ฤทธิ์ในการต้านเชื้อแบคทีเรียของกรดอุสนิคต่อเชื้อบาซิลัส ซีเรียสและบาซิลัส ซับติลิส จากการศึกษาในหลอดทดลองและแบบในกายโดยใช้หนอนไหมไทย

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จุฬาลงกรณ์มหาวิทยาลัย
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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIMICROBIAL ACTIVITIES OF (+)–USNIC ACID ON *BACILLUS CEREUS* AND *BACILLUS SUBTILIS IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL

Miss Pornthiwa Thawongma

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A Thesis Submitted in Partial Fulfillment of the Requirements
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พรธิวา ทาวงศ์มา : ฤทธิ์ในการต้านเชื้อแบคทีเรียของกรดอุสนิคต่อเชื้อบาซิลัส ซีเรียส และบาซิลัส ซับติลิส จากการศึกษาในหลอดทดลองและแบบในกายโดยใช้หนอนไหมไทย. (ANTIMICROBIAL ACTIVITIES OF (+)–USNIC ACID ON *BACILLUS CEREUS* AND *BACILLUS SUBTILIS IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ภก ดร. สันทัด จันทร์ประภาพ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ชนิดา พลานุเวช, 66 หน้า.

บาซิลลัส ซีเรียส และบาซิลลัส ซับติลิส เป็นเชื้อแบคทีเรียที่นอกจากจะมีปัญหาเกี่ยวกับอาหารเป็นพิษแล้ว ปัจจุบันพบว่ามีการดื้อยาของเชื้อแบคทีเรียเหล่านี้มากขึ้น ซึ่งปัจจัยสำคัญที่ทำให้เกิดการดื้อยาของเชื้อคือความสามารถในการสร้างไบโอฟิล์มของเชื้อแบคทีเรีย ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาฤทธิ์ในการต้านเชื้อจุลชีพ ฤทธิ์ยับยั้งการสร้างไบโอฟิลม์ รวมทั้งระยะเวลาในการฆ่าเชื้อ *B. cereus* และ *B. subtilils* ของกรดอุสนิค จากการทดสอบฤทธิ์ต้านจุลชีพโดยวิธี broth microdilution พบว่า ความเข้มข้นต่ำสุดของกรดอุสนิค (1MIC) ที่สามารถยับยั้งการเจริญของเชื้อ *B. cereus* และ *B. subtilils* มีค่าเท่ากับ 62.50 ไมโครกรัม/มิลลิลิตร เท่ากัน ในขณะที่ค่า MBC คือ 125 และ 250 ไมโครกรัม/มิลลิลิตร ตามลำดับ กรดอุสนิคมีฤทธิ์ในการยับยั้งการเจริญของเชื้อ *B.cereus* มากกว่า *B. subtilils* ส่วนการศึกษา time kill พบว่ากรดอุสนิคออกฤทธิ์ได้ทั้งแบบ bacteriostatic และ bactericidal โดยการออกฤทธิ์เป็นแบบขึ้นกับความเข้มข้นและระยะเวลาที่ใช้ในการบ่ม จากผลการทดลองแสดงว่าการออกฤทธิ์แบบ bacteriostatic และ bactericidal ของกรดอุสนิคและแอมพิซิลินเป็นแบบขึ้นกับความเข้มข้น นอกจากนี้กรดอุสนิคยังสามารถยับยั้งการสร้างไบโอฟิลม์ โดยความสามารถในการยับยั้งการสร้างไบโอฟิลม์ขึ้นกับความเข้มข้นของกรดอุสนิค กรดอุสนิคที่ความเข้มข้นตั้งแต่ 1/2 MIC ขึ้นไป สามารถยับยั้งการสร้างไบโอฟิลม์ของเชื้อ *B. cereus* และ *B. subtilils* ได้อย่างมีนัยสำคัญทางสถิติ ($p< 0.05$) เมื่อเปรียบเทียบกับกลุ่มควบคุม ส่วนการศึกษาในสัตว์ทดลอง ซึ่งได้นำหนอนไหมไทยมาใช้เพื่อทดสอบประสิทธิภาพในการต้านเชื้อของกรดอุสนิคและแอมพิซิลิน พบว่าความเข้มข้นของกรดอุสนิคและแอมพิซิลินที่ทำให้หนอนไหมรอดชีวิตจากการติดเชื้อ *B. cereus* ได้ 50% มีค่าเท่ากับ 3.56 มิลลิกรัม/มิลลิลิตร และ 0.23 มิลลิกรัม/มิลลิลิตร ตามลำดับ ส่วนกรดอุสนิคและแอมพิซิลินที่ทำให้หนอนไหมติดเชื้อ *B. subtilis* ได้ 50% มีค่าเท่ากับ 3.92 มิลลิกรัม/มิลลิลิตร และ 1.01 มิลลิกรัม/มิลลิลิตร ตามลำดับ ดังนั้นจากการศึกษาสรุปได้ว่ากรดอุสนิคและแอมพิซิลินมีฤทธิ์ในการต้านเชื้อ *B. cereus* และ *B. subtilils* ทั้งในหลอดทดลองและในสัตว์ทดลองโดยมีกลไกการต้านเชื้อส่วนหนึ่งผ่านทางการยับยั้งการสร้างไบโอฟิลม์

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

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PORNTHIWA THAWONGMA: ANTIMICROBIAL ACTIVITIES OF (+)–USNIC ACID ON *BACILLUS CEREUS* AND *BACILLUS SUBTILIS IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL. ADVISOR: SANTAD CHANPRAPAPH, Ph.D., CO-ADVISOR: CHANIDA PALANUVEJ, Ph.D., 66 pp.

B. cereus and *B. subtilis* not only continue to cause serious clinical problems through food poisoning, but also show natural resistance to some antimicrobial drugs. One of the key factors enabling pathogen to resist to antimicrobial drugs is the ability to form biofilm. This study was aimed to evaluate the antimicrobial activity, inhibitory effect of biofilm formation including time to kill *B. cereus* and *B. subtilis* of (+)-usnic acid. Antimicrobial activity was determined by broth microdilution method. MIC of (+)-usnic acid for *B. cereus* and *B. subtilis* was the same (62.50 µg/ml), whereas MBC of (+)-usnic acid for *B. cereus* and *B. subtilis* were 125 and 250 µg/ml, respectively. The antimicrobial activities of (+)-usnic acid against *B. cereus* was more effective than that on *B. subtilis.* Time kill studies showed that (+)-usnic acid exerted both bacteriostatic and bactericidal depending on concentration used and incubation time observed against *B. cereus* and *B. subtilis*. The result also indicated that bacteriostatic and bactericidal activity of (+)-usnic acid and ampicillin were concentration dependent. Furthermore, (+)-usnic acid had inhibitory effect on biofilm formation in a concentration dependent manner as well ranging from concentration equal or greater than half of MIC value with statistical significance comparing with negative control ($p<0.05$). Therefore, this study showed that (+)-usnic acid had both antimicrobial and antibiofilm formation activities on *B.cereus* and *B.subtilis*. *In vivo* study, silkworm were utilized for efficacy testing of compounds. ED_{50} of (+)usnic acid and ampicillin were 3.56 mg/ml and 0.23 mg/ml respectively for *B. cereus,* whereas ED_{50} of the(+)-usnic acid and ampicillin for *B. subtilis* were 3.92 mg/ml and 1.01 mg/ml respectively. Therefore, these findings showed that (+)-usnic acid had antimicrobial activities against *B. cereus* and *B. subtilis* both in *in vitro* and *in vivo.* Inhibition of biofilm formation may play an important role for the mechanism of action for antimicrobial activity shown.

Field of Study: Pharmacology

Academic Year: 2013

Student's Signature .....

Advisor's Signature .....

Co-Advisor's Signature .....

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B. subtilis

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
B. cereus	*Bacillus cereus*
B. subtilis	*Bacillus subtilis*
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
DMSO	dimethyl sulfoxide
ED_{50}	Median effective concentration
EPS	extracellular polymer substance
et al.	et alibi (and others)
etc.	et cetera (and other things)
h	hour (s)
LD_{50}	Median lethal dose
Log	Decimal logarithm
MBC	Minimum bactericidal concentration
mg	milligram
mg/g larva	milligram per gram larva
mg/ml	milligram per millititer

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MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration
min	minute
ml	milliliter
mm	millimetres
NaCl	sodium chloride
nm	nanometer
^{O}C	Degree Celsius
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
spp.	Species
v/v	volume by volume (ml/ml)
w/v	weight by volume (g/ml)
%	percent
µg	Microgram
µl	microliter
µm	micrometer

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CHAPTER I

INTRODUCTION

Background and Rationale

The genus *Bacillus* such as *Bacillus cereus* and *Bacillus subtilis*, is a large gram-positive rod-shaped occurring in chains. It is an aerobic, spore-forming bacteria and common soil inhabitant recognized as an opportunistic pathogen. It is often present in a variety of food, such as rice, spices, milk and dairy products etc. (Larsen & Jorgensen, 1997). *B. cereus* can grow in food and produce several toxins including emetic toxin and enterotoxins that are considered as the primary virulence factors in *B. cereus* diarrhea (Granum, O'Sullivan, & Lund, 1999). Moreover, *B. cereus* was linked to 14 outbreaks and caused 691 reported cases of foodborne illness in the United States (Lahuada & Mcclain, 1998). For *B. subtilis*, it can produce protease and amylase enzymes that were widely used as a broad spectrum antibiotic. However, some enzyme such as subtilisin (protease) which can cause allergic reactions after repeated exposure in high concentrations (HERA, 2007).

Furthermore, biofilm of *Bacillus* spp. are important contaminants in many food industry settings and are recognized as a serious problem (Frank, 2000). The wild strains of endospore-forming (e.g. *B. subtilis*) form robust biofilm both at liquid/air interfaces and on solid surface. They are also highly resistant to many conditions that are used to kill cells. This biofilms often cause serious illness and failure of the medical device (Ingraham, Maalee, & Meldhardt, 2008).

Although various chemicals have been used for the treatment of infection disease since the 17^{th} century (Jawetz, Melnick, & Adelberg, 1989). Now, Because of increasing resistance to antibiotics and problems through food poisoning of *Bacillus species,* researchers have increasing interest in the new antimicrobial agents. In previous study, *Usnea siamensis* Wainio was reported to be able to inhibit the growth of some pathogens and the major active compound found in this plant is usnic acid. In addition, usnic acid has been shown the inhibition zone against *B. cereus*, *B. subtilis*, *S. aureus*, *S. epidermis* (Chaowuttikul, 2013). Usnic acid, a complex polycyclic phenolic compound is one of the most abundant characteristic secondary metabolite of lichens. It has been used for various purposes worldwide. Usnic acid is a yellow cortical pigment and occurs in two enantiomeric forms; (+) D-usnic acid and (-) L-usnic acid, indicating an R or S projection of the angular-CH_3 group at position 9b. In Thailand, *Usnea exasperate* Motyka and *Usnea siamensis* Wainio are important species that can be found (Chaowuttikul, 2013; Huneck, Akinniyi, Cameron, Connolly,

& Mulhol-land, 1981). Usnic acid from plant extract and pure compound has been shown antimicrobial activity against Gram-positive organism *in vitro* (Lauterwein, Oethinger, Belsner, Peters, & Marre, 1995; Stoll, Brack, & Renz, 1950). It may also have some antifungal and antiparasitic activity. However, usnic acid has a few *in vivo* studies on efficacy and safety yet. For *in vivo* study, vertebrate animal such as mice, rat, rabbit, guinea pig and monkey etc. are often involved with several limitation. One of such limitations is cost and ethical issue. Therefore, in order to solve these problems, several research topics have been developed to use invertebrate animal as alternative infection model for candidate compounds screening. Recently, the researchers from the University of Tokyo, Japan have established silkworm (*Bombyx mori*) as an alternative animal infection model for efficacy testing of microorganisms that are pathogenic in humans including antibiotic clinically used in humans to treat microorganisms also have therapeutic effects in silkworm (Hamamoto *et al*., 2004; Kaito, Akimitsu, Watanabe, & Sekimizu, 2002). Therefore, the present study was aimed to evaluate antimicrobial activity of (+)-usnic acid against *B. cereus* and *B. subtilis*. For *in vivo* study, silkworm was used as an animal infection model for studying *B. cereus* and *B. subtilis* pathogenicity and the therapeutic effect.

Objective of the study

The purpose of this study was to evaluate the antimicrobial activities and antibiofilm activities of (+)-usnic acid against *B. cereus* and *B. subtilis in vitro* study and *in vivo* Thai silkworm model.

Hypothesis

(+)-Usnic acid can inhibit growth and biofilm formation of *B. cereus* and *B. subtilis in vitro* and show therapeutic effect in silkworm model for *in vivo* study.

Research design

Experimental research

Scope of the study

This study was performed both *in vitro* and *in vivo* animal model. 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 *B. cereus* and *B. subtilis*. For *in vivo* study, silkworm was used as an animal infection model for studying *B. cereus* and *B. subtilis* pathogenicity and the therapeutic effect of (+)-usnic acid against these microorganisms.

Benefits from the study

Benefit of the study is to obtain the information of (+)-usnic acid on antimicrobial and antibiofilm activities against *B. cereus* which is superior to *B. subtilis* in *in vitro* and *in vivo* study. Therefore, (+)-usnic acid showed the potential for developing as an antimicrobial and antibiofilm formation agent against *B. cereus* superior to *B. subtilis*. Further study is required to evaluate antimicrobial and antibiofilm activities of (+)-usnic acid against *B. cereus* and *B. subtilis* in mammalian model such as rat, mice etc.

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CHAPTER II
LITERATURE REVIEWS

2.1 MIcroorganism

2.1.1 Description of the genera and morphology

The genus *Bacillus* includes large gram-positive rods occurring in chains. They form spore and are aerobes or (under some conditions) anaerobic bacteria widely found in soil and water and form a unique type of resting cell called an endospore. In order to accommodate former members of the genus *Bacillus* covered in this chapter, its title has been changed to Gram-positive aerobic or facultative endospore-forming bacteria. Endospore formation universally found in the group, is thought to be a strategy for survival in the soil environment, where in these bacteria predominate. The unusual resistance of their endospores to chemical and physical agents, the developmental cycle of endospore formation, the production of antibiotics, the toxicity of their spores and protein crystals for many insects and pathogenicity. The *Bacillus* can contaminate in foods and produce an enterotoxin that cause food poisoning. This organism rarely produces disease in humans for example meningitis, endocarditis, endophythalmitis and acute gastroenteritis (Grande *et al*., 2006). *B. anthracis* and *B. cereus* are the predominant pathogens of medical importance. *B. subtilis*, *B. megaterium*, *B. coagulans*, *Brevibacillus laterosporus*, *B. sphaericus, B. circulans, Brevibacillus brevis*, *B. licheniformis*, *P. macerans*, *B. pumilus* and *B. thuringiensis* have been occasionally isolated from human infections (Todar, 2006).

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2.1.2 Endospores

Endospores were first described by Cohn in *Bacillus subtilis* and later by Koch in the pathogen, *Bacillus anthracis*. Cohn demonstrated the heat resistance of endospores in *B. subtilis*, and Koch described the developmental cycle of spore formation in *B. anthracis*. Endospores are so named because they are formed intracellularly, although they are eventually released from this mother cell or sporangium as free spores. Endospores have proven to be the most durable type of cell found in nature, and in their cryptobiotic state of dormancy they can remain viable for extremely long periods of time, perhaps millions of years. Endospores of

living bacilli appear edged in black and are very bright and refractile. Endospores strongly resist application of simple stains or dyes and hence appear as nonstaining entities in Gram-stain preparations. However, once stained, endospores are quite resistant to decolorization. This is the basis of several spore stains such as the Schaeffer-Fulton staining method which also differentiates the spores from sporangia and vegetative cells (Ngamwongsatit *et al*., 2008; Todar, 2006).

