STUDY OF HERBAL EXTRACTS FOR ANTIBACTERIAL ACTIVITY AND TYPE THREE SECRETION SYSTEM INHIBITOR IN BACTERIA

PATIPAT THINWANG

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in Biomedical Sciences
2022

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By Patipat Thinwang

has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy in Biomedical Sciences of Naresuan University

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Academic Paper	Ph.D. Dissertation in Biomedical Sciences, Naresuan University, 2022
Keywords	Garlic, Turmeric, Chantaleela, Type Three Secretion System, T3SS inhibitors, Thai herbs

ABSTRACT

Nowadays, Thai herbal extracts including garlic, turmeric, chantaleela and king of bitters have been used widely for basic spice in Thai food or traditional medicine in Thailand. These herbs were reported that they have antibacterial activity. However, the previous study showed that some herbs are potential as not only antibacterial activity but also anti-T3SS activity for interruption of bacterial pathogenesis. Therefore, the aim of this study is to study Thai herbs including garlic, chantaleela, turmeric, and king of bitters for being as T3SS inhibitors. The SPI-1 T3SS of *Salmonella* Typhimurium was used as a model in this study. First of all, anti-T3SS activity of herbs were screened and determining of the minimal concentration of the herbs for inhibition of SPI-1 T3SS effector protein secretion from engineering *S.* Typhimurium by Western blotting. The result showed that garlic, turmeric and Chantaleela inhibited the secretion of SipA-strep tag protein from engineered *Salomonella* in a dose-dependent manner. The minimal concentrations of garlic, turmeric and Chantaleela were 100 µg/ml, 75 µg/ml and 150 µg/ml, respectively. Moreover, result of invasion assay showed that herbs were able to inhibit invasion activity of *S.* Typhimurium into host cells. In addition, effective dose from 3 herbs did not affect bacterial growth and harm to HeLa cells, respectively. For anti-T3SS mechanism, RNA transcriptional level in regulatory cascade of SPI-1 T3SS illustrated that herbs suppressed the transcription of SPI-1 T3SS regulator gene, *HilD*. As this

reason, the reduction of *HilD* expression affected the ability for SPI-1 T3S. Therefore, garlic, turmeric and chantaleela were identified as potential for novel T3SS inhibitors against SPI-1 T3SS of *S.* Typhimurium.

ACKNOWLEDGEMENTS

First of all, I would like to extend my deepest gratitude to my advisor, Assistant Professor Rerngwit Boonyom, Ph.D., and co-advisor, Napaporn Apiratmateekul, Ph.D., for suggestion, support, patience, encouragement and invaluable help throughout the course of this study.

I would like to express my deepest thanks to Assistant Professor Wichet Leelamanit, Ph.D. that works at faculty of pharmacy in Mahidol University. He contributed the herbal extracts for my study and also adviced me about the preparation of the herbal extracts.

I would also like to express my deepest thanks to Sittiruk Roytrakul, Ph.D., Associate Professor Ratchada Cressey, Ph.D. and Assistant Professor Natpasit Chaianantakul, Ph.D. for their kindness to be my committee.

I would like to express my gratitude to all laboratory staffs in both Department of Medical Technology and Central research laboratoy in Allied Health Sciences for kind suggestion, technical support and facilities. And I would also like to thank all RB members especially, Natthapon Samakchan, Ph.D, and BMS graduate students for friendship, suggestion and helping me everything.

In addition, I would like to thank National Research Council of Thailand. Because this thesis is sponsored and supported by National Research Council of Thailand.

Finally, I would like to thank my parents and my friends for all their support throughout the period of this study. I am very grateful to myself for fight, patience, encouragement through this study.

Patipat Thinwang

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CHAPTER I

INTRODUCTION

World Health Organization WHO and Centers for Disease control and Prevention CDC have reported that antibiotic resistant bacteria as superbugs are the one of major problem and threat to human health worldwide at present (1, 2). The antibiotic resistant bacteria are increasing and leading infectious diseases, complication, mortality and cost of treatment. Moreover, the antibiotic resistant bacteria are one of the top 10 global public health threats facing humanity. They made infectious patients die approximately 1.2 million people every year and also would lead to 10 million people dying in 2050 (3, 4). Therefore, the antibiotic resistance bacteria seriously affect worldwide public health to develop antibiotics or discover novel substances for inhibition of virulence from the bacteria.

