ฤทธิ์ในการต้านจุลชีพและต้านการสร้างไบโอฟิล์มของสารคริซาซินต่อเชื้อสปีชีส์บาซิลลัสจากการศึกษาทั้งแบบนอกกายและแบบในกาย โดยใช้หนอนไหมไทย

นางสาวมาลิออน มิสเซอเลอร์

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ANTIMICROBIAL AND ANTIBIOFILM FORMATION ACTIVITIES OF CHRYSAZIN ON *BACILLUS* SPECIES *IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL

Miss Marion Micheler

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Thesis Title	ANTIMICROBIAL AND ANTIBIOFILM FORMATION ACTIVITIES OF CHRYSAZIN ON *BACILLUS* SPECIES *IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL
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มาลิออน มิสเซอเลอร์ : ฤทธิ์ในการต้านจุลชีพและต้านการสร้างไบโอฟิล์มของสารคริซาซิน ต่อเชื้อสปีชีส์บาซิลลัสจากการศึกษาทั้งแบบนอกกายและแบบในกาย โดยใช้หนอนไหมไทย (ANTIMICROBIAL AND ANTIBIOFILM FORMATION ACTIVITIES OF CHRYSAZIN ON *BACILLUS* SPECIES *IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.สันทัด จันทร์ประภาพ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ชนิดา พลานุเวช, 73 หน้า.

เชื้อสปีชีส์บาซิลลัสก่อให้เกิดภาวะอาหารเป็นพิษ การติดเชื้อเฉพาะที่ และการติดเชื้อแบบกระจายไปทั่วอวัยวะในร่างกาย เชื้อนี้สามารถเพิ่มอัตราการดื้อยาปฏิชีวนะโดยการสร้างไบโอฟิล์ม การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ในการต้านจุลชีพและฤทธิ์ต้านการสร้างไบโอฟิล์มของสารคริซาซินซึ่งเป็นสารออกฤทธิ์ทางชีวภาพหลักที่พบในกระถินทุ่งต่อเชื้อ *Bacillus cereus* และ *Bacillus subtilis* โดยทำการศึกษาทั้งแบบนอกกายและแบบในกายโดยใช้หนอนไหมไทย สำหรับการทดสอบฤทธิ์ในการต้านจุลชีพของสารโดยใช้วิธี broth microdilution พบว่าเชื้อ *B. cereus* มีความไวต่อสารคริซาซินมากกว่าเชื้อ *B. subtilis* ซึ่งความเข้มข้นต่ำสุดของสารคริซาซินที่สามารถยับยั้งเชื้อ *B. cereus* และ *B. subtilis* มีค่าเท่ากับ 7.81 และ 15.63 µg/ml ตามลำดับ ยิ่งไปกว่านั้นผลจากการศึกษา Time-Kill Assay ชี้ให้เห็นว่าคริซาซินมีฤทธิ์เป็นสารยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียทั้งสองชนิด นอกจากนั้นยังพบว่าสารคริซาซินมีฤทธิ์ในการยับยั้งการสร้างไบโอฟิล์มของเชื้อทั้งสองชนิดด้วยเป็นแบบขึ้นกับความเข้มข้น โดยมีค่าการยับยั้งสูงสุดเท่ากับ 85.15% และ 73.86% สำหรับเชื้อ *B. cereus* และ *B. subtilis* ตามลำดับ ที่ความเข้มข้น 500 µg/ml จากการศึกษาในหนอนไหมที่ติดเชื้อ *B. cereus* และ *B. subtilis* แสดงให้เห็นว่าคริซาซินมีฤทธิ์ต้านเชื้อแบคทีเรียในหนอนไหม โดยมีเปอร์เซ็นต์การรอดชีวิตในอัตรา 60.33±0.33 และ 50.67±0.33 ตามลำดับ ที่ความเข้มข้น 1,000 µg/ml โดยสรุปการศึกษานี้แสดงให้เห็นว่าคริซาซินมีศักยภาพในการพัฒนาเป็นยาต้านเชื้อแบคทีเรีย และสารต้านการสร้างไบโอฟิล์มของเชื้อแบคทีเรียสองชนิดนี้

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

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MARION MICHELER: ANTIMICROBIAL AND ANTIBIOFILM FORMATION ACTIVITIES OF CHRYSAZIN ON *BACILLUS* SPECIES *IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL. ADVISOR: SANTAD CHANPRAPAPH, Ph.D., CO-ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., 73 pp.

Bacillus species cause food poisoning, localized infections and systemic infections. They also can become more resistant to antibiotic by forming of biofilms. The aim of this study, therefore was to investigate antimicrobial and antibiofilm activities of chrysazin, a major bioactive compound in *Xyris indica* L. on *Bacillus cereus* and *Bacillus subtilis* using both *in vitro* studies and *in vivo* studies using Thai silkworm model. From broth microdilution method, we observed that *B. cereus* was more susceptible to chrysazin than *B. subtilis* with MIC value of 7.81 and 15.63 µg/ml, respectively. Furthermore, results from Time-Kill Assay indicated that chrysazin was bacteriostatic agent against both microorganisms. In addition chrysazin also exerted antibiofilm formation activity against both *B. cereus* and *B. subtilis* in a concentration-dependent manner with maximum % inhibition of 85.15 and 73.86, for *B. cereus* and *B. subtilis*, respectively at concentration 500 µg/ml. Chrysazin demonstrated antibacterial activity in Thai silkworm infection model on both *B. cereus* and *B. subtilis* with the maximum % survival of 60.33±0.33 and 50.67±0.33, respectively at concentration 1,000 µg/ml. In conclusion this study showed that chrysazin has its potential for developing as an antibacterial drug and antibiofilm agent against *B. cereus* and *B. subtilis.*

Field of Study: Pharmacology
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Student's Signature .....
Advisor's Signature .....
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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
B. cereus	*Bacillus cereus*
B. subtilis	*Bacillus subtilis*
B. megaterium	*Bacillus megaterium*
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DMH	1,2-dimethylhydrazine
DMSO	Dimethyl sulfoxide
ED_{50}	Median effective dose
LD_{50}	Median lethal dose
Log	Logarithm
MBC	Minimal bactericidal concentration
mg	milligram
MHA	Muller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimal inhibitory concentration
ml	milliliter
NSS	normal saline solution
ºC	Degree Celsius
S. aureus	*Staphylococcus aureus*
%	percent
µg	microgram

CHAPTER I

INTRODUCTION

Background and Rationale

Public Health England reports that the genus Bacillus currently comprises 268 species and 7 subspecies, generally found in the environment and as laboratory contaminants but a few of the species have been known to cause infections in humans (1). *Bacillus cereus* is one of the most interesting species for its pathogenicity. It is associated with food poisoning and eye infection. Moreover, Bottone described that the spectrum of infections include fulminant bacteremia, central nervous system (CNS) involvement (meningitis and brain abscesses), endophthalmitis, pneumonia, and gas gangrene-like cutaneous infections (2). Houry and coworkers mentioned that the persistency of this pathogen in various environment is resulted from the formation of spores and of biofilms (3). In many food industry segments, biofilms are serious problems (4). Furthermore, Wang and coworkers described that *Bacillus subtilis* could differentiate into multiple phenotypes with different functions during biofilm formation, such as motile, matrix-producing, and sporulating types (5). There have been reported by Fatemeh and coworkers that *B. subtilis* is a nosocomial bacteria. It could cause secondary diseases and infections, such as bacteremia, septicemia, and wound infection in hospitalized patients, especially patients who have the impaired immune system (6). There is information about *Bacillus* species resistant to various antibiotics.

Luna and coworkers observed that the majority of the *B. cereus* and all of the *Bacillus thuringiensis* isolates are resistant to amoxicillin, ampicillin, ceftriaxone, penicillin and oxacillin (7). Moreover, Oggioni and coworkers observed that *B. subtilis* strains which isolated during fever episodes showed resistance to penicillin, erythromycin, and chloramphenicol (8). As a result of increasing resistance to some

antibacterial agents, Bioresources are the important source of bioactive compounds for the discovery of lead compounds as drug candidates in clinical use as an active ingredient and starting material to produce semi-synthetic drugs (9).

The interesting plant in this study is *Xyris indica* L. (Xyridaceae), which is grass-like plant, and this plant has been named locally as Kra thin thung. It is widespread in Thailand and is a native plant of east India. *Xyris indica* L. flowering heads are used as crude drug in traditional Thai medicine for cure of ringworm, constipation and flatulence. Khuniad and coworkers performed antimicrobial activity screening of *Xyris indica* L. extract. They observed that the ethanol extract from the dry *Xyris indica* L. flowering heads inhibited the growth of tested gram positive (*Bacillus cereus* ATCC11778, *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC6538P, and *Micrococcus luteus* ATCC9341) and gram negative bacteria (*Escherichia coli* ATCC25922, *Enterobacter aerogenes* ATCC13048, *Salmonella typhimurium* ATCC13311) except *Pseudomonas aeruginosa* ATCC9027 whereas, the petroleum ether extract was only active against *B. cereus* and *B. subtilis*. Moreover, they described from the phytochemical studies of *Xyris indica* L. flowering heads that the heads contained two isocoumarins, two sterols and three anthraquinones; chrysazin as a main compound, 3-methoxychrysazin and 3-hydroxychrysazin (10). Owing to very few studies that have evaluated the antimicrobial activity of chrysazin, in this study we therefore investigated chrysazin, the major bioactive compound found in *Xyris indica* L., for its antimicrobial activity, inhibitory effect on the biofilm formation, and *in vitro* killing time of *Bacillus cereus* and *Bacillus subtilis*. Since most drug candidates screened by *in vitro* systems have problems in pharmacokinetics including absorption, distribution, metabolism, and excretion. Hence they did not show therapeutic effects in disease models with mammals such as mice and rats (the most commonly used animal models) (11). The sacrifice of large number of mammals is a major concern in ethical problem so that it

is going to be a key factor to slow down the speed of drug development in industrialized countries. Recently, to solve these problems, use of silkworm as model animals with a large numbers and low costs in the screening of new drug candidates has been employed (12, 13). Therefore, we also used silkworms as an infection model for evaluation of antimicrobial activities of chrysazin against both microorganisms.

Objective of this study

The aim of this study was to evaluate the antimicrobial and antibiofilm activities of chrysazin, the bioactive compound of *Xyris indica* L., against *B. cereus* and *B. subtilis in vitro* study and *in vivo* study using Thai silkworm model.

Research design

Experimental research

Scope of the study

In this study, we performed both *in vitro* and *in vivo* studies. *In vitro* study, it was aimed to determine antimicrobial and antibiofilm activities of chrysazin against *Bacillus cereus* and *Bacillus subtilis* using antimicrobial susceptibility testing, Time kill assay, and Inhibition of biofilm formation assay. *In vivo* study, Thai silkworms were used as an animal infection model for determine pathogenicity and therapeutic effect of chrysazin on both microorganisms.

Benefits from this study

Benefits from this study is the information of chrysazin that exerts both antimicrobial and antibiofilm activities against *B. cereus* and *B. subtilis in vitro* as well as *in vivo* study and this information may lead to further step for development of antimicrobial drugs in mammalian models.

CHAPTER II

LITERATURE REVIEWS

Bacteria

Taxonomy

Kingdom: Bacteria; Phylum: Firmicutes; Class: Bacilli; Order: Bacillales; Family: Bacillaceae; Genus: *Bacillus* (14).

Structure and Classification

The bacterial genus *Bacillus* are rod-shaped, endospore-forming aerobic or facultatively anaerobic, and gram-positive bacteria. They can grow at optimal temperatures ranging from 25 to 37°C, although thermophilic and psychrophilic members are capable of growth at temperatures as high as 75°C and as low as 3°C, respectively. Some species can flourish at extremes of acidity and alkalinity, ranging from pH 2 to 10 (15, 16). Moreover, they exhibit a broad range of physiologic abilities that permit them to live in every environment and only one endospore is formed per cell. The spores are resistant to heat, cold, radiation, desiccation, and disinfectants (17).

Structures on the surface of *Bacillus* species, they consist of S-Layers, Capsules, Flagella, Cell Walls, Macrofibers, and Membranes. Slepecky and Hemphill described that crystalline surface layers of protein or glycoprotein subunits, which called S-layers are found in the bacterial genus *Bacillus* (18). S-layers of individual strains of *Bacillus* have been shown to differ in molecular weight (40–200 kDa), the degree of glycosylation of the subunits, and the geometry of the S-layer lattice (19). Not all *Bacillus* species contain S-layers and some strains may lack such a layer. Furthermore, the type of lattice may vary from species to species and within strains of a species.

B. cereus, *Bacillus fastidiosus*, *Bacillus megaterium*, *Bacillus psychrophilus*, and *Bacillus schlegelii* present a square lattice (20). Other bacilli, such as *B. subtilis*, *B. megaterium*, and *Bacillus licheniformis*, possess capsules containing the homopolypeptide of D- or L-glutamic acid (21). Most *Bacillus* species possess peritrichous flagella. (20). In addition to peptidoglycan in the cell wall, all *Bacillus* species contain large amounts of an anionic polymer, such as teichoic acid (a glycerol or ribitol-based polymer joined together by phosphodiester linkages to form a flexible linear strand) or teichuronic acid (uronic acid-based polymer) which are bonded to muramic acid residues. The type of this anionic polymer present depends on the levels of phosphate and magnesium in the growth medium. The glycerol teichoic acids vary a great deal between Bacillus species and within species (19). Macrofibers are multicellular and multistranded structures, hundreds of micrometers in length, produced by autocatalytic mutants of *B. subtilis* (22). There is great diversity in the range and type of lipids in *Bacillus* membranes and wide variation in the fatty acids are found. The main phospholipids present are phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylethanolamine. The major isoprenoid quinones are menaquinone, and most species contain menaquinones with seven isoprenoid units (MK-7) (23).