A fixed smear is flooded with a solution of malachite green and placed over boiling water for 5 minutes. After rinsing, the smear is counterstained with safranine. Mature spores stain green, whether free or still in the vegetative sporangium; vegetative cells and sporangia stain red. Mature spores have no detectable metabolism, a state that is described as cryptobiotic. They are highly resistant to environmental stresses such as high temperature (some endospores can be boiled for several hours and retain their viability), irradiation, strong acids, disinfectants, etc. Although cryptobiotic, they retain viability indefinitely such that under appropriate environmental conditions, they germinate into vegetative cells. Endospores are formed by vegetative cells in response to environmental signals that indicate a limiting factor for vegetative growth, such as exhaustion of an essential nutrient. They germinate and become vegetative cells when the environmental stress is relieved. Hence, endospore-formation is a mechanism of survival rather than a mechanism of reproduction (Todar, 2006).

2.1.3 Ecology

Due to the resistance of their endospores to environmental stress, as well as their long-term survival under adverse conditions, most aerobic spore formers are ubiquitous and can be isolated from a wide variety of sources. Hence, the occurrence of spore forming bacteria in a certain environment is not necessarily an indication of habitat. However, it is generally accepted that the primary habitat of the aerobic endospore-forming bacilli is the soil. The Great Russian microbiologist, Winogradsky, considered them as "normal flora" of the soil. In the soil environment the bacteria become metabolically-active when suitable substrates for their growth are available, and presumably they form spores when their nutrients become exhausted. This is a strategy used by other microbes in the soil habitat, including the

filamentous fungi and the actinomycetes, which also predominate in the aerobic soil habitat. It is probably not a coincidence, rather an example of convergent evolution that these three dissimilar groups of microbes live in the soil, form resting structures (spores), and produce antibiotics in association with their sporulation processes.

Since many endospore forming species can effectively degrade a series of biopolymers (proteins, starch, pectin, etc.), they are assumed to play a significant role in the biological cycles of carbon and nitrogen. From soil, by direct contact or air-borne dust, endospores can contaminate just about anything that is not maintained in a sterile environment. They may play a biodegradative role in whatever they contaminate, and thereby they may be agents of unwanted decomposition and decay. Several *Bacillus* species are especially important as food spoilage organisms (David, Kirkby, & Noble, 1994).

2.1.4 Pathogenicity

A pathogen is a microorganism that is able to cause disease in a plant, animal or insect. Pathogenicity is the ability to produce disease in a host organism. Microbes express their pathogenicity by means of their virulence, a term which refers to the degree of pathogenicity of the microbe. Hence, the determinants of virulence of a pathogen are any of its genetic or biochemical or structural features that enable it to produce disease in a host. In *Bacillus species*, *B. anthracis* and *B. cereus* are the predominant pathogens of medical importance. *B.subtilis, B. megaterium, B. coagulans, Brevibacillus laterosporus, B. sphaericus, B. circulans*, *Brevibacillus brevis, B. licheniformis, P. macerans, B. pumilus* and *B. thuringiensis* have been occasionally isolated from human infections (Todar, 2006).

2.1.5 *Bacillus cereus*

B. cereus causes two types of food-borne intoxications. One type is characterized by nausea and vomiting and abdominal cramps and has an incubation period of 1 to 6 hours. It resembles *Staphylococcus aureus* food poisoning in its symptoms and incubation period. This is the "short-incubation" or emetic form of the disease. The second type is manifested primarily by abdominal cramps and diarrhea with an incubation period of 8 to 16 hours. Diarrhea may be a small volume or profuse and watery. This type is referred to as the "long-incubation" or diarrheal form of the disease and it resembles more food poisoning caused by *Clostridium perfringens*. In either type, the illness usually lasts less than 24 hours after onset. The short-incubation form of disease is caused by a preformed heat-stable enterotoxin.

The mechanism and site of action of this toxin are unknown. The long-incubation form of illness is mediated by a heat-labile enterotoxin, which apparently activates intestinal adenylatecyclase and causes intestinal fluid secretion (Todar, 2006).

Bacillus cereus produces one emetic toxin and three different enterotoxins: Hemolysin BL (HBL), Non hemolytic enterotoxin (Nhe) and Enterotoxin K (EntK). Two of the three enterotoxins are involved in food poisoning. They both consist of three different protein subunits that act together. One of these enterotoxins (HBL) is also a hemolysin; the second enterotoxin (Nhe) is not a hemolysin. The third enterotoxin is enterotoxin K (EntK) is a single component protein that has not been shown to be involved in food poisoning. EntK is similar to the β-toxin of *Clostridium perfringens* and was associated with a French outbreak of necrotic enteritis in which three people died. All three enterotoxins are cytotoxic and cell membrane active toxins that will make holes or channels in membranes. The emetic toxin is a ring-shaped structure of three repeats of four amino acids with a molecular weight of 1.2 kDa. It is a K^+ ionophoric channel, highly resistant to pH between 2 and 11, to heat, and to proteolytic cleavage. The nonhemolytic enterotoxin (Nhe) is one of the three-component enterotoxins responsible for diarrhea in *Bacillus cereus* food poisoning. Nhe is composed of NheA, NheB and NheC. The three genes encoding the Nhe components constitute an operon. The *nhe* genes have been cloned separately, and expressed in either *Bacillus subtilis* or *Escherichia coli*. Separate expression showed that all three components are required for biological activity (Ngamwongsatit *et al*., 2008).

Drug susceptibility

B. cereus is susceptible to imipenem and vancomycin, and most strains are sensitive to chloramphenicol, aminoglycosides, ciprofloxacin, erythromycin, and gentamicin (David et al., 1994; Murray, Baron, Jorgensen, Landry, & Pfaller, 2007). Some strains were moderately sensitive to clindamycin and tetracycline (Murray *et al*., 2007). Clindamycin with gentamicin, given early, is the best treatment for ophthalmic infections from *B. cereus* (Logan & Rodrigez-Diaz, 2006).

Drug resistance

B. cereus produce large amounts of β lactamase and are resistant to penicillin, ampicillin, cephalosporins, trimethoprim (Logan & Rodrigez-Diaz, 2006).

2.1.6 *Bacillus subtilis*

Bacillus subtilis grow as a unicellular rod and seldom as chains. They grow in nonacid food under aerobic conditions. Much of the information we have on the biology, biochemistry and genetics of the Gram-positive cell, indeed of bacteria in general, has been derived from the study of *B. subtilis.* Furthermore, *B. subtilis* is only known to cause disease in severely immunocompromised patients, and can conversely be used as a probiotic in healthy individuals (Oggioni *et al.*, 1998) It rarely causes food poisoning. Some *B. subtilis* strains produce the proteolytic enzyme subtilis in. *B. subtilis* spores can survive the extreme heat to which it is exposed during cooking. Some *B. subtilis* strains are responsible for causing ropiness, a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides in spoiled bread dough. Antibiotics which appear especially useful in the treatment of Bacillus infections are clindamycin and vancomycin, to which the vast majority of strains are susceptible *in vitro* (David *et al*., 1994).

2.2 Biofilm

One of the key factors in the pathogenesis and bring about of survive of *B. cereus* and *B. subtilis*. Biofilm of *Bacillus sp.* is important contaminants in many food industry settings and recognized as a serious problem (Frank, 2000). For example, spore-forming bacteria survive and accumulate on pipelines and joint in the processing environment. The wild strains of endospore-forming (e.g. *B. subtilis*) form robust biofilm both at liquid/air interfaces and on solid surfaces (Elhariry, 2008). *B. cereus* can also cause problems in food industry because of its capacity to form biofilms on several surfaces (Auger, Krin, Aymerich, & Gohar, 2006). A biofilm is a multicellular complex, formed of microorganisms that are attached to a surface and embedded in a matrix, consisting of exopolymeric substances (EPS). Cells within a biofilm are surrounded by the EPS matrix and cells in the outerlayers of the biofilm, protecting them from harsh influences from the environment, thereby making them more resistant to clenching agents and other antimicrobial substances (Peng, Tsai , & Chou, 2002). Biofilm formation can cause economic loss by equipment failure and shorten the time between cleaning. Contamination of products *via* biofilm cells can cause subsequent limitation of shelf life and raise safety concerns (Flint, Bremer, & Brooks, 1997; Stoodley, Sauer, Davies, & Costerton, 2002).

Biofilm associated resistance mechanisms

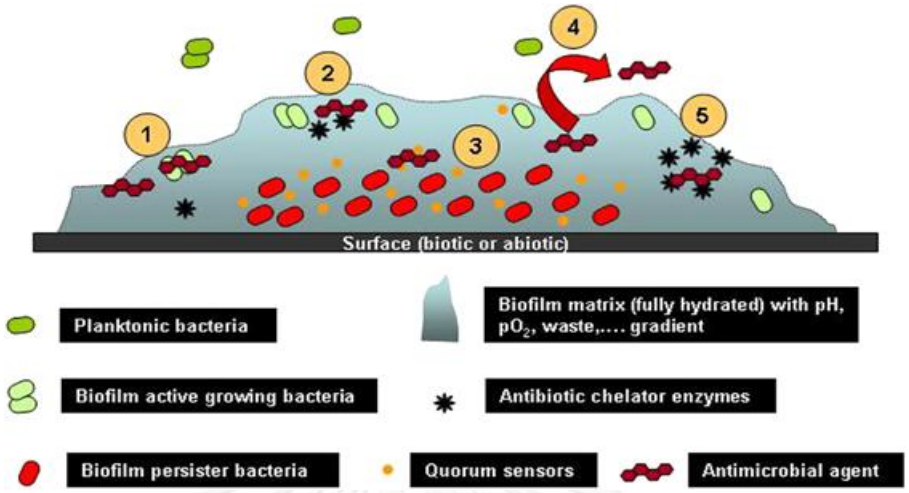

Figure 1 Some proposed-biofilm associated resistance mechanisms (Pozo & Patel, 2013)

1) Antimicrobial agents may fail to penetrate beyond the surface layers of the biofilm. Outer layers of biofilm cells absorb damage (Figure 1). Antimicrobial agents action may be impaired in areas of waste accumulation or altered environment (pH, pCO_2, pO_2, etc.).
2) Antimicrobial agents may be trapped and destroyed by enzymes in the biofilm matrix.
3) 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.
4) 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.
5) Antimicrobial agents are increasing remove from biofilm by multi-drug resistance (MDR) efflux pumps.
6) 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 (Pozo & Patel, 2013).

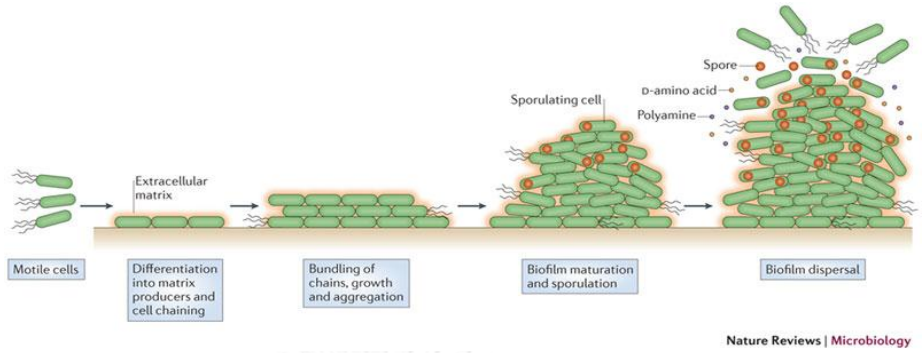

Figure 2 Stages in biofilm development of *bacillus* sp. (Vlamakis, Chai, Beauregard, Losick, & Kolter, 2013)

2.2.1 Stages involved in biofilm development of *bacillus* sp.

Stage 1: Initial attachment of cells to the surface (Figure 2). A bacterial biofilm begins to form when individual cells initially attach to a surface. The ability of a cell to perform this "initial attachment event" is controlled by both environmental factors, including nutrient levels, temperature, and pH, and genetic factors, including the presence of genes encoding motility functions, environmental sensors, adhesins, etc. Motile cells with flagella differentiate into non-motile.

Stage 2: Production of exopolysaccharide (EPS) matrix. After initial attachment, the cells begin to grow and spread as a monolayer on the surface to form microcolonies. Paramount among these changes is the production of the exopolysaccharide (EPS) matrix**.** They form long chains of non-motile cells that adhere to each other and to the surface by secreting an extracellular matrix. This substance is essential to the integrity of the biofilm, as it holds the community together.

Stage 3: Early development of biofilm architecture. During microcolony formation, the biofilm cells can develop and aggregate in surface.

Stage 4: Maturation of biofilm architecture. The matrix cells in biofilms are produce sporulation. These cells can develop and changes which give rise to the complex architecture of mature biofilm.

Stage 5: Dispersion of single cells from the biofilm. In aged biofilms, some cells secrete small molecules such as amino acids and polyamines, which break

down the extracellular matrix and allow the cells to disperse in the environment. The biofilm may spread into uninfected areas as environmental conditions allow and, occasionally, cells will detach from the biofilm and re-enter a planktonic mode. These planktonic cells can then repeat the cycle, infecting new surface (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995).

2.3 Phytochemical and pharmacological properties of Usnic acid

(+)-Usnic acid is a complex polycyclic phenolic compound. It is one of the most abundant characteristic secondary metabolite of lichens. It has been used for various purposes worldwide. Usnic acid is a yellow cortical pigment and occurs in two enantiomeric forms; (+) D-usnic acid and (-) L-usnic acid, indicating an R or S projection of the angular-CH_3 group at position 9b. (Figure 3). 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.

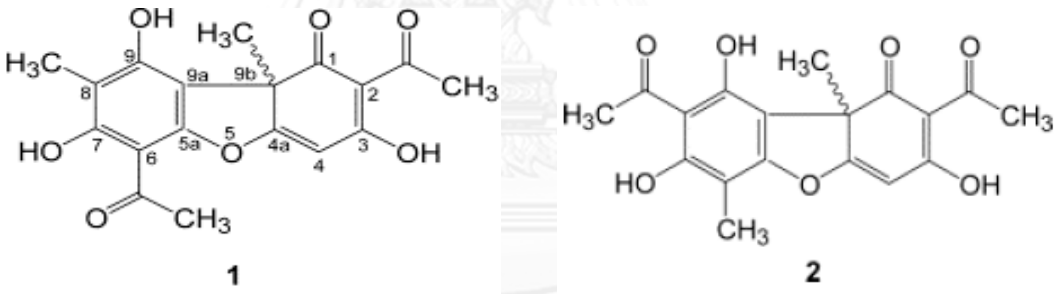

Figure 3 Structures of (+)-(9b-R)- and (-)-(9b-S)-usnic acids(1) and (+)-(9b-R) and (-)(9b-S)-isousnic acids (2) (Huneck et al., 1981)

Figure 4 Native of *Usnea* sp. and dye of *Usneasia mensis* Wainio (Chaowuttikul, 2013)

In Thailand *Usnea siamensis* Wainio is important species that can be found. *Usnea siamensis* Wainio is Thai herbal drug that used to treat diseases in folk medicine (Figure 4). The inhibition zones of (+)-usnic acid against *Bacillus cereus, Bacillus subtilis, Staphyolcoccus aureus, Staphylococcus epidermis, Micrococcus luteus* and *Candida albicans* were reported by Chayanon Chaowuttikul, College of Public Health Science Chulalongkorn University (Chaowuttikul, 2013; Huneck *et al.,* 1981). Usnic acid from plant extract and pure compound have been used as a topical antibacterial agent and it also showed antimicrobial activity against Gram-positive organism *in vitro* (Lauterwein et al., 1995; Stoll *et al*., 1950). It may also have some antifungal and antiparasitic activity. Usnic acid is poorly water soluble but more soluble in ethanol, acetonitrile, acetone, DMSO and dichloromethane etc. (Ingo´ lfsdo´ ttir, 2002). Irrespective of a long history of usnic acid containing products, only a few animal studies were conducted to evaluate the clinical safety of usnic acid. Acute toxicity studies: Mouse: LD_{50} oral = 838 mg/kg LD_{50} intravenous = 25 mg/kg LD_{50} subcutaneous = 75 mg/kg, Rabbit: LD_{50}oral >500 mg/kg. In an acute study where larvae of a herbivore insect (*Spodoptera littoralis*) received injections of both enantiomers of usnic acid in the hemolymph, (-) L-usnic acid was found to be 10 times more toxic than its (+) D-usnic acid (LD_{50} 8.6 versus 90.8 μmol) (Emmerich, Giez, Lange, & Proksch, 1993).

