3.34×10^{5}	2.10×10^{5}	3.75×10^{5}	3.13×10^{5}
2	3.20×10^{5}	8.83×10^{5}	6.92×10^{5}	3.63×10^{5}	2.30×10^{5}
4	2.40×10^{6}	1.12×10^{7}	4.79×10^{6}	2.81×10^{5}	1.84×10^{5}
6	2.22×10^{7}	1.90×10^{7}	3.47×10^{6}	2.63×10^{5}	1.77×10^{5}
8	2.85×10^{7}	1.99×10^{7}	4.17×10^{6}	3.39×10^{5}	1.62×10^{5}
10	4.10×10^{7}	4.48×10^{7}	7.94×10^{6}	5.50×10^{5}	2.70×10^{5}
12	4.20×10^{7}	4.59×10^{7}	1.59×10^{7}	6.76×10^{5}	4.68×10^{5}
24	9.00×10^{7}	4.07×10^{7}	2.72×10^{7}	3.24×10^{6}	2.09×10^{7}
48	1.33×10^{8}	6.50×10^{7}	5.05×10^{7}	1.14×10^{8}	2.13×10^{8}

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Table 6 Survival colonies of *S. epidermidis* received ampicillin

Hours	Survival colonies (CFU/ml)				
	Growth control	Ampicillin 0.78 µg/ml (1/2MIC)	Ampicillin 1.56 µg/ml (1MIC)	Ampicillin 3.12 µg/ml (2MIC)	Ampicillin 6.25 µg/ml (4MIC)
0	2.58×10^{5}	1.17×10^{5}	2.21×10^{5}	2.84×10^{5}	2.34×10^{5}
2	5.34×10^{5}	5.55×10^{4}	4.00×10^{4}	3.28×10^{4}	3.84×10^{4}
4	1.89×10^{6}	1.35×10^{4}	1.34×10^{4}	1.83×10^{4}	2.01×10^{4}
6	9.32×10^{6}	3.40×10^{3}	2.43×10^{3}	4.23×10^{3}	2.60×10^{3}
8	2.97×10^{7}	1.70×10^{3}	1.57×10^{3}	1.41×10^{3}	1.54×10^{3}
10	3.95×10^{7}	1.00×10^{3}	9.50×10^{2}	1.03×10^{3}	1.07×10^{3}
12	3.22×10^{7}	1.14×10^{3}	3.00×10^{2}	2.83×10^{2}	2.20×10^{2}
24	6.01×10^{7}	1.45×10^{5}	1.70×10^{2}	1.00×10^{2}	0.00
48	8.06×10^{7}	3.50×10^{6}	2.63×10^{5}	3.20×10^{3}	0.00

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Table 7 Survival colonies of *S. epidermidis* received (+)-usnic acid

Hours	Survival colonies (CFU/ml)				
	Growth control	(+)-Usnic acid 31.25 µg/ml (1/2MIC)	(+)-Usnic acid 62.50 µg/ml (1MIC)	(+)-Usnic acid 125 µg/ml (2MIC)	(+)-Usnic acid 250 µg/ml (4MIC)
0	2.69×10^{5}	1.86×10^{5}	1.05×10^{5}	1.62×10^{5}	2.02×10^{5}
2	1.26×10^{6}	5.58×10^{5}	1.58×10^{5}	1.31×10^{5}	2.83×10^{5}
4	4.68×10^{6}	3.80×10^{5}	1.84×10^{5}	2.97×10^{5}	4.41×10^{5}
6	7.08×10^{6}	2.02×10^{6}	5.53×10^{5}	2.04×10^{5}	3.72×10^{5}
8	1.36×10^{7}	7.62×10^{6}	7.13×10^{5}	1.38×10^{5}	1.17×10^{5}
10	3.00×10^{7}	1.01×10^{7}	5.20×10^{5}	7.58×10^{4}	4.68×10^{4}
12	4.50×10^{7}	1.35×10^{7}	3.78×10^{5}	7.09×10^{4}	1.55×10^{4}
24	4.50×10^{7}	3.50×10^{7}	2.43×10^{5}	5.60×10^{4}	1.60×10^{4}
48	1.13×10^{8}	2.95×10^{7}	4.05×10^{6}	1.91×10^{5}	6.35×10^{4}

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Table 8 Percentage of inhibition of biofilm formation of *S. aureus* received ampicillin

Ampicillin (µg/ml)	Percentage of inhibition of biofilm formation (Mean ± SEM)
0.05 (1/2MIC)	97.44 ± 0.15
0.10 (1MIC)	98.83 ± 0.16
0.20 (2MIC)	98.99 ± 0.15
0.40 (4MIC)	97.45 ± 0.15
negative control	0.00