Nutrition, Growth and Metabolism

Most *Bacillus* species grow well on ordinary nutrient media and they improved growth in the presence of glucose. Many species are aerobic, but some species, such as *Bacillus cereus*, *Bacillus anthracis*, *Bacillus licheniformis*, and *Bacillus coagulans* can grow anaerobically. Furthermore, the morphologic appearance of colonies is influenced by the media on which they are growing. Colonies of *Bacillus* species are usually large, flat, and dull with a ground-glass appearance, whereas colonies of *Bacillus cereus* are α- or β-hemolytic with a lavender color. Besides, they may be small,

shiny, and compact or large and feathery (24). Balows and coworkers described that *Bacillus* species form pigment, which may be brown, yellow, orange, pink, or black depending on the media on which they are growing. Pigment formation is increased by carbohydrate in the media. It is a variable, late-appearing feature and of little differential value. On carbohydrate media, there are most species produce acid only. Ordinarily fermented sugars include glucose, maltose, and sucrose, whereas lactose is rarely utilized. The end products of glucose fermentation vary. Some species produce lactic acid. *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus cereus* produce 2, 3-butanediol and glycerol (25).

Endospores

Losick and Youngman described that the *Bacillus* endospore is a dormant cell form that resists extremes of environment but, under appropriate conditions, rapidly germinates into a dividing cell. The endospores of *Bacillus* are formed intracellular as a consequence of a sporulation-specific cell-division pathway. A *Bacillus* vegetative cell propagates through binary fission, in which a symmetrically positioned septum partitions the dividing cell into daughters of equivalent size. A cell entering the sporulation process, in contrast, partitions asymmetrically without cell division, thereby forming two compartments, which have divergent developmental fates. The smaller compartment (the forespore) becomes the endospore, and the larger compartment (the mother cell) eventually lyses after participating in the maturation of the endospore. Growth and sporulation can therefore be thought of as two interlocking cycles of cell-division events with formation of an asymmetric septum being a hallmark of the sporulation process (26).

Resistance of Spores

Slepecky and Hemphill described that the resting forms of bacteria are usually more resistant to various environmental stresses than their counterpart vegetative forms. The structure and composition of the resting (and dormant) form of endospore forming bacteria. However, they are quite different from other bacterial-resting forms. The core or protoplast containing the heat labile DNA, RNA, ribosomes, enzymes, and other proteins is surrounded by a primitive (germ cell wall). Moving toward the surface, there is a layer called the cortex, which consists of peptidoglycan of a similar nature to that of the vegetative cell wall but with less cross linking in the peptides among other differences. A second cell membrane surrounds the cortex. The protoplast, cortex and their membranes are enclosed by layers of protein coat. A loose-fitting exosporium, appendages and internal protein crystals may be found in some species. Compared with vegetative cells, spores are more resistant to heat by a factor of 105 or more, to UV and ionizing radiation by 100-fold or more, and to desiccation, antibiotics, disinfectants, and other chemicals (19, 27).

Bacillus cereus

B. cereus is a ubiquitous, 1.4 μm gram-positive rods, endospore-forming and aerobic or facultatively anaerobic bacteria (28). It has colonial morphology of about 2-7 mm in diameter, and have a white granular texture. Moreover, *B. cereus* grows above 10-20 °C and below 35-45 °C with an optimum temperature of about 37 °C (29). The reservoirs of *B. cereus* in the environment are composed of decaying organic matter, fresh and marine waters, vegetables and fomites (30). As a result, soil and food products may become contaminated leading to the transient colonization of the human intestine (31). *B. cereus* has a saprophytic life cycle in which spores germinate in soil with the production of a vegetative bacillus, which could then sporulate and maintain the life cycle (2, 32).

Bacillus subtilis

B. subtilis is a gram-positive motile rod-shaped bacterium, aerobic, spore-forming soil bacterium and ubiquitous in the environment. Colonial morphology of *B. subtilis* has irregular shape, large size with a wavy margin, umbonate elevation, and white rough texture. Its optimum growth temperature is between 30-37 ºC, and it will produce fibrinolytic enzyme at 37 ºC (33). *B. subtilis* is best known for its ability to become competent and undergo sporulation in response to starvation and high population densities (34, 35). Tam and coworkers reported that upon exiting the stomach, *B. subtilis* spores can germinate, proliferate, and then resporulate (36).

Pathogenesis

The pathogenicity of *B. cereus*, whether intestinal or nonintestinal, was intimately associated with tissue-destructive/reactive exoenzyme production. These secreted toxins were four hemolysins (37), three distinct phospholipases, an emesis-inducing toxin, and three pore-forming enterotoxins: hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K. In the gastrointestinal tract (small intestine), vegetative cells, ingested as viable cells or spores produce and secrete a protein enterotoxin and induce a diarrheal syndrome, whereas emetic toxin, a plasmid-encoded cyclic peptide (cereulide) is produced in food products and ingested preformed. In addition to food poisoning, *B. cereus* causes a number of systemic and local infections in both immunologically compromised and immunocompetent individuals. Among those most commonly infected are neonates, intravenous drug abusers, patients sustaining traumatic or surgical wounds, and those with indwelling catheters. The spectrum of infections include fulminant bacteremia, central nervous system (CNS) involvement (meningitis and brain abscesses), endophthalmitis, pneumonia, and gas gangrene-like cutaneous infections (2).

B. subtilis produces an extracellular toxin known as subtilisin, a protease enzyme that has been reported to cause dermal allergic or hypersensitivity reactions and the oral, dermal and pulmonary acute toxicity in individuals repeatedly exposed to this enzyme in industrial settings (38). Logan cites more cases of *B. subtilis* infections in which identification of the bacterium appeared reliable. Infections include a case of endocarditis in a drug abuse patient; fatal pneumonia and bacteremia in three leukemic patients; septicemia in a patient with breast cancer; and infection of a necrotic axillary tumor in another breast cancer patient. Isolation of *B. subtilis* was also made from surgical wound-drainage sites, from a subphrenic abscess from breast prosthesis, and from two ventriculo-atrial shunt infections. Moreover, *B. subtilis* cause infection in vulnerable (young, aged, and immune-deficient or immune-compromised) individuals (39).

Antimicrobial Therapy

Based on *in vitro* data, the drug of choice for serious infections caused by Bacillus infections is vancomycin since *B. cereus* is the most common isolate. Based on clinical data, both vancomycin and clindamycin have been used successfully. Other drugs that are highly active and likely to be bactericidal include imipenem, ciprofloxacin and gentamicin. Tetracycline, chloramphenicol, clindamycin and erythromycin have activity against Bacillus species. Most Bacillus strains are resistant to broad spectrum cephalosporins and ticarcillin-clavulanate. Empiric coverage with the latter agents should be avoided in immunocompromised patients whose blood cultures yield gram positive aerobic Bacillus until susceptibility testing is available.

Serious infections caused by Bacillus species include ocular infections, endocarditis, bacteremia and septicemia, pneumonia, meningitis, musculoskeletal infections, and infections associated with injuries from motor vehicle accidents associated with road trauma and gunshot injuries. Systemic antibiotic therapy is usually

required in the treatment of most serious Bacillus infections. A self-limited illness which presents as food poisoning caused by *B. cereus* requires no antimicrobial therapy. Treatment is usually symptomatic and fluid replacement may be indicated for patients who are severely dehydrated.

In case of Ocular Infections which has *B. cereus* as a primary pathogen, endophthalmitis is a serious illness that can result in visual compromise within 12-48 hours after inoculation. Early diagnosis is important to achieve successful treatment. A high index of suspicion is important in the setting of a patient who presents with ocular infection after trauma or in the setting of drug abuse. Prompt recognition of the infection should allow initiation of appropriate therapy before permanent structural changes occur. In patients with post-traumatic endophthalmitis caused by *B. cereus*, if managed aggressively outcome may be associated with preservation of anatomic integrity and restoration of useful visual acuity. In the drug abuse setting both clindamycin and vancomycin have been used as a single agent. Intravitreal dexamethasone to control the destructive inflammation and early vitrectomy have recently been recommended in the management of sight-threatening endophthalmitis such as that induced by *B. cereu*s.

Because of the serious sequelae of panophthalmitis an aggressive approach with early vitrectomy and vitreal instillation of appropriate antibiotic is indicated. Both local and systemic antibiotics are used. Antibiotics administered systemically, intravitreally, topically, and via periocular routes are used in conjunction with surgical intervention. An aminoglycoside either gentamicin or tobramycin has been administered locally and systemically but is inadequate to eradicate the infection. Clindamycin or vancomycin with or without aminoglycoside is appropriate before the results of culture since *B. cereus* is the predictable isolate. Clindamycin and gentamicin seem to be favored by the ophthalmologists. Clindamycin has moderate to good

activity against *B. cereus* and when administered subconjunctivally or parenterally reaches therapeutic levels in the iris, choroid and vitreous. Intravitreal administration is favored and a combination of 200-400 μg of gentamicin and 450 μg of clindamycin is recommended. In addition, 8 μg/ml gentamicin and 9 μg/ml clindamycin can be added to the vitrectomy infusion fluid. Newer drugs (e.g., imipenem and quinolones) appear active, but more experience is needed in their use.

In case of Endocarditis caused by Bacillus organisms, a well-recognized complication of intravenous drug abuse, it has been rarely isolated from patients with underlying valvular disease. Since *B. cereus* is the most common isolate, empirical use of penicillin is usually not effective. Antibiotic therapy with vancomycin or clindamycin has achieved high cure rates in Bacillus endocarditis. Intravenous drug abusers with endocarditis caused by Bacillus species have responded well to clindamycin.

For Bacteremia, intravascular devices are the common source of positive blood cultures for Bacillus species. In patients with positive blood cultures for Bacillus, a decision has to be made whether the organism is causing disease. In most cases, especially if the patient is asymptomatic, the bacteremia is limited and requires no antimicrobial therapy which emphasizes that the process is relatively benign. A recent report on AIDS patient with *Bacillus cereus* bacteremia also emphasizes the low morbidity associated with this condition. However, there have been case reports of fulminant sepsis complicated by hemolysis in patients with acute leukemia. Appropriate therapy can be readily instituted once a decision has been made that a clinically significant infection is present. Usually infections have an indolent course and the institution of antimicrobial therapy can await specific sensitivity results. In the setting of immunosuppressed patients with chronic indwelling catheters, recent experience suggests that catheters should be promptly removed to prevent recurrent bacteremia in addition to administering antibiotic therapy. Depending on how ill the

patient, antibiotics e.g. vancomycin with or without gentamicin may be initiated until susceptibility studies become available.

Dosage and Duration of Therapy

For ocular infections, doses of clindamycin or vancomycin in combination with gentamicin will be similar to the treatment of deep seated infections as outlined above. In addition, as equally important is the intraocular administration of antibiotics as described earlier. It appears that clindamycin is preferred by ophthalmologists because of good penetration in ocular tissues and also based on limited *in vitro* synergy data. In patients with uncomplicated bacteremia dosages for either vancomycin or clindamycin will be as stated above. Duration of treatment can vary from 7-14 days depending on the severity of the illness and underlying host defense impairment.

Depending on the clinical setting, one may prefer to use vancomycin over clindamycin such as nosocomially acquired infection if there is a concern for *C. difficile* colitis as a complication. Newer drugs e.g. imipenem and quinolones appear active but more experience is needed in their use. There has been limited experience with the use of oral antibiotics in the treatment of *B. cereus* infections. A patient who developed severe wound infection with bacteremia caused by *B. cereus* was treated successfully with ciprofloxacin 750 mg every q 12 hours for almost 3 months. Doxycycline has been used in the treatment of bacteremia associated with Hickman catheters caused by B. cereus in a patient with non-Hodgkins lymphoma (40).

Biofilm

Biofilms are known to be the predominant mode of lifestyle of bacteria in all environmental niches. The development of bacterial biofilms follows five well-defined stages (1) initial reversible attachment of bacterium to substrate, (2) irreversible attachment and microcolony formation, (3) early development into biofilms, (4) biofilm maturation and (5) biofilm dispersion (41).

Vlamakis and coworkers reported that at the initiation of biofilm formation, motile cells with flagella differentiate into non-motile, matrix-producing cells that stop separating and form chains that are surrounded by extracellular matrix. In mature biofilms, matrix-producing cells sporulate. In aged biofilms, some cells secrete small molecules, such as d-amino acids and polyamines, which break down the extracellular matrix and allow the cells to disperse in the environment. It is important to note that although functionally distinct cell types exist within the biofilm, these cells are genetically identical, and differentiation into a specific cell type is not terminal and can be altered when environmental conditions change (42).

Donlan and Costerton described that the nature of biofilm structure and the physiological attributes of biofilm organisms confer an inherent resistance to antimicrobial agents, whether these antimicrobial agents are antibiotics, disinfectants, or germicides. Mechanisms responsible for resistance may be one or more of the following: (43).

1) Delayed penetration of the antimicrobial agent through the biofilm matrix

Antimicrobial molecules must diffuse through the biofilm matrix in order to inactivate the encased cells. The extracellular polymeric substances constituting this matrix present a diffusional barrier for these molecules by influencing either the rate

of transport of the molecule to the biofilm interior or the reaction of the antimicrobial material with the matrix material (44).

2) Altered Growth Rate of Biofilm Organisms

Biofilm-associated cells grow significantly more slowly than planktonic cells and, as a result, take up antimicrobial agents more slowly (45).

3) Other Physiological Changes Due to Biofilm Mode of Growth

Conditions, which elicit the slowing of bacterial growth, such as nutrient limitation and increases in toxic metabolite concentrations that favor the formation of biofilms. (43, 46).

Biofilms of Bacillus species are recognized as a serious problem due to the fact that they can cause enhanced risks of health threats (47) and are important contaminants in many food industry settings, such as fresh product, poultry, dairy, and red meat processing (48). Therefore, biofilm formation of several *B. cereus* strains are currently being studied to prevent potential food contamination and to ensure safety during production (49). *Bacillus subtilis* is able to form robust biofilms that are demonstrated by highly structured floating pellicles that grow on the surface of liquid cultures and colonies that grow on agar plates (50).

***Xyris indica* L.**

Xyris indica L. is the accepted name of a species in the genus *Xyris* (family: Xyridaceae, order: Commelinales, class: Liliopsida, phylum: Tracheophyta, and kingdom: Plantae). It is a perennial herb which is grass-like with yellow flowers packed between yellowish brown spike bracts. Its Thai name is Kra thin thung. It is used to treat ringworm, constipation and flatulence. *Xyris indica* L. is distributed in south and Southeast Asia, Australia and India. (51). The phytochemical studies of *Xyris indica* L. flowering heads indicating that they contain two isocoumarins (xyridin A and xyridin B),

two sterols (stigmasterol and spinasterol) and three anthraquinones (Chrysazin as the major bioactive compound found in this plant (58.90%), 3-methoxychrysazin and 3-hydroxychrysazin) (52).