2.4 Silkworm

The silkworm model is new alternative for identifying and screening useful for evaluating toxicity and pharmacokinetics of potential antibiotics. Silkworm are invertebrates animal and the scientific name is "*Bombyx mori*". Silkworm is domesticated form the wild silkmoth *Bombyx mandarina* which has a range from northern India to northern China, Korea, Japan, Thailand and the eastern regions of Russia. It is an economically important insect because of being a primary producer of silk. When silkworm has become putates, they will secrete a single protein strand as the silk from two glands on the head making the coccon is cropped for silk. Preferred food of silkworm is white mulberry leaves, but silkworm may also eat leaves of any other mulberry tree. Silkworms are classified in the Phylum of Arthropoda, Class of insect, Order of Lepidotera, Family of Bombycidae and *Bombyx mori* species.

2.4.1 The life cycle stages of silkworm

Silkworm is holometabolous insect (complete metamorphosis). Silkworm was born since time immemorial. The duration of the development stages can be controlled completely throughout the life cycle by regulating environmental conditions. In time about 6-8 weeks, they complete their life cycle of four different metamorphosing phases consist of egg, larva, pupa and adult (moth).

Bombyx mori composed of 4 major stages including egg, larva, pupa and moth. Silkworms make very nutritious live feeders for all your reptiles and all sizes of lizards: The Worm Lady silkworm sizes are 2^{nd} instar: Small, 3^{rd} instar: Med, 4^{th} instar: Large, 5^{th} instar: X Large. The life cycle of silkworm is shown in figure 5

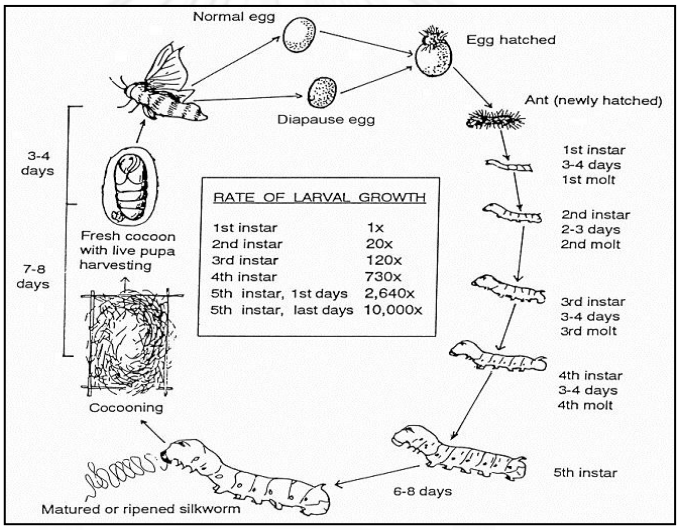

Figure 5 Life cycle of silkworm (Tahima, 1978)

The first stage of silkworm starts with a little egg laid by female moth. One female moth lays as many as 300 - 600 eggs in one night, during gestation. Then, the small eggs hatch and grow as the larva, the larva stage is growing through 5 steps consist of first instar to fifth instar larva. The young larva is about 10 mm in length. At about two days after hatching, the state on the body surface becomes less conspicuous. After one day the body length reaches about 20 mm. and the surface of the skin become glossy. The larva stops eating for 24 h (molting). During this time the larva produces a new cuticle and sheds the old one. This phenomenon of shedding the old skin is called ecdysis. Since molting is repeated 4 times during the

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

2.4.2 Administration of silkworm

Administration in silkworm have two routes, the first is intra hemolymph, which is similar to the intravenous injection in mouse and the second is intra midgut, which is similar to oral administration in mouse.

2.4.3 Anatomy and Physiology

The larva body

Regions of silkworm body are divided in three principle parts, namely, head, thorax and abdomen. Head has two sets of 6 ocelli which are situated just behind and downward on the left and right face. Antenae are situated a little under the ocelli. The antenna is composed of three short segments. The mouth parts are located downward and in front of the face and are composed of a pair of mandibles

and maxillae with labrum and labium. The mandibles are used for mastication and consist of two hard pieces. The larva body of silkworm is shown in figure 6.

The maxilla consists of a single maxillary lobe and the maxillary palpi, which is made up of three segments. The labrum hangs down from the frontal portion to from a flap of the mouth. The labium is situated on the ventral region of the head and has a pair of labial palps which form a sensory organ. A spinneret is situated at the proximal position between the two labial palpi. The thorax comprises three segments with one pair of spiracles and three pairs of thoracic legs. The abdomen is constructed of 11 segments with 8 pairs of spiracles, 4 pairs of abdominal legs, 1 pair of caudal legs and 1 caudal horn (Tahima, 1978).

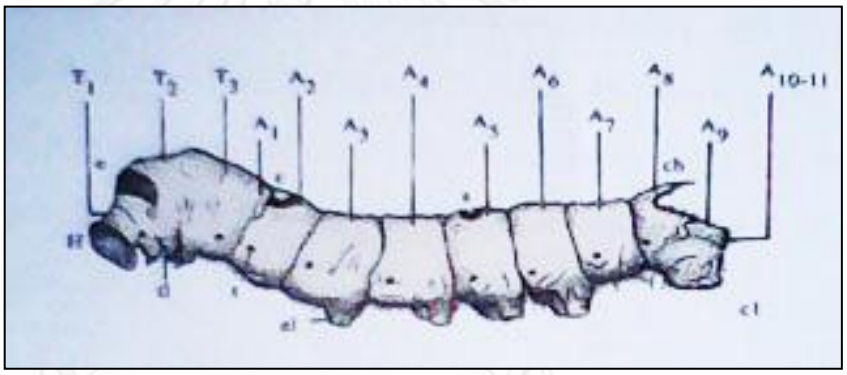

Figure 6 The larva body. T_1-T_3 ; Thoracic segment, A_1-A_{11} ; Abdominal segment, H ; Head, e; Eye spot, C; Crescent, S ; Star spot, tl ; Thoracic legs, ch ; Caudal horn

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Internal anatomy of the larva

Main internal organs is the alimentary canal (fore-gut, mid-gut and hind-gut), trachea, nervous system, dorsal vessel, malpighian tube, silk gland and others. The inner organ of a full-grown larva at the 5th instar is shown in Figure 7

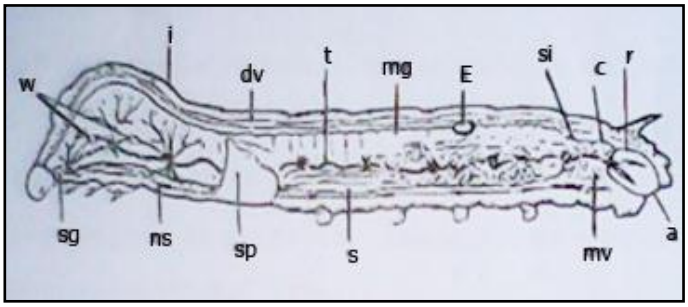

Figure 7 Internal organ of silkworm. a:anus, c:colon, dv:dorsal vessel, g:gonad, i: integument, mg:mid-gut, mv: malpighian vessel, ns:nervous system, r: rectum, si:small intestine, sg: salivary gland, sp: spiracle, t:trachea, w:wing disc (Tahima, 1978).

The alimentary canal

The alimentary canal in silkworms is divided into three main regions include the foregut (stomodaeum), which is ectodermal in origin, the midgut (mesenteron), which is endodermal and the hindgut (protodaeum), which is again ectodermal. In silkworms, these regions are subdivided into various functional parts.The of foregut is composed of pharynx, oesophagus and salivary glands. It is commonly concerned with the storage of food and sometimes helps to fragment the food before it passes to the midgut. The salivary glands can secrete digestive enzymes can hydrolyze protein, starch, sucrose, maltose and trehalose.

The midgut is the largest organ, extending over 8 of the 13 body segments. It is primarily concerned with the production of enzymes and the absorption of the products of digestion. The midgut tissue comprises globlet cells, columnar cells and a basement membrane. The globlet cells and columnar cells are concerned with enzyme secretion (such as protease, lipase, amylase and maltase etc.) and with the absorption of products of digestion. The basement membranes of cells adjoin to the haemocoel with few openining to hemolymph.

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

The blood circulation system

The dorsal vessel consists of an abdominal portion called the heart and a thoracic portion known as the aorta. The heart is the pulsating part of the tube with 9 pairs of opening, called the ostia, for admission of the blood. As for the circulatory mechanism of silkworm larva, the blood flows backwards through the body cavity, enters the ostia at the dorsal Bessel of abdominal 6^{th} to 11^{th} segments, and leaves the dorsal vessel at the Ostia of 2^{nd} to 5^{th} segments. The blood or hemolymph circulates round the body cavity between the various. It consists of a fluid plasma in which are suspended the blood cells or haemocytes. The content of water in the blood is about 90 to 95% and potential of hydrogen ion(PH) of the blood in 5^{th} instar larva is 6.3 to 6.5. The blood of the silkworm plays a major part in distribution of nutrition to the tissue and carries waste product from them. The blood also has an important role in respiration. The substances dissolved in the blood include proteins, amino acids, carbohydrates and inorganic component. The percentage of the blood based on body weight ranged from 21 to 25% in the larva stage. No significant difference among the values at various development stages and between those of males and females was found.

The respiratory system

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

Sex discrimination at the larval stage

The male has one spot of the heroldimaginal gland in the Ishiwataimaginal gland at ventral side of the 11^{th} and 12^{th} segments (Figure 8). The best time for discrimination between the two sexes is the early 5^{th} instar just after the 4^{th} molt. Discrimination is more or less difficult, depending on the strains.

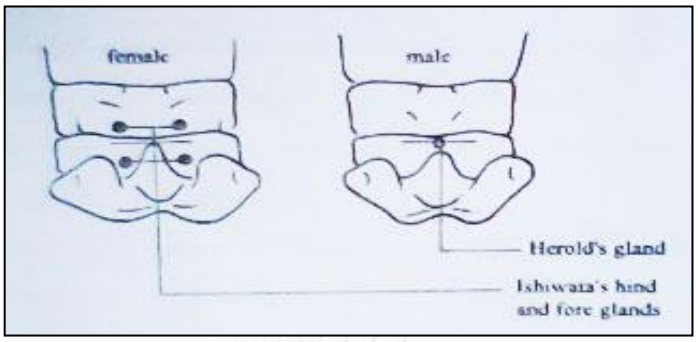

Figure 8 Sex discrimination of silkworm (Tahima, 1978).

The immune system of silkworm

Silkworm has powerful innate immune system against invading pathogens or adulterated thing during their existence. From previous studies it was reported that the innate immune system of vertebrates (Vilmos & Kurucz, 1998a). Innate immunity of silkworms is composed of two major types is humoral and cellular immune response. Humoral immune response involves antimicrobial proteins (AMPs), soluble proteins in the hemolymph. In families of AMPs have main activity for against Gram-positive and Gram-negative bacteria, whereas cellular immune response involve process of phagocytosis which is regulated by hemocytes of silkworm (Tanaka & Yamakawa, 2011).

2.5 Silkworm infection model

Mammalian model such as mice and rat are used for evaluation of the therapeutic effects of antibiotics for decades. However, mammalian experimentation is costly, time consuming, and requires full ethical consideration. If an initial toxicity and efficacy screen could be achieved in financially more attractive and ethically more acceptable alternative *in vivo* model. This could reduce the use of mammals and overall costs associated with the early stages of novel antimicrobial discovery and development. As a result, invertebrate animal models of infection have been developed and introduced, including insect and nematode models. Also, the innate immunity, the major pathways of host defense which covered of the response to microbial pathogen, has the similarities between vertebrates and invertebrates (Vilmos & Kurucz, 1998b).

On the contrary, 5th instar larvae of silkworm can be utilized for infection assay because they have several advantages as an animal model for studying bacterial pathogenicity and the therapeutic effect of handle, sample solution of pathogens and candidate samples can be injected into the hemolymph (mimics mounse intravenous administration) or midgut (mimics mouse oral administration) of the larvae. Silkworms are easy to raise the number of larvae and they have less of ethical issues compared with the use of mammals. Furthermore, they also ease the rearing and maintenance of silkworms in laboratory. Recently, silkworms were utilized as an alternative animal model for efficacy testing of candidate compounds. Therefore, silkworm models are proving to be valuable tools for studying microbial pathogenicity and preclinical assessment of new antimicrobial agents (Kaito, Yoshikai, & Sekimizu, 2012). In was previously reported that silkworm larvae were infected with bacteria such as *S. aureus*, *P. aeruginosa*, *E. coli* (O-157) and *V. cholera* etc. have lethal effects in silkworms within 2 days when injected into their hemolymph. When 3×10^{7}cells of *S. aureus* (RN4220, Smith, methicillin-sensitive *S. aureus*; MSSA, methicillin-resistant *S. aureus*; MRSA) were injected, over 90% of the larvae were dead within 2 days (Figure 9). The larvae injected with these microorganisms had decrease in movement, then they stopped eating and finally their skin color turned dark. Furthermore, the proliferation of *S. aureus* was observed in blood and tissues of silkworm larvae, and the bacteria were counted. In both blood and tissues, *S. aureus* increased to more than 1×10^{8} cells/larva within 2 days (Figure 10). The number of *S. aureus* just after injection was approximately 5×10^{5} cells/larva, although the injected number of *S. aureus* was 3×10^{7}cells/larva.

The result indicates that the killing effect requires bacterial growth in the body of silkworm larvae. *S. aureus* was increased exponentially both in the hemolymp and tissue of silkworm after injection. However, immunostaining analysis revealed that *S. aureus* proliferated at the epithelial cell surface of the midgut. These results indicate that silkworm larvae were killed by infection with bacteria pathogens. Infection of silkworm larvae by these pathogens were cured by antibiotics (for example ampicillin and vancomycin etc.), clinically used in humans (Kaito *et al.*, 2002).

