ฤทธิ์ในการต้านเชื้อแบคทีเรียและฤทธิ์ในการยับยั้งการสร้างไบโอฟิล์มของซินนามาลดีไฮด์ต่อเชื้อบาซิลลัส ซีเรียสและบาซิลลัส ซับทิลิส จากการศึกษาทั้งแบบนอกกายและแบบในกายโดยใช้หนอนไหมไทย

นางสาวกรรณิการ์ สิงห์เรืองรัตนสิทธิ์

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ANTIBACTERIAL AND ANTIBIOFILM FORMATION ACTIVITIES OF CINNAMALDEHYDE ON *BACILLUS CEREUS* AND *BACILLUS SUBTILIS IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL

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Thesis Title	ANTIBACTERIAL AND ANTIBIOFILM FORMATION ACTIVITIES OF CINNAMALDEHYDE ON *BACILLUS CEREUS* AND *BACILLUS SUBTILIS IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL
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กรรณิการ์ สิงห์เรืองรัตนสิทธิ์ : ฤทธิ์ในการต้านเชื้อแบคทีเรียและฤทธิ์ในการยับยั้งการสร้างไบโอฟิล์มของซินนามาลดีไฮด์ต่อเชื้อบาซิลลัส ซีเรียสและบาซิลลัส ซับทิลิส จากการศึกษาทั้งแบบนอกกายและแบบในกายโดยใช้หนอนไหมไทย (ANTIBACTERIAL AND ANTIBIOFILM FORMATION ACTIVITIES OF CINNAMALDEHYDE ON *BACILLUS CEREUS* AND *BACILLUS SUBTILIS IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: สันทัด จันทร์ประภาพ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ชนิดา พลานุเวช, 100 หน้า.

Bacillus species เป็นเชื้อที่สามารถทำให้เกิดอาการอาเจียนหรืออุจจาระร่วงได้ในผู้ที่มีภาวะอาหารเป็นพิษ และพบว่าเชื้อนี้มีการดื้อต่อยาปฏิชีวนะและสารที่มีฤทธิ์ต้านเชื้อแบคทีเรียตัวอื่นๆเพิ่มขึ้นด้วย ดังนั้นจึงมีความจำเป็นที่จะต้องวิจัยหายาต้านเชื้อแบคทีเรียตัวใหม่ๆ วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาฤทธิ์ในการต้านเชื้อแบคทีเรียและยับยั้งการสร้างไบโอฟิล์มของซินนามาลดีไฮด์ต่อเชื้อ *Bacillus* species โดยศึกษาทั้งแบบนอกกายและในกายโดยใช้หนอนไหมไทย ในการศึกษาฤทธิ์ต้านเชื้อแบคทีเรียใช้วิธี broth microdilution พบว่าความเข้มข้นต่ำสุดของซินนามาลดีไฮด์ที่สามารถยับยั้งการเจริญของเชื้อ *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 และ *B. cereus* Isolate 2 มีค่าเท่ากับ 49.21, 98.43, 196.87 และ 196.87 µg/ml ตามลำดับ ในขณะที่ความเข้มข้นต่ำสุดที่สามารถฆ่าเชื้อได้มีค่าเท่ากับ 196.87, 1,575, 196.87 และ 196.87 µg/ml ตามลำดับ ในการศึกษา time kill assay พบว่าซินนามาลดีไฮด์ออกฤทธิ์ได้ทั้งแบบฆ่าเชื้อและยับยั้งเชื้อ ขึ้นกับความเข้มข้น นอกจากนี้ซินนามาลดีไฮด์ยังมีฤทธิ์ในการยับยั้งการสร้างไบโอฟิล์มโดยสามารถยับยั้งการสร้างไบโอฟิล์มแบบขึ้นกับความเข้มข้น โดยมีค่าเปอร์เซ็นต์การยับยั้งสูงสุดต่อเชื้อ *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 และ *B. cereus* Isolate 2 เท่ากับ 95.94, 75.83, 97.27 และ 95.83 ตามลำดับ ที่ความเข้มข้น 1,575 µg/ml ในส่วนของการศึกษาในหนอนไหมไทยที่ติดเชื้อ *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 และ *B. cereus* Isolate 2 พบว่าซินนามาลดีไฮด์มีฤทธิ์ในการต้านการติดเชื้อโดยมีค่า ED_{50} เท่ากับ 438.16, 994.56, 389.50 และ 396.03 µg/ml ตามลำดับ โดยสรุปซินนามาลดีไฮด์มีฤทธิ์ทั้งต้านเชื้อแบคทีเรียและยับยั้งการสร้างไบโอฟิล์ม และสามารถนำไปศึกษาต่อเพื่อพัฒนาเป็นยาได้

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

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KANNIKA SINGRUEANGRATSIT: ANTIBACTERIAL AND ANTIBIOFILM FORMATION ACTIVITIES OF CINNAMALDEHYDE ON *BACILLUS CEREUS* AND *BACILLUS SUBTILIS IN VITRO* AND *IN VIVO* USING THAI SILKWORM MODEL. ADVISOR: SANTAD CHANPRAPAPH, Ph.D., CO-ADVISOR: ASSISTANT PROFESSOR CHANIDA PALANUVEJ, Ph.D., 100 pp.

Bacillus species can cause an emetic or a diarrhoeal type of food-associated illness. They are increasingly resistant to antibiotics and other antibacterial substances. Therefore, the new antibacterial agent is needed. The purpose of this study, therefore, was to evaluate antibacterial activities and antibiofilm activities of cinnamaldehyde against tested *bacillus* species both *in vitro* and *in vivo* studies using Thai silkworm infection model. Antibacterial activities were determined by broth microdilution method. MIC values of cinnamaldehyde for *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 were 49.21, 98.43, 196.87 and 196.87 µg/ml, whereas MBC values of cinnamaldehyde were 196.87, 1,575, 196.87 and 196.87 µg/ml, respectively. Time kill studies showed that cinnamaldehyde exerted both bactericidal and bacteriostatic depending on concentration. Moreover, cinnamaldehyde also showed the inhibitory effect on biofilm formation in concentration dependent manner with highest percent inhibitory effect (at concentration 1,575 µg/ml) of 95.94, 75.83, 97.27 and 95.83 against *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2, respectively. In Thai silkworm infection model, cinnamaldehyde was effective in preventing the infection in silkworm with *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 with ED_{50} of 438.16, 994.56, 389.50 and 396.03 µg/ml, respectively. In conclusion, cinnamaldehyde had both antibacterial activities and antibiofilm activities against tested *bacillus* spp. and can be further studied for the drug development.

Field of Study: Pharmacology
Academic Year: 2016

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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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
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 milliliter
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth

MIC	Minimum inhibitory concentration
min	minute
ml	milliliter
mm	millimeters
NaCl	sodium chloride
nm	nanometer
°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

CHAPTER I

INTRODUCTION

Background and Rationale

Bacillus species are aerobic spore forming rods that stain gram positive (1). These organisms are usually found in decaying organic matter, dust, vegetable and water. Some species are part of the normal flora (2). The clinical spectrum of infections caused by *Bacillus* spp. includes self-limited food poisoning, localized infections related to trauma (e.g. ocular infections), deep seated soft tissue infections, and systemic infections (e.g. endocarditis, bacteremia and meningitis). The various species implicated in serious infections include *B. cereus*, *B. subtilis*, *B. sphaericus*, *B. alvei*, *B. laterosporus*, *B. licheniformis*, *B.megaterium* and *B. pumilus*.

B. cereus and *B. subtilis* are infectious causes of foodborne illness. The bacterium causes two types of gastrointestinal disease. The diarrheal type is characterized by diarrhea and abdominal pain occurring 8 to 16 hours after consumption of the contaminated food. It is associated with a variety of foods, including meat and vegetable dishes, sauces, pastas, desserts, and dairy products. In emetic disease, on the other hand, nausea and vomiting begin 1 to 5 hours after the contaminated food is eaten.

Moreover, the ability of *Bacillus* spp. to form biofilms on surfaces can cause potential contaminate problems within the food industry. It is now broadly recognized that bacteria have the widespread capacity to form surface-associated multicellular aggregates, commonly referred to as biofilms (3), (4). Although it is not entirely known how microbes benefit from life within these structures, biofilm-associated microbes exhibit marked metabolic and physiological differences compared with their planktonic brethren, including decreased susceptibility to antimicrobial agents (5).

Although various antibiotics have been used for the treatment of infectious disease, but antibacterial drug resistance and problems through food poisoning of *Bacillus* spp. are still existing. Cinnamaldehtyde is a major component found in cinnamon bark oil. It has been used in pharmaceuticals, foods and cosmetics for a long time. *In vitro* antimicrobial activity studies found that cinnamon bark oil was active against Gram positive and Gram negative bacteria. Cinnamaldehyde has been reported to have several pharmacological effects such as antimicrobial, antioxidant, anti-inflammatory, anticancer, antiulcer, hypoglycemic and hypolipidemic potential (6).

Most drug candidates obtained by *in vitro* screening are inappropriate as medicines due to their toxicity and pharmacokinetics in humans. Preclinical tests in animal models are essential for evaluating the therapeutic effects of drug candidates for further development. Mammals, such as mouse, rat, marmot, rabbit, dog, and monkey, are commonly used as drug-screening models (7). Evaluation of the therapeutic effects of potential antibiotics has been performed using mammalian models, but conventional methods using a large number of mammals are problematic due to high costs and ethical concerns. Therefore, the development of a non-vertebrate infectious model to test drug efficacy in the early stages of development is highly desirable (8). The silkworm (*Bombyx mori*) is an easily bred invertebrate animal used for basic studies because of its importance in sericulture. Silkworm is large enough to inject sample solutions into the hemolymph. Recently, it might be possible to quantitatively evaluate the virulence of various bacteria or true fungi and to identify their virulence genes in the silkworm infection model (9). Therefore, we also used silkworms as an infection model for evaluation of antibacterial activities of cinnamaldehyde against both microorganisms.

In this research, we investigate cinnamaldehyde for its antibacterial and antibiofilm formation activities on *B. cereus* and *B. subtilis* both *in vitro* and *in vivo*

studies. *In vitro* studies, the minimum inhibition concentration (MIC) was determined using the serial two-fold dilution and MIC value represents the lowest concentration at which an antibacterial agent inhibits a particular microorganism. The minimum bactericidal concentration (MBC) is determined by a series of steps, undertaken after completion of MIC test. While the bactericidal activity is assessed by time kill assay *in vitro*. In addition, the biofilm assay is a useful method for assessing bacterial attachment by measuring the staining of the adherent biomass. Moreover, we consider the applicability of the silkworm as an infection model animal for quantitative measurement of the therapeutic effects of antibacterial agent against *Bacillus* species infection. This study indicates the qualitative information and then the next step, the quantitative information of cinnamaldehyde against *B. cereus* and *B. subtilis*. Moreover, cinnamaldehyde have not been studied *in vitro* and *in vivo* against *Bacillus* species on efficacy and safety yet. So, the aim of this work is to investigate the antibacterial activity of the cinnamaldehyde to *B. cereus* and *B. subtilis in vitro* and *in vivo*.

Objective

The purpose of this study is to evaluate the antibacterial activities and antibiofilm activities of cinnamaldehyde against *B. cereus* and *B. subtilis in vitro* and *in vivo* using Thai silkworm model.

Hypothesis

Cinnamaldehyde can inhibit growth and biofilm formation of *B. cereus* and *B. subtilis in vitro* and show therapeutic effect in silkworm model *in vivo*.

Research design

Experimental Research

Scope of the study

This study was performed both *in vitro* and *in vivo*. For *in vitro* study, the present study was aimed to evaluate antibacterial activity, inhibitory effect of biofilm formation and time of killing of cinnamaldehyde against *Bacillus* species. For *in vivo* study, silkworms were used as an animal model to study pathogenicity of *B. cereus* and *B. subtilis*, toxicity of cinnamaldehyde and therapeutic effects of cinnamaldehyde against these microorganisms.

Benefits from this study

The information of cinnamaldehyde on antibacterial and antibiofilm activities, time of killing and inhibition of biofilm formation against *Bacillus* species *in vitro* and *in vivo* using Thai silkworm model may lead to further step for development of antibacterial drugs in vertebrate models such as mouse, rabbit and rat *etc*.

CHAPTER II

LITERATURE REVIEWS

2.1 Microbiology

2.1.1 Description of the general and morphology

The genus *Bacillus* is a Gram-positive, motile (flagellated), spore-forming, facultative anaerobic, rod shaped bacterium that belongs to the *Bacillus* genus. Strains of organisms vary widely in their growth and survival characteristics temperature bacterial growth range from 4-55°C, pH range from 4.9-10.0 and water activity of 0.93-0.99 (10), (11).

2.1.2 Ecology

B. cereus is widespread in the soil and the food industry, in foods such as, herbs, spices, milk and vegetables. Many of these foods may contain *B. cereus* since spores of this organism are heat-resistant. Studies have shown that *B. cereus* spores can survive even recommended cooking temperatures. Keeping food on warmers may also prove to be ineffective in reducing *B. cereus*, as spores can still germinate as food is cooled, passing through the "danger zone" of 140°F to 41°F. Likewise *B. subtilis* is most commonly found in soil environments and on plant undergrowth. *B. subtilis* can be isolated from many environments-terrestrial and aquatic-making as this species is ubiquitous and broadly adapted to grow in diverse settings within the biosphere. However, like all members of the genus *Bacillus*, *B. subtilis* can form highly resistant dormant endospores in response to nutrient deprivation and other environmental stresses (12).

2.1.3 Endospores

An endospore is a dormant, tough, non-reproductive structure produced by a small number of bacteria from the Firmicute family. Endospores can survive without nutrients. They are resistant to ultraviolet radiation, desiccation, high temperature, extreme freezing and chemical disinfectants. They are commonly found in soil and water, where they may survive for long periods of time. Bacteria produce a single endospore internally. Then extraordinary resistance properties of endospores make them a particular importance because they are not readily killed by many antimicrobial treatments.

2.1.4 Pathogenicity

Although *Bacillus* spp. has not been recognized as major human pathogens, with recent advances in medical technology and increased number of immunosuppressed patients, they have been increasingly recognized as an opportunistic pathogen in the hospitalized patients. The different species of *Bacillus* produce a variety of extracellular products including antimicrobial substances, enzymes, pigments, and toxins in few species (13). Enzymes that can be found on culture include amylase, collagenase, hemolysin, lecithinase, phospholipase, protease, and urease. Two different types of enterotoxins are produced by *Bacillus* spp. during exponential growth: the enterotoxin that causes diarrhea and the emetic toxin.

2.1.4.1 *Bacillus cereus*

There is limited information on resistance *B. cereus* to various antibiotics, and a growing concern over the transfer of antibiotic resistance genes. *B. cereus* is a well-known cause of food-borne illness. *B. cereus* causes gastrointestinal distress, necrotic enteritis, liver failure, bacteremia, endocarditis, meningitis, pneumonia and skin lesions (14), (15), (16). The emetic syndrome is caused by emetic toxin produced by the bacteria during the growth phase in the food. The diarrhoeal

syndrome is caused by diarrhoeal toxins produced during growth of the bacteria in the small intestine (17). Three types of enterotoxins are associated with the diarrhoeal form of disease. These are the three components enterotoxin hemolysin BL (HBL), the three component of non-hemolytic enterotoxin (NHE) and the single component enterotoxin cytotoxin K. After consumption of food containing *B. cereus*, the enterotoxins are released into the small intestine during vegetative growth following spore germination (18). The diarrhoeal enterotoxins can be produced in the temperature range of 10-43°C (19), (20). Such diarrhoeal enterotoxin production occurs between pH 5.5-10, with an optimum of pH (21). The diarrhoeal enterotoxins are stable at pH 4-11 and inactivated by heating at 56°C for 5 minutes (22). Maltodextrin is known to stimulate growth of *B. cereus* and to aid diarrhoeal enterotoxin production in reconstituted and stored infant milk formulae (23). It has also been shown that *B. cereus* produces more HBL and NHE under conditions of oxygen tension (low oxygen reduction potential) that simulate the anaerobic, highly reducing fermentative conditions encountered in the small intestine (24).

Ocular infections

B. cereus has been recently recognized as a primary pathogen of ocular infections in humans (25). Endophthalmitis is a serious illness that can result in visual compromise within 12-48 hours after inoculation (26). 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 (27). 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 (28).

Endocarditis

Endocarditis caused by *Bacillus* organisms is a well-recognized complication of intravenous drug abuse (29). Rarely, it has been isolated from patients

with underlying valvular disease. Since *B. cereus* is the most common isolate, empirical use of penicillin is usually not effective.

Bacteremia

Intravascular devices are the common source of positive blood cultures for *Bacillus* spp. In patients with positive blood cultures for *Bacillus*, a decision has to be made whether the organism is pathogenic. In most cases, especially in the asymptomatic patient, the bacteremia is limited and requires no antimicrobial therapy, emphasizing that the process is relatively benign. A recent report on AIDS patient with *B. cereus* bacteremia also emphasizes the low morbidity associated with this condition (30). However, there have been case reports of fulminant sepsis complicated by hemolysis in patients with acute leukemia.