Chrysazin

Chrysazin is also known as 1,8-dihydroxyanthraquinone or Danthron. It is an anthraquinone compound $C_{14}H_6O_2(OH)_2$ that exists at room temperature as a reddish or orange crystalline powder. It is practically insoluble in water, soluble in acetone, chloroform, diethyl ether, and ethanol, and very soluble in alkaline hydroxide solutions. Chrysazin is stable under normal temperatures and pressures (53). The physical and chemical properties of chrysazin are shown in Table 1.

Table 1 Physical and chemical properties of Chrysazin (53)

Property	Information
Molecular weight	240.2
Specific gravity	1.54 g/cm^3
Melting point	193 °C
Boiling point	sublimes
Log K_{ow}	3.94
Water solubility	9 mg/l
Vapor pressure	7.6 × 10^{-11} mm Hg
Vapor density relative to air	8.3

Pharmacological and Toxicological Properties of Chrysazin

In 1988, Sjöberg and coworkers observed that groups of 30 male Sprague-Dawley rats, 50 days of age, received a single subcutaneous injection of 1,2-dimethylhydrazine (DMH) at 150 mg/kg bw. After one week, they were fed chrysazin (purity, ~ 97%) at 0, 60 or 240 mg/g diet, which the average daily intakes were

approximately 30 and 60 mg/kg bw. After 26 weeks, all of them were killed. Two additional groups of 30 male rats received either no treatment or were given the diet with the higher concentration of chrysazin alone. There was no significant difference in mean body weight gain between treated and control group. The rats that treated with DMH plus chrysazin, the combined incidences of intestinal adenomas and adenocarcinomas were 2/30 in the low-dose and 4/30 in the high-dose group. The incidences of intestinal adenocarcinomas were 0/30 in untreated controls, 0/30 in the group, which treated with chrysazin alone and 2/30 in the group, which treated with DMH alone. The difference in tumour incidence between the group, which treated with DMH alone and DMH plus chrysazin was not significant (54).

Later in 1989, Chrysazin used as laxative had an incident of the small intestine cancer related to the use of it in clinic. Patel and coworkers reported that the data available from studies in humans were inadequate to evaluate the relationship between human cancer and exposure specifically to chrysazin. That case report was identified the occurrence of cancer of the small intestine (leiomyosarcoma) in an 18-year-old girl with a history of prolonged exposure to chrysazin. In this case they observed a very rare tumor in a child who had prolonged exposure to chrysazin (since the age of 14 months, She was treated with the laxative Dorbanex (chrysazin 25 mg with poloxalkol 188-200 mg) 5 ml orally at night and this treatment was continued until her fifth or sixth year of age. Regular treatment was then discontinued but she took Dorbanex intermittently for constipation throughout the rest of her life. This clearly is not sufficient evidence to postulate a causal relationship. However, the biological and experimental background suggests that the possibility of a causal relationship should be considered. In rodents, the data are less extensive, with small numbers of animals, and they do not constitute conventional carcinogenicity studies (55).

In 2009, the effect of chrysazin on the cell migration and invasion of human brain glioblastoma multiforme was reported. Lin and coworkers observed that chrysazin inhibited the levels of matrix metalloproteinase-9 (MMP-9), rho-associated, coiled-coil-containing protein kinase 1 (ROCK-1), focal adhesion kinase (FAK) and urokinase plasminogen activator (uPA) leading to decreased proliferation, migration and invasion of human brain glioblastoma multiforme GBM 8401 cells in vitro. Thus, these findings indicated that chrysazin could serve as a therapeutic agent to inhibit migration and invasion in cancer cells of patients (56). Later in 2010, Rossi and coworkers suggested that chrysazin and quinizarin possessed significant antineoplastic properties, probably through the induction of intracellular transglutaminase activity (57). In 2013, Zhou and coworkers observed that chrysazin (0.1, 1, and 10 μmol/L) dose-dependently promoted the phosphorylation of AMPK and acetyl-CoA carboxylase (ACC) in both HepG2 and C2C12 cells. Chrysazin also effectively reduced intracellular lipid contents and enhanced glucose consumption *in vitro* via activation of AMPK signaling pathway (58).

For antimicrobial activity, flowering heads of *Xyris indica* L. from 15 different sources in Thailand were investigated for pharmacognostic specification by Khuniad and coworkers. They observed that the ethanol extracted chrysazins inhibited the growth of tested gram positive (*B. cereus* ATCC 11778, *B. subtilis* ATCC 6633 and *S. aureus* ATCC6538P) and gram negative bacteria (*Escherichia coli* ATCC25922) but not *P. aeruginosa* ATCC 9027 while the petroleum ether extracted chrysazins were only active against *B. cereus* ATCC 11778 (MIC= 250 μg/ml and MBC= 2000 μg/ml), and *B. subtilis* ATCC 6633 (MIC= 250 μg/ml and MBC= 1000 μg/ml). The ethanol extracted chrysazins displayed large inhibition zone on *B. cereus* and *B. subtilis* and showed the lowest MIC on *S. aureus* ATCC6538P (MIC= 125 μg/ml and MBC= >2000 μg/ml). The

MIC and MBC demonstrated that both of chrysazin extracts were bacteriostatic agent (10).

Silkworm

General Information

Silkworm is invertebrate's animal and scientific name is *Bombyx mori*. It is classified in the Phylum of Arthropoda, Class of Insect, Family of Bombycidae and Kingdom of Animalia. It is native to Asia that spins a cocoon of fine, strong, lustrous fiber, which is the source of commercial silk. The culture of silkworms is called sericulture. The various species are distinguished by the quality of the silk they produce. Silkworms feed on the leaves of the mulberries (genus *Morus*) and sometimes on the Osage orange (Maclura pomifera). The life cycle of a silkworm, the first stage of silkworm starts with a little egg laid by female moth. Then, the small eggs hatch and grow as 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. In the fifth instar, larva attains a maximum length of about 20-60 mm and it eats voraciously. It is interesting that the fifth instar larva body grows completely and actively functions. This stage has a long period about 20-24 days. It goes through 4 moths before reaching maturity and takes 48-72 hours to spin its cocoon. In 2-3 days it transforms into a pupa and another 10 days emerges as a moth. Emergence takes place in the morning when copulation also occurs. The female lays eggs from evening to next morning and dies within 4-5 days after emergence. The moth lays 500-700eggs (100 eggs weigh about 60mg). Under the natural cycle the silkworm which produces end generation a year is called univoltine, bivoltine if there are two and multivoltine if there are more. In thropical zones the common variety is multivoltine: under favourable rearing conditions the larvae are very resistant but they produce small cocoons and consequently the quality of the silk is lower than the temperate varieties which are usually uni- or bi-voltine (59).

The circulatory system and immune system in silkworm

The silkworm has an open circulatory system containing of hemocyte and hemolymph. The function of hemocyte is to phagocytosis, coagulation, and encapsulation of foreign objects such as parasite (60). Hemolymph is the circulating fluid of silkworm is similar to mammalian blood. Compared with mammalian blood, insect hemolymph differs in the absence of erythrocytes and has a high concentration of several types of free amino acids. It serves important roles in the immune system, lubricant for tissues, storage (amino acids and glycerol), protection (reflex bleeding) and in transport of hormones, nutrients, and metabolites (61). Hemolymph is composed of 90% Water, Inorganic ions such as Na, Ca, and K, Nitrogenous wastes, uric acid, Carbohydrates-Alpha trehalose (insect blood sugar), glucose, glycerol (antifreeze), Lipids, Proteins and enzymes (MFO's), Antifreeze proteins, Pigments and Hormones (60).

Tanaka and Yamakawa reported that insects possessed an effective innate immune system against foreign microorganisms. Innate immunity of insects is divided into two major reaction types: humoral and cellular reactions. Humoral reactions involve soluble proteins in the hemolymph such as phenoloxidase, antimicrobial proteins (AMPs), lysozymes, and lectins, whereas hemocytes mediate cellular reactions such as phagocytosis, encapsulation and nodule formation. In *Bombyx mori*, six different families of AMPs have been identified: Cecropin, Attacin, Lebocin, Moricin, Gloverin, and Defensin. One lysozyme and three lysozyme-like proteins, one of which is involved in elimination of invading pathogens, are also found in the silkworm. Both lysine-containing peptidoglycan (Lys-PGN) and meso-diaminopimelic acid containing peptidoglycan (DAP-PGN) trigger expression of AMP genes, probably through the Toll and IMD pathways, respectively. DAP-PGN has stronger elicitor activity than Lys-PGN in Bombyx mori because of the difference in transcriptional activity between BmRelishes

and BmRels, which are effectors of the IMD and Toll pathways, respectively. Furthermore, two recognition proteins and a serine protease are involved in activation of prophenoloxidase for melanization, and several C-type lectins, which participated in cellular reactions, were identified in Bombyx mori. Moreover, a paralytic peptide was reported to play important roles in silkworm immunity (62).

Silkworm infection model

Hamamoto and coworkers demonstrated that silkworms were big enough to be used in injection experiments, preparations of the hemolymph to be made, and organs such as midgut to be isolated. The volume of the hemolymph of a silkworm with a body weight of 1.8 g was estimated to be 0.67 ml. These are necessary methods for studying the pharmacodynamics of drugs in individual bodies. The lower cost and smaller space required for the maintenance of silkworms compared to mice or rat allow us to handle a larger number of animals in limited facilities. Owing to the long history of the silk industry, the method for taking care of silkworms is well established; therefore, silkworms have the potential to serve as a large-scale drug-screening system (63).

Routes of drug administration can be well controlled in silkworms, the first route is Intra hemolymph, that is similar to the intravenous injection in mouse and the second route is Intra midgut that is similar to oral administration in mouse (64).

In a previous study by Kaito and coworkers, they examined silkworm as an animal model of human infection with pathogenic bacteria. They observed that when 3×10^7 cells of Staphylococcus aureus (*S. aureus*), Pseudomonas aeruginosa, and Vibrio cholerae were injected into the hemolymph of fifth instar silkworm larvae, over 90% of silkworms died within 2 days, whereas over 90% survived for 5 days after injection of the same amount of *Escherichia coli*. Growth of *S. aureus* was observed in larvae blood and tissues, which Immunostaining analysis revealed that *S. aureus* proliferated

at the surface of the midgut. Furthermore, infection of silkworm larvae by methicillin-sensitive *S. aureus* was cured by ampicillin, oxacillin, and vancomycin, whereas infection by methicillin-resistant *S. aureus* was not cured by ampicillin or oxacillin, although vancomycin was effective. In addition to disinfectants were not effective because of toxicity against the larvae. They suggested that silkworm are useful for evaluating antibiotics for pathogenic bacterial infection in humans (65).

Hamamoto and coworkers had proposed that the silkworm larva was an excellent animal model to evaluate the therapeutic effects of antibiotics. In this report, they selected compounds that do not require specific transporters and examined the transport rates of these compounds through the silkworm larva midgut membrane. The transport rate of antibiotics that are transported via a non-specific route in the silkworm midgut is compared with bioavailability in humans and with permeability in an intestinal epithelial cell culture model, Caco-2, in Table 2.

Table 2 Comparison of antibiotics transported via non-specific transport in silkworm larva midgut model and in other models (66).

	Silkworm: transport (% of total/30 min)	**Humans: oral Bioavailability (%)**	**Caco-2 cells: P_{app} ($\times 10^{-5}$ cm/s)**
Tetracycline	1.3	70	0.162
CFPN-PI	1.2	40	0.785
Vancomycin	<1	<1	<0.01

The permeability of the compounds through the silkworm midgut was dependent on both their molecular mass and their hydrophobicity, as is the case in the mammalian intestine. Therefore, the absorption mechanism in the midgut (intestine) via a non-specific pathway might be similar between silkworm larvae and mammals, although the structures are morphologically different. Antibiotics such as vancomycin (MW1485 Da) and kanamycin (MW484 Da) do not permeate the midgut and have no therapeutic effect in the silkworm larva infection model owing to their hydrophilicity and high molecular mass, consistent with their lack of effectiveness when administered orally. Compounds with a molecular mass less than 400 Da might pass through a paracellular pathway (66).

In 2009, Hamamoto and coworkers evaluated the feasibility of using the silkworm as an animal model for screening drug candidates. They observed that the lethal dose levels of cytotoxic chemicals in silkworms were consistent with those in mammals. Furthermore, they examined the fate of model drugs such as 4-methyl umbelliferone, umbelliferone, and 7-ethoxycoumarine in silkworm larvae. The half-life of 4-methyl umbelliferone in the hemolymph of silkworm was 7.0±0.1 min, similar to that in mouse blood. In silkworm larvae, 4-methyl umbelliferone was conjugated with glucose, whereas in mammals it is conjugated with glucuronate or sulfate. Their results are consistent with a previous report that UDP-glucosyltransferase catalyzes the conjugation of 4-methyl umbelliferone. The glucose-conjugation reaction of 4-methyl umbelliferone was observed in microsomal fractions of fat bodies isolated from silkworms. Moreover, most umbelliferone and 7-ethoxycoumarine injected into the hemolymph of silkworms was eliminated through the feces in the glucose-conjugated form. They suggested that chemicals are metabolized through a pathway common to both mammals and silkworms: reaction with cytochrome P450, conjugation with hydroxylated compounds, and excretion (67).

In recent studies of silkworm infection model, Usui and coworkers performed an acute oral toxicity test of 59 compounds in silkworms. These compounds are listed in OECD guidelines as standard substances for a cytotoxicity test and acute oral LD_{50} values in mammals are listed in OECD guidelines. They observed that R^2 for the correlation between LD_{50} values in mammals and LD_{50} values in silkworms was 0.66. In addition, the acute oral toxicity test in silkworms was performed by two different facilities, and test results from the facilities were highly reproducible. Their findings suggested that an acute oral toxicity test in silkworms is a useful way to evaluate the toxicity of compounds in mammals (68).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Bacterial strain

Bacillus cereus (ATCC 11778) and *Bacillus subtilis* subsp. spizizenii (ATCC 6633) were provided by College of Public Health Sciences, Chulalongkorn University. Furthermore, *Staphylococcus aureus* (ATCC 29213) was used for quality control.