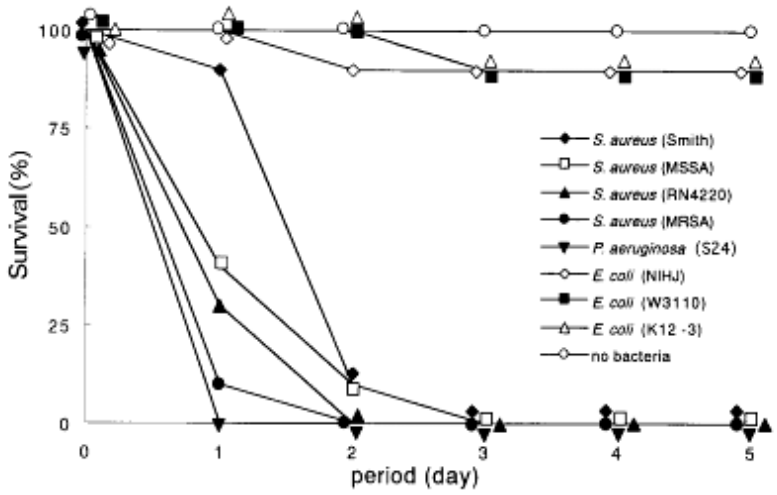

Figure 9 Survival of the silkworm larvae injected with *S. aureus*, *P. aeruginosa*,or *E. coli*. Fifth instar larvae of silkworms (n=10) were injected with 3×10^{7} cells of *S. aureus* (RN4220, Smith, MSSA, MRSA), *P. aeruginosa* (S24) or *E. coli* (K12-3, W3110, NIHJ). The numbers of surviving larvae are shown (Kaito *et al*., 2002)

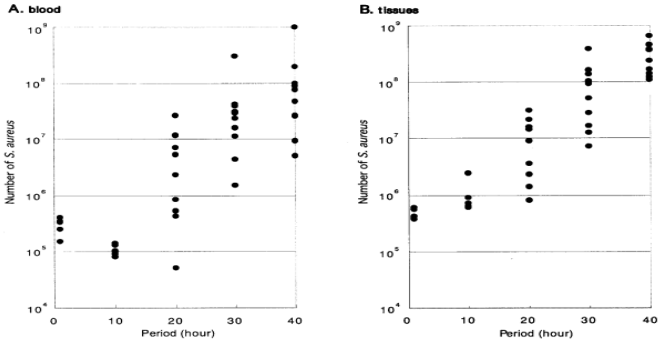

Figure 10 Proliferation of *S. aureus* in blood and tissues of silkworm larvae. Silkworm larvae were injected with 3×10^{7} cells of *S. aureus* (MSSA). Animals were sacrificed and blood (A) and tissues (B) were collected. After appropriate dilution, samples were spread on mannit agar plates, incubated at 37℃ overnight, and the number of colonies was counted (Kaito *et al*., 2002)

The silkworm infection model can also be applied to evaluate the pharmacokinetics of antibiotics, based on the ED_{50} per MIC ratio. Smaller values indicate that the antibiotics functioned effectively in animal bodies, similar to their functioning in the culture medium in which the MICs were determined. The ED_{50} per MIC values of clinically used antibiotics are <10 (Hamamoto *et al*., 2004). Silkworm was used as an animal model for evaluated of the therapeutic effects of antibiotics. This research showed the ED_{50} per MIC ratio was less than 10 for all the samples tested. The ED_{50} per MIC ratios obtained from silkworms were consistent with those obtained from mice in previous studies (Table 1) (Hamamoto *et al*., 2004).

Table 1 ED_{50} of antibiotics in a silkworm infection model with *S. aureus* and *S. maltophilia* (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.

Furthermore, Bacterial infections research topics at the University of Tokyo, Japan, have been studied therapeutic effects and pharmacokinetics of antibacterial chromogenic agents in a silkworm model, Show that rifampicin had a therapeutic effect and ED_{50} value was 0.08 μg/g.larva (Table 2). This is consistent with the value in a murine model (0.062 μg/g) (Rothstein, Irving, Walden, & Yearsley, 2006).

Table 2 Antibacterial activity, toxicity, and therapeutic effect of dyes in the silkworm model of *S. aureus* infection (Fujiyuki, Imamura, Hamamoto, & Sekimizu, 2010)

Dye	MIC (μg/mL)	tEDmini (μg/g·larva)	LD_{50} (μg/g·larva)	ED_{50} (μg/g·larva)	ED_{50}/MIC
Rifampicin	0.02	0.05	ND	0.08	4
Amidol	19	48	54	> 9	> 0.5
Pyronin G	2	5	11	> 13	> 7
Safranin	10	25	34	> 23	> 2
Fuchsin basic	6	15	100-500	> 230	> 38
Methyl Green	13	33	530	> 160	> 12
CBB R250	4	10	> 660	> 83	> 17
CBB G250	7	18	> 800	> 72	> 10
Cresyl Blue	4	10	530	> 150	> 39
Nigrosin	5	13	490	> 170	> 33
Crystal Violet	0.2	0.5	15	> 9	> 45
Malachite Green	0.3	0.8	15	> 10	> 33
Toluidine Blue O	14	35	1,000	> 700	> 50
Vancomycin	1	2	ND	0.3*	0.3*

*; the values are cited from Hamamoto *et al.*, 2004. tEDmini; theoretical minimal effective dose required for therapeutic effect in the infected silkworm. ND; not determined.

The absorption system of drug in the silkworm larva midgut is much simpler compared with 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. The general features of the non-specific transport route are similar between silkworm and mammals.

For example, the transport of chloramphenicol and vancomycin through midgut membranes in living silkworm. The ineffectiveness of vancomycin by the oral pathway in the silkworm model is due to the inability of this drug to penetrate midgut membranes (Figure 11). The results demonstrated that the concentration of vancomycin in the hemolymph did not increase when 500 μg of the drug was injected into the midgut whereas chloramphenicol rapidly increased and was maintained at a high level when 100 μg of the drug was injected. In addition, The ED_{50} obtained for these antibiotics by i.m. administration were similar to those obtained by injection into the hemolymph (Table 3). On the other hand, vancomycin and kanamycin were ineffective when they were injected into the midgut. Vancomycin (MW 1485 Da) does not permeate the midgut and have no therapeutic effect in the silkworm

larva infection model. Vancomycin is high molecular mass, consistent with their lack of effectiveness when administered orally (Hamamoto *et al*., 2004).

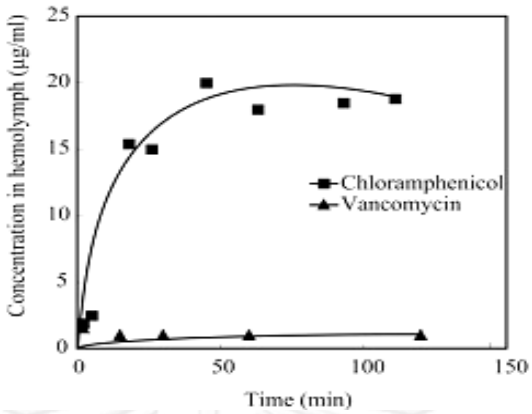

Figure 11 Transport of chloramphenicol and vancomycin from the midgut to the hemolymph in living silkworms (Hamamoto *et al*., 2004)

Table 3 Oral administration of vancomycin or kanamycin does not have therapeutic effects in a silkworm mode (Hamamoto *et al*., 2004)

Antibiotic	ED_{50} (μg of antibiotic/g of larva) of drug administered by the following route:		
	i.h.	i.m.	p.o.
Chloramphenicol	9	11	40
Tetracycline	0.4	1	8
Vancomycin	0.3	>700	>400
Kanamycin	3	>700	>500

In the next study, silkworm was used as an animal model for evaluated of the therapeutic effects and pharmacokinetics of antibacterial chromogenic agents, the concentrations of CBB R250, CBB G250, cresyl blue and nigrosin in the hemolymph were higher than the MIC values even 2 days after injection, but they still did not show the therapeutic effects (Table 4). Therefore, the researcher examined whether the antibacterial activity of CBB R250, CBB G250, cresyl blue and nigrosin were inhibited in the presence of the silkworm plasma. Adding the silkworm plasma fraction increased the MICs of these four dyes. Adding 25mg/ml BSA also increased the MICs of the dyes, whereas adding the silkworm plasma or BSA did not affect the MIC of vancomycin (Table 5). These dyes lost their antibacterial activity in the presence of silkworm plasma or BSA, which suggested that these dyes less

distributes or bind to silkworm plasma proteins and the concentrations of the free compounds are too small in the hemolymph to produce therapeutic effects in the silkworm infection model (Fujiyuki *et al*., 2010).

Table 4 Dye concentration in the silkworm hemolymph after injection (Fujiyuki *et al*., 2010)

Dye	MIC (μg/mL)	Concentration in hemolymph after 2 days (μg/mL)
Fuchsin basic	6	0 ± 0 (n = 5)
Methyl green	13	1 ± 1 (n = 5)
CBB R250	4	14 ± 5 (n = 7)
CBB G250	7	7 ± 3 (n = 5)
Cresyl blue	4	12 ± 4 (n = 4)
Nigrosin	5	9 ± 1 (n = 4)

Table 5 Effect of the presence of the silkworm plasma (25%) and BSA(25 mg/ml) on the antibacterial activity of dyes (Fujiyuki *et al*., 2010)

Dye	None	Plasma	BSA
CBB R250	7 ± 2 (n = 3)	> 263 (n = 3)	> 1,050 (n = 3)
CBB G250	7 ± 0 (n = 3)	600 ± 184 (n = 3)	> 900 (n = 3)
Cresyl Blue	5 ± 1 (n = 4)	> 16 (n = 4)	> 131 (n = 4)
Nigrosin	3 ± 0 (n = 3)	> 163 (n = 4)	> 325 (n = 3)
Vancomycin	1 ± 0 (n = 2)	1 ± 0 (n = 2)	1 ± 0 (n = 2)

The MICs of the dyes are shown (μg/mL). A CBB R250 reagent was obtained from the supplier and used without purification in this experiment. Values are presented as mean ± S.E.M.

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The metabolizations of the chemicals in mammals and silkworm have a reaction with cytochrome P450, conjugation with hydroxylated compounds and excretion. To determine the metabolic pathways of chemicals in silkworms were used 4-methyl umbelliferone as model drug. The fluorescens (4-methyl umbelliferone) is converted to a non-fluorescent from metabolic modification with P450 in mammalian livers. The half-life of 4-methyl umbelliferone in silkworm larvae was 7.0 ±0.1 min and 9 min in mouse blood, respectively. Therefore, the stability of 4-methyl umbelliferone in silkworm hemolymph is similar to that in mouse. The

metabolite of 4-methyl umbelliferone in silkworm was determined by HPLC analysis. Glucose-conjugation form of umbelliferone was determined by mass spectrometry analysis. The result showed that, A peak absorbance at a retention time of 13 min, which was distinguishable from the 4-methyl umbelliferone standard retention time (17 min), was detected in the sample of silkworm hemolymph after injected with umbelliferone 20 min (Figure 12). The molecular mass of this compound was determined to be 338. It is different from the molecular mass of 4-methyl umbelliferone (176). This suggested that 4-methyl umbelliferone was conjugated with glucose in silkworm, indicated that 4-methyl umbelliferone was metabolized into a glucose-conjugated form in silkworm (Hamamoto, Tonoike, Narushima, Horie, & Sekimizu, 2009).

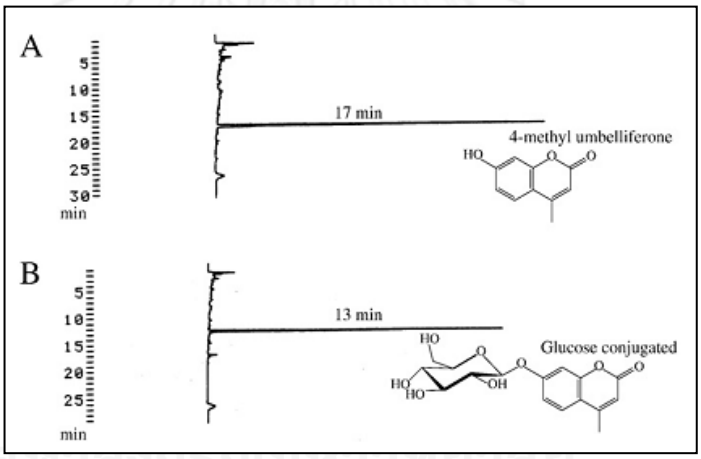
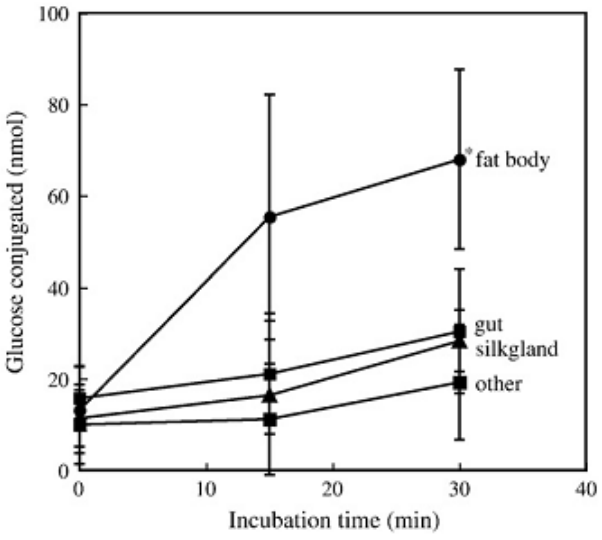


Figure 12 HPLC analysis of a 4-methyl umbelliferone metabolite in silkworms. Glucose-conjugation form of umbelliferone (A). hemolymph collected immediately after injection of 4-methyl umbelliferone (B) (Hamamoto *et al*., 2009)

Fat body of the silkworm plays a role metabolizes substances. Fat body scattered throughout the body of the silkworm, like the liver in mammals. The concentration of the glucose-conjugated from increased in the fat body culture medium in a time-dependent manner (Figure 13). The level of the glucose-conjugated from of umbelliferone was much lower in culture of isolated silk gland,

gut and mixture of other tissues than that in culture of fat bodies. The chemicals were metabolized by a reaction with cytochrome P450, followed by glucose conjugation. Follow the excretion, In larva, The malpighin tube of silkworm is well-known organs for excretion. The terminal portions of the tubules are intimately associated with the wall of rectum. The terminal portion of malpighin tubeles in the body cavity directly absorbs the other substances from the blood and then waste products are excreted as urine (Candy and Kilby, 1975). Then, the excretion of 4-methyl umbelliferone from silkworm larva was excreted into the hindgut *via* the malpighian tubules (Figure 14). Umbelliferone was injection into the silkworm hemolymph, and feces were collected at 3, 6, 9 and 12 h. after injection. Unaltered and glucose-conjugated umbelliferone was extracted drug were excreted into the faces in the glucose-conjugated from within 3 h.

As a result, the chemiclas are metabolized through a pathway common to both mammals and silkworm, reaction with cytochrome P450, conjugation with hydroxylated and excretion (Hamamoto *et al*., 2009). Therefore, silkworm infection model can be evaluated the pharmacokinetics of antibiotics and utilized as an alternative animal model for efficacy testing of candidate compounds (Kaito & Sekimizu, 2007).