Meningitis

A wide variety of *Bacillus* spp. have been isolated from cerebrospinal fluid of patients who had spinal anesthesia, subdural hematoma, ventricular shunts and parameningeal foci of infections e.g. otitis and mastoiditis. Removal of any foreign body such as a ventricular shunt is necessary to eradicate the infection. Initial therapy is intravenous vancomycin with or without an aminoglycoside which can be adjusted when susceptibility result becomes available. Supplemental intraventricular or intrathecal instillation of vancomycin may be required in patients with poor response. Intrathecal doses of 3-5 mg of vancomycin may be used in addition to parenteral antibiotics (31).

2.1.4.2 *Bacillus subtilis*

Effect on human health: in items of human health, reviewers found the *B. subtilis* bacteria to be relatively benign. *B. subtilis* produces the enzyme subtilisin, which has been reported to cause dermal allergic or hypersensitivity reactions in individuals repeatedly exposed to this enzyme in industrial settings. The symptoms of food poisoning caused by *B. subtilis* are less well defined. Diarrhea and/or nausea occur

within 1 to 14 hours after consumption of the contaminated food. A wide variety of food types have proved responsible in recorded instances (32).

2.1.5 Drugs susceptibility

The drug of choice for serious infectious cause of foodborne illness by *B. cereus* is susceptible to aminoglycosides, chloramphenicol, clindamycin, erythromycin, tetracyclin and vancomycin (33). In 2007 Luna and his colleague performed susceptibility test of *bacillus* spp. to antimicrobial agents including chloramphenicol, ciprofloxacin, gatifloxacin, levofloxacin, moxifloxacin, gentamicin, linezolid, rifampicin, streptomycin, tetracycline, tigecycline and vancomycin (34). Moreover, *B. cereus* isolates are usually susceptible to susceptible to imipenem, chloramphenicol, vancomycin, gentamicin and ciprofloxacin (35).

2.1.6 Drugs resistance

Recently, difficulties have been reported in treating *B. cereus* infections, with some strains resistant to the antibiotics erythromycin, tetracyclines, carbapenems, clindamycin, and many of the other cell-wall active antibiotics including ampicillin, penicillins, cephalosporins, cephalothin and methicillin (32), (34), (35). Moreover, *B. cereus* isolates were resistant to ampicillin, and almost all were resistant to cephalosporins (35).

2.1.7 Gentamicin (Positive control)

Gentamicin a water soluble antibiotic of the aminoglycoside group, is derived from *Micromonospora purpurea*, and actinomycete.

2.1.7.1 Mechanism of action

Gentamicin, an aminoglycoside, interferes with protein synthesis in susceptible microorganisms. It is thought that drug is actively transported across the bacterial cell membrane, then binds to a specific receptor protein on the 30S subunit of bacterial ribosomes and interferes with an initiation complex between mRNA (messenger RNA) and the 30S subunit, resulting in inhibition of protein synthesis.

2.1.7.2 Concentration-dependent killing

Gentamicin is concentration dependent antibiotics, meaning that as gentamicin concentration increases, the rate and extent of bacterial killing increases. For drugs with concentration-dependent bactericidal action, such as aminoglycosides the rate of bactericidal activity is maximal at the Cmax. This dependence on both the magnitude and the duration of exposure of bactericidal concentrations implies that concentration-dependent drugs are influenced by both the Cmax and AUC, whereas for drugs with time-dependent activity, the extent of bactericidal activity depends solely on the duration of drug exposure. The importance of pharmacodynamic factors in developing optimal treatment strategies has been confirmed in many studies in *in vitro* models and in models of infection in experimental animals that simulate human infections, and in clinical studies. The requirement for bactericidal therapy of antimicrobial therapy, for obtaining Cmax/MIC ratios that are ≥10 (36).

2.2 Biofilm

Bacterial biofilms cause serious problems, such as antibiotic resistance and medical device-related infections. Recent reports indicate that *Bacillus* spp. potentially forms biofilms causing nosocomial bacteremia via catheter infection (37) and forms biofilms on surfaces causing potential contamination problems in the food industry. A biofilm is a multicellular complex, formed by microorganisms that are attached to a surface and embedded in a matrix, consisting of exopolymeric substances (EPS). Bacterial cells within a biofilm are surrounded by the EPS matrix and cells in the outer layers of the biofilm, protecting them from harsh influences from the environment. Thereby biofilms make them more resistant to cleaning agents and other antimicrobial substances (38).

2.2.1 *B. cereus* biofilm-associated infection

Biofilm formation from several *B. cereus* strains is currently being studied to prevent potential food contamination and to ensure safety during food production. In a recent study, microliter assay and assays on stainless steel were completely or

partially submerged in liquid in order to observe *B. cereus* biofilm formation. Since stainless steel is commonly used for pipes and tanks in the food industry, additional tests were conducted to investigate *B. cereus* biofilm formation from spores on stainless steel equipments. The results from both tests were similar. It appears that *B. cereus* biofilms preferentially form within the air-liquid interface. This tendency is due to the availability of oxygen in this region, causing bacterial movement toward of oxygen. In addition, spore formation was more rapid in the suspension phase of biofilm formation suggesting that biofilms are a cavity for sporulation. The results show that *B. cereus* biofilms may develop within storage and piping systems when either partially filled or when liquid residues remain during production. In addition, increase in spore formation by *B. cereus* within biofilms can potentially cause recontamination during food production (39). *B. cereus* can also cause problems in food industry because of its capacity to form biofilms on several substrata. *B. cereus* has been found to account for 12.4% of the constitutive microflora, growing in biofilms, in a commercial dairy plant (40).

2.2.2 *B. subtilis* biofilm-associated infection

B. subtilis, which is a non-pathogenic Gram-positive bacterium, has emerged as an alternative model organism for studying the molecular basis of biofilm formation (41). *B. subtilis* is an industrially important bacterium. It forms rough biofilms at the air-liquid interface rather than on the surface of a solid phase in a liquid, due to the aerotaxis of the cells. Biofilm formation by *B. subtilis* and related species permits the control of infection caused by plant pathogens, the reduction of mild steel corrosion, and the exploration of novel compounds. Although it is obviously important to control harmful biofilm formation, the exploitation of beneficial biofilms formed by such industrial bacteria may lead to a new biotechnology (42). *B. subtilis* is traditionally considered as a soil-dwelling organism and can be found preferentially in association with plant roots in the upper rhizosphere. Then, a central question arises as to whether the lifestyle of *B. subtilis* in its natural habital entitles biofilm formation, and if so, whether those biofilms are structurally similar to the ones they are currently studying

in the laboratory. It has been recently shown that *B. subtilis* colonizes plant roots, as well as plant leaves in a biofilm-dependent manner, and the presence of the biofilms increases protection of the plants form a variety of pathogenic insults. Importantly, the regulatory networks and structural components of the biofilms unraveled in laboratories are now proven important for the development of plant-associated biofilms (43).

2.2.3 The molecular basis of biofilm formation in *bacillus* spp.

Research performed in many biofilm-forming organisms has revealed that the development of a biofilm is a 3-step process involving an initial attachment and a subsequent maturation phase, which are physiologically different from each other and require phase-specific factors. A final detachment (or dispersal) phase involves the detachment of single cells or cell clusters by various mechanisms and is believed to be crucial for the dissemination of the bacteria, in the case of pathogens to new infection sites in the human body.

2.2.3.1 Attachment

In the human body, the attachment to human matrix proteins represents the first step of biofilm formation. *Bacillus* spp. express dozens of so-called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that have the capacity to bind to human matrix proteins such as fibrinogen or fibronectin, and often combine binding capacity for several different matrix proteins (44). MSCRAMMs have a common structure that includes an exposed binding domain, a cell-wall spanning domain, which often has a repeat structure, and a domain that is responsible for the covalent or non-covalent attachment to the bacterial surface. Covalent attachment is catalyzed by a family of enzymes called sortases that link a conserved motif of the MSCRAMMS to peptidoglycan (45).

2.2.3.2 Maturation

The maturation phase of biofilm formation is characterized by intercellular aggregation that can be accomplished by a variety of molecules such as adhesive proteins or–usually polysaccharide-based–exopolymers. This intercellular

aggregation of biofilm producing structuring forces that can lead to the typical 3-dimensional appearance of mature biofilms with its mushroom-like cell towers surrounding fluid-filled channels.

2.2.3.3 Detachment

Biofilm detachment is crucial for the dissemination of bacteria to other colonization sites. It may occur by the detachment of single cells or larger cell clusters. Several factors may contribute to detachment: mechanical forces such as flow in a blood vessel, cessation of the production of biofilm building material, such as exopolysaccharide, detachment factors sensu stricto, such as enzymes that destroy the matrix, or surfactants. For all that we know, the latter factors are not different from those discussed as biofilm structuring agents. When produced at a high rate, these factors will cause detachment, especially at the biofilm surface area. In fact, controlled detachment maintains a certain biofilm thickness and governs a specific rate of biofilm dissemination (46).

2.3 Cinnamaldehyde

Cinnamaldehyde is cinnamon oils isolated from cinnamon trees found in China and other Asian countries (47). It is used primarily to impart a cinnamon flavor in foods and beverages (including liquors, cordials and medicinal) and to impart a cinnamon fragrance in medical products, perfumes and cosmetics. Cinnamaldehyde is an aromatic aldehyde, occurs naturally in the bark of cinnamon trees of the genus *Cinnamomum* (48). The essential oil contains approximately 70 to 90 percent cinnamaldehyde. Cinnamaldehyde or IUPAC name 3-phenylprop-2-enal, molecular formula: C_9H_8O, is an oily yellow liquid at room temperature with a boiling point of 246°C. The molecular weight is 132.15922 g/mol. It is poorly soluble in water but very soluble in alcohol and ether. Cinnamaldehyde has been identified and utilized as a non-toxic, food grade antimicrobial agent. It is generally regarded as safe by the US Food and Drug Administration (US FDA). Only at high concentrations for prolonged exposures have been shown to cause detrimental physiological changes in mammals (49). Cinnamaldehyde, along with other spice oils, can be expected to be present in a

variety of cinnamon containing foods and other edible preparations (50). While the mechanisms of action of these essential oil components have not been accurately elucidated, some studies have indicated their mechanisms of action through their interactions with the bacterial cell surface (51) and inhibition of energy metabolism (52). Recently, chemical genetic approaches have been used to understand the mode of action of these essential oils (53), (54). Most of the essential oil components studied probably shares some commonality in their antibacterial mode of action. Incidences of significant resistance against cinnamaldehyde (and other spice oils) have not been reported; on the other hand, bacterial strains that have developed antibiotic resistance have been shown to be susceptible to these oils (55).

2.3.1 Antimicrobial effect of cinnamaldehyde

In 2009 Al-Bayati and his colleagues isolated cinnamaldehyde, the most important bioactive compound, from *Cinnamomum zeylanicum* L. barkoil. The plant essential oil was extracted via steam distillation. Cinnamaldehyde was separated using a separating funnel and identified according to Tollen's test followed by detection on TLC plates in comparison with standard cinnamaldehyde that served as positive control. The isolated material was investigated for its antibacterial activity against six selected pathogenic bacteria. The Gram-positive bacteria were *S. aureus* and *B. cereus*; Gram-negative bacteria included *E. coli*, *Proteus mirabilis*, *Klebsiella pneumonia* and *P. aeruginosa*. Cinnamadehyde at different concentrations (1:1, 1:5, 1:10 and 1:20) was active against all tested bacteria and the highest inhibitory effect was observed against *B. cereus* (zone of inhibition 25.3 mm) using the disk diffusion method. The minimal inhibitory concentration (MIC) of cinnamaldehyde was determined using a broth microdilution method in 96-well microtiter plates. MIC values ranged from 31.2 to 125.0 µg/ml. The most promising result was observed against *B.cereus*, while *S. aureus*, *E. coli* and *K. pneumonia* ranked next (MIC: 62.5 µg/ml) followed by *P. mirabilis* and *P. aeruginosa* with a MIC of 125.0 µg/ml (56). Furthermore, in 2011 Wei and his colleagues evaluated the antibacterial activities of cinnamaldehyde against *B. subtilis*, *E. coli* and *Saccharomyces cerevisiae*. Antimicrobial activities were

evaluated by measuring the inhibition zone diameter (IZD) of the tested microorganisms. The cinnamaldehyde concentrations used were 200 mg/ml, 400 mg/ml and 600 mg/ml. Cinnamaldehyde was more active against *B. subtilis* than *E. coli* and *S. cerevisiae* (57). Moreover, in 2013 Ghosh and his colleagues demonstrated the antibacterial effect of cinnamaldehyde against a variety of pathogenic bacterial species, by micro broth dilution assays. Cinnamaldehyde inhibited Gram-positive (such as *B. cereus*, *S. aureus*) and Gram-negative (such as *E. coli*, *P. aeruginosa*) strains to similar extents. MIC values of cinnamaldehyde against *B. cereus* was 328 µg/ml (58). In 2014 Huang and his colleagues evaluated the antibacterial activity of essential oil against *S. aureus*, *B. subtilis*, *S. typhimurium* and *E. coli*. The majority of components of the essential oil from *Cinnamomum cassia* bark was cinnamaldehyde in about 68.52% identified by gas chromatography and mass-spectrometry (GC-MS). The results showed that it has stronger effect against *S. aureus* with the largest zone of inhibition of 27.4 mm and the MIC of 2.5 mg/ml and MBC of 5.0 mg/ml, respectively (59). Furthermore, in 2015 Raeisi and his colleagues determined the antibacterial effects of cinnamon essential oil against *S. aureus* and *E. coli.* MIC determination in this study revealed that cinnamon essential oil had antibacterial effect against both *S. aureus* and *E. coli* with MIC of 2,500 µg/ml and 625 µg/ml, while the MBCs were 2,500 µg/ml and 625 µg/ml, respectively (60). Lastly, in 2016 Zhang and his colleagues demonstrated that cinnamon essential oil exhibited effective antibacterial activity against foodborne spoilage and pathogenic bacteria using *E. coli* and *S. aureus*. The MICs of cinnamon essential oil were similar for both bacteria (1.0 mg/ml) while the MBCs were 4.0 mg/ml and 2.0 mg/ml for *E. coli* and *S. aureus*, consecutively. Much effort was focused on elucidating the mechanism of antibacterial action of cinnamon essential oil against *E. coli* and *S. aureus* by observing the changes of cell microstructure using scanning electron microscope, determining of cell permeability, membrane integrity and membrane potential. After adding cinnamon essential oil at MIC levels, there were obvious changes in the bacteria cells morphology indicating cell damage. When cinnamon essential oil were added at MBC levels, the cell were destroyed (61).

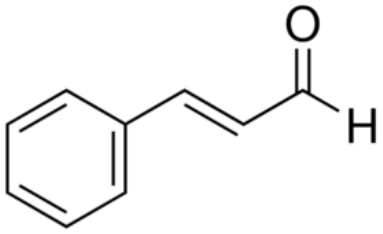

Figure 1 Chemical Structure of cinnamaldehyde

2.3.2 Toxicological effect of cinnamaldehyde

In 2006 Ooi and his colleagues, reported that cinnamaldehyde is non-toxic and non-carcinogenic to mammals. In the summary report of EMEA (the European Agency for the Evaluation of Medicinal Products, Veterinary Medicines and Information Technology Unit, January, 2000) on the toxicity test in rats as an example, the oral LD_{50} of cinnamaldehyde in rats is 2,220 mg/kg body weight and the oral tolerated doses of cinnamaldehyde for rats without any symptom of toxicity is at 70 mg for 8 week (62). Moreover, in 2010 Sivakumar and his colleagues evaluated the effects on certain behavioral parameters of cinnamaldehyde in rats. Rats were given cinnamaldehyde orally by gavage at the dose levels of 2.14, 6.96, 22.62 and 73.5 mg/kg body weight/day for the period of 10, 30 or 90 days. Only the group of rats treated with cinnamaldehyde at the dose of 73.5 mg/kg body weight/day for 90 days showed significant changes in the olfactory discrimination, auditory startle response and negative geotaxis behavior. No treatment related impairment of cliff avoidance behavior was noted. Thus cinnamaldehyde has no effect on the neuromuscular system. This is due to changes in acetyl cholinesterase (AChE) and creatine kinase activities in the serum of these rats (63).

2.4 Silkworm

Silkworm is an invertebrate animal (*Bombyx mori*). Silk moths belong to Phylum-Artropoda, Class–Insecta, Order–Lepidoptera, Super family–Bombycoidea.

Silkworm was born since time immemorial. The duration of the development stages can be controlled completely throughout the life cycle by regulating environmental conditions. Silkworm is complete metamorphosis. Within about 6-8 weeks. Silkworms live by consuming the leaves of the mulberries and other genera in this family.

2.4.1 The cycle stages of silkworm

Life cycle of *Bombyx mori* demonstrates the most advanced form of metamorphosis. The serial progressions of four distinct stages of development complete one generation of the species; egg (ova), larvae, pupa and adult moth.

The first stage of a silkworm's life cycle is egg. The female moth lays eggs about the size of an ink dot during summer or the early fall. The silkworm eggs are round and white. The weight of newly laid 2,000 eggs is about 1.0 g. It measures 1-1.3 mm in length and 0.9-1.2 mm in width. With time, eggs become darker. Races producing with cocoons lay pale yellow eggs; while races producing yellow cocoons lay deep yellow eggs.