2. Chrysazin and gentamicin

Chrysazin stock solution was prepared at 1,000 µg/ml in 10% dimethyl sulfoxide (DMSO) by dissolving 0.1 g of Chrysazin powder (96% purity, Sigma-Aldrich, USA) in 10 ml of Dimethyl sulfoxide (99.9% purity, Labscan, Thailand), then 1 ml of the solution was added in the flask, which was containing of normal saline solution 9 ml. Gentamicin stock solution was prepared at 1,000 µg/ml in DI water by taking 0.25 ml of gentamicin 40 mg/ml (the original stock) and adding 9.75 ml of DI water.

3. Silkworms

Thai silkworm, the first day of fifth-instar larvae weighting about 1 g/larvae and were supplied by The Queen Sirikit Department of Sericulture, under the Ministry of Agriculture and Cooperatives.

4. Chemicals

4.1 70% Ethyl alcohol

4.2 1% (v/v) Crystal violet

4.3 Dimethyl sulfoxide (Labscan, Thailand)

4.4 Gentamicin injectable solution (T.P. Drug Laboratories Co.,Ltd., Thailand)

4.5 Glacial acetic acid (BDH Laboratory, England)

4.6 Methanol (RCI Labscan, Thailand)

4.7 Mueller Hinton Agar (BBL, USA)

4.8 Mueller Hinton Broth (BBL, USA)

4.9 0.9% NaCl

4.10 Silkmate

5. Instruments

5.1 Stainless alcohol lamp

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

5.3 Cylinders

5.4 1 milliliter Disposable syringe (Nipro, Thailand)

5.5 Glass tank

5.6 Hot air oven (WTB binder, Germany)

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

5.8 Incubator (Memmert, Germany)

5.9 Larminar airflow cabinet (Astec, Thailand)

5.10 Loop sterilizer (Lab Scientific, USA)

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

5.12 Microplate reader (Lab Scientific, USA)

5.13 Multichannel pipette (Biohit, Finland)

5.14 Petri dish (Pyrex, USA)

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

5.16 Plastic cuvette 2.5-4.0 ml (NP Chemical, Thailand)

5.17 Shaker bath

5.18 Spreader

5.19 UV/Visible spectrophotometer

5.20 96 Well microtiter plate (Costar, USA)

5.21 Inoculating loop

Methods

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

Inoculum preparation for dilution tests

Prepare the inoculum by streaking *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 29213) on Muller-Hinton agar medium (MHA). Then, incubated at 37°C for 24 hours. After incubation, the well-isolated colonies of the same morphological type from an agar plate culture of each bacterium were selected and transferred into a tube containing 10 mL of normal saline solution (NSS). The turbidity of bacterial suspensions were adjusted to an optical density of 0.08-0.10 (corresponding to 0.5 McFarland) at a wavelength of 625 nm, using UV/Visible spectrophotometer (equivalent to 1×10^8 CFU/ml). This suspension was diluted 1:100 with Mueller Hinton Broth (MHB) medium, then inoculums containing approximately 1×10^6 CFU/ml (69).

1.1 Determination of the minimal inhibitory concentration (MIC) by Broth

1.1.1 Microdilution method

(n=2, experiments were performed in triplicate)

MICs were determined using a microdilution method recommended by the M07-A9 document (69). One hundred microliters of each concentration of serially 2-folded dilution of chrysazin stock solution were added into the wells number 1-12 of the 96-well microplate (the final concentration of chrysazin ranging from 0.24-500 µg/ml). Then, one hundred microliters of freshly bacterial suspensions from procedure 1.1 were added and mixed in each well (the final concentration of bacteria were 5×10^5 CFU/ml in all wells). In other wells, it consisted of control procedures, such as growth control, which contained 100 µl of bacterial suspensions from procedure 1.1 with 100 µl of MHB medium. Negative control contained 100 µl of 10% DMSO (solvent of chrysazin solution) or 100 µl of Sterile distilled water with 100 µl of bacterial suspensions from procedure 1.1. Positive controls contained 100 µl of gentamicin 0.12 µg/ml with 100 µl of bacterial suspensions from procedure 1.1. All of these test plates were cultured at 37°C for 24 hours. The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibits visible growth of the organism in the microdilution wells. In addition, *Staphylococcus aureus* (ATCC 29213) is used for quality control strain (70).

1.1.2 Determination of the minimal bactericidal concentration (MBC) (n=2, experiments were performed in triplicate)

The suspension in the wells from MIC and higher concentrations (from the wells that showed no apparent bacteria growth) were spotted on MHA medium. Then, incubated all plates at 37°C for 24 hours. The lowest concentration of chrysazin that prevented any growth of bacteria was recorded as the MBC of chrysazin. (99.9% killing).

1.1.3 Evaluation of bactericidal and bacteriostatic property

MBC/MIC ratios were calculated using MIC and MBC from 1.2 and 1.3 to determine the action of chrysazin on the bacterial strains. If the ratio MBC/MIC was equal to 1 or 2, chrysazin was considered to have bactericidal effect against *B. cereus* and *B. subtilis*. On the other hand, if the ratio was greater than or equal to 4, chrysazin was considered to have bacteriostatic effect against *B. cereus* and *B. subtilis* (71).

1.2 Determination of time to kill bacteria by Time - Kill Assay

(n=2, experiments were performed in triplicate)

Bacteria were grown overnight at 37°C in MHB medium as well as procedure 1.1. The overnight broth were adjusted to an optical density of 0.08-0.10 (corresponding to 0.5 McFarland) at a wavelength of 625 nm, by using UV/Visible spectrophotometer (equivalent to 1×10^8 CFU/ml). Bacterial suspensions were diluted with MHB medium (1:100) to make approximately 1×10^6 CFU/ml. A stock solution of positive control (gentamicin) and chrysazin were diluted with sterile distilled water and 10% DMSO, respectively to concentrations as exact multiples of the MIC value at 1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, 1 MIC, and 2 MIC for gentamicin as well as 1/2 MIC, 1 MIC, 2 MIC, 4 MIC, and 8 MIC for chrysazin. Each isolate was inoculated in to sterile test tubes that containing of positive control (gentamicin, which the concentration ranging

from 0.0075 - 0.24 µg/ml) or chrysazin (the concentration ranging from 3.91-125 µg/ml). While the growth control consisted of bacterial suspensions with MHB medium and the negative control consisted of 10% DMSO (solvent of chrysazin solution) with bacterial suspensions. All of these test tubes were incubated with shaking on shaker water bath at 37°C. One hundred microliters of an aliquot was removed from the tube at 0, 2, 4, 6, 8, 10, 12 and 24 hour and diluted serially. Finally, 100 µl of the serial diluted samples were spread on MHA plates. Then incubated at 37°C, for 24 hour for the determination of surviving bacteria. Data were analyzed as killing curves by plotting the log10 colony forming unit per milliliter (CFU/ml) versus time (hours) as well as the change in bacterial concentration was determined. (72, 73). The time-kill end point determination as the antibiotics that reduced the original inoculum by ≥3 $\log_{10}$ were considered bactericidal, whereas antibiotics that reduced the original inoculum by ≤2 $\log_{10}$ were considered bacteriostatic (74).

1.3 Inhibition of biofilm formation assay

(n=2, experiments were performed in triplicate)

Freshly bacterial suspensions from procedure 1.1 were diluted with 1.5% glucose of MHB (1:100) (inoculums approximately 1.0 x 10^6 CFU/ml). The 96-well plate were filled with 100 µl of each strain and 100 µl of chrysazin (the concentration ranging from 3.91-500 µg/ml) or 100 µl of positive control (Gentamicin, which the concentration ranging from 0.06-3.91 µg/ml) that the concentrations as exact multiples of the MIC value at 1/2MIC, 1MIC, 2MIC, 4MIC, 8MIC, 16MIC and 32MIC. While the growth control consisted of 100 µl of bacterial suspensions with 100 µl of 1.5% glucose of MHB (1:100) medium and the negative control consisted of 100 µl of 10% DMSO (solvent of chrysazin solution) with 100 µl of bacterial suspension. All of these test plates were incubated at 37°C for 24 hour. The medium were removed after 24 hour and the wells were washed three times with 300 µl of sterile deionized water. Bacteria

were fixed with 250 µl of methanol per well. After 15 min, test plates were emptied and air dried. Biofilm cells were stained with 250 µl of 1% crystal violet for 15 min. The wells were washed twice with 250 µl sterile deionized water. 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 (75). The data were analyzed using the following formula.

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

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

2.1 Pathogenicity study of bacteria in silkworms

(Experiments were performed in triplicate)

Silkworms were fed with an antibacterial-free artificial food for one day before the experiment began. The first day of fifth-instar larvae (n=10) were injected with 50 µl of bacterial suspensions ranging from 1×10^1 to 1×10^8 CFU/ml into hemolymph. While the control group (n=10) was injected with 50 µl of normal saline solution into hemolymph. The mortality rate of silkworms were observed at 48 hour after injection, then determine the lethal dose 50 (LD_{50}). LD_{50} is the amount of Bacterial suspension, given all at once, which causes the death of 50% (one half) of a group of silkworm (65).

2.2 Determination of LD_{50} of chrysazin

(Experiments were performed in triplicate)

Silkworms were fed with an antibacterial-free artificial food for one day before the experiment began. The first day of fifth-instar larvae (n=10) were injected with 50 µl of chrysazin solution (dissolved in 5% DMSO) at concentration 5, 25, 125, 625, and 1,000 µg/ml. While the control group (n=10) was injected with 50 µl of 5% DMSO into

hemolymph. The mortality rate of silkworms were observed at 48 hour after injection. Determined the lethal dose 50 (LD_{50}). LD_{50} is the amount of chrysazin, given all at once, which causes the death of 50% (one half) of a group of test silkworms. LD_{50} was calculated by using Graph Pad Prism v5.0 through non-linear regression. The LD_{50} of positive control (gentamicin was dissolved in DI water at concentration 0.01, 0.06, 0.32, 1.60, 8.00, 40.00, 200.00, and 1,000 µg/ml) was performed the same experiment as determined the LD_{50} of chrysazin. While the control group (n=10) was injected with 50 µl of DI water (65).

2.3 Determination of ED_{50} of Chrysazin

(Experiments were performed in triplicate)

Silkworms were fed with an antibacterial-free artificial food for one day before the experiment began. Suspension of bacteria that kill all silkworms from the previous experiment in procedure 2.1 (*B. cereus* 1×10^8 CFU/ml and *B. subtilis* 1×10^6 CFU/ml) 50 µl were injected into hemolymph of the first day of fifth-instar larvae (n=10). Chrysazin solution (dissolved in 5% DMSO) at concentration 5, 25, 125, 625, and 1,000 µg/ml as well as positive control (gentamicin solution, which dissolved in DI water at concentration 0.01, 0.06, 0.32, 1.60, 8.00, 40.00, 200.00, and 1,000 µg/ml) 50 µl were injected into hemolymph immediately after injection of bacterial suspension. While the negative control group (n=10) was injected with 50 µl of 5% DMSO into hemolymph immediately after injection of bacterial suspension. The number of surviving silkworms were observed at 48 hour after injection. Determined the mean effective dose (ED_{50}). ED_{50} is the amount of a drug that is therapeutic in 50 percent of silkworms in which it is tested (65). ED_{50} was calculated by using Graph Pad Prism v5.0 through non-linear regression.

Statistical Analysis

Data from *in vivo* studies were shown as Mean ± SEM. Statistical significance between groups was evaluated using one-way ANOVA. A P-value of <0.05 was considered statistically significant.

CHAPTER IV

RESULTS

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

1.1 Determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC)

Antimicrobial susceptibility testing of chrysazin was performed by using broth microdilution method recommended by the M07-A9 document (CLSI, 2012). The overall results were represented as three independent experiments that they are presented in Table 3.

Table 3 *In vitro* antibacterial activity of Chrysazin against *B. cereus* and *B. subtilis*

Substances	Bacteria	MIC (μg/ml)	MBC (μg/ml)	MBC/MIC
Chrysazin	*B. cereus* (ATCC 11778)	7.81	>500	>64
Gentamicin (Positive Control)	*B. cereus* (ATCC 11778)	0.12	0.12	1
Chrysazin	*B. subtilis* (ATCC 6633)	15.63	>500	>32
Gentamicin (Positive Control)	*B. subtilis* (ATCC 6633)	0.12	0.12	1
Gentamicin (Quality Control)	*S. aureus* (ATCC 29213)	0.06	0.06	1

From the results, they are indicated that chrysazin exhibited antibacterial activities against *B. cereus* ATCC 11778 and *B. subtilis* ATCC 6633, with the MIC values

of 7.81 and 15.63 µg/ml, respectively. Therefore, *B. cereus* was more susceptible to chrysazin than *B. subtilis*. The MBC values of chrysazin against both microorganisms were greater than 500 µg/ml. Then, MBC/MIC ratios of chrysazin against *B. cereus* ATCC 11778 and *B. subtilis* ATCC 6633 were greater than 64 and 32, respectively. To control of this study, we used gentamicin as the positive control, and *S. aureus* ATCC 29213 as the quality control. MIC and MBC values of gentamicin against both microorganisms were 0.12 µg/ml. Moreover, MIC and MBC values of gentamicin against *S. aureus* ATCC 29213 were 0.06 µg/ml. In addition to MBC/MIC ratios of gentamicin against *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, and *S. aureus* ATCC 29213 were equal to 1.

1.2 Determination of time to kill bacteria by Time - Kill Assay

The results were presented in Figure 1 - 4 as the killing curves by plotting between the $\log_{10}$ colony forming unit per milliliter (CFU/ml) and time (hours). The Time-kill end point determination as the antibiotics that reduced the original inoculum by ≥3 $\log_{10}$ were considered bactericidal, whereas antibiotics that reduced the original inoculum by ≤2 $\log_{10}$ were considered bacteriostatic (74).