In recent years, there have been several studies that attempt to develop the novel antibiotics for struggle of antibiotic resistant bacteria (5). However, these developed novel antibiotics are not sufficient to combat with the rising antibacterial resistance worldwide (6). Because the antibiotics that inhibit cell wall synthesis, DNA replication and protein synthesis have decreased the efficiency for inhibition of bacterial growth or survival. Therefore, there is another strategy that has investigated natural or chemical substances for inhibition of virulence or pathogenesis from bacteria instead of bacterial growth, for example, toxin, pili and secretion system (7, 8).

Bacterial secretion systems are used to translocate proteins from bacteria into the environment for bacterial pathogenesis. There are different types of secretion system responsible for pathogenesis. Among the system, Type three secretion system (T3SS) is mostly connected to pathogenesis from bacteria (8). The structure of T3SS is assembled from more than 20 proteins to form needle like shape complexes. The function of T3SS is secretion of effector proteins into host cell to modulate a variety of cellular functions such as actin rearrangement to facilitate bacterial invasion into host cells. The T3SS is a key of virulence in many gram-negative bacteria including *Shigella* spp., Enteropathogenic *Escherichia coli* (EPEC) *Yersinia* spp. and *Salmonella* spp. (9). Moreover, The T3SS has high similarity in both of structures and functions (10). Therefore, it is interested as a target for development of therapeutic drugs. The drugs are called T3SS inhibitors that could inhibit activity of the T3SS for virulence from bacteria rather than inhibition of the bacterial growth. Since 2002, there are several compounds that are identified as T3SS inhibitors including Salicylidene Acyl Hydrazides and Cytosporone B (11, 12).

As mentioned earlier, many studies have attempted to discover novel antibiotic or T3SS inhibitors. They used natural products including bacteria, fungus and plants, for discovery of the antibiotics and the T3SS inhibitors (13-15). However, this study focused on the plant, especially Thai herbs. The herbs are the plants which have been used in medicine for a long time around the world (16). Using herbs for protection and treatment of the diseases, called herbal medicine, is categorized the one of traditional medicines (17). The herbs contain a variety of phytochemicals, such as flavonoids, that lead them to prevention and treatment of the several diseases (18, 19). For the infectious diseases, there are a lot of herbs around the world that are able to inhibit bacterial growth, for example, *Aquilaria crassna. A. crassna* or agarwood

leaves were extracted and screened antibacterial activities by Disk diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The results showed that the extracts are an antibacterial agent against *Staphylococcus epidermidis* and probably other gram-positive bacteria (20). Moreover, some herbs are able to inhibit activity of T3SS, such as *Scutellaria baicalensis*. *S. baicalensis* has been used as Traditional Chinese Medicines (TCMs). It has been found that baicalein, a specific flavonoid, is able to inhibit activity of SPI-1 T3SS in *S.* Typhimurium resulting in unable to secretion of effecter proteins and invasion into the host cells (21). These results demonstrate that phytochemicals in the herbs have properties for treatment of infectious diseases including the antibacterial activity and anti-T3SS activity.

From all the reasons above, the herbs are potential as both antibacterial activity for inhibition of bacterial growth and anti-T3SS activity for interruption of bacterial pathogenesis. Therefore, this study interested whether Thai herbs as the antibacterial agents can also inhibit T3SS activity as the T3SS inhibitors. The Thai herbs in this study consist of garlic, turmeric, chantaleela and king of bitter. Moreover, SPI-1 T3SS of *S.* Typhimurium was used as a model for monitoring activity of T3SS. Firstly, Thai herbal extracts for inhibition of SPI-1 T3SS were screened and a minimal concentration of the Thai herbal extracts for inhibition of SPI-1 T3SS was measured by Western blotting. Secondly, antibacterial activity of Thai herbal extracts against *S.* Typhimurium was evaluated by Time-kill assay. Then, cytotoxicity of Thai herbal extracts to Hela cells was measured by MTT assay. After that, Thai herbal extracts for inhibiting bacterial invasion of *S.* Typhimurium were detected by invasion assay. Finally, mechanisms of Thai herbal extracts for inhibition of SPI-1 T3SS were detected by measurement of transcription level of genes regulating the SPI-1 T3SS. Therefore, the results of this study demonstrated which Thai herbs could inhibit SPI-1 T3SS of *S.* Typhimurium for being T3SS inhibitors and mechanisms of Thai herbs for inhibition of SPI-1 T3SS. Moreover, this study may be used for development of therapeutic drugs against antibiotic resistant bacteria in the future.