The second stage of silkworm is larvae. The larvae is the vegetative stage where growth takes place. The larva of *Bombyx mori* is host specific to mulberry. During growth, the larva molts 4 times. The period between successive molts is called an instar. There are 5 feeding periods or instars. The Worm Lady silkworms sizes are 2nd instar: small, 3rd instar: med, 4th instar: large and 5th instar: x-large. Young silkworms can only be fed on tender mulberry leaves. The larval stage lasts for about 27 days and the silkworm goes through five growth stages called instars, during this time. During the first molting, the silkworm sheds all its hair and gains a smooth skin.

The third stage of silkworm is pupa. The silk cocoon serves as protection for the pupa. Cocoons are shades of white, cream and yellow depending on silkworm genetics. After a final molt inside the cocoon, the larva develops into the brown, chitin covered structure called the pupa. Metamorphic changes of the pupa result in an emerging moth. Cocoon is the stage in which the larva spins silk threads around it, to protect itself from its predators. The larva traps itself inside the cocoon in order to pupate. The second molting occurs inside the cocoon, when the larva turns into a brown

pupa. It takes about 2-3 weeks for the pupa to metamorphose into an adult moth. The adult stage completes the life cycle of *Bombyx mori*. It is the reproductive stage where adults mate and females lay eggs. Moths are flightless and lack functional mouth parts, so are unable to consume the food/nutrition.

2.4.2 Drug administration in silkworm

There are 2 routes of drug administration in silkworms, the first routes is intra hemolymph, which is similar to the intravenous injection in animals and the second route is intra midgut, which is similar to oral administration in animals.

2.4.3 Anatomy and physiology

2.4.3.1 Internal anatomy of the larva

Main internal organs in the alimentary canal (fore-gut, mid-gut and hind-gut) are trachea, nervous system, dorsal vessel, malpighian tubule, silk gland and others.

2.4.3.2 The alimentary canal

Alimentary canal in silkworms is divided into three main regions include the foregut, which is ectodermal in origin, the midgut, which is endodermal and the hindgut, which is again ectodermal. In silkworms, these regions are subdivided into various functional parts. The foregut is composed of pharynx, esophagus and salivary glands. It is commonly involved in the storage of food and sometimes helps to the fragment the food before it passes to the midgut. The salivary glands can secrete digestive enzymes to hydrolyze protein, starch, sucrose, maltose and trehalose. The midgut is the largest organ, extending over 8 of the 13 body segments. It is primarily involved in the production of enzymes and the absorption of digestive content. The midgut tissue comprises of globlet cells, columnar cells and a basement membrane. The globlet cells and columnar cells are involved in or functioned in enzyme secretion and the absorption of digestive products. The basement membranes of cells adjoin to the hemocoel with few opening to hemolymph. The hindgut is composed of epithelial cells forming numerous projections, each of which is made up of two or three large cuneiform cells. Four regions are recognizable in this part include the small intestine, the colon, the

rectum and anus. The hindgut conducts undigested food to the exterior via the anus, but also has other functions. In particular, the rectum is involved in salt and water.

2.4.3.3 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, entering the ostia at the dorsal Bessel of abdominal 6^{th} to 11^{th} segments, and leaving the dorsal vessel at the ostia of 2^{nd} to 5^{th} segments. The blood or hemolymph circulates around the body cavity between the various organs. It consists of fluid plasma in which is suspended the blood cells or hemocytes. The content of water in the blood is about 90 to 95% and potential of hydrogen ion of the blood in 5^{th} instar larva is 6.3 to 6.5%. The blood of the silkworm plays a major part in the distribution of nutritions to the tissues and carries waste products from them. The blood also plays an important role in respiration. The substances dissolved in 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.

2.4.3.4 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 “ventilation control” caused by variations in the frequency and intensity of respiratory movements of the tracheal system. The latter movements include opening or closing of tracheal system which are influenced by oxygen and carbon dioxide concentrations.

2.4.3.5 The immune system of silkworm

Silkworm has powerful innate immune system against invading pathogens or adulterated things during its existence. From previous studies it was reported that. Innate immunity of silkworms is composed of two major types; humoral and cellular immune response. Humoral immune response involves antimicrobial proteins (AMPs), soluble proteins in the hemolymph. In families of AMPs have main activity against Gram-positive and Gram-negative bacteria, whereas cellular immune response involves process of phagocytosis which is regulated by hemocytes of silkworms.

2.4.4 Silkworm model of infection for antibiotic screening

Silkworm is, despite their appearance, biologically similar to human in many respects, such as possessing analogous tissues or organs, having similar sensitivities to pathogens, and exhibiting comparable drug effects (64). The silkworm model shows many advantages as an animal model. First, the cost is much lower than mice. Second, there is no/less few ethical problems and no biohazards. In addition, it is easier to inject samples into their body fluids and gut accurately since they are less motile.

In previous study by Kaito and his colleagues, they examined silkworms as an animal model of human infection with pathogenic bacteria. When $3x10^7$ cells of *S. aureus*, *P. aeruginosa*, or *Vibrio cholerae* were injected into the blood of fifth instar silkworm larvae, over 90% of the larvae died within 2 days, whereas over 90% survived for 5 days after injection of the same amount of *E. coli*. Growth of *S. aureus* was observed in larvae blood and tissues. Immunostaining analysis revealed that *S. aureus* proliferated at the surface of the midgut. 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. Disinfectants were not effective because of toxicity against the larvae. Thus, silkworm larvae are useful for evaluating antibiotics for pathogenic bacterial infection in humans (65).

Although invertebrates do not have acquired immune systems, they have developed innate immunity, such as that expressed by Toll-like receptors (66), which is highly conserved and important for resistance to infection by microorganisms. In 2009 Sekimizu and his colleagues considered screening for innate immunity stimulants using the silkworm. There are two different systems of protection in mammals: acquired immunity, which depends on antibodies, and innate immunity, which is independent of them. Stimulation of innate immunity is useful for killing cancer cells, since this system is able to recognize them. For this purpose, they use silkworm muscle contraction as an index. If a sample stimulates innate immunity, then the silkworm muscle contracts. This is a unique phenomenon, in which the muscle of the silkworm contracts and the silkworm's length gradually decreases. When stimulants of innate immunity activate immune cells, reactive oxygen species (ROS) are released. These act on serine proteases, which activate BmPP (*Bombyx mori* paralytic peptide), which in turn induces muscle contraction. Therefore, by observing the circumstances of muscle contraction valuable stimulants of innate immunity can be discovered (67).

In addition, in 2007 Kaito and his colleagues also determined the larval tissues in which *S. aureus* proliferated were examined using immune-staining techniques. A vertically cut section of the larvae at 40 h after *S. aureus* injection. Strong fluorescence was observed at the epithelial cell surface of the midgut. Fluorescence was not observed in tissues from the larvae injected with saline, or in the samples that were not exposed to the *S. aureus* antibody. These results indicated that silkworm larvae were killed by *S. aureus* infection. Kaito and his colleagues determined whether *S. aureus* proliferated in larval bodies. The blood and tissues from the larvae injected with *S. aureus* were sampled, and the bacteria were counted. In both blood and tissues, *S. aureus* increased to more than 1×10^8 cells/larva within 2 days. They also examined if various disinfectants suppressed the killing effect of *S. aureus* (MSSA). First they compared the MIC values against MSSA on Luria-Bertani agar and the LD_{50} values of silkworm larvae (Table 1). Because the LD_{50} values of ethanol and povidone-

iodine were lower than the MIC values, curing the *S. aureus* infection with these disinfectants would not be expected. The MIC value of benzalkonium chloride was lower than the LD_{50} value. It did not, however, suppress the killing effect of *S. aureus* in silkworms. Thus, they concluded that these disinfectants were not effective against *S. aureus* infection of the silkworms (68).

Table 1 Antibacterial effects and toxic effects of various drugs on silkworm larvae.

	MIC on plate (μg/ml)		IC_{50} in larva (μg/ml)		Larva LD_{50} (μg/ml)
	MSSA	**MRSA**	**MSSA**	**MRSA**	
Ampicillin	2	130	<130	>650	$>7\times10^3$
Oxacillin	<0.5	16	<130	>650	5×10^3
Vancomycin	1	1	<130	<130	5×10^3
Benzalkonium chloride	1	2	>70	N.D.	100
EtOH	1.4×10^5	1.4×10^5	N.D.	N.D.	2×10^4
Povidone iodine	5.6×10^5	5.6×10^5	N.D.	N.D.	900

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. In mammals, drugs absorbed from the intestine are carried by the portal vein and first pass through the liver, but this pathway does not exist in silkworm larvae. The 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. The general features of the non-specific transport route are similar between silkworm and mammals (69).

Table 2 Comparison of antibiotics transported via non-specific transport in silkworm larva midgut model and in other models (7).

	Silkworm: transport (% of total/30 min)	**Humans: oral bioavailability (%)[a]**	**Caco-2 cells: P_{app} ($x10^{-5}$ cm/s)[a]**
Tetracycline	1.3	70	0.162
CFPN-PI	1.2	40	0.785
Vancomycin	<1	<1	<0.01

CFPN-PI, cefcapene pivoxil.

[a]The data regarding oral bioavailability in humans and the apparent permeability coefficient (P_{app}) in Caco-2 cells were from.

In 2005 Hamamoto and his colleagues evaluated the feasibility of using the silkworm as a model animal for screening drug candidates. They examined whether the lethal dose of cytotoxic chemicals in silkworm, Bombyx mori, were consistent with those in mammals, and also compared the metabolic pathways of these drugs between silkworms and mice. It has been founded that the lethal dose levels of cytotoxic chemicals in silkworms were consistent with those in mammals. Then they examined further the fate of model drugs, 4-methyl umbellife rone, umbellife rone, and 7-ethoxycoumarine, in silkworm larvae. The half-life of 4-methyl umbelliferone in the silkworm larvae hemolymph was 7.0 ± 0.1 min, similar to that in mouse blood. In

silkworm larvae, 4-methyl umbellife rone was conjugated with glucose, whereas in mammals it is conjugated with glucuronate or sulfate. Furthermore, most umbelliferone and 7-ethoxycoumarin e injected into the hemolymph of silk-worms was eliminated through the feces in the glucose-conjugated form. These findings suggest that chemicals are metabolized through a pathway common to both mammals and silkworms: reaction with cytochrome P450, conjugation with hydroxylated compounds, and excretion (7).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Animals

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

2. Bacterial strains

The bacterial strains used throughout this study were *B. cereus* (Isolate) 2 strains from Department of Microbiology, Faculty of Medicine, Chulalongkorn University, *B. cereus* (ATCC 11778) and *B. subtilis* (ATCC 6633) from College of Public Health Science, Chulalongkorn University and Department of Medical Sciences. *S. aureus* (ATCC 29213) was used for quality control.

3. Preparation of cinnamaldehyde

Cinnamaldehyde stock solution was prepared at 3,150 µg/ml with 0.7% ethanol. By dissolving 30 µl of cinnamaldehyde oil with 70 µl ethanol (99.9%), the solution then was further diluted 100 fold by MHB medium (Molecular weight 132.16 g/mol, Sigma, USA).

4. Chemicals

4.1 70% alcohol

4.2 Cinnamaldehyde (Sigma, USA)

4.3 1% crystal violet

4.4 Ethanol 99.9% (RCI Labscan, Thailand)

4.5 Gentamicin (T.P. Drug Laboratories., Ltd., Thailand)

4.6 Glacial acetic acid (BDH Laboratory, England)

4.7 Methanol (RCI Labscan, Thailand)

4.8 Mueller Hinton Agar (BBL, USA)

4.9 Mueller Hinton Broth (BBL, USA)

4.10 0.9% NaCl

4.11 Silkmate

4.12 Sterile distilled water

5. Instruments

5.1 stainless steel alcohol lamp

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

5.3 Cylinderes

5.4 1 milliliter disposable syringes (Nipro, Thailand)

5.5 Hot air oven (WTB binder, Germany)

5.6 Hypodermic needle 27G×1" (Nipro, Thailand)

5.7 Incubater (Memmert, Germany)

5.8 Larminar airflow cabinet (Astec, Thailand)

5.9 Loop Sterilizer (LabScientific, USA)

5.10 Microcentrifuge tubes (Corning incorporated, Mixico)

5.11 Micropipette size 10, 100 and 1000 microliter (Gilson, France)

5.12 Microplate reader (LabSciencetific, USA)

5.13 Multichannel pipette (Biohit, Finland)

5.14 Petri dishes (Pyrex, USA)

5.15 Pipette tips size 0.1-10 microliter and 100-1000 microliter (Corning incorporated, Mexico)

5.16 Shaker bath

5.17 Spreader

5.18 Sterile loop

5.19 T60 Visible spectrophotometer

5.20 96 well microliter plate (Costar, USA)

Methods

1. *In vitro* antibacterial activity testing of cinnamaldehyde

1.1 Inoculum preparation for dilution tests

The inoculum was prepared by streaking *B. cereus* (Isolate) 2 strains, *B. cereus* (ATCC 11778), *B. subtilis* (ATCC 6633) and *S. aureus* (ATCC 29213) on MHA plates and incubated at 37°C for 24 h. The colonies were touched with a loop and transferred to a tube containing 10 mL of 0.9% NSS. The suspension was adjusted to a turbidity equivalent 0.5 McFarland standard by optical density (OD) of 0.08-0.10 at 625 nm using UV/Visible spectrophotometer (equivalent to $1x10^8$ CFU/ml). This suspension was further diluted 1:100 with MHB (inoculums approximately $1×10^6$ CFU/ml). After that, freshly bacterial suspension was used for each experiment of antimicrobial activity testing (70).

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

MICs were determined using a microdilution method recommended by the CLSI document M07-A9 (70). One hundred microliters of each concentration of serially diluted 2-folded from cinnamaldehyde stock solution were added into the wells number 1-12 of the 96-well microplate (the final concentration of cinnamaldehyde ranging from 0.76-1,575 µg/ml). Gentamicin stock solution were also added into the wells number 1-12 of the 96-well microplate (the final concentration of gentamicin ranging from 0.0018-3.84 µg/ml). Then, one hundred microliters of freshly bacterial suspension from procedure 1.1 were added and mixed in each well (the final concentration of bacteria are $5×10^5$ CFU/ml in all wells). In other wells they consist of control procedures such as growth control, containing 100 µl of bacterial suspension from procedure 1.1 with 100 µl of MHB medium, negative control containing 100 µl of 0.7% ethanol and DI water (solvent of cinnamaldehyde and gentamicin solution).

All test plates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of antibacterial agent that completely inhibited visible growth of the organism in the microdilution wells. In addition, *S. aureus* (ATCC 29213) was used for quality control (71) (All experiments were performed in triplicate).

1.3 Determination of minimum bactericidal concentration (MBC)

The suspension in the wells from MIC and higher concentrations (from the wells that showed no apparent bacteria growth) were spotted on MHA medium. After spotting, the MHA plate was incubated at 37°C for 24 h. The lowest concentration of cinnamaldehyde that prevents any growth of bacteria was recorded as the MBC of cinnamaldehyde. (99.9% killing) (n=3, experiments were performed in triplicate).

1.4 Evaluation of bactericidal and bacteriostatic activity

Actions of an antibacterial agent on the bacterial strains were characterized with two parameters, MIC and MBC. According to the MBC/MIC ratio, we appreciated antibacterial activity. If the MBC/MIC ratio of 1-4, the effect was considered as bactericidal, but if the MBC/MIC ratio of >4, the effect was defined as bacteriostatic (72).

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

Microorganisms were grown shaking overnight at 37°C in MHB medium. The overnight broth was 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). Bacterial suspension was diluted with MHB medium (1:100) that they were containing approximately 1×10^6 CFU/ml. Stock solutions of cinnamaldehyde and gentamicin were diluted with sterile 0.7% ethanol and DI water, respectively to concentrations as exact multiples of the MIC value at 1/2MIC, 1MIC, 2MIC, 4MIC, 8MIC and 16MIC for cinnamaldehyde and 1MIC for gentamicin. Each isolate was inoculated into sterile test tubes containing cinnamaldehyde

(concentrations ranging from 24.60 - 1,575 µg/ml) and positive control (gentamicin 0.12 µg/ml). While the growth control consisted of bacterial suspension with MHB medium and the negative control consisted of 0.7% ethanol and DI water with bacterial suspension. The tube were incubated with shaking on shaker water bath at 37°C. 100 µl aliquots were removed from the tubes at 0, 2, 4, 6, 8, 10, 12 and 24 h and diluted 1: 100 serially. Finally, 100 µl of the serial diluted samples was spread on MHA plates and incubated at 37°C for 24 h for the determination of surviving bacteria. The growth control tube was composed of bacterial pathogen and MHB. The positive control and negative control were composed of gentamicin and 0.7% ethanol, respectively. These control tube was performed in the same experiment as mentioned above. Total viable bacteria were presented as colony forming unit per milliliter (CFU/ml) and the time kill curve was plotted between **Δ**log10 of the viable colonies against time. (All experiments were performed in triplicate).