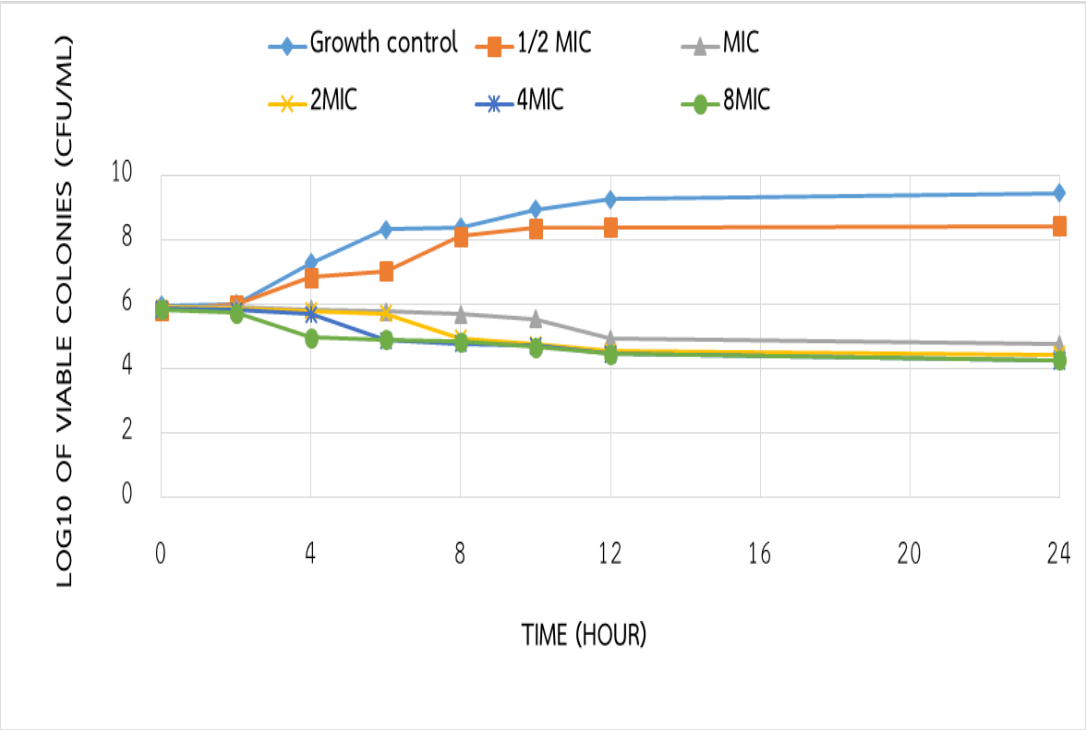

Figure 1 Time kill curve of chrysazin against *B. cereus*. Data were the means of triplicate experiments.

In figure 1, we summarized that chrysazin possessed bacteriostatic effect against *B. cereus* ATCC 11778, since the reduction of the original inoculum was ≤2 log10. The results showed the time in which chrysazin started inhibiting *B. cereus* ATCC 11778 in approximately 1 log scale reduction compared with the original inoculum, such as chrysazin 7.81 µg/ml (1 MIC), 15.63 µg/ml (2 MIC), 31.25 µg/ml (4 MIC), and 62.50 µg/ml (8 MIC) were at 12, 8, 6, and 4 hours, respectively.

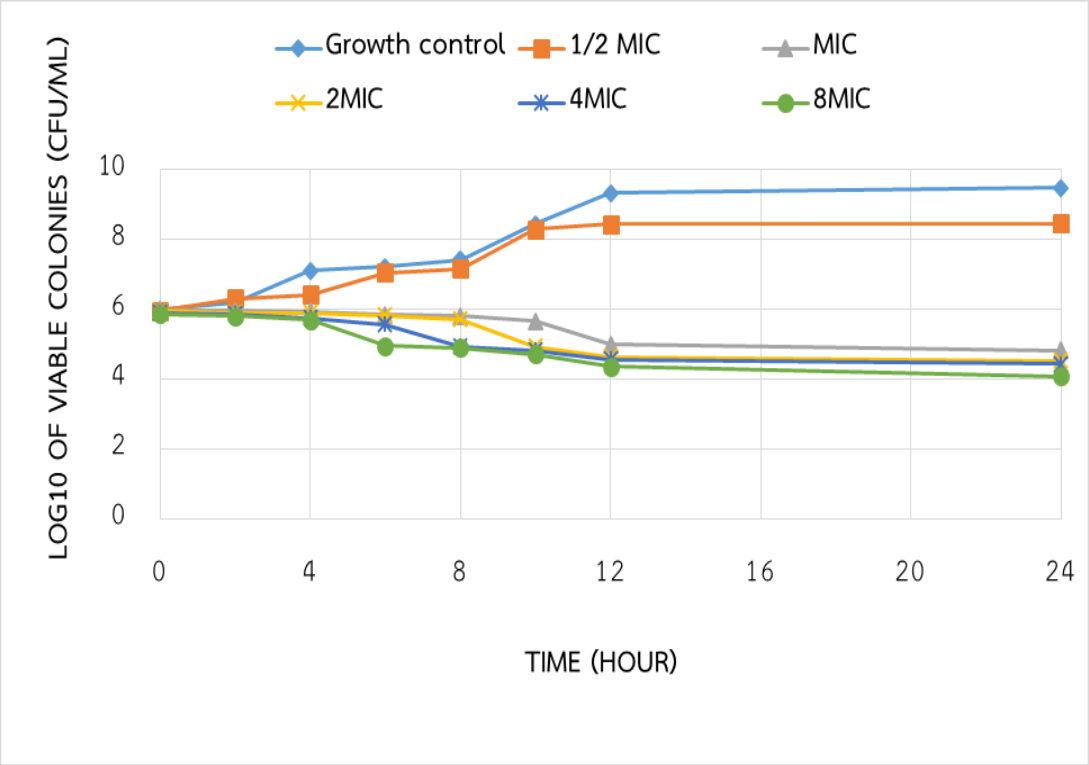

Figure 2 Time kill curve of chrysazin against *B. subtilis*. Data were the means of triplicate experiments.

The results showed the time in which chrysazin started inhibiting *B. subtilis* ATCC 6633 in approximately 1 log scale reduction compared with the original inoculum at concentrations of 15.63 µg/ml (1 MIC), 31.25 µg/ml (2 MIC), 62.50 µg/ml (4 MIC), and 125 µg/ml (8 MIC) were 12, 10, 8, and 6 hours, respectively. We can summarized that chrysazin possessed bacteriostatic effect against *B. subtilis* ATCC 6633, since the reduction of the original inoculum was ≤2 $\log_{10}$.

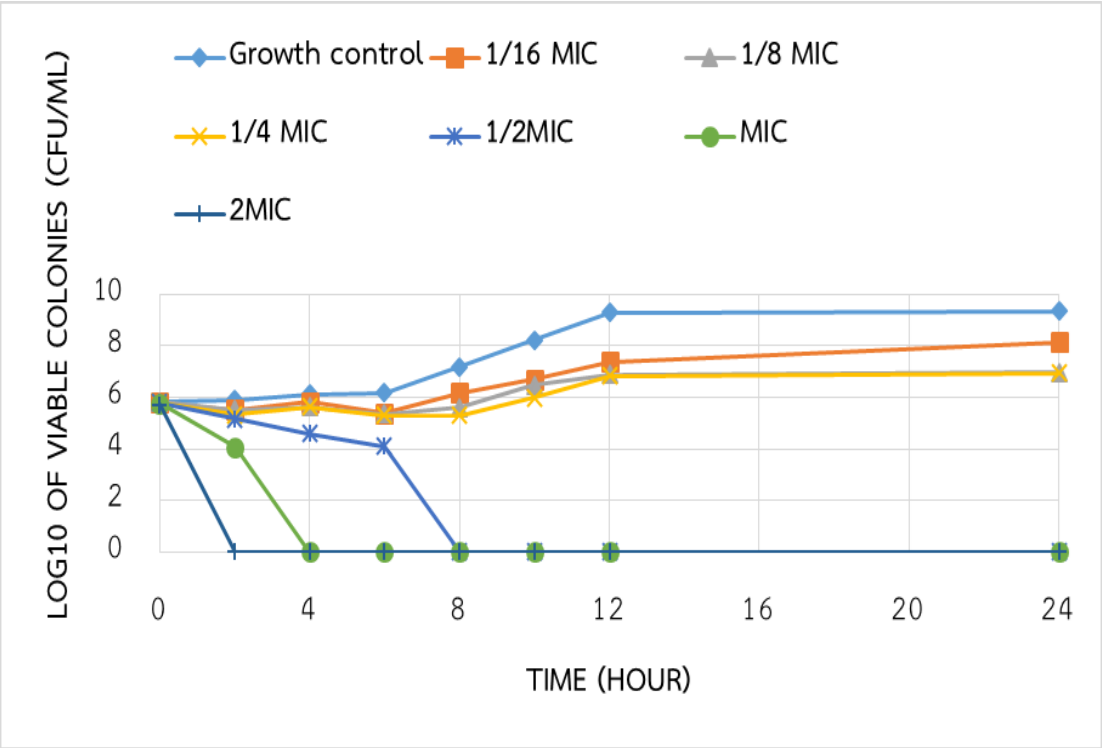

Figure 3 Time kill curve of gentamicin (positive control) against *B. cereus*. Data were the means of triplicate experiments.

Figure 3, we summarized that gentamicin possessed bactericidal effect against *B. cereus* ATCC 11778, since the reduction of the original inoculum was ≥3 $\log_{10}$. The results showed the time in which gentamicin killed *B. cereus* ATCC 11778 in approximately ≥3 log scale reduction compared with the original inoculum at concentrations of 0.06 µg/ml (1/2 MIC), 0.12 µg/ml (MIC), 0.24 µg/ml (2 MIC) were 8, 4, and 2 hours, respectively.

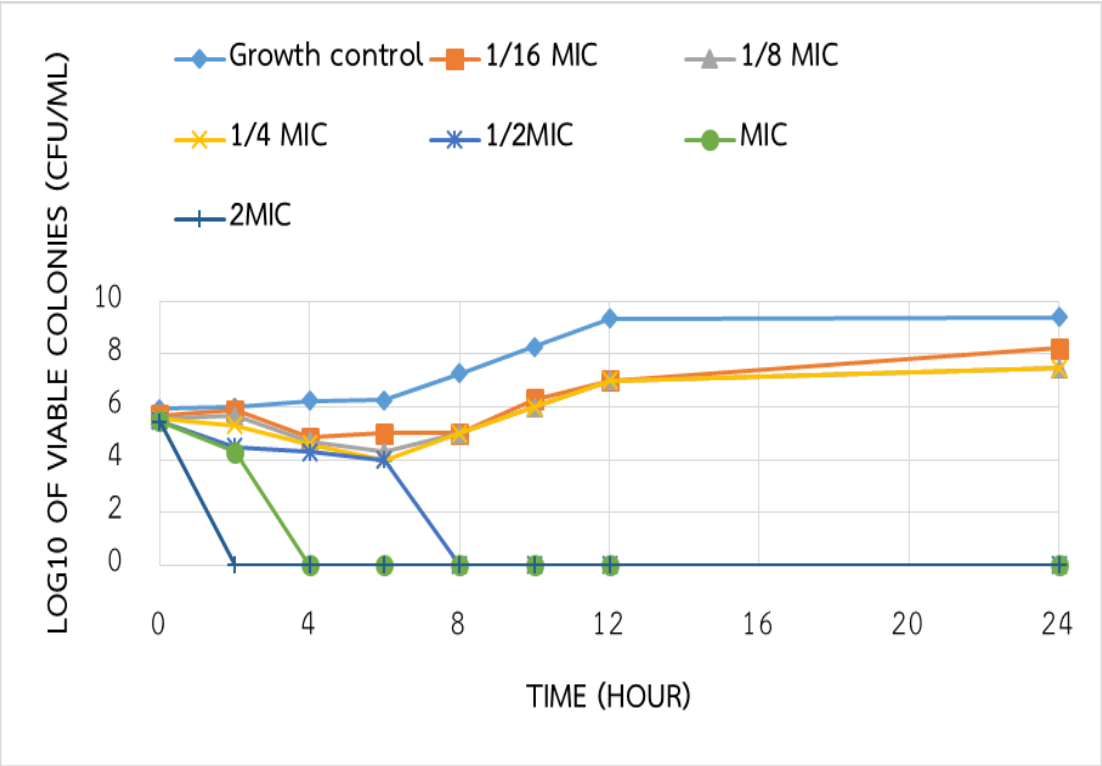

Figure 4 Time kill curve of gentamicin (positive control) against *B. subtilis*. Data were the means of triplicate experiments.

The results showed the time in which gentamicin killed *B. subtilis* ATCC 6633 in approximately ≥3 log scale reduction compared with the original inoculum at concentrations of 0.06 µg/ml (1/2 MIC), 0.12 µg/ml (MIC), 0.24 µg/ml (2 MIC) were 8, 4, and 2 hours, respectively. We can summarize that gentamicin possessed bactericidal effect against *B. subtilis* ATCC 6633.

1.3 Inhibition of biofilm formation assay

The antibiofilm potentials of chrysazin and gentamicin, which were used as positive control were analyzed at various concentrations (1/2 - 32 MIC). The results were expressed in terms of the percentage of biofilm inhibited in comparison to negative control group, which were displayed in Figure 5-8.