Figture 13 Glucose-conjugation reaction of umbelliferone in isolated organs (Hamamoto *et al*., 2009)

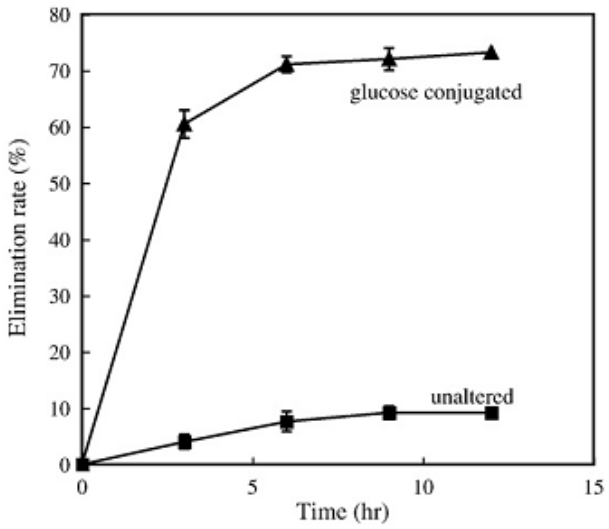


Figure 14 Elimination rate of umbelliferone into feces in the glucose-conjugated or unaltered form (Hamamoto *et al*., 2009)

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CHAPTER III

MATERIALS AND METHODS

Materials

1. Animals

Fertilized silkworm eggs of silkworm, (Bombyx mori, Hu·Yo × Tukuba·Ne) were purchased from Ehime Sanshu Co., Ltd. (Surin, Thailand). Hatched larvae were fed artificial food, Silkmate 2S (Nosan Corporation, Yokohama, Japan) at 27°C. All of the animals used in this study are fifth instar larvae weighting 0.4 -1.0 g was supplied from The Queen Sirikit Department of Sericulture, Ministry of Agriculture and Cooperatives.

2. Bacterial strains

The bacterial strains used throughout this study were *B. cereus* (ATCC 11778) and *B. subtilis* (ATCC 6633) derived from The college of Public Health Sciences, Chulalongkorn university. *S. aureus* (ATCC 29213) was used for quality control test.

3. Chemicals

3.1) Ampicillin (T.P. Drug Laboratories Co.,Ltd.,Thailand)
3.2) 70% Alcohol
3.3) 1% (v/v) Crystal violet
3.4) Dimethyl sulfoxide (Labscan, Thailand)
3.5) Glacial acetic acid (BDH Laboratory, England)
3.6) Mueller Hinton Agar (BBL, USA)
3.7) Mueller Hinton Broth (BBL, USA)
3.8) Methanol (RCI Labscan, Thailand)
3.9) 0.9% NaCl
3.10) Silkmate
3.11) Sterile distilled water
3.12) (+)-Usnic acid (sigma, USA)

4. Instruments

4.1) Autoclave (ALP Co., Ltd., Japan)
4.2) Corkborker
4.3) Cylinders
4.4) Disposable syringe size 1 milliliter (Nipro, Thailand)

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4.5) Glass tank
4.6) Hot air oven (WTB binder, Germany)
4.7) Hypodermic needle 27G x 1” (Nipro, Thailand)
4.8) Incubater (Memmert, Germany)
4.9) Larminar airflow cabinet (Astec, Thailand)
4.10) Loop Sterile (LabScientific, USA)
4.11) Microcentrifuge tubes (Corning incorporated, Mexico)
4.12) Micropipette size 10.100 and 1,000 microliter (Gilson, France)
4.13) Microplate reader (LabScientific, USA)
4.14) Multichannel pipette (Biohit, Finland)
4.15) Petri dish (Pyrex, USA)
4.16) Pipette tip size 0.1-10 and 100 -1,000 microliter
4.17) Shaker bath
4.18) Spectrometer
4.19) Spreader
4.20) Sterile loop
4.21) UV/Visible spectrophotometer
4.22) 96 well microtiter plate (Costar, USA)

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Methods

1. Culture conditions and bacterial strains

Bacillus cereus (ATCC 11778), *Bacillus subtilis* (ATCC 6633) and *S. aureus* (ATCC 29213) were streaked on Mueller Hinton agar (MHA), cultured at 37^0 C for 18-24 h. Full growth *B. cereus and B. subtilis* culture were used for *in vitro* and *in vivo* experiment for determination of antimicrobial activity and antibiofilm activities for *in vitro* experiment. For *in vivo*, silkworm was animal model for studies bacterial infection.

2. Antimicrobial activities testing of usnic acid *in vitro* study

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

A review of activities of usnic acid in disc diffusion method and determination of zone of inhibition by TLC-bioautography method showed the inhibition zone of (+)-usnic acid against *Bacillus cereus* and *Bacillus subtilis* (Chaowuttikul, 2013). Therefore, the present studies begin evaluating antimicrobial activities by MICs. MICs were determined using a microdilution method; this can be accomplished either by the growth method or direct colony suspension. For the growth method, a loop was used to touch the top of three to five colonies of same morphological type form an agar plate culture. The turbidity of the actively growing cells is adjusted to the 0.5 McFarland standards using sterile saline (Schwalbe, Steele, & Goodwin, 2007). The OD_{625} nm requited to 0.5 McFarland value use between 0.08-0.1 (1×10^8) CFU/mL. Diluted bacterial culture with MHB medium 1 mL: 10 µL of bacterial culture which adjusted to the 0.5 McFarland. Each concentration (100 µl) was serially diluted 2-fold of the (+)-usnic acid or ampicillin was placed in a 96-well microplate (final concentration of (+)-usnic acid and ampicillin were 0.00195 mg/ml - 4 mg/ml), and 100 µl of bacterial culture in MHB medium was added, and cultured at 37°C for 18 to 24 h. The MIC was defined as the lowest concentration that inhibited the bacterial growth. The bacteria strains for quality test was *S. aureus* (ATCC 29213).

2.2 Determination of the minimal bactericidal concentration (MBC)

The minimal bactericidal concentration (MBC) was defined as the lowest concentration of an antibiotic killing the majority (99.9%) of a bacterial inoculum**.** Since MIC was the ability of inhibitory status (Schwalbe *et al*., 2007). Following a broth dilution MIC test, from each concentration that shows no growth, a loop was used to touch the suspension and streaked on MHA medium and incubate the plates at 37°C for 18-24 h. Observe bacteria growing from each of the concentration and the number of colonies it not growth in MHA medium was recorded as the MBC (99.9% killing)

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

B. cereus and B. subtilis suspension were adjusted to a 0.5 McFarland standard (approximately 1.0 x 10^8 CFU/ml) in MHB and diluted 1:100 with MHB (inoculums approximately 1.0 x 10^6 CFU/ml). A stock solution of (+)-usnic acid was diluted in 5%DMSO to concentrations as exact multiples of the MIC value at 1/2MIC (31.25 µg/ml), 1MIC (62.50 µg/ml), 2MIC (125 µg/ml) and 4MIC (250 µg/ml). The concentrations of ampicillin were 1/2MIC (3.91 µg/ml), 1MIC (7.81 µg/ml), 2MIC (15.62 µg/ml) and 4MIC (31.25 µg/ml). Each isolate was inoculated into sterile test tubes (each tube contains final bacterial concentration approximately 1×10^5 CFU/ml). The tube were incubated with shaking on shaker water bath at 37° C. 100 µl aliquot was removed from the tube at 0, 2, 4, 6, 8, 10, 12, 24, and 48 hour and diluted serially. Finally, 100 µl of the serial diluted samples were spread on MHA plates after that incubated at 37° C, 24 hour for the determination of surviving bacteria. The growth control tube was composed of bacterial pathogen and MHB. The positive control tube was composed of ampicillin. These control tube was performed the same experiment as mentioned above. Time kill assay was calculated to give colony forming unit per milliliter (CFU/ml) and the time kill curve was plotted between Δlog10 of the viable colonies against time (Arthur *et al*., 1999; Ogunmwonyi *et al*., 2010). The regrowth of bacteria was defined as maintenance of the growth of increase ≥1 log scale from nadir (Hanberger, 1992).

2.4 Biofilm formation assay

B. cereus and *B. subtilis* were harvested from an overnight culture of cells in nutrient broth at 37°C (approx. 300 rpm.). The overnight culture of bacteria was diluted 1:100 with 1.5 % glucose of MHB (inoculums approximately 1.0× 10^6 CFU/ml). The wells of sterile 96-well polystyrene microtiter-plate were filled with 100 µl of each strain and (+)-usnic acid each concentration at 1/2MIC (31.25 µg/ml), 1MIC (62.50 µg/ml), 2MIC (125 µg/ml) and 4MIC (250 µg/ml) for both *B. cereus* and *B.*

subtilis (The growth control wells contained bacteria only). The concentrations of ampicillin were used at 1/2MIC (3.91 µg/ml), 1MIC (7.81 µg/ml), 2MIC (15.62 µg/ml) and 4MIC (31.25 µg/ml) for *B. cereus.* The concentrations of ampicillin were used at 1/2MIC (7.81 µg/ml), 1MIC (15.62 µg/ml), 2MIC (31.25 µg/ml) and 4MIC (62.50 µg/ml) for *B. subtilis.* The plates were incubated at 37°C for 24 h. The medium were removed after 24 h and the wells were washed three times with 300 µl of water sterile. The remaining attached bacteria were fixed with 250 µl of methanol per well. After 15 min microtiter-plates were emptied and air dried. Biofilm cells were stained with 250 µl of 1% crystal violet for 15 min. After these 15 min, the wells were washed twice with 250 µl sterile deionized water to remove unbound crystal violet. After the microtiter-plates was air dried, the dye bound to the adherent cells were extracted with 33% (v/v) glacial acetic acid per well. The absorbance of each well were measured at 570 nm using a UV/Visible spectrophotometer. The percentage inhibition was determined using follow Equation (Sandasi, Leonard, Van, & Viljoen, 2011).

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

3. Antimicrobial activities testing of (+)-usnic acid (*in vivo* study)

3.1 Pathogenicity of bacteria to silkworm

Silkworm (n=10) were injected with 50 µl of bacterial suspension ranging from 1×10^{1} to 1×10^{9} CFU/ml inject into hemolymph of silkworm. Control group (n=10) was also inject with 0.9% NaCl saline into hemolymph. The mortality rate of silkworm was observed at 48 hour after injection. Pathogenicity of bacterial to silkworm was performed at three times independent experiments per microorganism. The LD_{50} was determined as the doses of *B. cereus* and *B. subtilis* that killed half of the silkworms (Kaito *et al*., 2002).

3.2 Determination of LD_{50} of (+)-Usnic acid and Ampicillin

Silkworm (n=10) were injected with 50 µl of (+)-usnic acid solution (concentration 0.05, 0.5, 5, 50, 500, 1,000, 2,000, 4,000, 5,000, 10,000, 25,000 µg/ml) or ampicillin (concentration 2.5, 5, 10, 20, 40, 80 mg/ml). The control group (n=10)

was injected *via* intra-hemolymph of silkworm with 50 µl of 5%DMSO. The mortality rate of silkworm was observed at 48 hour after injection. The LD_{50} of ampicillin was performed the same experiment as mentioned above. The LD_{50} of (+)-usnic acid and ampicillin was performed at three times independent experiment. The LD_{50} value was determined as the doses of (+)-usnic acid or ampicillin that killed half of the silkworms (Fujiyuki *et al*., 2010; Kaito *et al*., 2002).

3.3 Determination of ED_{50} of the (+)-Usnic acid

Suspension of bacterial pathogen in 0.9% NaCl (50 µl) that kill all silkworms from the previous experiment were injected into hemolymph. The (+)-usnic acid (concentration 0.7, 70, 700, 1,000, 2,000, 3,000, 4,000 µg/ml) or ampicillin (concentration 0.5, 50, 500, 1,000, 2,000, 4,000, 6,000, 8,000 µg/ml) were injected into hemolymph immediately after injection of *B. cereus* or *B. subtilis***.** Similarly, the positive control group and the negative control group are injected with 50 µl of ampicillin and 5% DMSO, respectively in intra-hemolymph of silkworm. The number of surviving silkworms was observed at 48 hour after injection. The ED_{50} of (+)-usnic acid or ampicillin was performed at three times independent experiments. The ED_{50} was determined as the doses of the (+)-usnic acid or ampicillin required producing a silkworm survival rate of 50% (Fujiyuki *et al*., 2010; Kaito *et al*., 2002).

Statistical analysis

Data was shown as mean ± standard error of the mean obtained from three independent experiments. Statistical significance between groups was evaluated using one-way ANOVA. A *P*-value of <0.05 was considered statistically significant.

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CHAPTER IV

RESULTS

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

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

The antimicrobial activity of the (+)-usnic acid against target pathogens was examined quantitatively by broth microdilution method. Result of MIC and MBC values given in Table 6, the results showed that (+)-usnic acid exhibited antimicrobial activities against the bacterial pathogens at the MIC values equal to 62.50 µg/ml for both *B. cereus* and *B. subtilis*, whereas MBC of (+)-usnic acid for *B. cereus* and *B. subtilis* were 125 and 250 µg/ml, respectively. The antimicrobial activities of (+)-usnic acid on *B. cereus* was more effective than that on *B. subtilis*. Ampicillin was used as a positive control for these bacterial pathogens. MIC values of ampicillin against *B. cereus* and *B. subtilis* were 7.81 µg/ml and 15.62 µg/ml respectively, whereas MBC of ampicillin for *B. cereus* and *B. subtilis* were 15.62 and 62.50 µg/ml. respectively. The antimicrobial activities of ampicillin on *B. cereus* were more effective than that on *B. subtilis.* However, the bacteria strains for quality test was *S. aureus* (ATCC 29213) (Table 7). MIC values of ampicillin against *S. aureus* (ATCC 29213) were 0.12 µg/ml.

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Table 6 MIC and MBC values of the (+)-usnic acid against *B. cereus* (ATCC 11778) and *B. subtilis* (ATCC 6633)

Substances	*B. cereus* (ATCC 11778)		*B. subtilis* (ATCC 6633)	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
(+)-Usnic acid	62.5	125	62.5	250
Ampicillin	7.81	15.62	15.62	62.50
Negative control (5% DMS0 or DI water)	NA	NA	NA	NA

NA = No Activity

Table 7 MIC and MBC values of the ampicillin against *S. aureus* (ATCC 29213) (quality test)

Substance	*S. aureus* (ATCC 29213)	
	MIC (µg/ml)	MBC (µg/ml)
Ampicillin	0.12	0.12

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1.2 Determination of time to kill bacteria by 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). Figure 15-16, time-kill curves of (+)-usnic acid against *B. cereus* and *B. subtilis*.

For *B. cereus*, (+)-usnic acid at 62.50 µg/ml (1MIC) inhibited the growth of *B. cereus* with the reduction of bacterial 1 to < 3 log scale at 24 h, which showed bacteriostatic activity and the regrowth was observed as 48 h of incubation. (+)-Usnic acid at 125 µg/ml (2MIC) inhibited the growth of *B. cereus* with the reduction of bacterial 1 to < 3 log scale after 6 h of incubation, which showed bacteriostatic activity. After 8 h of incubation, (+)-usnic acid at 125 µg/ml (2MIC) inhibited the growth of *B. cereus* with the reduction of bacterial ≥ 3 log scale which showed bactericidal activity, the viable colonies of *B. cereus* were not seen after 8 h of incubation (Figure 15). (+)-Usnic acid at 250 µg/ml (4MIC) inhibited the growth of *B. cereus* with the reduction of bacterial 1 to < 3 log scale after 4 h of incubation, which showed bacteriostatic activity. After 6 h of incubation, (+)-usnic acid at 250 µg/ml (4MIC) inhibited the growth of *B. cereus* with the reduction of bacterial ≥ 3 log scale which showed bactericidal activity, the viable colonies of *B. cereus* were not seen after 6 h of incubation.