1.6 Antibiofilm formation assay

Bacterial suspensions from an overnight culture of cells in nutrient broth at 37°C were diluted 1:100 with 1.5% glucose of MHB (inoculums approximately 1×10^6 CFU/ml). All the wells of sterile 96-well polystyrene microliter-plate was filled with 100 µl of each strain cinnamaldehyde and gentamicin was diluted in 0.7% ethanol and DI water, respectively. Sterile 0.7% ethanol and DI water were used as the negative control. The final bacterial concentration in 96-well microliter plate was $5x10^5$ CFU/ml. The plates was incubated at 37°C for 24 h. The medium was removed after 24 h and the wells were washed three times with 300 µl of DI water sterile. The remaining attached bacteria were fixed with 250 µl of methanol per well. After 15 min, microliter-plates were emptied and air dried. Biofilm cells were stained with 250 µl of 1% crystal violet for 15 min. After that, the wells were washed twice with 250 µl of sterile DI water to remove unbound crystal violet. After the microliter plates was air dried, the dye

bound to the adherent cells was extracted with 33% (v/v) glacial acetic acid per well. The absorbance of each well was measured at 570 nm using a UV/Visible spectrophotometer. The percent inhibition were determined using follow Equation (n=3, experiments were performed in triplicate) (73).

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

2. *In vivo* antibacterial activity testing of cinnamaldehyde

2.1 Pathogenicity of bacteria to silkworms

Silkworms were fed with an antibacterial-free artificial food for one day before the experiment began. On the first day, 10 fifth-instar larvae 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 0.9% NSS into hemolymph. The mortality rate of silkworms were observed at 48 hour after injection, then LD_{50} was determined(74). (Experiments were performed in triplicate).

2.2 Determination of LD_{50} of cinnamaldehyde and gentamicin

Silkworms were fed with an antibacterial-free artificial food for one day before the experiment began. On the first day, 10 fifth-instar larvae were injected with 50 µl of cinnamaldehyde and gentamicin solution (dissolved in 0.7% ethanol and DI water) at concentrations of 10, 50, 500, 1,000, 1,500 and 2,000 µg/ml. While the negative control group was injected with 50 µl of 0.7% ethanol into hemolymph. After injection, survival rate of silkworms was observed at 48 h. The LD_{50} was determined by the survival curve as the dose of cinnamaldehyde and gentamicin that killed half of the larvae (LD_{50})

2.3 Determination of ED_{50} of cinnamaldehyde and gentamicin

Silkworms were fed with an antibacterial-free artificial food for one day before the experiment began. 50 µl of suspension of bacteria concentrations that kill all

silkworms from the previous experiment in procedure 2.1 for 1×10^8, 1×10^6, 1×10^7 and 1×10^7 CFU/ml (*B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* isolate1 and *B. cereus* isolate 2, respectively) were injected into hemolymph of the first day of fifth-instar larvae (n=10). 50 µl of cinnamaldehyde solution at concentrations of 10, 50, 100, 500, 1,000 and 1,500 µg/ml as well as gentamicin solution at concentrations of 0.01, 0.1, 1.0, 5.0, 500 and 1,500 µg/ml 50 µl were injected into hemolymph immediately after injection of bacterial suspension. While the negative control group was injected with 50 µl of 0.7% ethanol into hemolymph immediately after injection of bacterial suspension. The number of surviving silkworms were observed at 48 h after injection. ED_{50} was determined from the concentrations of cinnamaldehyde and gentamicin that cured in 50 percent of the silkworms tested (74) (Experiments were performed in triplicate).

Statistical Analysis

Data from *in vitro* and *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. *In vitro* antibacterial activities testing

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

The antibacterial activity of the cinnamaldehyde was evaluated by using broth microdilution method recommended by the CLSI document M07-A9 (70) against medically important pathogens. *In vitro* antibacterial activity of cinnamaldehyde against *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 were shown in Table 3.

Table 3 *In vitro* antibacterial activity of cinnamaldehyde against *B. cereus* ATCC 11778*, B. subtilis* ATCC 6633*, B. cereus* Isolate 1 *and B. cereus* Isolate 2

Bacteria	Substances	MIC (μg/ml)	MBC (μg/ml)	MBC/MIC
B. cereus (ATCC 11778)	Cinnamaldehyde	49.21	196.87	4
B. cereus (ATCC 11778)	Gentamicin*	0.12	0.12	1
B. subtilis (ATCC 6633)	Cinnamaldehyde	98.43	1,575	16
B. subtilis (ATCC 6633)	Gentamicin*	0.12	0.12	1
B. cereus (Isolate 1)	Cinnamaldehyde	196.87	196.87	1
B. cereus (Isolate 1)	Gentamicin*	0.12	0.12	1

Bacteria	Substances	MIC (µg/ml)	MBC (µg/ml)	MBC/MIC
B. cereus (Isolate 2)	Cinnamaldehyde	196.87	196.87	1
B. cereus (Isolate 2)	Gentamicin*	0.12	0.12	1
S. aureus ATCC 29213**	Gentamicin*	0.06	0.06	1

* Positive Control / ** Quality Control

From Table 3, the results showed that cinnamaldehyde exhibited antibacterial activity against the bacterial pathogens at MIC values of 49.21, 98.43, 196.87 and 196.87 µg/ml for *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2, respectively. Whereas MBC values were 196.87 1,575, 196.87 and 196.87 µg/ml, respectively. The MBC/MIC ratios of cinnamaldehyde against *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 were 4, 16, 1 and 1, respectively. Gentamicin was used as a positive control for these bacterial pathogens. MIC and MBC values of gentamicin were the same (0.12 µg/ml) for all microorganisms. Furthermore, *S. aureus* ATCC 29213 was used for quality control. MIC values of gentamicin against *S. aureus* ATCC 29213 was 0.06 µg/ml.

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

The time kill curve was used to determine the bactericidal or bacteriostatic activity of antibacterial. It was analyzed by plotting log 10 CFU/ml versus time. Bactericidal activity was defined as a reduction of greater than or equal to 3 log10 of the total number of CFU/ml in the original inoculum. Bacteriostatic activity was defined as a reduction of less than 3 log10 of the total number of CFU/ml in the original inoculum (75).

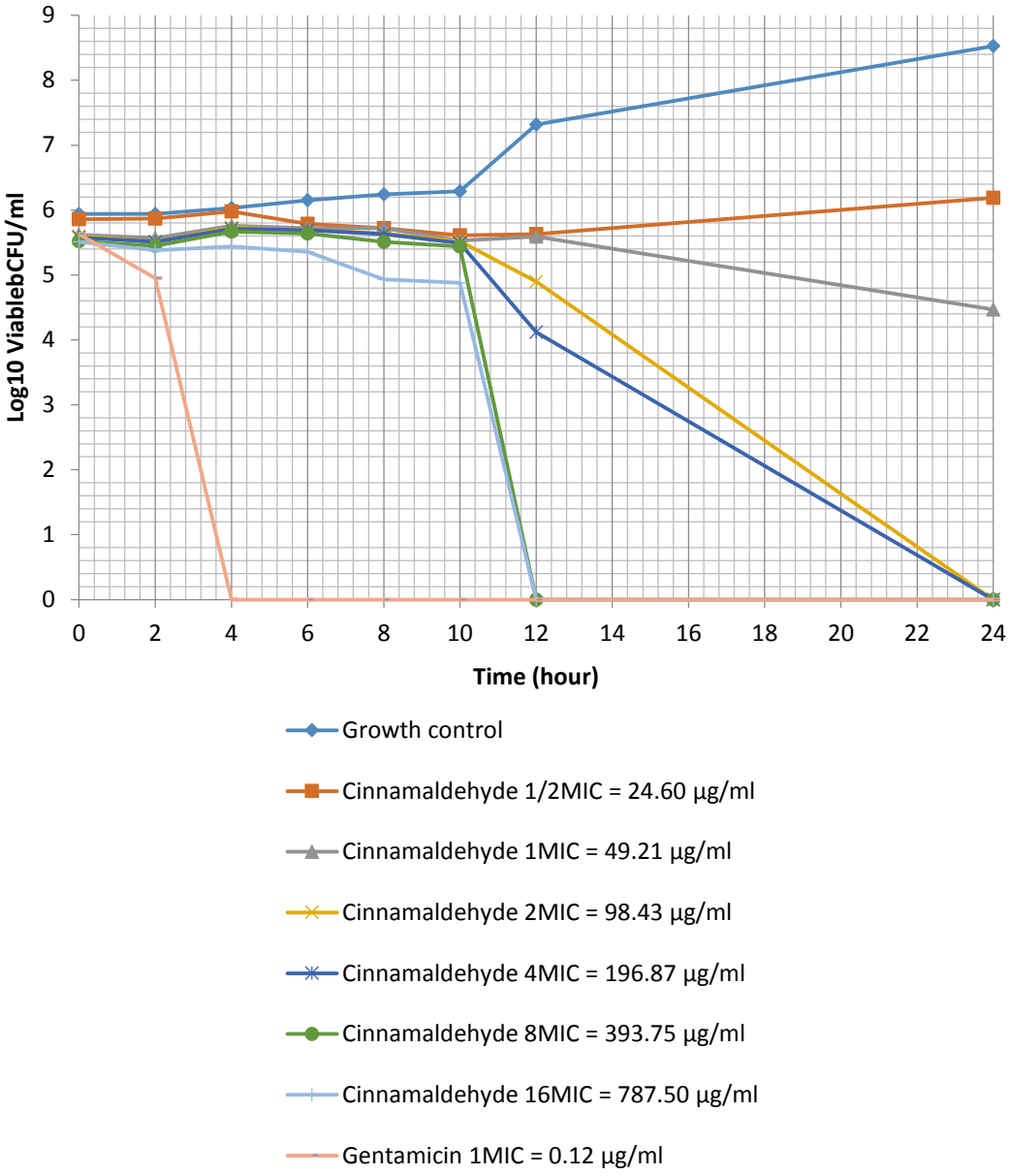

Figure 2 Time kill curve of cinnamaldehyde and gentamicin (Positive control) against *B. cereus* ATCC 11778. Data were the means of triplicate experiments.

In figure 2, cinnamaldehyde at 1/2MIC (24.60 µg/ml) inhibited the growth of *B. cereus* ATCC 11778 with the reduction of <1 log10 which showed bacteriostatic activity at 12 h of incubation and the regrowth was observed at 24 h of incubation. Cinnamaldehyde at 1MIC (49.21 µg/ml) inhibited the growth of microorganism with the reduction of <3 log10 of the total number of CFU/ml in the original inoculum showing bacteriostatic activity at 24 h. Cinnamaldehyde at 2MIC (98.43 µg/ml) and 4MIC (196.87 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum, showing bactericidal effects at 24 h. Cinnamaldehyde at 8MIC (393.75 µg/ml) and 16MIC (787.50 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum, showing bactericidal effects at 12 h. While the positive control, gentamicin at 1MIC (0.12 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum, showing bactericidal effects at 4 h. In addition, when the concentrations of cinnamaldehyde were increased up from 2MIC to 4MIC, 8MIC and the last 16MIC, the onset of killing effect were much faster to occur (24, 24, 12 and 12 h, respectively).

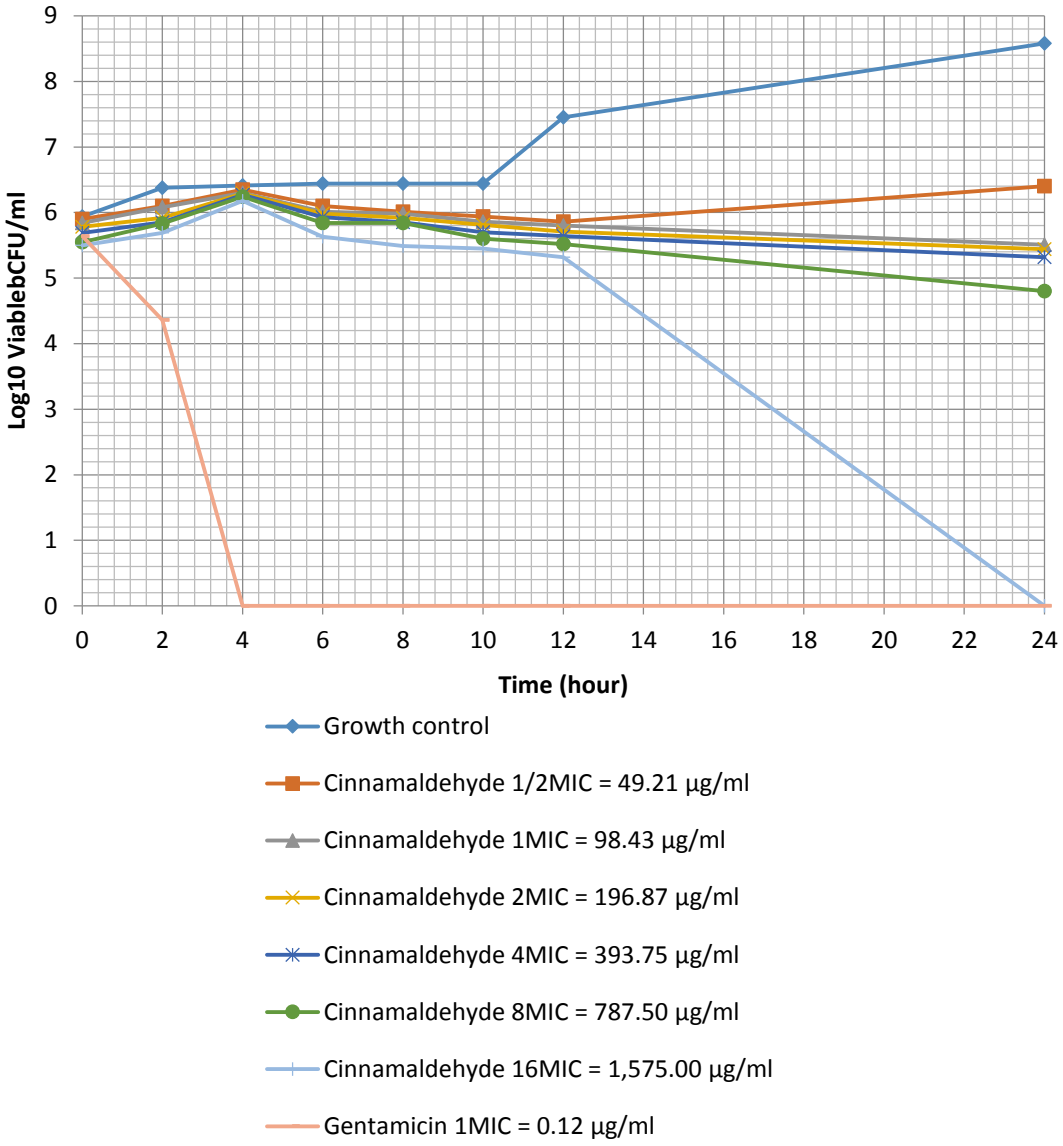

Figure 3 Time kill curve of cinnamaldehyde and gentamicin (Positive control) against *B. subtilis* ATCC 6633. Data were the means of triplicate experiments.

In figure 3, cinnamaldehyde at 1/2MIC (49.21 µg/ml) inhibited the growth of *B. subtilis* ATCC 6633 with the reduction of <1 log10, showing bacteriostatic activity at 12 h of incubation and the regrowth was observed at 24 h of incubation. Cinnamaldehyde at 1MIC (98.43 µg/ml), 2MIC (196.87 µg/ml) and 4MIC (393.75 µg/ml) inhibited the growth of microorganism with the reduction of <1 log10 and 8MIC (787.50 µg/ml) with the reduction of <3 log10 of the total number of CFU/ml in the original inoculum, showing bacteriostatic activity at 24 h. Cinnamaldehyde at 16MIC (1,575 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum, showing bactericidal effects at 24 h. While positive control, gentamicin at 1MIC (0.12 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum showing bactericidal effects at 4 h. In addition, when the concentrations of cinnamaldehyde were increased up to 16MIC, the onset of killing effect were occurred at 24 h.