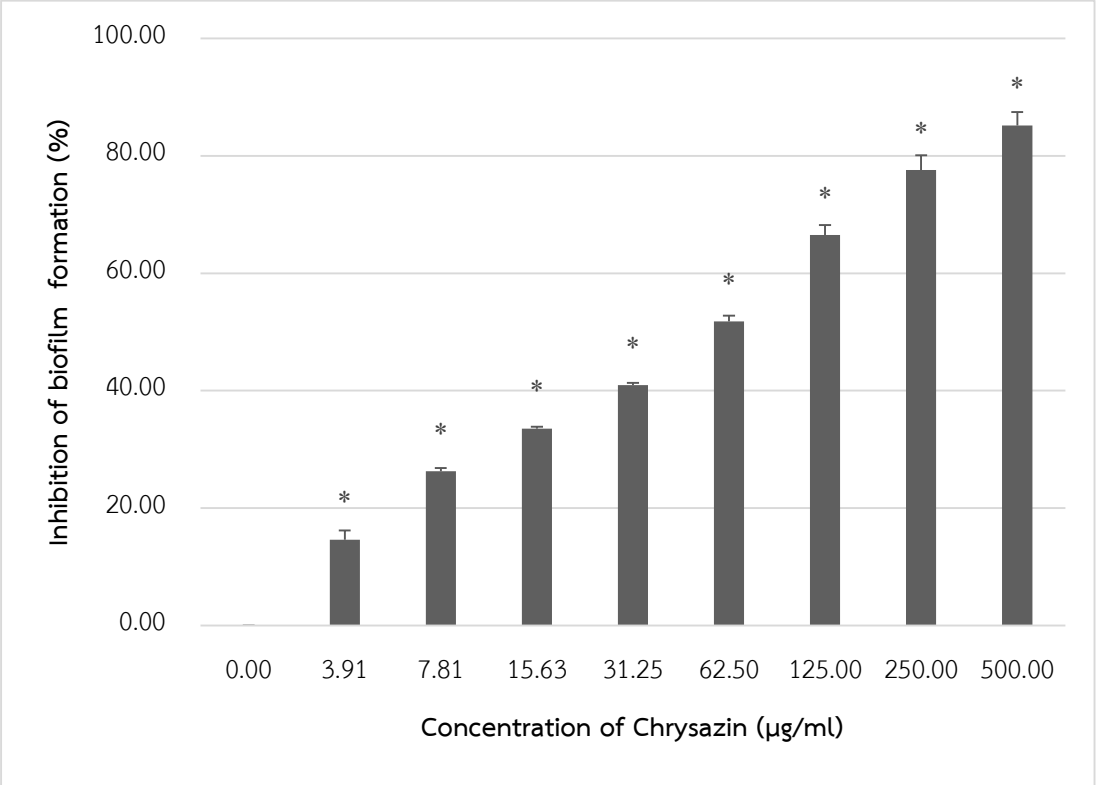

Figure 5 The inhibitory effect on the biofilm formation of chrysazin against *B. cereus*, Error bars represent the mean ± SEM, *p<0.05; chrysazin groups were compared with negative control group (5% DMSO). (0.00 µg/ml = control, 3.91 µg/ml = 1/2MIC, 7.81 µg/ml = 1MIC, 15.63 µg/ml = 2MIC, 31.25 µg/ml = 4MIC, 62.50 µg/ml = 8MIC, 125.00 µg/ml = 16MIC, 250.00 µg/ml = 32MIC, and 500.00 µg/ml = 64MIC).

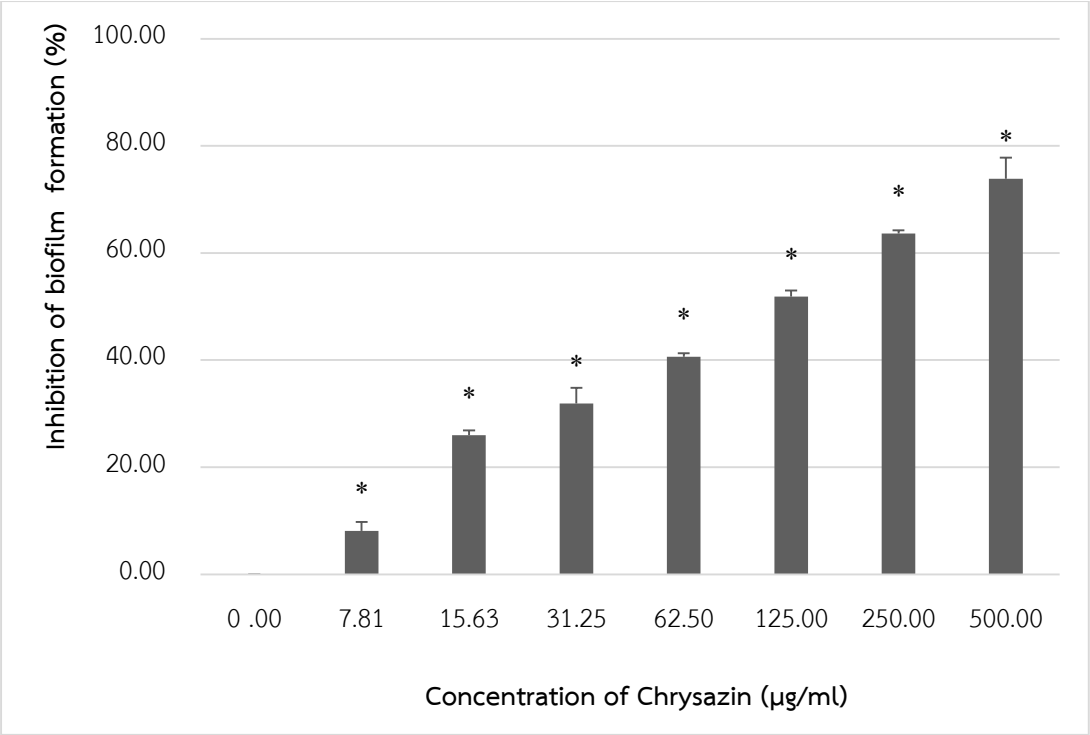

Figure 6 The inhibitory effect on the biofilm formation of chrysazin against *B. subtilis*, Error bars represent the mean ± SEM, *p<0.05; chrysazin groups were compared with negative control group (5% DMSO). (0.00 µg/ml = control, 7.81 µg/ml = 1/2MIC, 15.63 µg/ml = 1MIC, 31.25 µg/ml = 2MIC, 62.50 µg/ml = 4MIC, 125.00 µg/ml = 8MIC, 250.00 µg/ml = 16MIC, and 500.00 µg/ml = 32MIC).

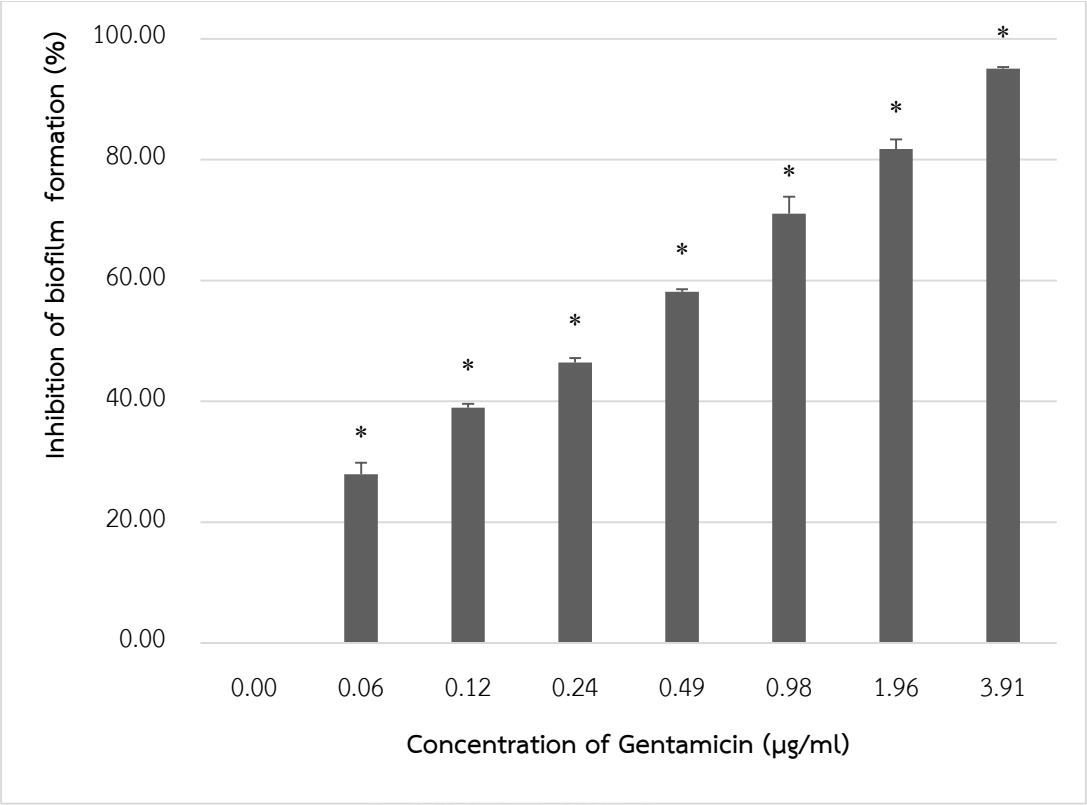

Figure 7 The inhibitory effect on the biofilm formation of gentamicin (positive control) against *B. cereus*, Error bars represent the mean ± SEM, *p<0.05; gentamicin groups (positive control) were compared with negative control group (DI water). (0.00 µg/ml = control, 0.06 µg/ml = 1/2MIC, 0.12 µg/ml = 1MIC, 0.24 µg/ml = 2MIC, 0.49 µg/ml = 4MIC, 0.98 µg/ml = 8MIC, 1.96 µg/ml = 16MIC, and 3.91 µg/ml = 32MIC).

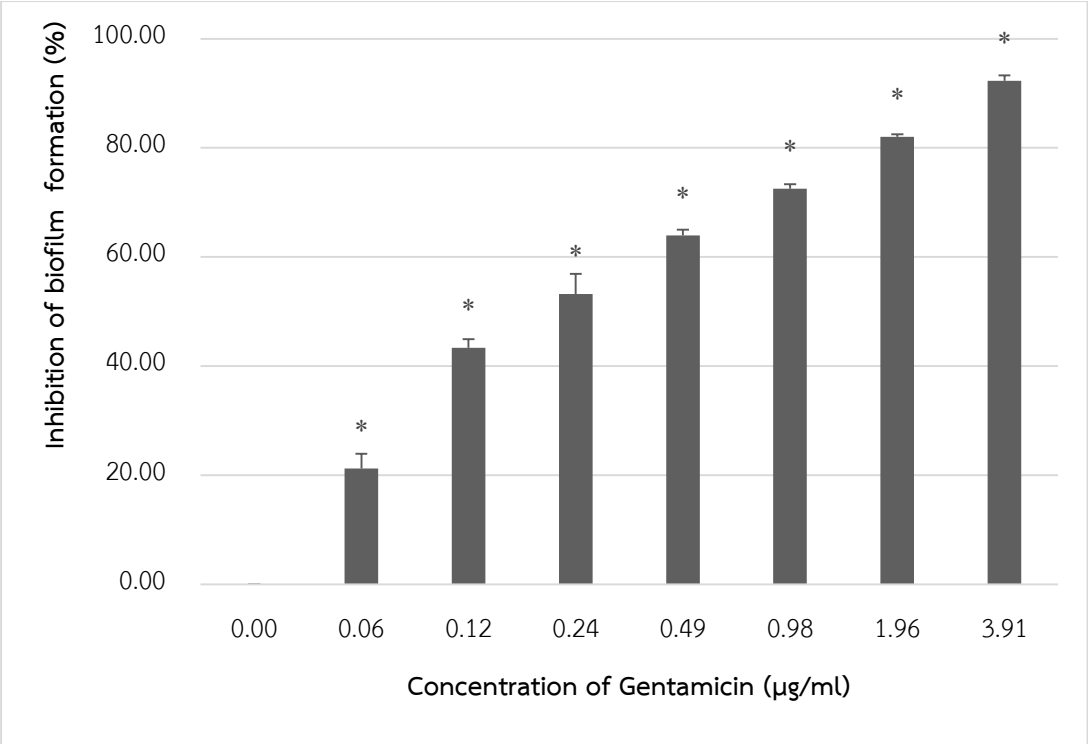

Figure 8 The inhibitory effect on the biofilm formation of gentamicin (positive control) against *B. subtilis*, Error bars represent the mean ± SEM, *p<0.05; gentamicin groups (positive control) were compared with negative control group (DI water). (0.00 µg/ml = control, 0.06 µg/ml = 1/2MIC, 0.12 µg/ml = 1MIC, 0.24 µg/ml = 2MIC, 0.49 µg/ml = 4MIC, 0.98 µg/ml = 8MIC, 1.96 µg/ml = 16MIC, and 3.91 µg/ml = 32MIC).

In Figure 5-8, the results exhibited in the same manner. We summarized that chrysazin and gentamicin (positive control) inhibited biofilm formation of both microorganisms in a concentration dependent manner. Chrysazin at the highest concentration (500 µg/ml) showed the maximum inhibition on biofilm formation with the percentage of inhibition for *B. cereus* and *B. subtilis* 85.15% and 73.86%, respectively, whereas gentamicin at the highest concentration (32 MIC) showed the maximum inhibition on biofilm formation with the percentage of inhibition for *B. cereus* and *B. subtilis* 95.05% and 92.29%, respectively.

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

2.1 Pathogenicity study of bacteria in silkworms

This study aims to determine the ability of *B. cereus* and *B. subtilis* which are the known human pathogens to cause disease and kill silkworm at last. The results were presented in Figure 9 and 10 as the sigmoid curves by plotting between the percent mortality of silkworms and Log concentration of *B. cereus* (CFU/ml). LD_{50} was calculated by using Graph Pad Prism v5.0 through non-linear regression. The data were fit to the equation $Y = D + (A - D)/(1 + 10^{((\log C - X)*b}$. For the four parameters in logistic equation, Y is the observed effect, D is the bottom, A is the top, C is the LD_{50}, X is the log of the concentration and b is the Hill slope. (76).

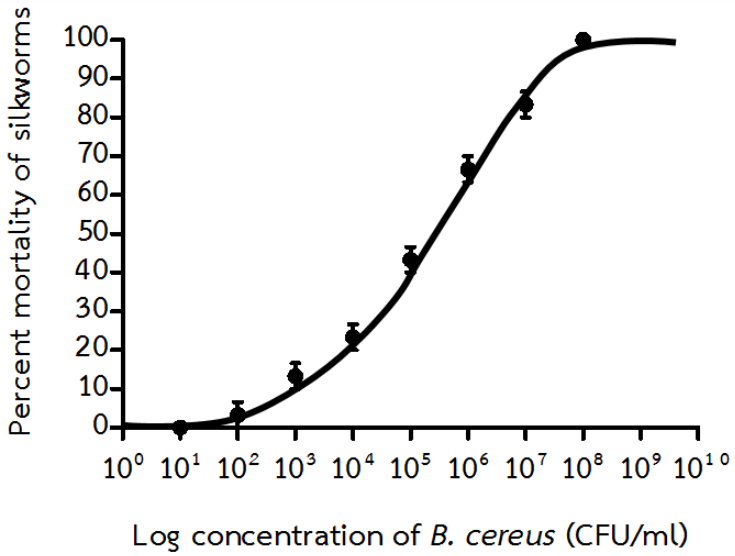

Figure 9 Pathogenicity of *B. cereus* in silkworms. The lethal dose 50 (LD_{50}) was 2.24×10^5 CFU/ml.