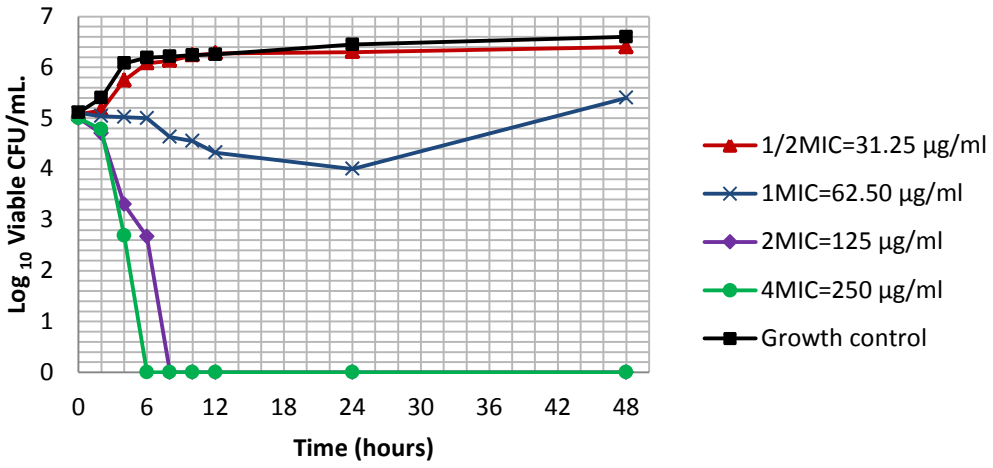

Figure 15 Time-kill curves of (+)-usnic acid against *B. cereus*. For the time kill end point determination; bacteriostatic activity was defined as a reduction of 0-3 log scale and bactericidal activity was defined as a reduction of ≥ 3 log scale.

For *B. subtilis*, (+)-usnic acid at 62.50 µg/ml (1MIC) inhibited the growth of *B. subtilis* with the reduction of bacterial 1 to < 3 log scale after 10 h of incubation, which showed bacteriostatic activity and the regrowth was observed as 48 h of incubation. (+)-Usnic acid at 125 µg/ml (2MIC) inhibited the growth of *B. subtilis* with the reduction of bacterial 1 to < 3 log scale after 10 h of incubation, which showed bacteriostatic activity. After 24 h of incubation, (+)-usnic acid at 125 µg/ml (2MIC) inhibited the growth of *B. subtilis* with the reduction of bacterial ≥ 3 log scale which showed bactericidal activity (Figure 16). (+)-Usnic acid at 250 µg/ml (4MIC) inhibited the growth of *B. subtilis* with the reduction of bacterial 1 to < 3 log scale after 4 h of incubation, which showed bacteriostatic activity. After 8 h of incubation, (+)-usnic acid at 250 µg/ml (4MIC) inhibited the growth of *B. subtilis* with the reduction of bacterial ≥ 3 log scale which showed bactericidal activity, the viable colonies of *B. cereus* were not seen after 8 h of incubation.

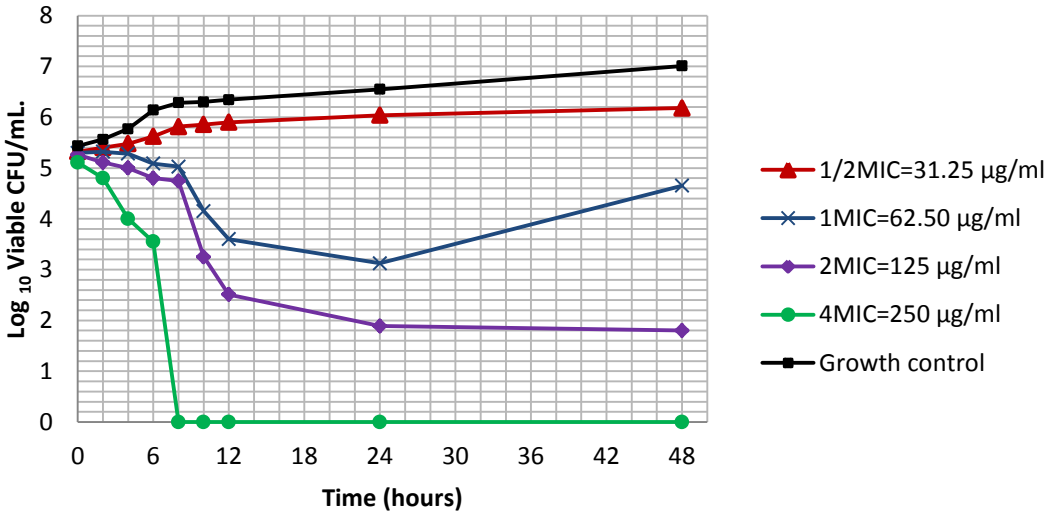

Figure 16 Time-kill curves of (+)-usnic acid against *B. subtilis*. For the time kill end point determination; bacteriostatic activity was defined as a reduction of 0-3 log scale and bactericidal activity was defined as a reduction of ≥ 3 log scale.

Figure 17-18, time-kill curves of ampicillin against *B. cereus* and *B. subtilis.* For *B. cereus*, ampicillin at 7.81 µg/ml (1MIC) inhibited the growth of *B. cereus* with the reduction of bacterial 1 to < 3 log scale at 24 h, which showed bacteriostatic activity and the regrowth was observed as 48 h of incubation. Ampicillin at 15.62 µg/ml (2MIC) inhibited the growth of *B. cereus* with the reduction of bacterial 1 to < 3 log scale after 4 h of incubation, which showed bacteriostatic activity. After 12 h of incubation, ampicillin at 15.62 µg/ml (2MIC) inhibited the growth of *B. cereus* with the reduction of bacterial ≥ 3 log scale which showed bactericidal activity, the viable colonies of *B. cereus* were not seen after 12 h of incubation. Ampicillin at 31.25 µg/ml (4MIC) inhibited the growth of *B. cereus* with the reduction of bacterial 1 to < 3 log scale after 4 h of incubation, which showed bacteriostatic activity. After 10 h of incubation, ampicillin at 31.25 µg/ml (4MIC) inhibited the growth of *B. cereus* with the reduction of bacterial ≥ 3 log scale which showed bactericidal activity, the viable colonies of *B. cereus* were not seen after 10 h of incubation.

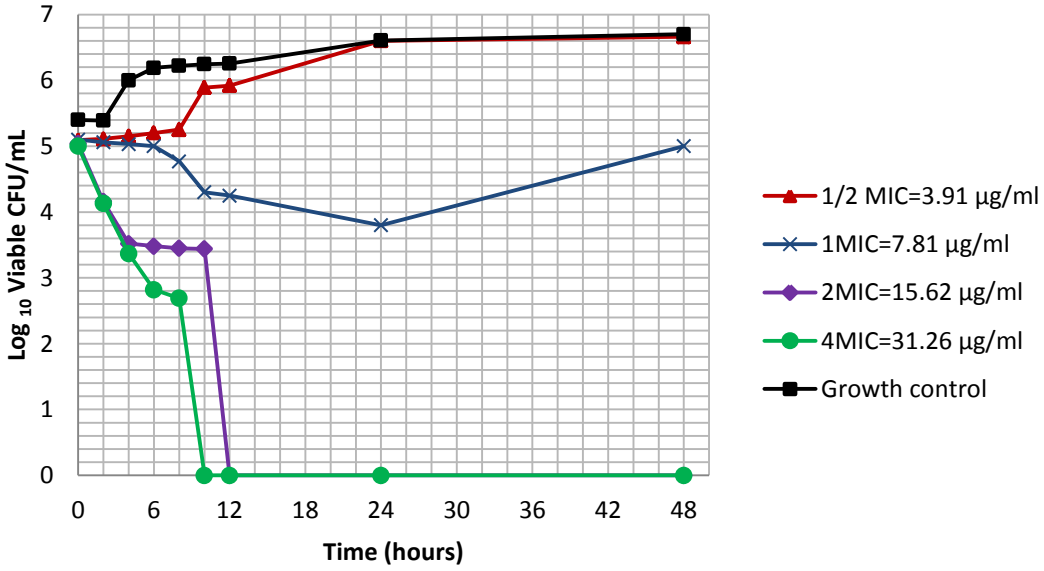

Figure 17 Time-kill curves of ampicillin against *B. cereus* (positive control). For the time kill end point determination; bacteriostatic activity was defined as a reduction of 0-3 log scale and bactericidal activity was defined as a reduction of ≥ 3 log scale.

For *B. subtilis*, ampicillin at 15.62 µg/ml (1MIC) inhibited the growth of *B. subtilis* with the reduction of bacterial 1 to < 3 log scale after 4 h of incubation, which showed bacteriostatic activity and the regrowth was observed as 48 h of incubation. Ampicillin at 31.25 µg/ml (2MIC) inhibited the growth of *B. subtilis* with the reduction of bacterial 1 to < 3 log scale after 4 h of incubation, which showed bacteriostatic activity. After 24 h of incubation, ampicillin at 31.25 µg/ml (2MIC) inhibited the growth of *B. subtilis* with the reduction of bacterial ≥ 3 log scale which showed bactericidal activity (Figure 18). Ampicillin at 62.50 µg/ml (4MIC) inhibited the growth of *B. subtilis* with the reduction of bacterial 1 to < 3 log scale after 4 h of incubation, which showed bacteriostatic activity. After 6 h of incubation, ampicillin at 62.50 µg/ml (4MIC) inhibited the growth of *B. subtilis* with the reduction of bacterial ≥ 3 log scale which showed bactericidal activity, the viable colonies of *B. cereus* were not seen after 6 h of incubation.

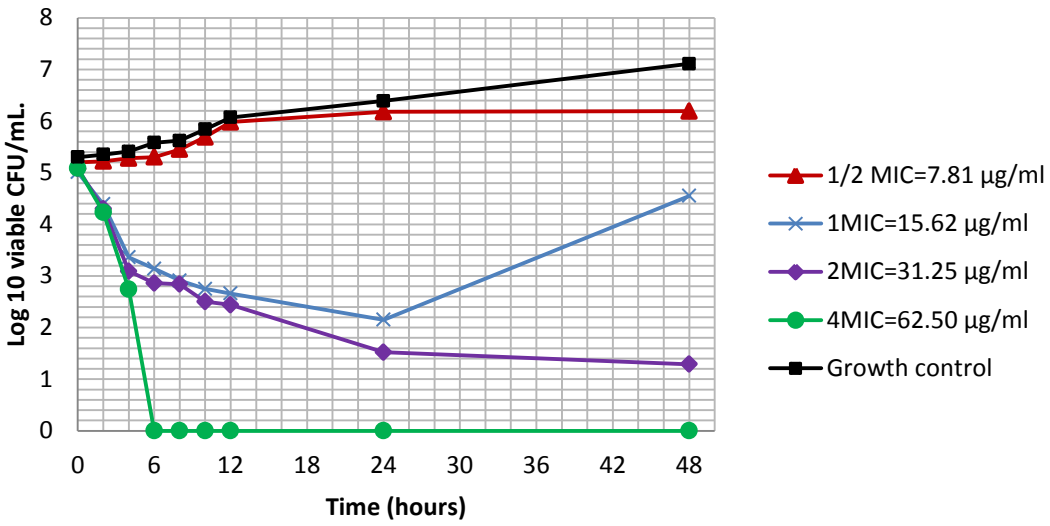

Figure 18 Time-kill curves of ampicillin against *B. subtilis* (positive control). For the time kill end point determination; bacteriostatic activity was defined as a reduction of 0-3 log scale and bactericidal activity was defined as a reduction of ≥ 3 log scale.

1.3 Biofilm formation assay

This study tested the activity of the (+)-usnic acid and ampicillin on bacterial biofilm formation. The result expressed as percentages of inhibition of biofilm formation and compared difference statistical significant with negative control group. Figure22, Inhibition of biofilm formation of *B. cereus* and *B. subtilis* by (+)-usnic acid. The effect of (+)-usnic acid on inhibition of biofilm formation of *B. cereus* and *B. subtilis* was concentration dependent manner at concentration equal or greater than half of MIC value with statistical significance comparing with 5% DMSO ($p < 0.05$). The results also showed that at same concentration 31.25 µg/ml (1/2MIC), 62.50 µg/ml (1MIC), 125 µg/ml (2MIC) and 250 µg/ml (4MIC) µg/ml. (+)-usnic acid exhibited more antibiofilm activity against *B. cereus* than *B. subtilis* (Figure 19).

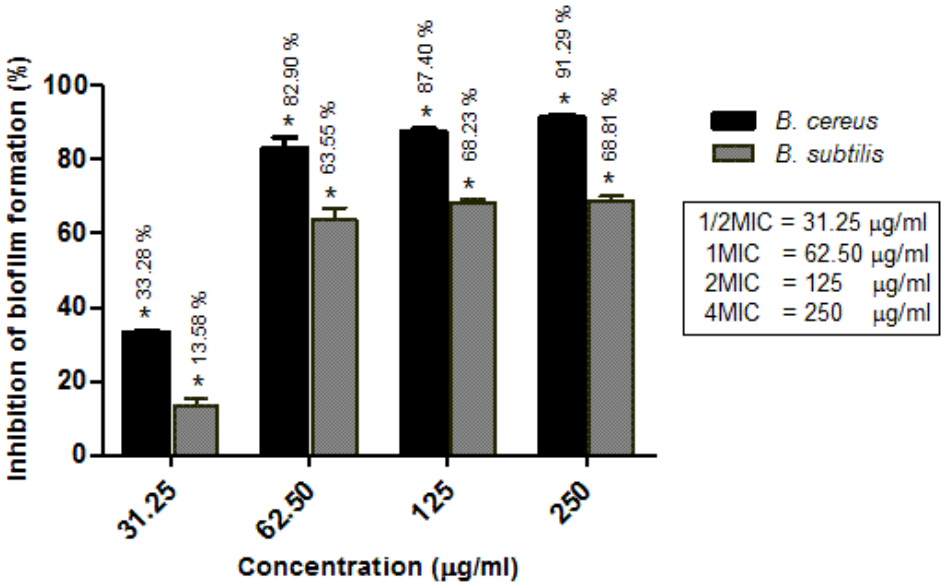

Figure 19 Inhibition of biofilm formation of *B. cereus* and *B. subtilis* by (+)-usnic acid. Error bars represent the mean ± SEM, *p<0.05.

Figure 20-21, the result also showed that ampicillin exhibited antibiofilm activity against both *B. cereus* and *B. subtilis* was concentration dependent manner at concentration equal or greater than half of MIC value with statistical significance comparing with DI water ($p < 0.05$). Figure 23-24, the results also showed that same concentration 7.81, 15.62 and 31.25 µg/ml. ampicillin exhibited more antibiofilm activity against *B. cereus* than *B. subtilis*.

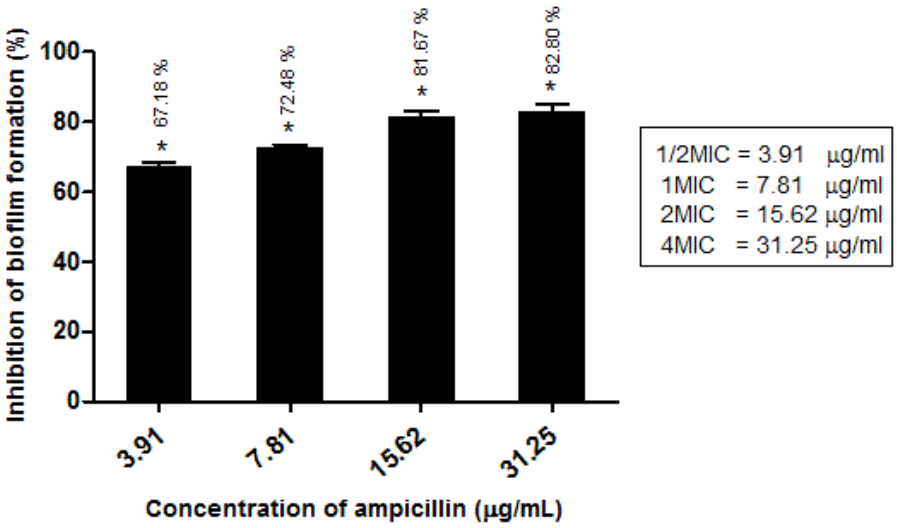

Figure 20 Inhibition of biofilm formation of *B. cereus* by ampicillin. Error bars represent the mean ± SEM, *p<0.05.