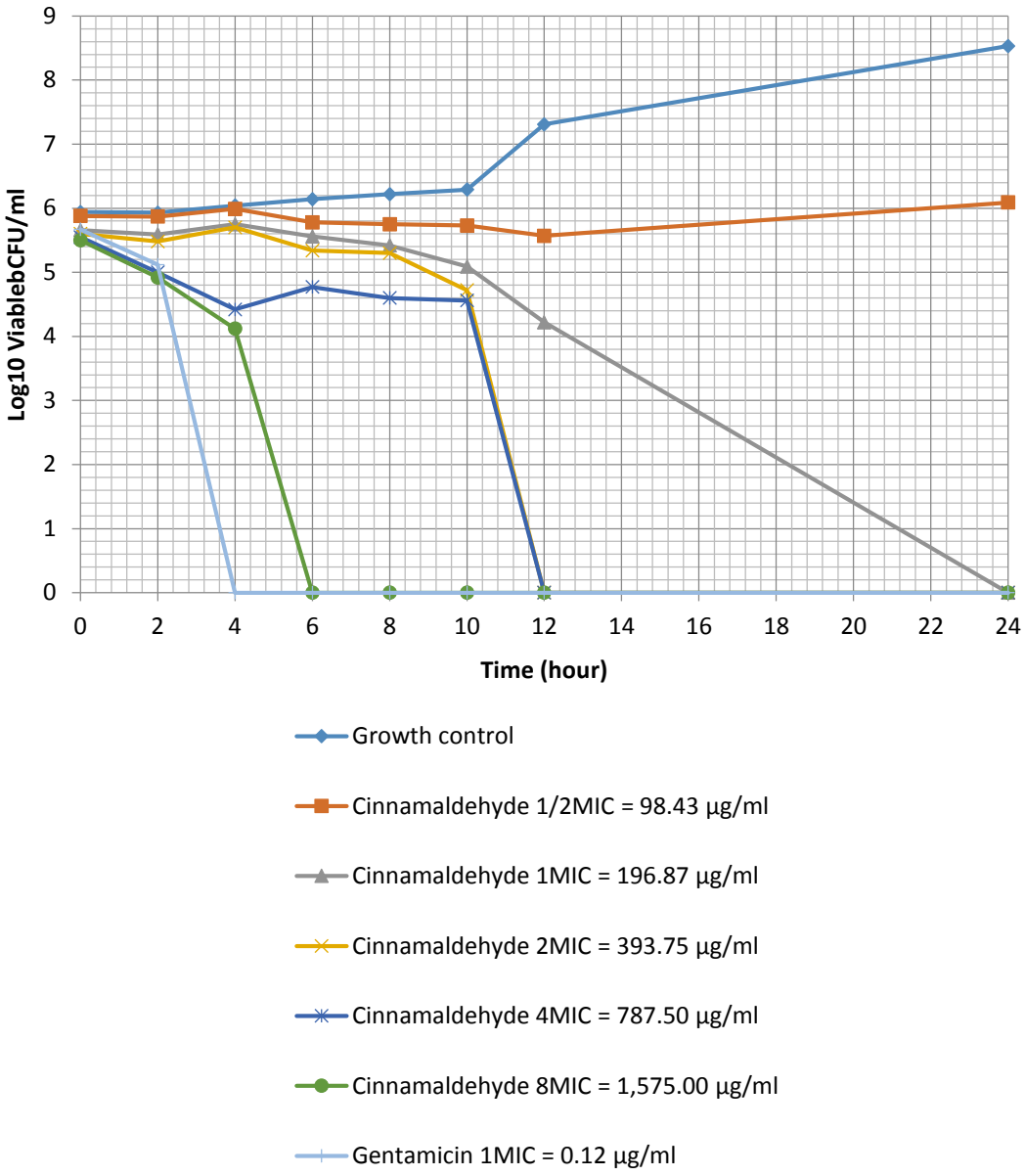

Figure 4 Time kill curve of cinnamaldehyde and gentamicin (Positive control) against *B. cereus* Isolate 1. Data were the means of triplicate experiments.

In figure 4, cinnamaldehyde at 1/2MIC (98.43 µg/ml) inhibited the growth of *B. cereus* Isolate 1 with the reduction of <1 log10 which showed bacteriostatic activity at 12 h of incubation and the regrowth was observed at 24 h of incubation. Cinnamaldehyde at 1MIC (196.87 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum showing bactericidal activity at 24 h. Cinnamaldehyde at 2MIC (393.75 µg/ml) and 4MIC (787.50 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum, showing bactericidal activity at 12 h. Cinnamaldehyde at 8MIC (1,575 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum showing bactericidal activity at 6 h. While positive control, gentamicin at 0.12 µg/ml (1MIC) inhibited the growth of microorganism with the reduction >3 log10 of the total number of CFU/ml in the original inoculum showing bactericidal activity at 4 h. In addition, when the concentrations of cinnamaldehyde were increased up from 1MIC to 2MIC, 4MIC and the last 8MIC, the onset of killing effect were much faster to occur (24, 12, 12 and 6 h, respectively).

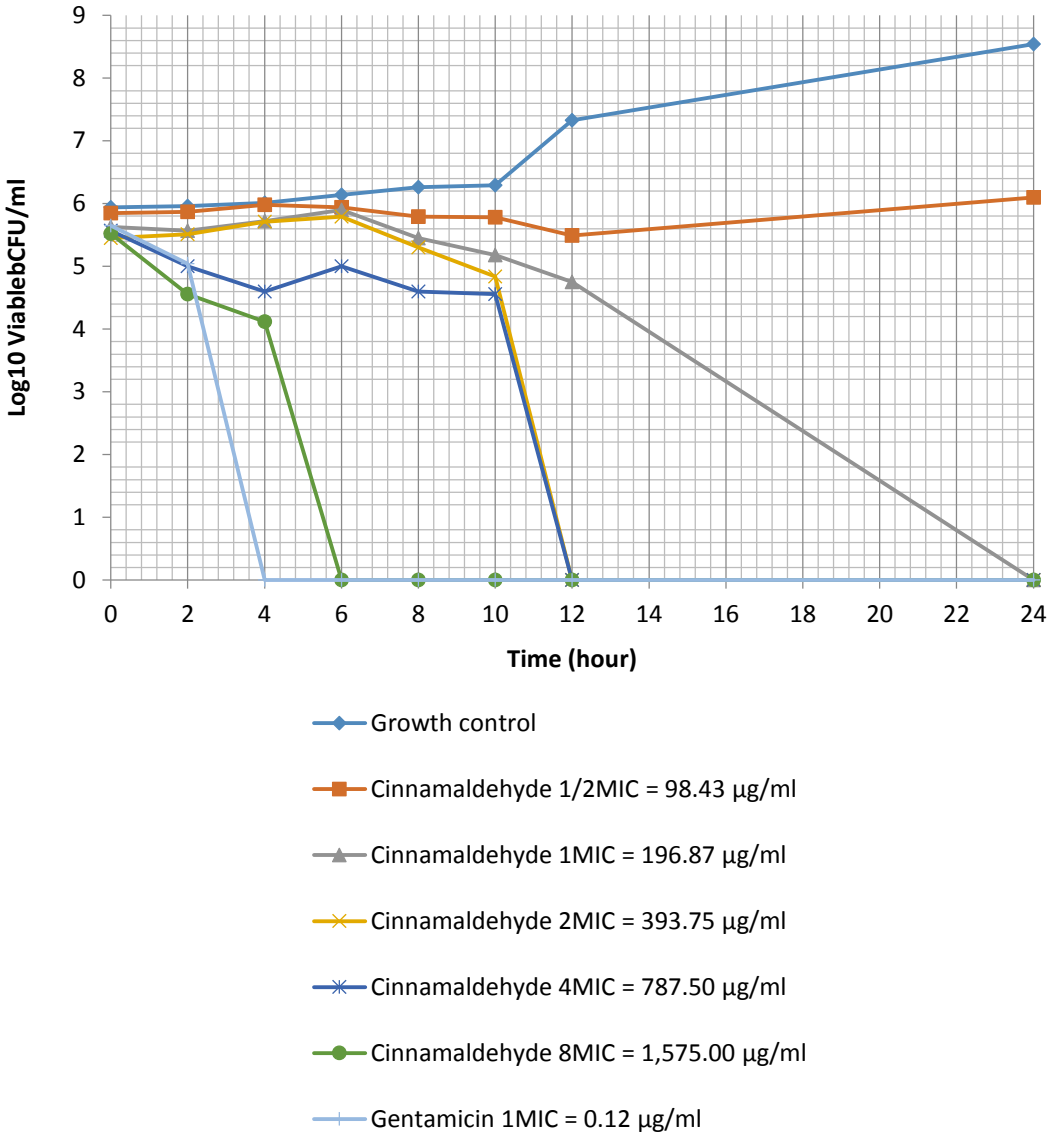

Figure 5 Time kill curve of cinnamaldehyde and gentamicin (Positive control) against *B. cereus* Isolate 2. Data were the means of triplicate experiments.

In figure 5, cinnamaldehyde at 1/2MIC (98.43 µg/ml) inhibited the growth of *B. cereus* Isolate 1 with the reduction of <1 log10 which showed bacteriostatic activity at 12 h of incubation and the regrowth was observed at 24 h of incubation. Cinnamaldehyde at 1MIC (196.87 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum showing bactericidal activity at 24 h. Cinnamaldehyde at 2MIC (393.75 µg/ml) and 4MIC (787.50 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum, showing bactericidal activity at 12 h. Cinnamaldehyde at 8MIC (1,575 µg/ml) inhibited the growth of microorganism with the reduction of >3 log10 of the total number of CFU/ml in the original inoculum showing bactericidal activity at 6 h. While positive control, gentamicin at 0.12 µg/ml (1MIC) inhibited the growth of microorganism with the reduction >3 log10 of the total number of CFU/ml in the original inoculum showing bactericidal activity at 4 h. In addition, when the concentrations of cinnamaldehyde were increased up from 1MIC to 2MIC, 4MIC and the last 8MIC, the onset of killing effect were much faster to occur (24, 12, 12 and 6 h, respectively).

1.3 Inhibition of biofilm formation assay

The results showed that both cinnamaldehyde and gentamicin exerted the inhibitory effects on biofilm formation in concentration dependent manner at concentration equal and greater than of 1MIC with statistical significance comparing with negative control (0.7% ethanol group) (Figure 6-13).

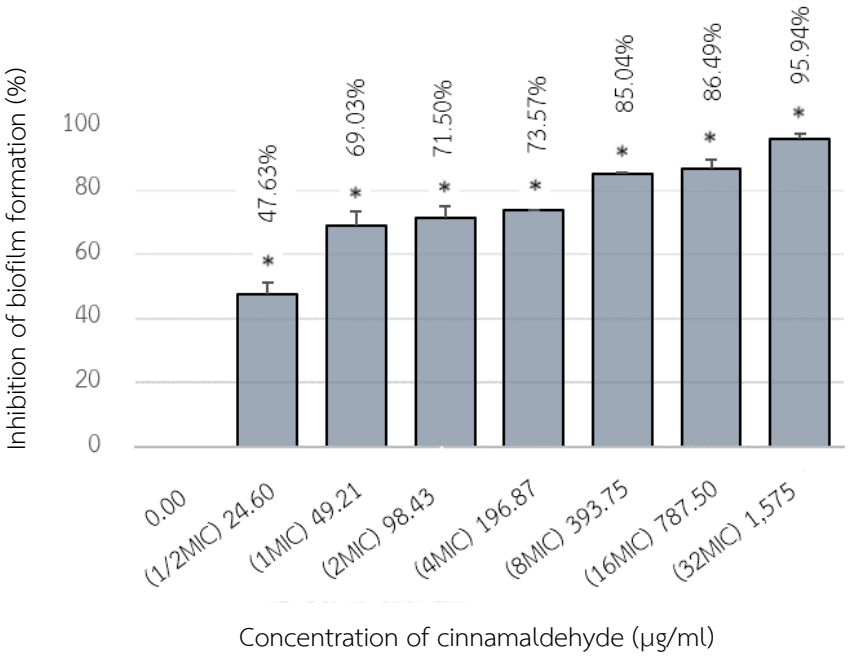

Figure 6 The inhibition effect of cinnamaldehyde on the biofilm formation by *B. cereus* ATCC 11778, error bars represent the mean ± SEM, *p<0.05.

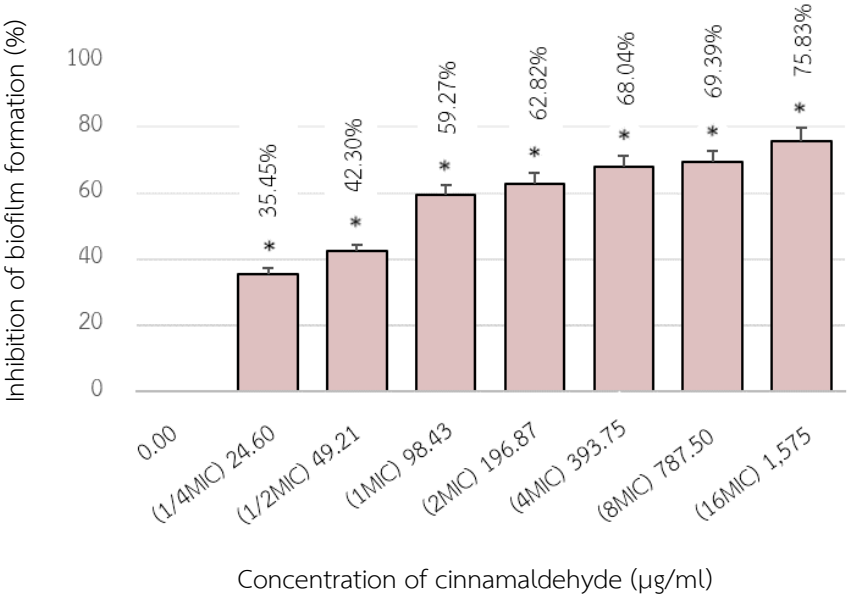

Figure 7 The inhibition effect of cinnamaldehyde on the biofilm formation by *B. subtilis* ATCC 6633, error bars represent the mean ± SEM, *p<0.05.

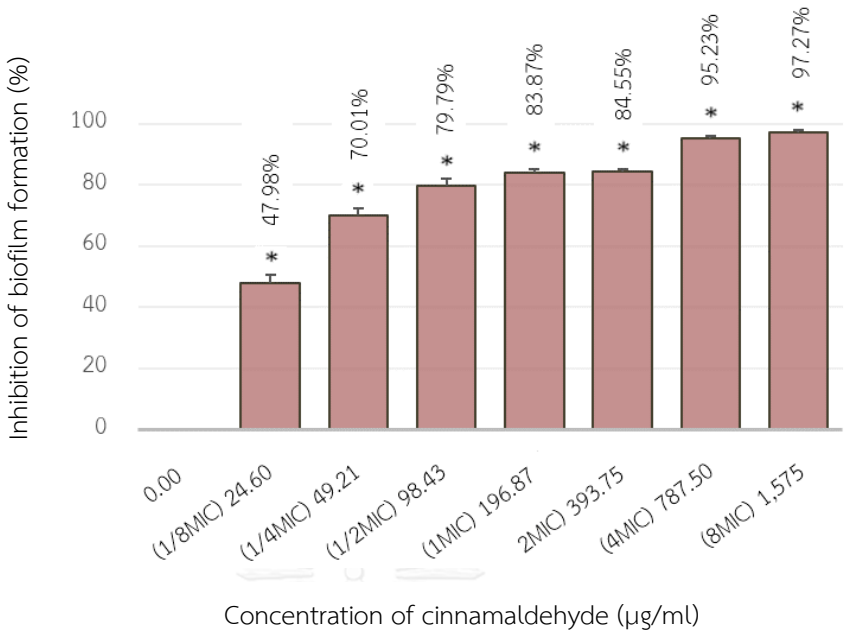

Figure 8 The inhibition effect of cinnamaldehyde on the biofilm formation by *B. cereus* Isolate 1, error bars represent the mean ± SEM, **p*<0.05.

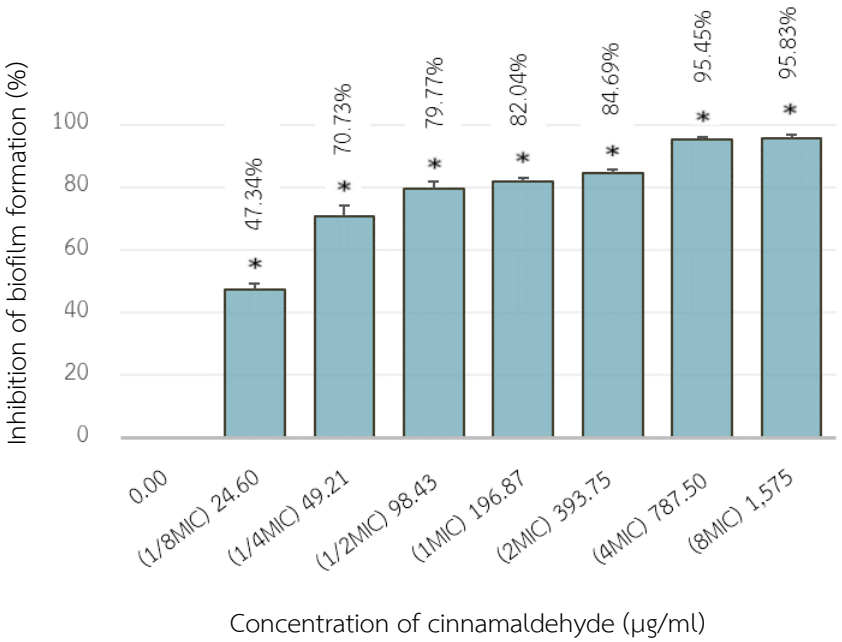

Figure 9 The inhibition effect of cinnamaldehyde on the biofilm formation by *B. cereus* Isolate 2, error bars represent the mean ± SEM, **p*<0.05.

Cinnamaldehyde at the highest concentration 1,575 µg/ml showed the maximum inhibition on biofilm formation with the percentage of inhibition of 95.94%, 75.83%, 97.27% and 95.83% for *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2, respectively.