Table 9 Percentage of inhibition of biofilm formation of *S. aureus* received (+)-usnic acid

(+)-Usnic acid (µg/ml)	Percentage of inhibition of biofilm formation (Mean ± SEM)
125 (1/2MIC)	22.14 ± 6.82
250 (1MIC)	36.68 ± 0.59
500 (2MIC)	47.44 ± 0.21
1,000 (4MIC)	66.54 ± 1.18
negative control	0.00

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Table 10 Percentage of inhibition of biofilm formation of *S. epidermidis* received ampicillin

Ampicillin (μg/ml)	Percentage of inhibition of biofilm formation (Mean ± SEM)
0.78 (1/2MIC)	96.66 ± 0.42
1.56 (1MIC)	96.66 ± 1.10
3.12 (2MIC)	96.25 ± 0.83
6.25 (4MIC)	97.50 ± 0.83
negative control	0.00

Table 11 Percentage of inhibition of biofilm formation of *S. epidermidis* received (+)-usnic acid

(+)-Usnic acid (μg/ml)	Percentage of inhibition of biofilm formation (Mean ± SEM)
31.25 (1/2MIC)	31.25 ± 11.70
62.50 (1MIC)	36.68 ± 7.20
125 (2MIC)	47.44 ± 9.38
250 (4MIC)	66.54 ± 0.83
negative control	0.00

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Appendix B

Data of Antimicrobial Activities Testing

(*in vivo* studies)

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Table 12 Survival rate of silkworm received (+)-usnic acid at various concentrations

(+)-Usnic acid (mg/ml)	Mean of number of silkworm survival (% survival)
0.005	100.00
0.05	100.00
0.5	100.00
2	100.00
4	100.00
5	50.00
10	27.50
25	0

Table 13 Survival rate of silkworm received ampicillin at various concentrations

Ampicillin (mg/ml)	Mean of number of silkworm survival (% survival)
5	100.00
10	100.00
20	90.00
40	90.00
80	86.67

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Table 14 Survival rate of silkworm received DMSO at various concentrations

concentration of DMSO	Mean of number of silkworm survival (% survival)
5%	100.00
10%	70.00
15%	26.67
20%	16.67
25%	13.33
50%	0.00
100%	0.00

Table 15 Survival rate of silkworm received *S. aureus* at various concentrations

S. aureus (CFU/ml)	Mean of number of silkworm death (% survival)
1.2×10^{1}	95.56
1.2×10^{2}	94.44
1.2×10^{3}	85.56
1.2×10^{4}	85.56
1.2×10^{5}	84.44
1.2×10^{6}	78.89
1.2×10^{7}	28.89
1.2×10^{8}	18.89
1.2×10^{9}	0.00

Table 16 Survival rate of silkworm received *S. epidermidis* at various concentrations

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S. epidermidis (CFU/ml)	Mean of number of silkworm death (% survival)
1.2×10^{1}	96.67
1.2×10^{2}	86.67
1.2×10^{3}	83.33
1.2×10^{4}	80.00
1.2×10^{5}	80.00
1.2×10^{6}	73.33
1.2×10^{7}	36.67
1.2×10^{8}	20.00
1.2×10^{9}	0.00

Table 17 Survival rate of silkworm infected with *S. aureus* received ampicillin at various concentrations

Ampicillin (mg/ml)	Mean of number of silkworm survival (% survival)
0.01	3.33
0.10	43.33
1.00	46.67
5.00	60.00
10.00	63.33

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Table 18 Survival rate of silkworm infected with *S. aureus* received (+)-usnic acid at various concentrations

(+)-usnic acid (mg/ml)	Mean of number of silkworm survival (% survival)
0.25	0.00
0.50	3.33
1.00	16.67
2.00	23.33
3.00	33.33
4.00	36.67

Table 19 Survival rate of silkworm infected with *S. epidermidis* received ampicillin at various concentrations

Ampicillin (mg/ml)	Mean of number of silkworm survival (% survival)
0.01	0.00
0.10	20.00
1.00	33.33
5.00	43.33
10.00	53.33

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Table 20 Survival rate of silkworm infected with *S. epidermidis* received (+)-usnic acid at various concentrations

(+)-usnic acid (mg/ml)	Mean of number of silkworm survival (% survival)
0.25	0.00
0.50	10.00
1.00	6.67
2.00	23.33
3.00	36.67
4.00	40.00

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VITA

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