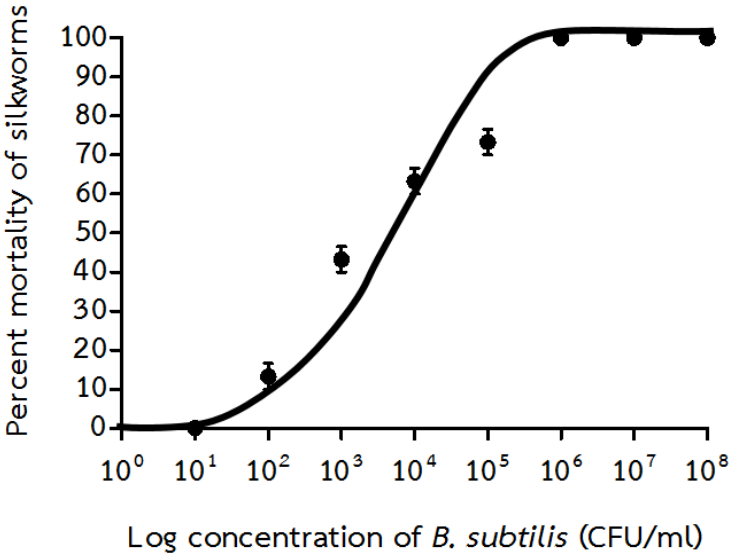

Figure 10 Pathogenicity of *B. subtilis* in silkworms. The lethal dose 50 (LD_{50}) was 8.16×10^3 CFU/ml.

2.2 Determination of LD_{50} of chrysazin in silkworms

We determined an acute toxicity of chrysazin in silkworms by the investigation of LD_{50} that measured of the toxicity of chrysazin that kill half of the test silkworms. The result showed that the value of LD_{50} for chrysazin and positive control (gentamicin) in silkworms were greater than 1,000 µg/ml. Therefore, chrysazin and gentamicin were safe in silkworms.

Table 4 Lethal dose 50 (LD_{50}) of chrysazin

Substances	LD_{50} (µg/ml)
Chrysazin	>1,000
Gentamicin (Positive control)	>1,000

2.3 Determination of ED_{50} of chrysazin

The median effective dose (ED_{50}) was used in tests as the quantitative analysis of chrysazin that produced the wanted result or effect occur in half of the test silkworms. The results were presented in Figure 11-14 as the sigmoid curves by plotting between the percent survival of silkworms and concentration of chrysazin (µg/ml) as well as were determined by Graph Pad Prism. ED_{50} was calculated by using Graph Pad Prism v5.0 through non-linear regression. The data were fit to the equation $Y = D + (A - D)/(1 + 10^{(\log C - X)*b}$. For the four parameters in logistic equation, Y is the observed effect, D is the bottom, A is the top, C is the ED_{50}, X is the log of the concentration and b is the Hill slope. (76).

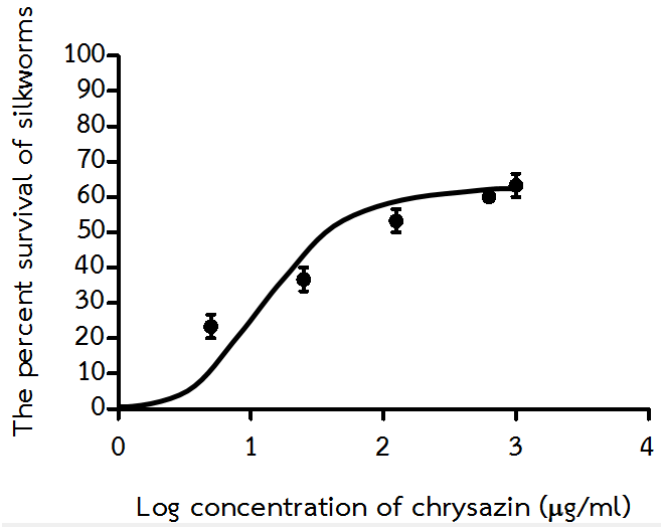

Figure 11 Median Effective Dose (ED_{50}) of chrysazin in silkworm infected with *B. cereus*. The number of surviving silkworms were observed at 48 hour after injection and were performed in triplicate, Error bars represent the mean ± SEM, *$p<0.05$; chrysazin groups were compared with negative control group (5% DMSO). The result showed that ED_{50} was 34.56 µg/ml.

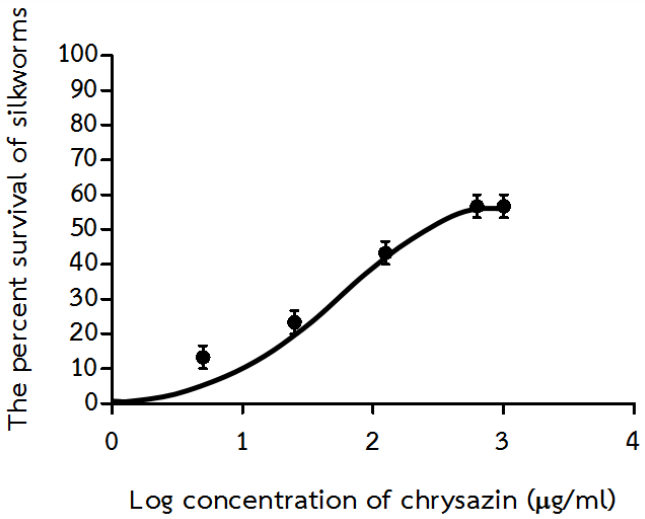

Figure 12 Median Effective Dose (ED_{50}) of chrysazin in silkworm infected with *B. subtilis*. The number of surviving silkworms were observed at 48 hour after injection and were performed in triplicate, Error bars represent the mean ± SEM, *p<0.05; chrysazin groups were compared with negative control group (5% DMSO). The result showed that ED_{50} was 145.53 µg/ml.

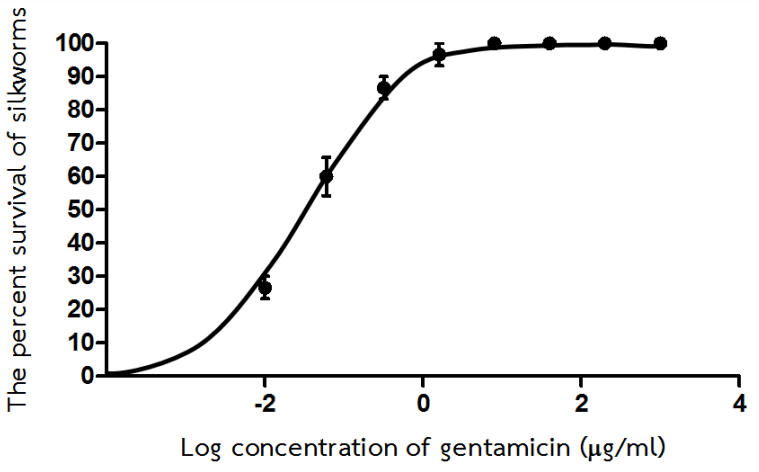

Figure 13 Median Effective Dose (ED_{50}) of gentamicin in silkworm infected with *B. cereus*. The number of surviving silkworms were observed at 48 hour after injection and were performed in triplicate, Error bars represent the mean ± SEM, *p<0.05; gentamicin groups were compared with negative control group (DI water). The result showed that ED_{50} was 0.03 µg/ml.

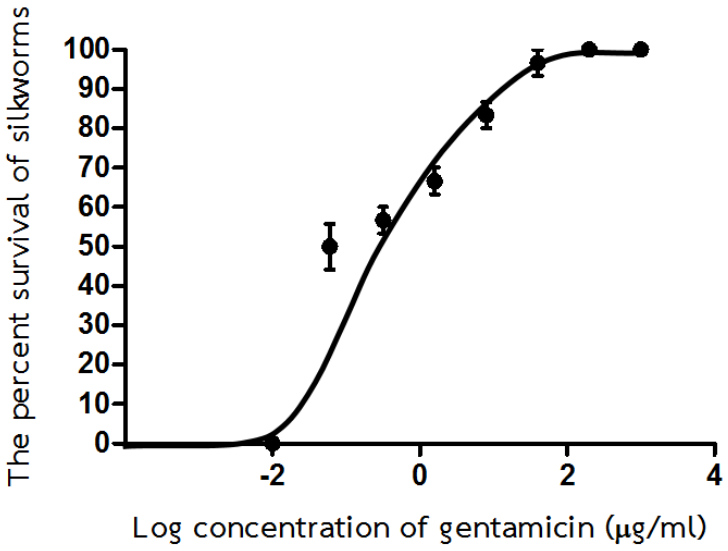

Figure 14 Median Effective Dose (ED_{50}) of gentamicin in silkworm infected with *B. subtilis*. The number of surviving silkworms were observed at 48 hour after injection and were performed in triplicate, Error bars represent the mean ± SEM, *$p<0.05$; gentamicin groups were compared with negative control group (DI water). The result showed that ED_{50} was 0.12 µg/ml.

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

The antimicrobial activity of chrysazin against *B. cereus* ATCC 11778 and *B. subtilis* ATCC 6633 using broth microdilution method (*in vitro* models) recommended by the M07-A9 document (69) showed that *B. cereus* ATCC 11778 was more susceptible to chrysazin than *B. subtilis* ATCC 6633. This study is consistent with the previous study by Thawongma and coworkers. They reported that the antimicrobial activities of (+)-usnic acid against *B. cereus* ATCC 11778 was more effective than that on *B. subtilis* ATCC 6633 (77). The MIC values of chrysazin were 7.81 µg/ml against *B. cereus* and 15.63 µg/ml against *B. subtilis,* respectively. Furthermore, the MBC values of chrysazin against both microorganisms were greater than 500 µg/ml (could not be determined within the range of concentrations used). In order to determine the action of chrysazin on the bacterial strains using MBC/MIC ratios. We exploited the following definition. If the ratio MBC/MIC is equal to 1 or 2, chrysazin will be considered as bactericidal agent. If the ratio is greater than or equal to 4, chrysazin will be considered as bacteriostatic agent (71, 78). Therefore, chrysazin possessed bacteriostatic effect against both *B. cereus* and *B. subtilis.* MBC/MIC ratios of chrysazin against *B. cereus* ATCC 11778 and *B. subtilis* ATCC 6633 were greater than 64 and 32, respectively. In addition, we used three control groups including positive control (gentamicin), negative control (5% DMSO and Sterile distilled water), and growth control (no treatment) to minimize the errors. Furthermore, in the experiments of MIC and MBC determination, we used *S. aureus* ATCC 29213 as the quality control as recommended by the M07-A9 document (70). The results of control groups showed that MIC and MBC values of gentamicin against both *B. cereus* ATCC 11778 and *B. subtilis* ATCC 6633 were 0.12 µg/ml. Moreover, MIC and MBC values of gentamicin against *S. aureus* ATCC 29213 were 0.06 µg/ml as well as MIC of gentamicin against *B. cereus* ATCC 11778, *B. subtilis*

ATCC 6633, and *S. aureus* ATCC 29213 conformed to the susceptibility breakpoint which defined by Clinical & Laboratory Standards Institute (CLSI) on January 2014 (70). These results suggested that strains of tested bacteria were considered susceptible to gentamicin and this study provided the accurate results of laboratory susceptibility testing. MBC/MIC ratios of gentamicin against *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, and *S. aureus* ATCC 29213 were equal to 1.

Kiem and Schentag mentioned that although MIC and MBC have been used as the most popular prediction tools for antimicrobial action, they have shortcomings. The MIC and MBC do not consider time-related antimicrobial effects, such as killing rate and post antibiotic effect (79). Therefore, we further performed Time - Kill Assay to determine the antimicrobial activity of chrysazin. In this study, Time-kill Assay were performed using chrysazin concentrations of 1/2MIC, 1MIC, 2MIC, 4MIC, and 8MIC as well as the initial inoculums of *B. cereus* ATCC 11778 and *B. subtilis* ATCC 6633 $\sim 1\times10^6$ CFU/ml. Serial samples were removed at 0, 2, 4, 6, 8, 10, 12, and 24 hour. Then, colony count determinations were diluted by 10-fold serial dilutions and spread on MHA plates. Later Time-Kill Curve was plotted between the $\log_{10}$ colonies forming unit per milliliter (CFU/ml) and time (hours) to determine whether chrysazin had bacteriostatic effect or bactericidal effect using the following definition. The agents that can reduce the original inoculum by ≥3 $\log_{10}$ is considered bactericidal, whereas the agents that can reduce the original inoculum by ≤2 $\log_{10}$ is considered bacteriostatic (74). In our study we observed that the results from Time - Kill Assay were consistent with the result of MIC/MBC ratio showing that chrysazin possessed bacteriostatic effect against both *B. cereus* and *B. subtilis*. On the contrary, gentamicin (positive control) possessed bactericidal effect against both *B. cereus* (ATCC 11778) and *B. subtilis* (ATCC 6633) in a concentration-dependent manner.

In addition we investigated *in vitro* antibiofilm activities of chrysazin against both *B. cereus* and *B. subtilis* and observed that chrysazin showed antibiofilm activity against both microorganisms in a concentration dependent manner and consistent with gentamicin (positive control). Chrysazin at the highest concentration (500 µg/ml) showed the maximum inhibition on biofilm formation with the percentage of inhibition for *B. cereus* and *B. subtilis* 85.15 % and 73.86%, respectively. This information may lead to further step for development of antibiofilm agent as antibiotic to overcome drug resistance or may be applied to cosmetic or healthcare industries. Moreover, antibiofilm agent can be applied in food industry settings to prevent and solve food contaminations and to ensure safety during production (51).