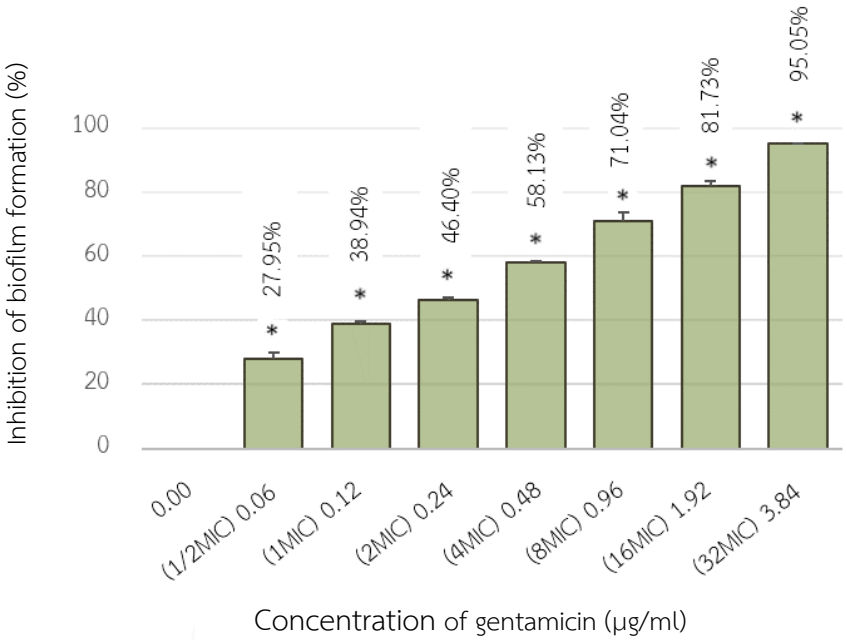

Figure 10 The inhibition effect of gentamicin on the biofilm formation by *B. cereus* ATCC 11778, error bars represent the mean ± SEM, *$p<0.05$.

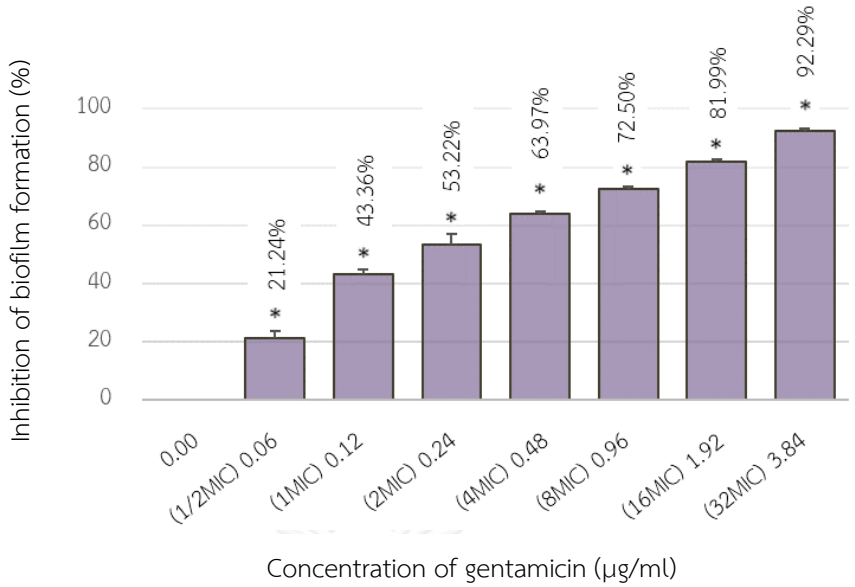

Figure 11 The inhibition effect of gentamicin on the biofilm formation by *B. subtilis* ATCC 6633, error bars represent the mean ± SEM, *p<0.05.

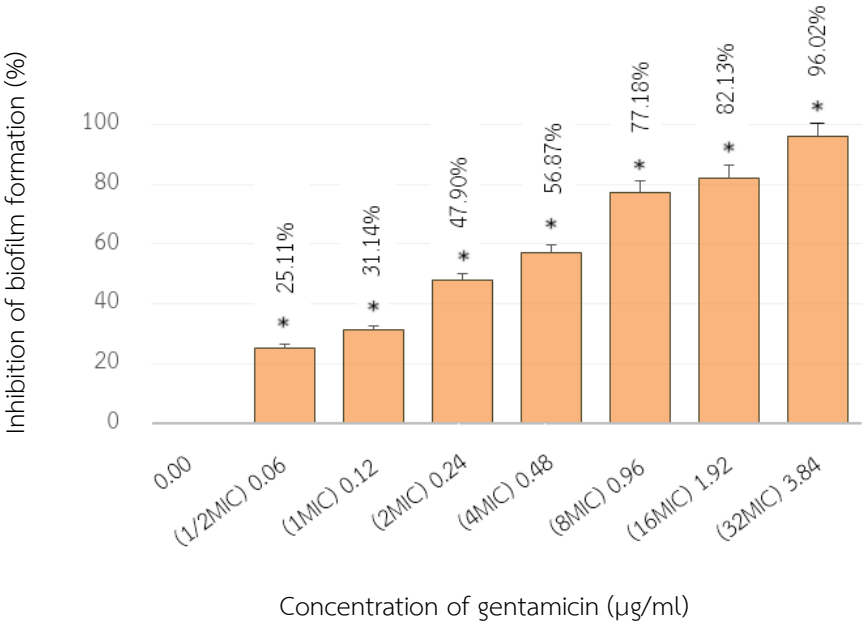

Figure 12 The inhibition effect of gentamicin on the biofilm formation by *B. cereus* Isolate 1, error bars represent the mean ± SEM, *p<0.05.

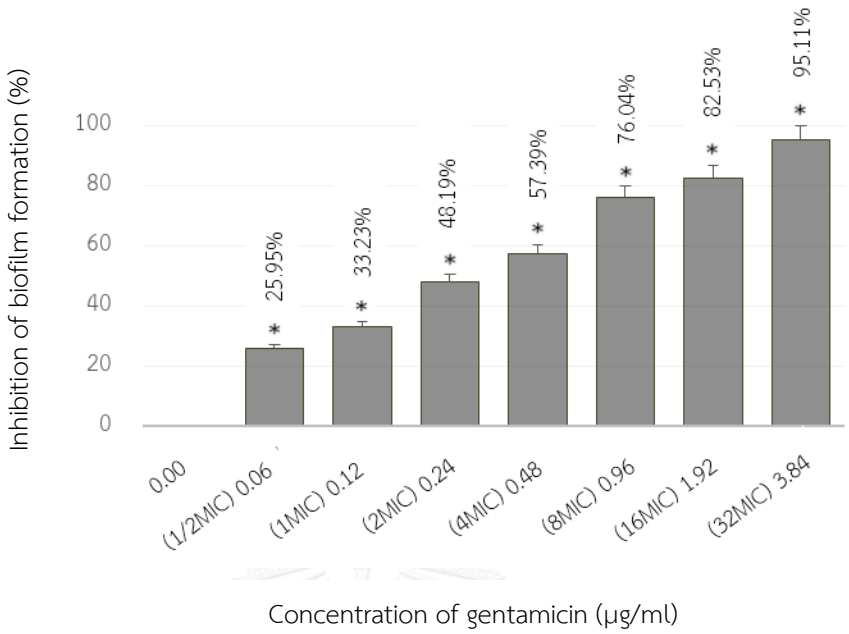

Figure 13 The inhibition effect of gentamicin on the biofilm formation by *B. cereus* Isolate 2, error bars represent the mean ± SEM, *p<0.05.

Figure 10-13, Gentamicin at the highest concentration 3.84 µg/ml showed the maximum inhibition on biofilm formation with the percentage of inhibition for *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 at 95.05%, 92.29%, 96.02% and 95.11%, respectively.

2. *In vivo* antibacterial activities testing

2.1 Pathogenicity study of bacteria in silkworms

This study examined whether injection of *Bacillus* spp. could kill silkworms. The results were shown as LD_{50}. LD_{50} of *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 were 5.32×10^5, 2.37×10^3, 2.61×10^5 and 1×10^5 CFU/ml, respectively (Figure 14 to 17). All silkworms died within 48 h.

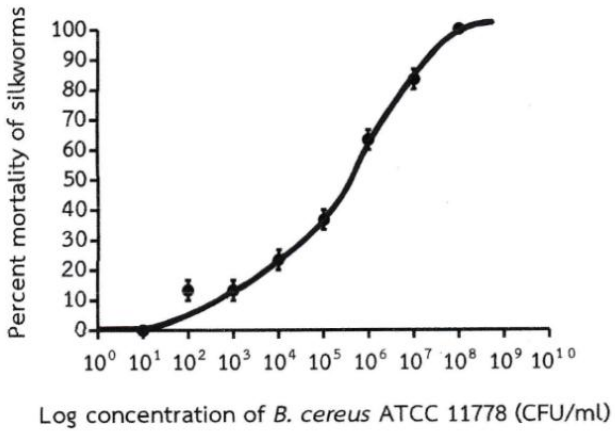

Figure 14 Pathogenicity of *B. cereus* ATCC 11778 in silkworms. The LD_{50} was 5.32×10^5 CFU/ml

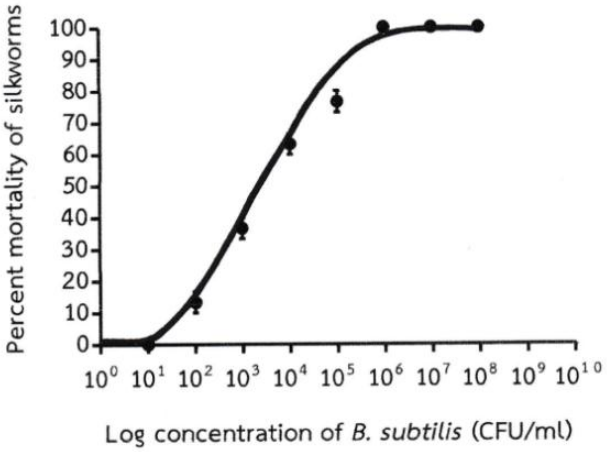

Figure 15 Pathogenicity of *B. subtilis* ATCC 6633 in silkworms. The LD_{50} was 2.37×10^3 CFU/ml

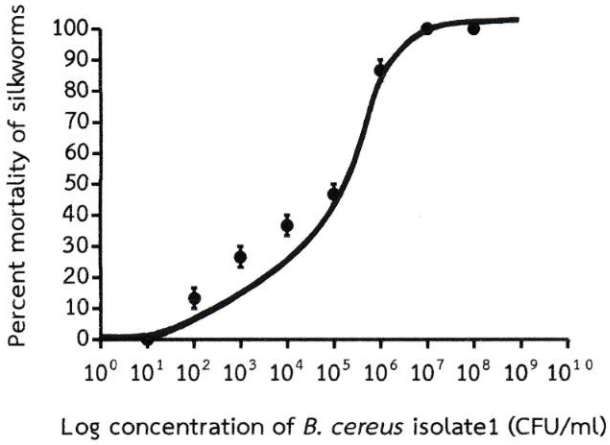

Figure 16 Pathogenicity of *B. cereus* Isolate 1 in silkworms. The LD_{50} was 2.61×10^5 CFU/ml

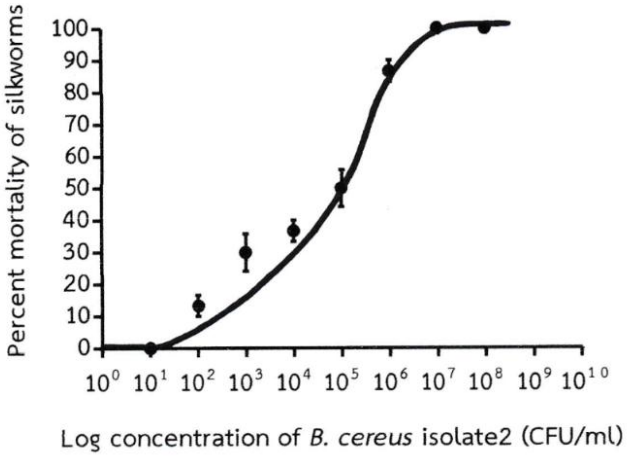

Figure 17 Pathogenicity of *B. cereus* Isolate 2 in silkworms. The LD_{50} was 1×10^5 CFU/ml

2.2 Determination of Lethal dose (LD_{50}) of cinnamaldehyde and gentamicin in silkworms

The results were shown as LD_{50} of cinnamaldehyde and gentamicin (Positive control). LD_{50} of cinnamaldehyde and gentamicin were greater than 2,000 µg/ml. Therefore, cinnamaldehyde and gentamicin were safe in silkworms.

Table 4 LD_{50} of cinnamaldehyde and gentamicin

Substances	LD_{50} (µg/ml)
Cinnamaldehyde	> 2,000
Gentamicin (Positive control)	> 2,000

2.3 Determination of Median Effective Dose (ED_{50}) of cinnamaldehyde and gentamicin

Cinnamaldehyde and gentamicin were used to determine the ED_{50} in silkworm infection model. ED_{50} values against *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 were 438.16, 994.56, 389.50 and 396.03 µg/ml (figure 18 to 21), respectively. ED_{50} values of gentamicin against *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 were 0.04, 0.27, 0.03 and 0.03 µg/ml (figure 22 to 25), respectively. Error bars represent the mean ± SEM, $*p < 0.05$; cinnamaldehyde and gentamicin groups were compared with negative control group.

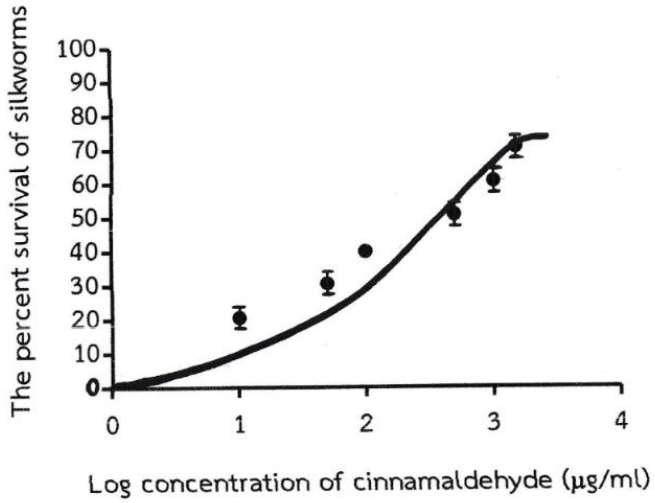

Figure 18 ED_{50} of cinnamaldehyde in silkworm infected with *B. cereus* ATCC 11778. The ED_{50} was 438.16 µg/ml.

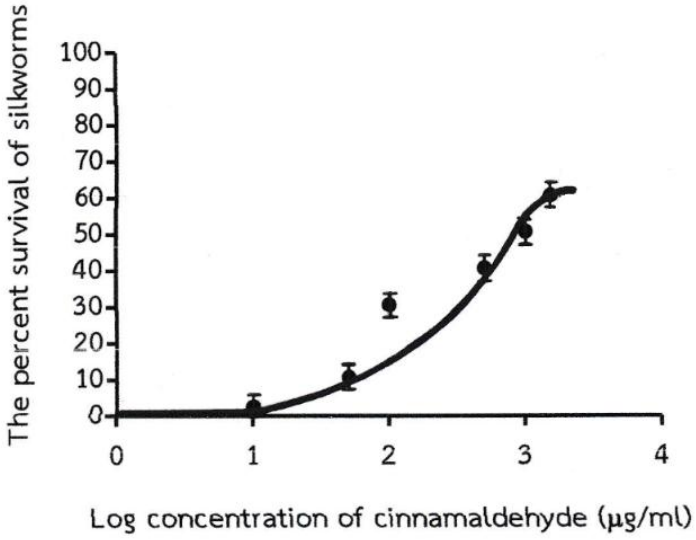

Figure 19 ED_{50} of cinnamaldehyde in silkworm infected with *B. subtilis* ATCC 6633. The ED_{50} was 994.56 µg/ml.

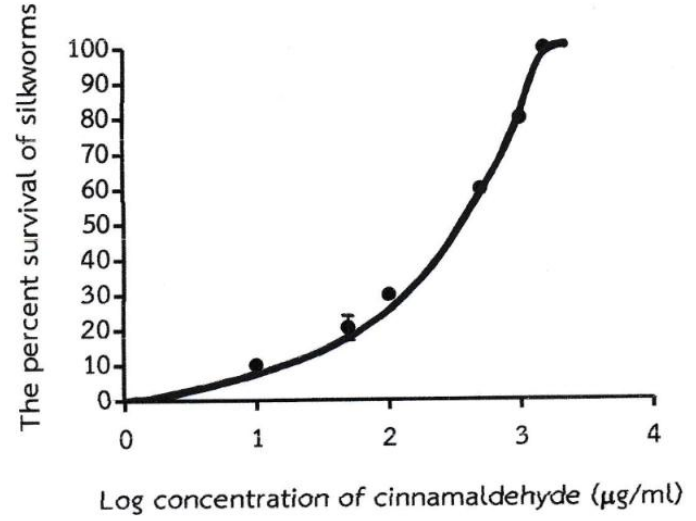

Figure 20 ED_{50} of cinnamaldehyde in silkworm infected with *B. cereus* Isolate 1. The ED_{50} was 389.50 µg/ml.

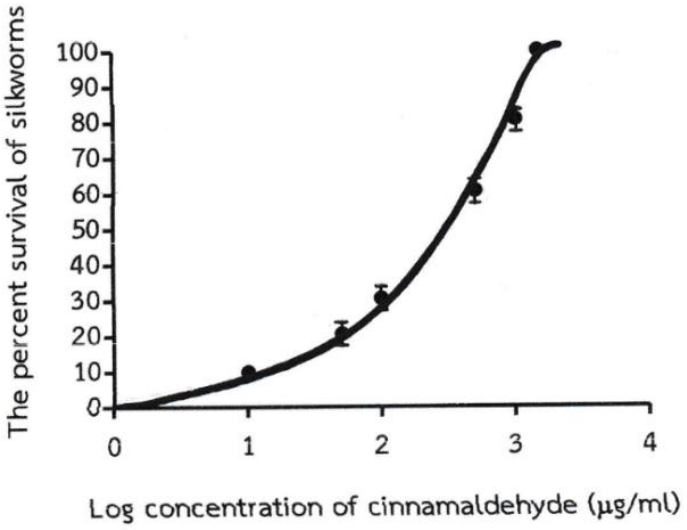

Figure 21 ED_{50} of cinnamaldehyde in silkworm infected with *B. cereus* Isolate 2. The ED_{50} was 396.03 µg/ml.