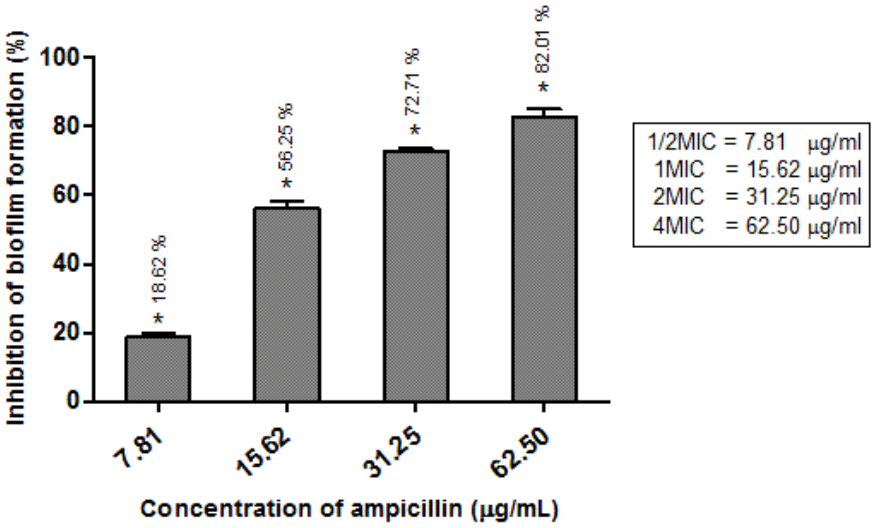

Figure 21 Inhibition of biofilm formation of *B. subtilis* by ampicillin. Error bars represent the mean ± SEM, *p<0.05.

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

2.1 Pathogenicity of bacterial to silkworm

This study was performed in order to examine whether injection of bacteria that are pathogenic to humans could kill silkworm. The result showed that silkworms injected with *B. cereus* or *B. subtilis* into hemolymph, had decreased activity and finally their skin color turned dark. The median lethal doses (LD_{50}) of *B. cereus* and *B. subtilis* were 1.24 × 10^7 CFU/ml (1.55 ×10^6 CFU/g larva) and 6.46 × 10^3 CFU/ml (8.08 ×10^2 CFU/g larva), respectively (Figure 22 -23). Furthermore, when 1 × 10^9 CFU/ml (1.25×10^8 CFU/g larva) of *B. cereus* or 1 × 10^6 CFU/ml (1.25×10^5 CFU/g larva) of *B. subtilis* was injected into silkworm hemolymph, all silkworms died within 48 h. These concentrations of the microorganisms were used for determination of efficacy of (+)-usnic acid. Silkworm larvae survived 48 h when injected with 0.9% NaCl.

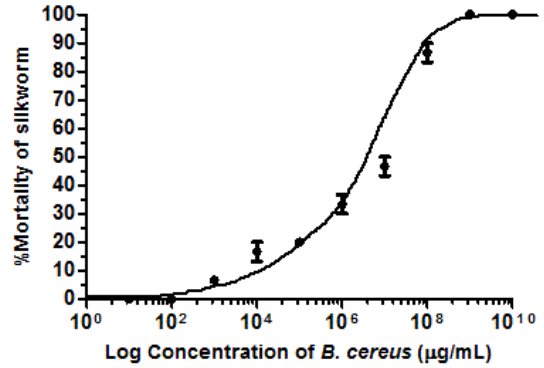

Figure 22 Median lethal doses (LD_{50}) of *B. cereus* in silkworm

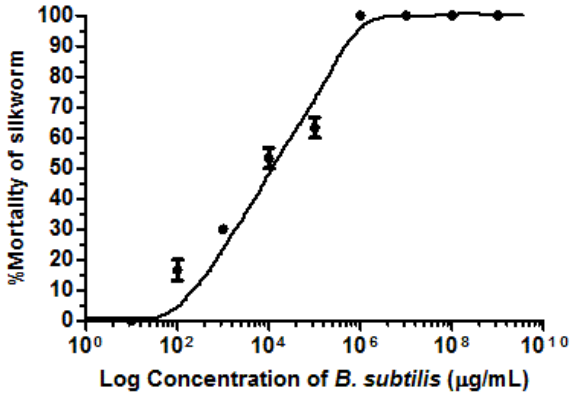

Figure 23 Median lethal doses (LD_{50}) of *B. subtilis* in silkworm

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

In order to investigate the toxicity of (+)-usnic acid to silkworm. The weight of 5^{th} instar larva was average 0.40 g/larva. They were injected with 50 µl of (+)-usnic acid in various concentrations into hemolymph and the mortality rate of silkworm was observed at 48 h after injection. The result showed that the LD_{50} value of the (+)-usnic acid in silkworm was 6.2 mg/ml (0.78 mg/g larva) (Figure 24). The LD_{50} value of ampicillin that killed half of the larvae was > 80 mg/ml (>10 mg/g larvae).

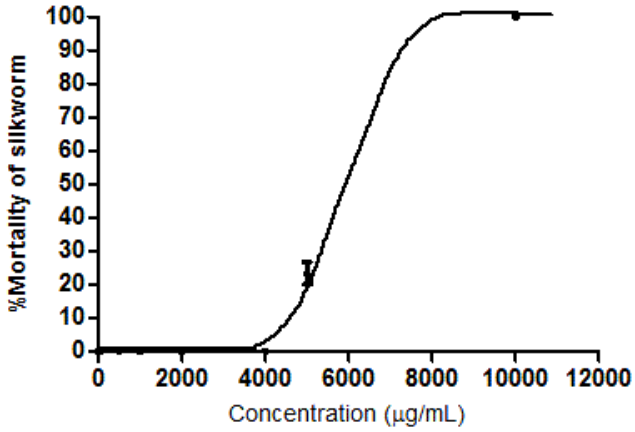

Figure 24 Median lethal dose (LD_{50}) of (+)-usnic acid in silkworm

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2.3 Determination of efficacy (ED_{50}) of the (+)-usnic acid and ampicillin

This study was investigate whether the (+)-usnic acid were effective in silkworms infected with *B. cereus* or *B. subtilis*. The survival of silkworms was determined 2 days after injection of the bacterial suspension and (+)-usnic acid or ampicillin into hemolymph. Concentrations of *B. cereus* and *B. subtilis* that could kill all of silkworms were used in control group. The ED_{50} values, concentration that showing 50% survival of the silkworms, the ED_{50} values of (+)-usnic acid against *B. cereus* and *B. subtilis* were presented in Figure 25-26 and the ED_{50} values of ampicillin against *B. cereus* and *B. subtilis* were presented in Figure 27-28, respectively. The ED_{50} values obtained from the (+)-usnic acid against *B. cereus* and *B. subtilis* were 3.56 mg/ml (0.45 mg/g larvae) and 3.92 mg/ml (0.49 mg/g larvae), respectively whereas the ED_{50} values of ampicillin against *B. cereus* and *B. subtilis* were 0.23 mg/ml (0.03 mg/g larvae) and 1.01 mg/ml (0.13 mg/g larvae), respectively.

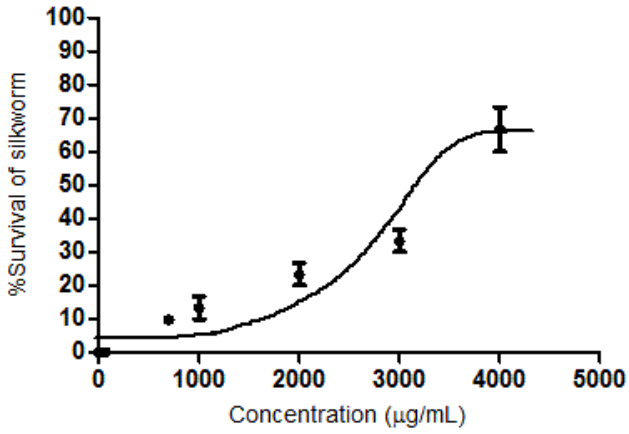

Figure 25 Median effective doses (ED_{50}) of (+)-usnic acid in silkworm infected *B. cereus*

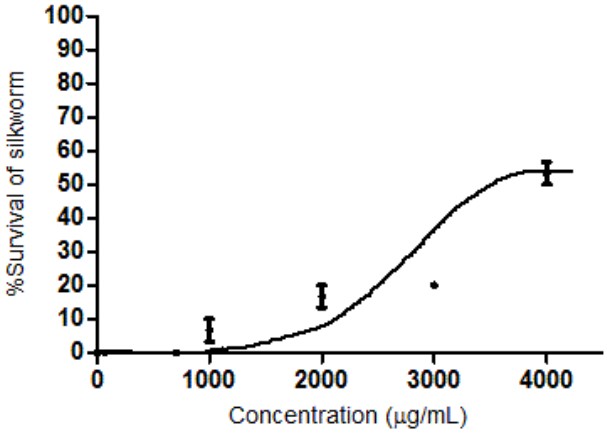

Figure 26 Median effective doses (ED_{50}) of (+)-usnic acid in silkworm infected *B. subtilis*

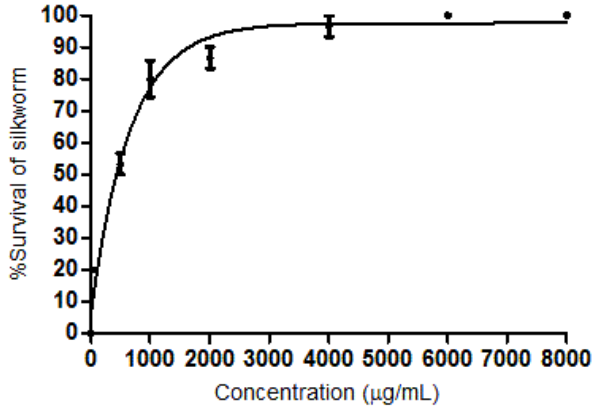

Figure 27 Median effective doses (ED_{50}) of ampicillin in silkworm infected *B. cereus*

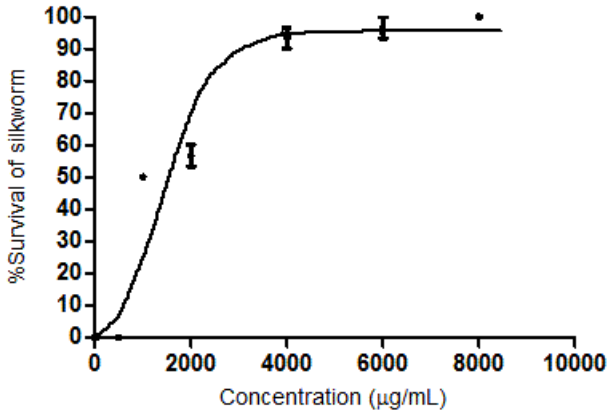

Figure 28 Median effective doses (ED_{50}) of ampicillin in silkworm infected *B. subtilis*

CHAPTER V
DISCUSSION AND CONCLUSION

B. cereus and *B. subtilis* not only continue to cause serious clinical problems through food poisoning, but also show natural resistance to some antimicrobial drugs. One of the key factors enabling pathogen to resist to antimicrobial drugs is the ability to form biofilm. In this study, the quantitative analysis of the antimicrobial activity was evaluated by broth microdilution method to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of (+)-usnic acid while the bactericidal and bacteriostatic activity including time of killing microorganism was assessed further by time kill assay. From the study, the results of the experiments both *in vitro* and *in vivo* were consistent.

Firstly, (+)-usnic acid was further estimated quantitatively by broth microdilution method. The results showed that (+)-usnic acid exhibited antimicrobial activities against the bacterial pathogens at the MIC values equal to 62.50 µg/ml for both *B. cereus* and *B. subtilis*, whereas MBC of (+)-usnic acid for *B. cereus* and *B. subtilis* were 125 and 250 µg/ml, respectively. The antimicrobial activities of (+)-usnic acid on *B. cereus* was more effective than that on *B. subtilis.* Ampicillin was used as a positive control for these bacterial pathogens. MIC values of ampicillin against *B. cereus* and *B. subtilis* were 7.81µg/ml and 15.62 µg/ml respectively, whereas MBC of ampicillin for *B. cereus* and *B. subtilis* were 15.62 and 62.50 µg/ml. respectively. The antimicrobial activities of ampicillin on *B. cereus* were more effective than that on *B. subtilis.* Furthermore, *S. aureus* (ATCC 29213) was used for quality control test. The MIC value of ampicillin against *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).

In time kill assay, *B. cereus* and *B. subtilis* were examined and samples were collected at 0, 2, 4, 6, 10, 12, 24 and 48 h, respectively. The results are presented in terms of the $\log_{10}$ CFU/ml of viable colonies (Ogunmwonyi *et al*., 2010; Soontornpas, Saraya, Chulasiri, Chindavijak, & Mootsikapun, 2005). Bacteriostatic activity was defined as 1 to 3 $\log_{10}$ scale reduction (90% to < 99.9%) compared with original

inoculum, whereas bactericidal activity was defined as a ≥ 3 log10 CFU/ml or greater reduction (≥ 99.9%) compared with original inoculum (NCCLS, 1999). In this study, at concentration equal MIC (1MIC) of (+)-usnic acid and ampicillin inhibited the growth of *B. cereus* and *B. subtilis* with the reduction of bacterial 1 to < 3 log scale which showed bacteriostatic activity. *B. cereus* and *B. subtilis* started to regrowth after 24 h of incubation. At concentration greater than MIC of (+)-usnic acid and ampicillin inhibited the growth of *B. cereus* and *B. subtilis* with the reduction of bacterial ≥ 3 log scale which showed bactericidal activity (Table 8). This result indicated that bacteriostatic activity and bactericidal activity of (+)-usnic acid and ampicillin were concentration dependent. Furthermore, The results also showed that at same concentration of (+)-usnic acid, For example, at 125 µg/ml (2MIC), (+)-usnic acid showed bacteriostatic effect against *B. cereus* at 6 h whereas (+)usnic acid showed bacteriostatic effect at the longer time (10 h) for *B. subtilis*. In addition, (+)-usnic acid exerted bactericidal effect against *B. cereus* at 8 h whereas it exerted bactericidal effect against *B. subtilis* in the longer time (24 h). At concentration 250 µg/ml (4MIC), (+)-usnic acid showed bacteriostatic effect against *B. cereus* and *B. subtilis* at the same time (4 h), whereas (+)-usnic acid exerted bactericidal effect against *B. cereus* at 6 h but it exerted bactericidal effect against *B. subtilis* in the longer time (8 h).

Table 8 Onset of bacteriostatic and bactericidal activity of (+)-usnic acid and ampicillin against *B. cereus* and *B. subtilis*

Antimicrobial effect	*B. cereus*		*B. subtilis*	
	Onset time for 125 µg/ml (hour)	Onset time for 250 µg/ml (hour)	Onset time for 125 µg/ml (hour)	Onset time for 250 µg/ml (hour)
Bacteriostatic	6	4	10	4
Bactericidal	8	6	24	8

In biofilm assay, inhibitory effects of biofilm formation of (+)-usnic acid and ampicillin were concentration dependent manner at concentration equal and greater than half of MIC value with statistical significance comparing with negative control (5% DMSO and DI water respectively) ($p< 0.05$). The results also showed that (+)-

usnic acid exhibited more antibiofilm activity against *B. cereus* than *B. subtilis* with a higher percentage of biofilm inhibition at concentration 31.25 (1/2MIC), 62.50 (1MIC), 125 (2MIC) and 250 (4MIC) (µg/ml). Furthermore, ampicillin at concentration 7.81, 15.62 and 31.25 µg/ml exerted more antibiofilm activity against *B. cereus* than *B. subtilis*. For all results presented above, it is indicated that ampicillin has more antimicrobial and antibiofilm activities against *B. cereus* than *B. subtilis*. The results from time kill assay were consistent with the antimicrobial activity by broth microdilution method and antibiofilm formation assay.