Most candidate drugs that are effective *in vitro* screening establish an ineffective *in vivo* studies because of their pharmacokinetic factors and toxicity for organisms that not having in the *in vitro* studies (80). We, therefore, utilized Thai silkworm model for *in vivo* drug screening to investigate toxicity and efficacy of chrysazin and solve the problem of using mammalian animals, such as mouse and rat that are more expensive with high cost for the maintenance and highly problematical with consider to ethical issues (67, 81, 82). In the study we observed that both *B. cereus* and *B. subtilis* known as human pathogens were able to kill silkworm larvae when injected into the hemolymph in silkworms. *B. subtilis* were more virulent to silkworm larvae than *B. cereus* with lethal dose 50 (LD_{50}) of 8.16×10^3 CFU/ml and 2.24×10^5 CFU/ml, respectively. This result is consistent with the fact that *B. subtilis* is a common pathogenic to silkworm in the field of mulberry cultivation. Furthermore, in *in vivo* toxicity study using chrysazin, we found that chrysazin showed very high safety profile for silkworm larvae with LD_{50} greater than 1,000 µg/ml and this figure is equal to the value of LD_{50} of gentamicin. Results from the efficacy study in Thai silkworm model showed that chrysazin had antibacterial activity against both *B. cereus* and *B. subtilis*

with ED_{50} about 34.56 µg/ml and 145.53 µg/ml, respectively. Chrysazin did not show 100% survival of silkworm lavae infected in both microorganism. This is due to the fact that the solubility of chrysazin is limited to 1,000 µg/ml. Therefore the highest percent survival of silkworm at 1,000 µg/ml was only 60.33% and 50.67% for *B. cereus* and *B. subtilis,* respectively. On the contrary, gentamicin showed antibacterial activity with 100% survival against both *B. cereus* and *B. subtilis*. Our results are in agreement with a study by Hamamoto and coworkers. They reported that silkworms were killed by injection of bacteria and true fungi that are pathogenic to humans as well as the antibiotics that clinically used for humans are effective for silkworms (83). Owing to the results from Time-Kill Assay, we suggest that further study may be needed such as synergy study for the combination of gentamicin with chrysazin using Time–Kill Assay. The synergy study will provide more information for the use of chrysazin in clinical setting. And the mechanism of action of chrysazin may also be elucidated as well.

In conclusion, we suggest that chrysazin has a potential as antibacterial agent together with antibiofilm formation agent in drug development and need further step to elucidate its activity in mammalian model such as mice or rat model. Silkworm infection model also is a useful animal model for screening stage prior in the development of new antibiotics. Moreover, it will reduce the amount of mammals, such as mice and rat which used in preclinical testing as well as will decrease costs (84). Despite the silkworm infection model has several advantages, such as very low cost, less of ethical problems, the body size is large enough to inject sample solutions, organs of silkworm larvae can be easily isolated to perform pharmacological tests (67), and they have an efficient innate immune system that can recognize organisms, it also has the limitations. For example, the sample solutions that are injected into the silkworm must be homogeneous solution and the maximum amount of substance that injected in silkworm larvae is 100 µl (50 µl per site); therefore, the concentration of

sample solution is less than that found in mammalian models. Another reason, Kaito and coworkers reported that disinfectants were not effective in silkworm because of toxicity against the larvae (65) as well as some microorganism did not infect silkworm, such as *Wolbachia etc.* (85).

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APPENDIX

APPENDIX A

Data of *In Vitro* Studies

Table 5 Survival colonies of *B. cereus* received chrysazin in Time kill assay

Hour	Survival colonies (log_{10} CFU/ml)					
	Growth control	Chrysazin 3.91 µg/ml (1/2 MIC)	Chrysazin 7.81 µg/ml (1 MIC)	Chrysazin 15.63 µg/ml (2 MIC)	Chrysazin 31.25 µg/ml (4 MIC)	Chrysazin 62.50 µg/ml (8 MIC)
0	9.27×10^5	6.80×10^5	8.37×10^5	8.10×10^5	7.53×10^5	7.07×10^5
2	1.03×10^6	1.01×10^6	8.13×10^5	7.37×10^5	6.67×10^5	5.47×10^5
4	2.01×10^6	7.00×10^6	7.07×10^5	6.33×10^5	5.07×10^5	9.60×10^4
6	2.21×10^6	1.11×10^7	5.97×10^5	5.27×10^5	7.90×10^4	8.23×10^4
8	2.50×10^7	1.33×10^8	4.90×10^5	8.87×10^4	6.17×10^4	7.10×10^4
10	8.90×10^8	2.35×10^8	3.63×10^5	5.87×10^4	5.17×10^4	4.87×10^4
12	1.86×10^9	2.57×10^8	8.73×10^4	3.63×10^4	2.97×10^4	2.97×10^4
24	2.97×10^9	2.71×10^8	5.90×10^4	2.83×10^4	1.83×10^4	1.87×10^4

Table 6 Survival colonies of *B. subtilis* received chrysazin in Time kill assay

Hour	Survival colonies (log_{10} CFU/ml)					
	Growth control	Chrysazin 7.81 µg/ml (1/2 MIC)	Chrysazin 15.63 µg/ml (1 MIC)	Chrysazin 31.25 µg/ml (2 MIC)	Chrysazin 62.50 µg/ml (4 MIC)	Chrysazin 125 µg/ml (8 MIC)
0	9.87×10^5	8.97×10^5	9.47×10^5	8.93×10^5	7.87×10^5	7.23×10^5
2	1.53×10^6	2.07×10^6	9.07×10^5	8.07×10^5	7.33×10^5	6.47×10^5
4	1.27×10^7	2.60×10^6	8.80×10^5	7.87×10^5	5.70×10^5	4.87×10^5
6	1.62×10^7	1.12×10^7	7.13×10^5	6.83×10^5	3.63×10^5	9.00×10^4
8	2.69×10^7	1.43×10^7	6.40×10^5	5.10×10^5	8.67×10^4	7.87×10^4
10	2.75×10^8	1.96×10^8	4.47×10^5	8.47×10^4	6.80×10^4	5.20×10^4
12	2.13×10^9	2.71×10^8	9.60×10^4	4.30×10^4	3.70×10^4	2.33×10^4
24	2.97×10^9	2.87×10^8	6.63×10^4	3.37×10^4	2.73×10^4	1.23×10^4

Table 7 Survival colonies of *B. cereus* received gentamicin in Time kill assay

Hour	Survival colonies (log_{10} CFU/ml)						
	Growth control	Gentamicin 0.0075 µg/ml (1/16 MIC)	Gentamicin 0.015 µg/ml (1/8 MIC)	Gentamicin 0.03 µg/ml (1/4 MIC)	Gentamicin 0.06 µg/ml (1/2 MIC)	Gentamicin 0.12 µg/ml (1 MIC)	Gentamicin 0.24 µg/ml (2 MIC)
0	6.50×10^5	6.40×10^5	6.50×10^5	6.20×10^5	6.20×10^5	5.90×10^5	5.10×10^5
2	8.00×10^5	3.40×10^5	3.30×10^5	2.10×10^5	1.50×10^5	1.20×10^4	0.00
4	1.35×10^6	6.50×10^5	4.40×10^5	4.20×10^5	4.00×10^4	0.00	0.00
6	1.49×10^6	2.40×10^5	2.30×10^5	2.00×10^5	1.30×10^4	0.00	0.00
8	1.60×10^7	1.50×10^6	4.00×10^5	2.00×10^5	0.00	0.00	0.00
10	1.69×10^8	5.00×10^6	3.00×10^6	1.00×10^6	0.00	0.00	0.00
12	1.97×10^9	2.40×10^7	8.00×10^6	7.00×10^6	0.00	0.00	0.00
24	2.31×10^9	1.42×10^8	9.10×10^6	8.60×10^6	0.00	0.00	0.00

Table 8 Survival colonies of *B. subtilis* received gentamicin in Time kill assay

Hour	Survival colonies (log_{10} CFU/ml)						
	Growth control	Gentamicin 0.0075 µg/ml (1/16 MIC)	Gentamicin 0.015 µg/ml (1/8 MIC)	Gentamicin 0.03 µg/ml (1/4 MIC)	Gentamicin 0.06 µg/ml (1/2 MIC)	Gentamicin 0.12 µg/ml (1 MIC)	Gentamicin 0.24 µg/ml (2 MIC)
0	8.60×10^5	4.80×10^5	3.60×10^5	3.50×10^5	3.00×10^5	2.90×10^5	2.70×10^5
2	1.00×10^6	7.60×10^5	4.50×10^5	2.00×10^5	3.00×10^4	2.00×10^4	0.00
4	1.69×10^6	7.00×10^4	5.00×10^4	4.00×10^4	2.00×10^4	0.00	0.00
6	1.80×10^6	9.80×10^4	2.00×10^4	1.00×10^4	1.00×10^4	0.00	0.00
8	1.89×10^7	1.00×10^5	1.00×10^5	1.00×10^5	0.00	0.00	0.00
10	1.97×10^8	2.00×10^6	1.00×10^6	1.00×10^6	0.00	0.00	0.00
12	2.23×10^9	1.00×10^7	1.00×10^7	1.00×10^7	0.00	0.00	0.00
24	2.58×10^9	1.70×10^8	3.00×10^7	3.00×10^7	0.00	0.00	0.00

Table 9 *In vitro* antibiofilm activity of chrysazin against *B. cereus*, expressed in terms of the percentage inhibition of biofilm formation

Chrysazin (μg/ml)		**The percentage inhibition of biofilm formation (Mean ± SEM)**
0.00	(control)	0.00 ± 0.00
3.91	(1/2 MIC)	14.59 ± 1.62
7.81	(MIC)	26.30 ± 0.55
15.63	(2MIC)	33.53 ± 0.33
31.25	(4MIC)	40.93 ± 0.40
62.50	(8MIC)	51.81 ± 0.99
125.00	(16MIC)	66.50 ± 1.73
250.00	(32MIC)	77.56 ± 2.56
500.00	(64MIC)	85.15 ± 2.33

Table 10 *In vitro* antibiofilm activity of chrysazin against *B. subtilis*, expressed in terms of the percentage inhibition of biofilm formation

Chrysazin (μg/ml)		**The percentage inhibition of biofilm formation (Mean ± SEM)**
0.00	(control)	0.00 ± 0.00
7.81	(1/2 MIC)	8.11 ± 1.68
15.63	(MIC)	25.95 ± 0.93
31.25	(2MIC)	31.89 ± 2.93
62.50	(4MIC)	40.62 ± 0.65
125.00	(8MIC)	51.87 ± 1.10
250.00	(16MIC)	63.62 ± 0.60
500.00	(32MIC)	73.86 ± 3.92

Table 11 *In vitro* antibiofilm activity of gentamicin against *B. cereus*, expressed in terms of the percentage inhibition of biofilm formation

Gentamicin (µg/ml)	The percentage inhibition of biofilm formation (Mean ± SEM)
0.00 (control)	0.00 ± 0.00
0.06 (1/2 MIC)	27.95 ± 1.89
0.12 (MIC)	38.94 ± 0.65
0.24 (2MIC)	46.40 ± 0.74
0.49 (4MIC)	58.13 ± 0.43
0.98 (8MIC)	71.04 ± 2.81
1.96 (16MIC)	81.73 ± 1.63
3.91 (32MIC)	95.05 ± 0.25

Table 12 *In vitro* antibiofilm activity of gentamicin against *B. subtilis*, expressed in terms of the percentage inhibition of biofilm formation

Gentamicin (µg/ml)	The percentage inhibition of biofilm formation (Mean ± SEM)
0.00 (control)	0.00 ± 0.00
0.06 (1/2 MIC)	21.24 ± 2.68
0.12 (MIC)	43.36 ± 1.60
0.24 (2MIC)	53.22 ± 3.71
0.49 (4MIC)	63.97 ± 1.01
0.98 (8MIC)	72.50 ± 0.81
1.96 (16MIC)	81.99 ± 0.48
3.91 (32MIC)	92.29 ± 0.99

APPENDIX B

Data of *In Vivo* Studies

Table 13 Pathogenicity of *B. cereus* in silkworms

***B. cereus* (CFU/ml)**	**The percent mortality of silkworms (Mean ± SEM)**
normal saline solution (control)	0.00 ± 0.00
1×10^{1}	0.00 ± 0.00
1×10^{2}	3.33 ± 3.33
1×10^{3}	13.33 ± 3.33
1×10^{4}	23.33 ± 3.33
1×10^{5}	43.33 ± 3.33
1×10^{6}	66.67 ± 3.33
1×10^{7}	83.33 ± 3.33
1×10^{8}	100.00 ± 0.00

Table 14 Pathogenicity of *B. subtilis* in silkworms

***B. subtilis* (CFU/ml)**	**The percent mortality of silkworms (Mean ± SEM)**
normal saline solution (control)	0.00 ± 0.00
1×10^{1}	0.00 ± 0.00
1×10^{2}	13.33 ± 3.33
1×10^{3}	43.33 ± 3.33
1×10^{4}	63.33 ± 3.33
1×10^{5}	73.33 ± 3.33
1×10^{6}	100.00 ± 0.00
1×10^{7}	100.00 ± 0.00
1×10^{8}	100.00 ± 0.00

Table 15 LD_{50} of chrysazin in silkworm model

Chrysazin (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
5% DMSO (control)	0.00±0.00
5	100.00±0.00
25	100.00±0.00
125	100.00±0.00
625	100.00±0.00
1,000	100.00±0.00

Table 16 LD_{50} of gentamicin in silkworm model

Gentamicin (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
DI water (control)	0.00±0.00
0.01	100.00±0.00
0.06	100.00±0.00
0.32	100.00±0.00
1.60	100.00±0.00
8.00	100.00±0.00
40.00	100.00±0.00
200.00	100.00±0.00
1,000.00	100.00±0.00

Table 17 ED_{50} of chrysazin in silkworm infected with *B. cereus*

Chrysazin (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
5% DMSO (control)	0.00±0.00
5	20.33±0.33
25	30.67±0.33
125	50.33±0.33
625	60.00±0.00
1,000	60.33±0.33

Table 18 ED_{50} of chrysazin in silkworm infected with *B. subtilis*

Chrysazin (µg/ml)	**Mean of number of silkworm survival (% survival) n=10**
5% DMSO (control)	0.00±0.00
5	10.33±0.33
25	20.33±0.33
125	40.33±0.33
625	50.67±0.33
1,000	50.67±0.33

Table 19 ED_{50} of gentamicin in silkworm infected with *B. cereus*

Gentamicin (µg/ml)	**Mean of number of silkworm survival (% survival) n=10**
DI water (control)	0.00±0.00
0.01	20.67±0.33
0.06	60.00±0.58
0.32	80.67±0.33
1.60	90.67±0.33
8.00	100.00±0.00
40.00	100.00±0.00
200.00	100.00±0.00
1,000.00	100.00±0.00

Table 20 ED_{50} of gentamicin in silkworm infected with *B. subtilis*

Gentamicin (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
DI water (control)	0.00±0.00
0.01	0.00±0.00
0.06	50.00±0.58
0.32	50.67±0.33
1.60	60.67±0.33
8.00	80.33±0.33
40.00	90.67±0.33
200.00	100.00±0.00
1,000.00	100.00±0.00

VITA

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