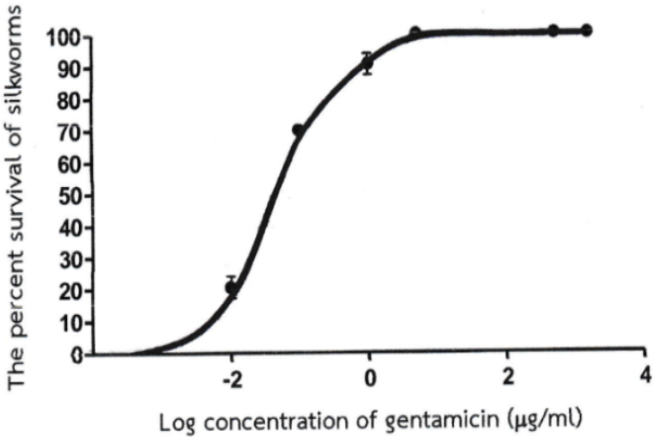

Figure 22 ED_{50} of gentamicin in silkworm infected with *B. cereus* ATCC 11778. The ED_{50} was 0.04 µg/ml.

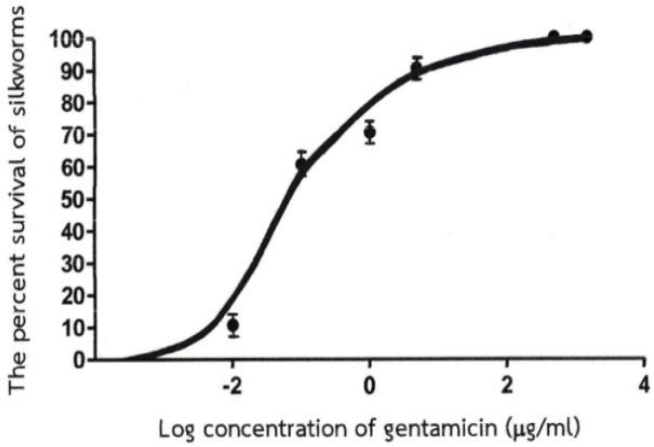

Figure 23 ED_{50} of gentamicin in silkworm infected with *B. subtilis* ATCC 6633. The ED_{50} was 0.27 µg/ml.

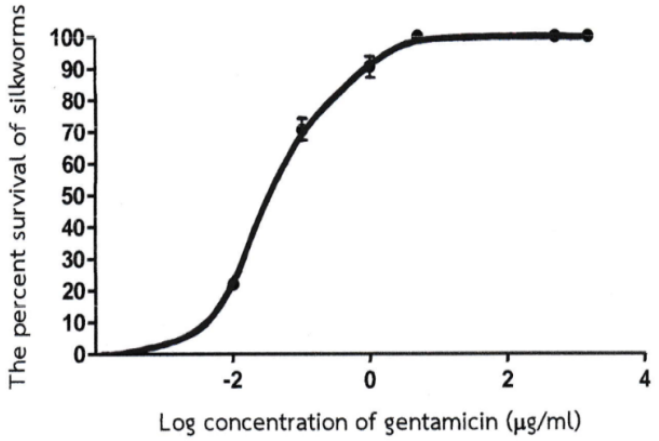

Figure 24 ED_{50} of gentamicin in silkworm infected with *B. cereus* Isolate 1. The ED_{50} was 0.03 µg/ml.

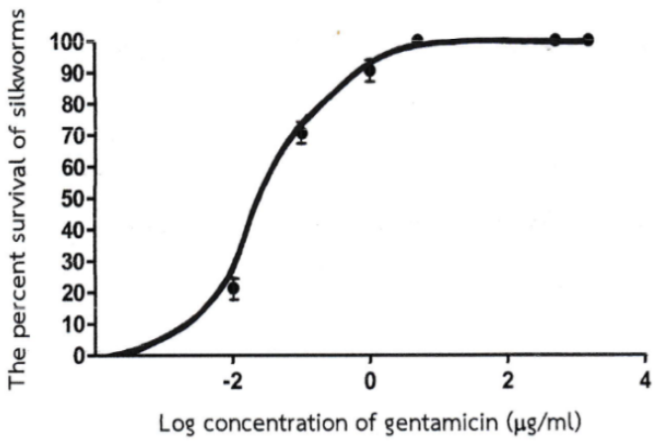

Figure 25 ED_{50} of gentamicin in silkworm infected with *B. cereus* Isolate 2. The ED_{50} was 0.03 µg/ml.

CHAPTER V

DISCUSSION AND CONCLUSION

Bacillus species associated foodborne illness occurs as two distinct intoxication syndromes: emetic and diarrhoeal. Recovery is rapid for both syndromes, usually within 12-24 hours. There are usually no long-term effects, but severe consequences, including fatalities, can occasionally occur. Although various antibiotics have been used for the treatment of infectious disease, but increasing drug resistance and problems through food poisoning of *Bacillus* spp. In this study, therefore, we investigated antibacterial activities both *in vitro* (MIC and MBC) and *in vivo* (Thai silkworm infection model) and also investigated antibiofilm formation activities of cinnamaldehyde in *Bacillus* spp. Furthermore the bactericidal activity was assessed using time kill assay *in vitro*.

The results showed that cinnamaldehyde exhibited antibacterial activity against all *bacillus* spp. used in this study at MIC values of 49.21, 98.43, 196.87 and 196.87 µg/ml for *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2, respectively. Whereas MBC values were 196.87, 1,575, 196.87 and 196.87 µg/ml respectively. Gentamicin was used as a positive control for these bacterial pathogens. MIC and MBC values of gentamicin were the same (0.12 µg/ml) for all tested *bacillus* spp. In order to determine whether cinnamaldehyde possesses bactericidal or bacteriostatic effect, value of MBC/MIC ratio, was calculated. From MBC/MIC ratio, it was indicated that cinnamaldehyde was bacteriostatic agent against *B. subtilis* ATCC 6633 with MBC/MIC ratio of 16, whereas cinnamaldehyde was bactericidal agent against *B. cereus* ATCC 11778, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 with the MBC/MIC ratio of 4, 1 and 1, respectively. Furthermore, *S. aureus* ATCC 29213 was used as quality

control recommended by CLSI document M07-A9 (70). Moreover, MIC and MBC values of gentamicin against *S. aureus* ATCC 29213 were 0.06 µg/ml and the value was consistent to the susceptibility breakpoint defined by CLSI document M07-A9 (70).

Al-Bayati and his colleagues investigated antibacterial activity of cinnamaldehyde 99% purity from *Cinnamomum zeylanicum* L. barkoil against pathogenic bacteria. A stock solution of cinnamaldehyde was prepared in 10% DMSO. The MIC of cinnamaldehyde was determined using a broth microdilution method in 96-well microtiter plates. MIC values ranged from 31.2 µg/ml. The most promising result was observed against *B.cereus* (56). This MIC value of *B. cereus* is much better than our MIC value of *B. cereus* (31.2 and 49.21 µg/ml, respectively). This may be due to the fact that percent purity of our cinnamaldehyde is a little bit lower than Al-Bayati's cinnamaldehyde (≥95% and 99%, respectively). In addition, Huang and his colleagues investigated antibacterial activity of cinnamaldehyde of bark extract of cinnamon. The extract was prepared in 5% DMSO. The extract was 68.52% in purity. The results showed its effect against *B. subtilis* with MIC and MBC value of 5,000 and 10,000 µg/ml, respectively (59). Whereas, our results showed that cinnamaldehyde with ≥95% in purity prepared in 0.7% ethanol exhibited antibacterial activity against *B. subtilis* ATCC 6633 had MIC and MBC values of 98.43 and 1,575 µg/ml for *B. subtilis* ATCC 6633. This may be due to the fact that percent purity of our cinnamaldehyde is much higher than Huang's cinnamaldehyde (≥95% and 99%, respectively).

MBC measurement is a one-dimensional endpoint determination based on a qualitative "yes" or "no" presumption; but concentration-killing experiments with antibacterial agent have shown the true response to concentration and this has clearly exposed a trend in the gradual reduction in the number of surviving CFU per plate (76). The time kill assay was used to determine whether the cinnamaldehyde was

bactericidal or bacteriostatic. In time kill assay, cinnamaldehyde possess bactericidal effect when concentrations increased from 2MIC to 4MIC, 8MIC and 16MIC for *B. cereus* ATCC 11778. Whereas cinnamaldehyde possess bactericidal effect when concentrations increased from 1MIC to 2MIC, 4MIC and 8MIC for both *B. cereus* Isolate 1 and *B. cereus* Isolate 2. But not for *B. subtilis,* cinnamaldehyde possess bactericidal effect only at the highest concentration (16MIC). This results of time kill assay were consistent with MIC and MBC value indicating that *B. cereus* is more susceptible to cinnamaldehyde than *B. subtilis*. For gentamicin (positive control) at 1MIC (0.12 µg/ml), it can kill all tested microorganisms within 4 h. These results indicated that cinnamaldehyde exhibited antibacterial activity in concentration dependent manner. Moreover, in 2016 Zhang and his colleagues elucidated the mechanism of antibacterial action of cinnamon essential oil against *E. coli* and *S. aureus* using scanning electron microscope. After adding cinnamon essential oil at MIC level, there were obvious changes in the morphology of bacteria cells indicating cell damage. When cinnamon essential oil were added at MBC levels, the cell were destroyed (61). This concrete results in morphological changes may apply for the mechanism of action of cinnamaldehyde on tested *Bacillus* spp. in our study.

Biofilm formations would be crucial to further step in the development of medical and dental implants as they are easily colonized by biofilm-forming pathogenic bacteria (77). Apart from antibacterial activities, cinnamaldehyde also possesses antibiofilm activity in concentration dependent manner (at concentration equal and greater than 1MIC) with statistical significance ($p<0.05$) comparing with negative control (0.7% ethanol group). The results also showed that cinnamaldehyde at the highest concentration (1,575 µg/ml) could inhibit biofilm formation of *B. cereus* ATCC 11778, *B. cereus* Isolate1 and *B. cereus* Isolate 2 better than *B. subtilis* ATCC 6633

with percent inhibition of 95.94, 97.27, 95.83 and 75.83, respectively. Again cinnamaldehyde inhibited biofilm formation against *B. cereus* better than *B. subtilis.*

In the course of developing drugs to treat infectious diseases, chemical compounds with antibacterial activity *in vitro* are tested for their therapeutic efficacy *in vivo* in animal infection models. A serious problem is that most of compounds that exhibit antibacterial activity *in vitro* do not have therapeutic effects in animal infection models due to toxicity and pharmacokinetic issues (8). Therefore, the development of a non-vertebrate infection model to test drug efficacy in the early stages of development is highly desirable (8). In the present study, we also examined both cytotoxic effects and the therapeutic effects of cinnamaldehyde and gentamicin in Thai silkworm infection model. The results showed that this model can be used to evaluate the toxicity of cinnamaldehyde and gentamicin and it has been found that cinnamaldehyde and gentamicin were very safe in Thai silkworm infection model with LD_{50} greater than 2,000 µg/ml. Furthermore, Thai silkworm infection model can also be used to evaluate the therapeutic effects of cinnamaldehyde and gentamicin. The results showed that cinnamaldehyde was effective in preventing the infection in silkworm with *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 with ED_{50} of 438.16, 994.56, 389.50 and 396.03 µg/ml, respectively. Gentamicin (positive control) also could prevent the infection in silkworm with *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2 with ED_{50} of 0.04, 0.27, 0.03 and 0.03 µg/ml, respectively.

When we investigated further the concentration level of cinnamaldehyde and gentamicin in silkworm hemolymph in effcicacy study with various injected doses, the corresponding concentration in hemolymp were shown below.

Calculated concentration of cinnamaldehyde in silkworm hemolymph corresponding to each injected dose

Solution concentration (µg/ml)	Cinnamaldehyde concentration in hemolymph in effcicacy study
10	0.5/470= 0.0011 µg/g larvae/ 0.0011 µg/µl = 1.10 µg/ml
50	2.5/470=0.0053 µg/g larvae/0.0053 µg/µl = 5.3 µg/ml
100	5/470= 0.011 µg/g larvae/0.011 µg/µl = 11 µg/ml
500	25/470= 0.053 µg/g larvae/0.053 µg/µl = 53 µg/ml
1,000	50/470= 0.11 µg/g larvae/0.11 µg/µl = 110 µg/ml
1,500	75/470= 0.159 µg/g of larvae/0.159µg/µl = 159 µg/ml

Calculated concentration of gentamicin in silkworm hemolymph corresponding to each injected dose

Solution concentration (µg/ml)	Gentamicin concentration in hemolymph in effcicacy study
0.01	0.0005/470= 0.0000011 µg/g larvae/0.0000011 µg/µl = 0.0011 µg/ml
0.1	0.005/470= 0.000011 µg/g larvae/0.000011 µg/µl = 0.011 µg/ml
1.0	0.05/470= 0.00011 µg/g larvae/0.00011 µg/µl = 0.11 µg/ml
5.0	0.25/470= 0.00053 µg/g larvae/0.00053 µg/µl = 0. 53 µg/ml
500	25x/470= 0.053 µg/g larvae/0.0532 µg/µl = 53.2 µg/ml
1,500	75/470= 0.159 µg/g larvae/0.159µg/µl = 159 µg/ml

For gentamicin, at injected dose 500 and 1,500 µg/ml 100% survival of silkworm was observed for all tested bacteria and concentration of gentamicin in silkworm hemolymph corresponding to such injected dose were 53.2 (~40MIC) and 159 (~120MIC) µg/ml, respectively. This result is in consistent with the nature of gentamicin having concentration dependent manner for antibacterial activity with concentration level ≥ 10MIC. However, In case of cinnamaldehyde, at all injected dose 10, 50, 100, 500, 1,000 and 1,500 µg/ml 100% survival of silkworm could not be observed for *B. cereus* ATCC 11778 and *B. subtilis* ATCC 6633. But *B. cereus* Isolate1 and *B. cereus* Isolate 2 at injected dose 1,500 µg/ml 100% survival of silkworm could be observed and concentration of cinnamaldehyde in silkworm hemolymph corresponding to such injected dose were 159 µg/ml. This 159 µg/ml of cinnamaldehyde in hemolymph is only 1MIC for *B. cereus* Isolate1 and *B. cereus* Isolate 2. From the result in our *in vitro* study cinnamaldehyde exhibited concentration dependent manner for antibacterial activity. Therefore cinnamaldehyde should not have antibacterial activity at low concentration as 1MIC (159 µg/ml) against *B. cereus* Isolate1 and *B. cereus* Isolate 2. It is possible that cinnamaldehyde may have another activity such as helper in strengthening the immune system and resulting in increasing the overall antibacterial activity. This *in vivo* study again confirmed that *B. cereus* is more susceptible to cinnamaldehyde than *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 (74).

Owing to results from Time kill assay, we suggest that further study may be needed such as synergy study for the combination of erythromycin (35) with cinnamaldehyde using time–kill assay. The result from synergy study will provide more

information on the proper use of cinnamaldehyde in combination with other antibiotics using in clinical setting.

In conclusion, this study reported that cinnamaldehyde had both antibacterial and antibiofilm formation activities on tested *Bacillus* spp. Moreover, it showed bactericidal activity against *B. cereus* ATCC 11778, *B. subtilis* ATCC 6633, *B. cereus* Isolate 1 and *B. cereus* Isolate 2. Therefore, cinnamaldehyde could be a compound used for treating infection, considering as a helper to increase the overall antibacterial activity. However, further studies may be needed such as pharmacodynamics and pharmacokinetics in mammalian model.