In *in vivo* study, silkworm model was used as an animal model to investigate toxicity and efficacy of (+)-usnic acid. It was performed in order to examine whether injection of bacteria that are pathogenic to humans could kill silkworms or not. The result demonstrated that silkworms were killed by injection of *B. cereus* or *B. subtilis* suspension within 48 h. Later, the toxicity study was performed to evaluate the LD_{50} of (+)-usnic acid and ampicillin in order to select dose range that are save for using in the efficacy study in silkworm infection model. Table 9 showed ED_{50}, MIC, ED_{50} per MIC, LD_{50}, ED_{50}/MIC of (+)-usnic acid and ampicillin in silkworm infection model for *B. cereus* and *B. subtilis.*

Teble 9 ED_{50}, MIC, ED_{50} per MIC, LD_{50}, ED_{50}/MIC values of (+)-usnic acid and ampicillin in silkworm infection model for *B. cereus* and *B. subtilis*

Microrganisms	Substance	ED_{50} (mg/ml)	MIC (µg/ml)	ED_{50} per MIC ratio	LD_{50} (mg/ml)	LD_{50} per ED_{50} ratio
B. cereus	(+)-Usnic acid	3.56	62.50	56.50	6.19	1.74
	Ampicillin	0.23	7.81	29.45	>80.00	>347.83
B. subtilis	(+)-Usnic acid	3.92	62.50	62.72	6.19	1.58
	Ampicillin	1.01	15.62	64.66	>80.00	>79.21

LD_{50} value of (+)-usnic acid was 6.19 mg/ml (0.78 mg/g larva) whereas LD_{50} value of ampicillin was >80 mg/ml (>10 mg/g larvae). In the last experiment, (+)-usnic acid was examined for the therapeutic effect (ED_{50}) against *B. cereus* and *B. subtilis*

by using silkworm infection model. A highest concentration of (+)-usnic acid and ampicillin for testing therapeutic effect should be lower than LD_{50} value. Therefore, the highest concentration of (+)-usnic acid use in efficacy testing was 4 mg/ml. We therefore selected the inoculum concentration of *B. cereus* at 1×10^{9} and *B. subtilis* at $1x10^{6}$ 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, ED_{50} values of (+)-usnic acid against *B. cereus* and *B. subtilis* were 3.56 mg/ml (0.45 mg/g larvae) and 3.92 mg/ml (0.49 mg/g larvae), respectively. The results also showed that (+)-usnic acid exhibited more therapeutic effect against *B. cereus* than *B. subtilis.* ED_{50} value of ampicillin against *B. cereus* and *B. subtilis* were 0.23 mg/ml (0.03 mg/g larvae) and 1.01 mg/ml (0.13 mg/g larvae), respectively (Table 9).

The ED_{50}/MIC ratio of (+)-usnic acid were 56.50 and 62.72 for *B. cereus* and *B. subtilis* respectively, whereas The ED_{50}/MIC ratio of ampicillin were 29.45 and 64.66 for *B. cereus* and *B. subtilis* respectively (Table 9). Theoretically, the higher the ratio of ED_{50}/MIC, the more impact the pharmacokinetic in silkworm larvae body involved. Such pharmacokinetics may include absorption, distribution, metabolism and excretion (Hamamoto *et al*., 2004). Based on the result from efficacy study, (+)-usnic acid and ampicillin were 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 role in silkworm model. Moreover, therapeutic index, ratio of LD_{50}/ED_{50}, of (+)-usnic acid was 1.74 and 1.58 for *B. cereus* and *B. subtilis* respectively 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 ampicillin was >347.83 and >79.21 for *B. cereus* and *B. subtilis* respectively which is broader therapeutic index comparing to the one of (+)-usnic acid.

In conclusion (+)-usnic acid had antimicrobial activities and antibiofilm formation against *B. cereus* and *B. subtilis* in all *in vitro* models, Furthermore, (+)-usnic acid have a potency both on *B. cereus* and *B. subtilis in vitro* and *in vivo*.

In addition, antibiofilm activity may play and important role for antimicrobial activity of (+)-usnic acid. Mechanism of action which may contribute to antibiofilm formation activities.

This study also suggested that (+)-usnic acid may be useful as an antimicrobial agent in the future. However, further study is required to evaluate antimicrobial and antibiofilm formation activities of (+)-usnic acid against *B. cereus* and *B. subtilis* in mammalian model.

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APPENDICES

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Appendix A

Data of Antimicrobial Activities Testing

(*In vitro* studies)

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Table 10 Survival colonies of *B. cereus* received (+)-usnic acid in Time kill assay

Hours	Survival colonies (Log_{10} CFU/ml)				
	Growth control	(+)-Usnic acid 31.25 µg/ml	(+)-Usnic acid 62.50 µg/ml	(+)-Usnic acid 125 µg/ml	(+)-Usnic acid 250 µg/ml
0	1.29×10^{5}	1.20×10^{5}	1.26×10^{5}	1.00×10^{5}	1.00×10^{5}
2	2.50×10^{5}	1.43×10^{5}	1.10×10^{5}	5.00×10^{4}	6.60×10^{4}
4	1.23×10^{6}	5.52×10^{5}	1.05×10^{5}	1.00×10^{3}	5.00×10^{2}
6	1.55×10^{6}	1.20×10^{6}	1.00×10^{5}	5.00×10^{2}	0.00
8	1.62×10^{6}	1.35×10^{6}	4.27×10^{4}	0.00	0.00
10	1.74×10^{6}	1.73×10^{6}	3.55×10^{4}	0.00	0.00
12	1.79×10^{6}	1.88×10^{6}	2.10×10^{4}	0.00	0.00
24	2.80×10^{6}	2.00×10^{6}	1.00×10^{4}	0.00	0.00
48	4.00×10^{6}	2.50×10^{6}	2.50×10^{5}	0.00	0.00

Table 11 Survival colonies of *B. cereus* received ampicillin in Time kill assay

Hours	Survival colonies (Log_{10} CFU/ml)				
	Growth control	Ampicillin 3.91 µg/ml	Ampicillin 7.81 µg/ml	Ampicillin 15.62 µg/ml	Ampicillin 31.25 µg/ml
0	2.51×10^{5}	1.23×10^{5}	1.26×10^{5}	1.10×10^{5}	1.00×10^{5}
2	2.45×10^{5}	1.29×10^{5}	1.15×10^{5}	1.45×10^{4}	2.70×10^{4}
4	1.00×10^{6}	1.41×10^{5}	1.08×10^{5}	3.31×10^{3}	2.34×10^{3}
6	1.55×10^{6}	1.58×10^{5}	1.00×10^{5}	2.82×10^{3}	6.60×10^{2}
8	1.66×10^{6}	1.35×10^{5}	5.59×10^{4}	2.75×10^{3}	4.89×10^{2}
10	1.76×10^{6}	1.78×10^{5}	2.0×10^{4}	2.45×10^{3}	0.00
12	1.80×10^{6}	8.32×10^{5}	1.78×10^{4}	0.00	0.00
24	4.03×10^{6}	3.99×10^{6}	6.31×10^{3}	0.00	0.00
48	5.01×10^{6}	4.57×10^{6}	1.00×10^{5}	0.00	0.00

Table 12 Survival colonies of *B. subtilis* received (+)-usnic acid in Time kill assay

Hours	Survival colonies (Log_{10} CFU/ml)				
	Growth control	(+)-Usnic acid 31.25 µg/ml	(+)-Usnic acid 62.50 µg/ml	(+)-Usnic acid 125 µg/ml	(+)-Usnic acid 250 µg/ml
0	2.72×10^{5}	2.09×10^{5}	2.00×10^{5}	1.82×10^{5}	1.29×10^{5}
2	3.65×10^{5}	2.52×10^{5}	2.09×10^{5}	1.29×10^{5}	6.34×10^{4}
4	5.90×10^{5}	3.00×10^{5}	1.92×10^{5}	1.00×10^{5}	1.00×10^{4}
6	1.38×10^{6}	4.19×10^{5}	1.22×10^{5}	6.01×10^{4}	3.55×10^{3}
8	1.93×10^{6}	6.53×10^{5}	1.06×10^{5}	5.55×10^{4}	0.000
10	2.00×10^{6}	7.18×10^{5}	1.42×10^{4}	1.78×10^{3}	0.000
12	2.22×10^{6}	7.94×10^{5}	3.99×10^{3}	3.23×10^{2}	0.000
24	3.55×10^{6}	1.10×10^{6}	1.33×10^{3}	7.76×10^{1}	0.000
48	1.02×10^{7}	1.52×10^{6}	4.49×10^{4}	6.31×10^{1}	0.000

Table 13 Survival colonies of *B. subtilis* received ampicillin in Time kill assay

Hours	Survival colonies (Log_{10} CFU/ml)				
	Growth control	Ampicillin 7.81 µg/ml	Ampicillin 15.62 µg/ml	Ampicillin 31.25 µg/ml	Ampicillin 62.50 µg/ml
0	2.00×10^{5}	1.58×10^{5}	1.02×10^{5}	1.20×10^{5}	1.22×10^{5}
2	2.24×10^{5}	1.66×10^{5}	2.45×10^{4}	1.99×10^{4}	1.70×10^{4}
4	2.57×10^{5}	1.90×10^{5}	2.30×10^{3}	1.23×10^{3}	5.50×10^{2}
6	3.80×10^{5}	1.99×10^{5}	1.38×10^{3}	7.24×10^{2}	0.00
8	4.17×10^{5}	2.82×10^{5}	8.13×10^{2}	6.92×10^{2}	0.00
10	6.92×10^{5}	4.89×10^{5}	5.62×10^{2}	3.16×10^{2}	0.00
12	1.17×10^{6}	9.55×10^{5}	4.57×10^{2}	2.75×10^{2}	0.00
24	2.45×10^{6}	1.51×10^{6}	1.41×10^{2}	3.31×10^{1}	0.00
48	1.29×10^{7}	1.55×10^{6}	3.55×10^{4}	1.93×10^{1}	0.00

Table 14 Percentage of inhibition of biofilm formation of *B. cereus* received the (+)-usnic acid

(+)-Usnic acid (µg/ml)	Percentage of inhibition of biofilm formation (Mean±SEM)
0.00	0.00
31.25	13.58±1.98
62.50	63.55±3.30
125	68.23±0.83
250	68.81±1.31

Table 15 Percentage of inhibition of biofilm formation of *B. cereus* received ampicillin

(+)-Usnic acid (µg/ml)	Percentage of inhibition of biofilm formation (Mean±SEM)
0.00	0.00
31.25	33.28±0.23
62.50	82.89±2.80
125	87.40±0.85
250	91.29±0.71

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Table 16 Percentage of inhibition of biofilm formation of *B. subtilis* received the (+)-usnic acid

Ampicillin (µg/ml)	Percentage of inhibition of biofilm formation (Mean±SEM)
0.00	0.00
3.90	67.18±1.16
7.81	72.48±1.0
15.62	81.67±1.58
31.25	82.80±2.32

Table 17 Percentage of inhibition of biofilm formation of *B. subtilis* received ampicillin

Ampicillin (µg/ml)	Percentage of inhibition of biofilm formation (Mean±SEM)
0.00	0.00
7.81	18.62±1.41
15.62	56.25±1.86
31.25	72.22± 0.28
62.50	82.57± 2.45

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Appendix B

Data of Antimicrobial Activities Testing

(*In vivo* studies)

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Table 18 Mortality rate of silkworm received *B. cereus* at various concentration

B. cereus **(CFU/mL)**	**Mean of number of silkworm death (% mortality)**
1×10^{1}	0.00±0.00
1×10^{2}	0.00±0.00
1×10^{3}	6.67±0.33
1×10^{4}	16.67±3.33
1×10^{5}	20.00±0.00
1×10^{6}	33.33±3.33
1×10^{7}	46.67±3.33
1×10^{8}	86.67±3.33
1×10^{9}	100.00±0.00
0.9% NaCl	100.00±0.00

Table 19 Mortality rate of silkworm received *B. subtilis* at various concentration

B. subtilis **(CFU/mL)**	**Mean of number of silkworm death (% mortality)**
1×10^{1}	0.00±0.00
1×10^{2}	16.67±3.33
1×10^{3}	30.00±0.00
1×10^{4}	53.37±3.33
1×10^{5}	63.33±3.33
1×10^{6}	100.00±0.00
1×10^{7}	100.00±0.00
1×10^{8}	100.00±0.00
1×10^{9}	100.00±0.00
0.9% NaCl	100.00±0.00

Table 20 Mortality rate of silkworm received the (+)-usnic acid at various concentrations

(+)-Usnic acid (µg/ml)	Mean of number of silkworm death (% mortality) n=10
0.05	0.00±0.00
0.5	0.00±0.00
5	0.00±0.00
50	0.00±0.00
500	0.00±0.00
1,000	0.00±0.00
2,000	0.00±0.00
4,000	0.00±0.00
5,000	23.33±3.33
10,000	100.00±0.00
25,000	100.00±0.00
5% DMSO	0.00±0.00

Table 21 Mortality rate of silkworm received ampicillin at various concentrations

Ampicillin (mg/ml)	Mean of number of silkworm death (% mortality) n=10
2.5	0.00±0.00
5.0	0.00±0.00
10.0	0.00±0.00
20.0	0.00±0.00
40.0	0.00±0.00
80.0	0.00±0.00
DI water	0.00±0.00

Table 22 Survival rate of silkworm infected with *B. cereus* received (+)-usnic acid at various concentrations

(+)-Usnic acid (µg/ml)	Mean of number of silkworm survival (% survival) n=10
0.7	0.00±0.00
70	0.00±0.00
700	9.60±0.33
1,000	13.33±3.33
2,000	23.33±3.33
3,000	33.33±3.33
4,000	66.66±6.66
Control	0.00±0.00

Table 23 Survival rate of silkworm infected with *B. cereus* received ampicillin at various concentrations

Ampicillin (µg/ml)	Mean of number of silkworm survival (% survival) n=10
0.5	0.00±0.00
50	20.00±0.00
500	50.33±3.33
1,000	80.00±5.77
2,000	86.66±3.33
4,000	96.66±3.33
6,000	100.00±0.00
8,000	100.00±0.00
Control	0.00±0.00

Table 24 Survival rate of silkworm infected with *B. subtilis* received (+)-usnic acid at various concentrations

(+)-Usnic acid (µg/ml)	Mean of number of silkworm survival (% survival) n=10
0.7	0.00±0.00
70	0.00±0.00
700	0.00±0.00
1,000	6.66±3.33
2,000	16.66±3.33
3,000	20.00±0.00
4,000	53.33±3.33
Control	0.00±0.00

Table 25 Survival rate of silkworm infected with *B. subtilis* received ampicillin at various concentrations

Ampicillin (µg/ml)	Mean of number of silkworm survival (% survival) n=10
0.5	0.00±0.00
50	0.00±0.00
500	0.00±0.00
1,000	50.00±0.00
2,000	56.66±3.33
4,000	93.33±3.33
6,000	96.66±3.33
8,000	100.00±0.00
Control	0.00±0.00

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VITA

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