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APPENDIX

APPENDIX A

Data of *In Vitro* Studies

Table 5 Survival colonies of *B. cereus* ATCC 11778 received cinnamaldehyde and gentamicin (Positive control) in Time kill assay

Hours	Survival colonies (Log_{10} CFU/ml)							
	Growth control	CNMA 1/2MIC (24.60 µg/ml)	CNMA 1MIC (49.21 µg/ml)	CNMA 2MIC (98.43 µg/ml)	CNMA 4MIC (196.87 µg/ml)	CNMA 8MIC (393.75 µg/ml)	CNMA 16MIC (787.50 µg/ml)	Gentamicin 1MIC (0.12 µg/ml)
0	$8.83x10^5$	$7.4x10^5$	$4.2x10^5$	$4.03x10^5$	$3.86x10^5$	$3.33x10^5$	$3.26x10^5$	$4.33x10^5$
2	$8.9x10^5$	$7.43x10^5$	$3.8x10^5$	$3.23x10^5$	$3.26x10^5$	$2.83x10^5$	$2.43x10^5$	$9x10^4$
4	$1.07x10^6$	$9.76x10^5$	$5.76x10^5$	$5.4x10^5$	$5.23x10^5$	$4.73x10^5$	$2.76x10^5$	0.00
6	$1.41x10^6$	$6.3x10^5$	$5.26x10^5$	$4.96x10^5$	$4.9x10^5$	$4.46x10^5$	$2.33x10^5$	0.00
8	$1.76x10^6$	$5.36x10^5$	$5.26x10^5$	$4.36x10^5$	$4.26x10^5$	$3.3x10^5$	$8.6x10^5$	0.00
10	$1.98x10^6$	$4.16x10^5$	$3.46x10^5$	$3.36x10^5$	$3.13x10^5$	$2.76x10^5$	$7.66x10^4$	0.00
12	$2.14x10^7$	$4.26x10^5$	$3.96x10^5$	$8x10^4$	$1.33x10^4$	0.00	0.00	0.00
24	$3.39x10^8$	$1.55x10^6$	$3x10^4$	0.00	0.00	0.00	0.00	0.00

*CNMA = Cinnamaldehyde

Table 6 Survival colonies of *B. subtilis* ATCC 6633 received cinnamaldehyde and gentamicin (Positive control) in Time kill assay

Hours	Survival colonies (Log_{10} CFU/ml)							
	Growth control	CNMA 1/2MIC (49.21 µg/ml)	CNMA 1MIC (98.43 µg/ml)	CNMA 2MIC (196.87 µg/ml)	CNMA 4MIC (393.75 µg/ml)	CNMA 8MIC (787.50 µg/ml)	CNMA 16MIC (1,575 µg/ml)	Gentamicin 1MIC (0.12 µg/ml)
0	$8.96x10^5$	$8.06x10^5$	$7x10^5$	$6.03x10^5$	$4.9x10^5$	$3.6x10^5$	$3.2x10^5$	$4.46x10^5$
2	$2.4x10^6$	$1.26x10^6$	$1.22x10^6$	$8.43x10^5$	$7.13x10^5$	$6.86x10^5$	$4.96x10^5$	$2.33x10^4$
4	$2.6x10^6$	$2.26x10^6$	$2.03x10^6$	$1.95x10^6$	$1.88x10^6$	$1.77x10^6$	$1.53x10^6$	0.00
6	$2.64x10^6$	$1.26x10^6$	$1.02x10^6$	$9.56x10^5$	$8.6x10^5$	$7.06x10^5$	$4.3x10^5$	0.00
8	$2.78x10^6$	$1.03x10^5$	$9.76x10^5$	$8.43x10^5$	$7.23x10^5$	$7.03x10^5$	$3.13x10^5$	0.00
10	$2.81x10^6$	$8.76x10^5$	$7.36x10^5$	$6.46x10^5$	$5.03x10^5$	$4.03x10^5$	$2.86x10^4$	0.00
12	$2.87x10^7$	$7.3x10^5$	$6.36x10^5$	$5.16x10^5$	$4.43x10^5$	$3.36x10^5$	$2.1x10^5$	0.00
24	$3.87x10^8$	$2.54x10^6$	$3.23x10^5$	$2.76x10^5$	$2.13x10^5$	$6.33x10^4$	0.00	0.00

*CNMA = Cinnamaldehyde

Table 7 Survival colonies of *B. cereus* Isolate 1 received cinnamaldehyde and gentamicin (Positive control) in Time kill assay

Hours	Survival colonies (Log_{10} CFU/ml)						
	Growth control	CNMA 1/2MIC (98.43 µg/ml)	CNMA 1MIC (196.87 µg/ml)	CNMA 2MIC (393.75 µg/ml)	CNMA 4MIC (787.50 µg/ml)	CNMA 8MIC (1,575 µg/ml)	Gentamici 1MIC (0.12 µg/ml)
0	$8.73x10^5$	$7.66x10^5$	$4.66x10^5$	$4.03x10^5$	$3.56x10^5$	$3.16x10^5$	$4.76x10^5$
2	$8.53x10^5$	$7.43x10^5$	$3.96x10^5$	$3.03x10^5$	$1x10^5$	$8.33x10^4$	$1.33x10^5$
4	$1.11x10^6$	$9.86x10^5$	$5.63x10^5$	$5.03x10^5$	$2.66x10^4$	$1.33x10^4$	0.00
6	$1.41x10^6$	$6.03x10^5$	$3.7x10^5$	$2.2x10^5$	$6x10^4$	0.00	0.00
8	$1.67x10^6$	$5.7x10^5$	$2.66x10^5$	$2x10^5$	$2x10^4$	0.00	0.00
10	$1.99x10^6$	$5.46x10^5$	$1.22x10^5$	$5.33x10^4$	$3.66x10^4$	0.00	0.00
12	$2.05x10^7$	$3.73x10^5$	$1.66x10^4$	0.00	0.00	0.00	0.00
24	$3.44x10^8$	$1.25x10^6$	0.00	0.00	0.00	0.00	0.00

*CNMA = Cinnamaldehyde

Table 8 Survival colonies of *B. cereus* Isolate 2 received cinnamaldehyde and gentamicin (Positive control) in Time kill assay

Hours	Survival colonies (Log_{10} CFU/ml)						
	Growth control	CNMA 1/2MIC (98.43 µg/ml)	CNMA 1MIC (196.87 µg/ml)	CNMA 2MIC (393.75 µg/ml)	CNMA 4MIC (787.50 µg/ml)	CNMA 8MIC (1,575 µg/ml)	Gentamicin 1MIC (0.12 µg/ml)
0	$8.76x10^5$	$7.23x10^5$	$4.3x10^5$	$2.86x10^5$	$3.76x10^5$	$3.36x10^5$	$4.56x10^5$
2	$9.23x10^5$	$7.56x10^5$	$3.73x10^5$	$3.23x10^5$	$1x10^5$	$3.66x10^4$	$1.1x10^5$
4	$1.04x10^6$	$9.6x10^5$	$5.36x10^5$	$5.16x10^5$	$4x10^4$	$1.33x10^4$	0.00
6	$1.4x10^6$	$8.73x10^5$	$8.1x10^5$	$6.26x10^5$	$1x10^5$	0.00	0.00
8	$1.82x10^6$	$6.26x10^5$	$2.83x10^5$	$2.03x10^5$	$4x10^4$	0.00	0.00
10	$1.96x10^6$	$6.1x10^5$	$1.53x10^5$	$7x10^4$	$3.66x10^4$	0.00	0.00
12	$2.17x10^7$	$3.1x10^5$	$5.66x10^4$	0.00	0.00	0.00	0.00
24	$3.49x10^8$	$1.26x10^6$	0.00	0.00	0.00	0.00	0.00

*CNMA = Cinnamaldehyde

Table 9 *In vitro* antibiofilm activity of cinnamaldehyde against *B. cereus* ATCC 11778, expressed in terms of the percentage inhibition of biofilm formation

cinnamaldehyde (µg/ml)	**The percentage inhibition of biofilm formation (Mean ± SEM)**
(control)	0.00
24.60	47.63 ± 3.62
49.21	69.03 ± 4.25
98.43	71.50 ± 3.38
196.87	73.57 ± 0.26
393.75	85.04 ± 0.61
787.50	86.49 ± 3.04
1,575.00	95.94 ± 1.67

Table 10 *In vitro* antibiofilm activity of cinnamaldehyde against *B. subtilis* ATCC 6633, expressed in terms of the percentage inhibition of biofilm formation

cinnamaldehyde (µg/ml)	**The percentage inhibition of biofilm formation (Mean ± SEM)**
(control)	0.00
24.60	35.45 ± 2.11
49.21	42.30 ± 3.35
98.43	59.27 ± 3.69
196.87	62.82 ± 4.27
393.75	68.04 ± 4.94
787.50	69.39 ± 4.11
1,575.00	75.83 ± 4.70

Table 11 *In vitro* antibiofilm activity of cinnamaldehyde against *B. cereus* Isolate 1, expressed in terms of the percentage inhibition of biofilm formation

cinnamaldehyde (µg/ml)	**The percentage inhibition of biofilm formation (Mean±SEM)**
(control)	0.00
24.60	47.98 ± 2.53
49.21	70.01 ± 2.37
98.43	79.79 ± 2.25
196.87	83.87 ± 1.18
393.75	84.55 ± 0.68
787.50	95.23 ± 0.68
1,575.00	97.27 ± 0.66

Table 12 *In vitro* antibiofilm activity of cinnamaldehyde against *B. cereus* Isolate 2, expressed in terms of the percentage inhibition of biofilm formation

cinnamaldehyde (µg/ml)	**The percentage inhibition of biofilm formation (Mean±SEM)**
(control)	0.00
24.60	47.34 ± 1.83
49.21	70.73 ± 3.61
98.43	79.77 ± 2.27
196.87	82.04 ± 1.14
393.75	84.69 ± 1.00
787.50	95.45 ± 0.66
1,575.00	95.83 ± 1.00

Table 13 *In vitro* antibiofilm activity of gentamicin against *B. cereus* ATCC 11778, expressed in terms of the percentage inhibition of biofilm formation

Gentamicin (µg/ml)	**The percentage inhibition of biofilm formation (Mean ± SEM)**
(control)	0.00
0.06	27.95 ± 1.89
0.12	38.94 ± 0.65
0.24	46.40 ± 0.74
0.48	58.13 ± 0.43
0.96	71.04 ± 2.81
1.92	81.73 ± 1.63
3.84	95.05 ± 0.25

Table 14 *In vitro* antibiofilm activity of gentamicin against *B. subtilis* ATCC 6633, expressed in terms of the percentage inhibition of biofilm formation

Gentamicin (µg/ml)	**The percentage inhibition of biofilm formation (Mean ± SEM)**
(control)	0.00
0.06	21.24 ± 2.68
0.12	43.36 ± 1.60
0.24	53.22 ± 3.71
0.48	63.97 ± 1.01
0.96	72.50 ± 0.81
1.92	81.99 ± 0.48
3.84	92.29 ± 0.99

Table 15 *In vitro* antibiofilm activity of gentamicin against *B. cereus* Isolate 1, expressed in terms of the percentage inhibition of biofilm formation

Gentamicin (µg/ml)	**The percentage inhibition of biofilm formation (Mean ± SEM)**
(control)	0.00
0.06	25.11 ± 0.67
0.12	31.14 ± 0.97
0.24	47.90 ± 0.11
0.48	56.87 ± 0.37
0.96	77.18 ± 1.61
1.92	82.13 ± 0.78
3.84	96.02 ± 0.45

Table 16 *In vitro* antibiofilm activity of gentamicin against *B. cereus* Isolate 2, expressed in terms of the percentage inhibition of biofilm formation

Gentamicin (µg/ml)	**The percentage inhibition of biofilm formation (Mean ± SEM)**
(control)	0.00
0.06	25.95 ± 1.79
0.12	33.23 ± 0.56
0.24	48.19 ± 0.23
0.48	57.39 ± 0.13
0.96	76.04 ± 2.56
1.92	82.53 ± 0.43
3.84	95.11 ± 0.65

APPENDIX B

Data of *In Vivo* Studies

Table 17 Pathogenicity of *B. cereus* ATCC 11778 in silkworms

***B. cereus* ATCC 11778** **(CFU/ml)**	**The percent mortality of silkworms** **(Mean ± SEM)**
Normal saline solution (control)	0.00 ± 0.00
10^1	0.00 ± 0.00
10^2	3.33 ± 3.33
10^3	13.33 ± 3.33
10^4	23.33 ± 3.33
10^5	36.67 ± 3.33
10^6	63.33 ± 3.33
10^7	83.33 ± 3.33
10^8	100.00 ± 0.00

Table 18 Pathogenicity of *B. subtilis* ATCC 6633 in silkworms

B. subtilis **ATCC 6633** **(CFU/ml)**	**The percent mortality of silkworms (Mean ± SEM)**
Normal saline solution (control)	0.00 ± 0.00
10^1	0.00 ± 0.00
10^2	13.33 ± 3.33
10^3	36.67 ± 3.33
10^4	66.67 ± 3.33
10^5	76.67 ± 3.33
10^6	100.00 ± 0.00
10^7	100.00 ± 0.00
10^8	100.00 ± 0.00

Table 19 Pathogenicity of *B. cereus* Isolate 1 in silkworms

***B. cereus* Isolate 1** **(CFU/ml)**	**The percent mortality of silkworms** **(Mean ± SEM)**
Normal saline solution (control)	0.00 ± 0.00
10^1	0.00 ± 0.00
10^2	13.33 ± 3.33
10^3	26.67 ± 3.33
10^4	36.67 ± 3.33
10^5	46.67 ± 3.33
10^6	86.67 ± 3.33
10^7	100.00 ± 0.00
10^8	100.00 ± 0.00

Table 20 Pathogenicity of *B. cereus* Isolate 2 in silkworms

***B. cereus* Isolate 2** **(CFU/ml)**	**The percent mortality of silkworms** **(Mean ± SEM)**
Normal saline solution (control)	0.00 ± 0.00
10^1	0.00 ± 0.00
10^2	13.33 ± 3.33
10^3	30.00 ± 5.77
10^4	36.67 ± 3.33
10^5	50.00 ± 5.77
10^6	86.67 ± 3.33
10^7	100.00 ± 0.00
10^8	100.00 ± 0.00

Table 21 LD_{50} of cinnamaldehyde in silkworm model

Cinnamaldehyde (µg/ml)	**Mean of number of silkworm survival (% survival) n=10**
0.7% Ethanol (control)	100.00 ± 0.00
10	100.00 ± 0.00
50	100.00 ± 0.00
500	100.00 ± 0.00
1,000	100.00 ± 0.00
1,500	100.00 ± 0.00
2,000	100.00 ± 0.00

Table 22 LD_{50} of gentamicin in silkworm model

Gentamicin (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
DI water (control)	100.00 ± 0.00
10	100.00 ± 0.00
50	100.00 ± 0.00
500	100.00 ± 0.00
1,000	100.00 ± 0.00
1,500	100.00 ± 0.00
2,000	100.00 ± 0.00

Table 23 ED_{50} of cinnamaldehyde in silkworm infected with *B. cereus* ATCC 11778

Cinnamaldehyde (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
0.7% Ethanol (control)	100.00 ± 0.00
10	20.67 ± 0.67
50	30.67 ± 0.67
100	40.00 ± 0.00
500	50.67 ± 0.67
1,000	60.67 ± 0.67
1,500	70.67 ± 0.67

Table 24 ED_{50} of cinnamaldehyde in silkworm infected with *B. subtilis* ATCC 6633

Cinnamaldehyde (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
0.7% Ethanol (control)	100.00 ± 0.00
10	2.67 ± 0.67
50	10.67± 0.67
100	30.67 ± 0.67
500	40.67 ± 0.67
1,000	50.67 ± 0.67
1,500	60.67 ± 0.67

Table 25 ED_{50} of cinnamaldehyde in silkworm infected with *B. cereus* Isolate 1

Cinnamaldehyde (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
0.7% Ethanol (control)	100.00 ± 0.00
10	10.00 ± 0.00
50	20.67 ± 0.67
100	30.00 ± 0.00
500	60.00 ± 0.00
1,000	82.00 ± 0.00
1,500	100.00 ± 0.00

Table 26 ED_{50} of cinnamaldehyde in silkworm infected with *B. cereus* Isolate 2

Cinnamaldehyde (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
0.7% Ethanol (control)	100.00 ± 0.00
10	10.00 ± 0.00
50	20.67 ± 0.67
100	30.67 ± 0.67
500	60.67 ± 0.67
1,000	80.67 ± 0.67
1,500	100.00 ± 0.00

Table 27 ED_{50} of gentamicin in silkworm infected with *B. cereus* ATCC 11778

Gentamicin (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
DI water (control)	100.00 ± 0.00
0.01	20.67 ± 0.67
0.1	70.00 ± 0.00
1.0	90.67± 0.67
5.0	100.00 ± 0.00
500	100.00 ± 0.00
1,500	100.00 ± 0.00

Table 28 ED_{50} of gentamicin in silkworm infected with *B. subtilis* ATCC 6633

Gentamicin (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
DI water (control)	100.00 ± 0.00
0.01	10.67± 0.67
0.1	60.67 ± 0.67
1.0	70.67 ± 0.67
5.0	90.67 ± 0.67
500	100.00 ± 0.00
1,500	100.00 ± 0.00

Table 29 ED_{50} of gentamicin in silkworm infected with *B. cereus* Isolate 1

Gentamicin (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
DI water (control)	100.00 ± 0.00
0.01	22.00 ± 0.00
0.1	70.67 ± 0.67
1.0	90.67 ± 0.67
5.0	100.00 ± 0.00
500	100.00 ± 0.00
1,500	100.00 ± 0.00

Table 30 ED_{50} of gentamicin in silkworm infected with *B. cereus* Isolate 2

Gentamicin (μg/ml)	**Mean of number of silkworm survival (% survival) n=10**
DI water (control)	100.00 ± 0.00
0.01	21.33 ± 0.67
0.1	70.67 ± 0.67
1.0	90.67 ± 0.67
5.0	100.00 ± 0.00
500	100.00 ± 0.00
1,500	100.00 ± 0.00

VITA

Miss Kannika Singrueangratsit was born on March 11, 1991 in Phichit, Thailand. She graduated with Bachelor Degree of Chemistry from Faculty of Science, UNIVERSITY OF PHAYAO. Consequently, she had enrolled for Master's Degree in Pharmacology at the Graduate School of Chulalongkorn University.