CHAPTER II

LITERATURE REVIEWS

1. Antibiotic resistant bacteria threat

Antibiotics, called antimicrobial agents, are “substances inhibit or destroy microorganisms” (22). The antibiotic for bacteria was discovered firstly by Sir Alexander Fleming in 1929. He found that purified penicillin from the culture filtrate of Penicillium notatum was able to kill some bacteria, such as streptococcus, meningococcus and the diphtheria bacillus. Then, penicillin was studied for future therapy in infected patients and firstly used to cure the patients since 1940s (23, 24). Therefore, the discovery of antibiotic from Sir Alexander Fleming led to begin widely to discover novel substances for treatment of the infected patients. Because of increasing the discovery of antibiotics, nowadays there are a lot of antibiotics being natural or semi-synthetized agents which are available in the market around the world for treatment of the infected patients (25). Mechanisms of the antibiotics are divided into 5 mechanisms to inhibit bacterial growth and kill the bacteria as shown in figure 1 (22).

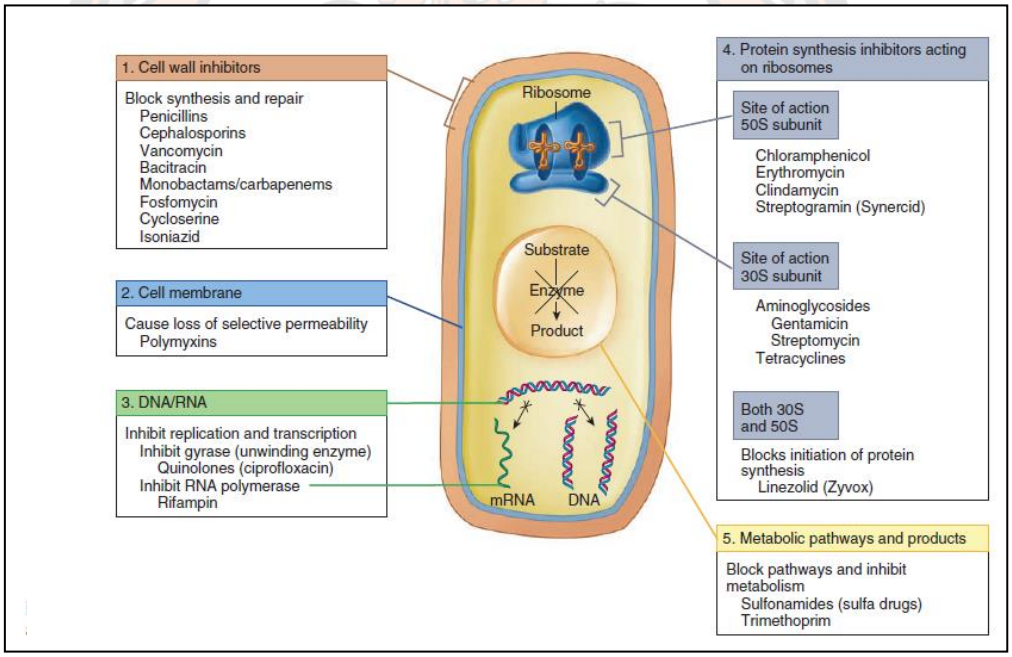


Figure 1 Mechanisms of the antibiotics

There are 5 targets that include cell wall, cell membrane, DNA/ RNA, protein synthesis and metabolic pathways. Moreover, examples of antibiotics in each mechanism are shown in this figure (22).

The first mechanism is inhibition of bacterial cell wall synthesis. This mechanism prevents peptidoglycan construction to be cell wall of bacteria and then cause bacteria to be died. The mechanism has a lot of antibiotics, for example, Penicillins, Cepharosporins, Vancomycin, Bacitracin and Carbarpenems. The Penicillins is the first antibiotic. It consists of beta-lactam ring in itself that is able to bind to penicillin binding protein (PBP) and then it leads PBP to unable function for cross-linking

between peptidoglycan monomers. At last it interrupts the completion of the cell wall and leads to undergoing lysis and cell death (22). Details of cell wall structure and penicillin action are shown in figure 2.

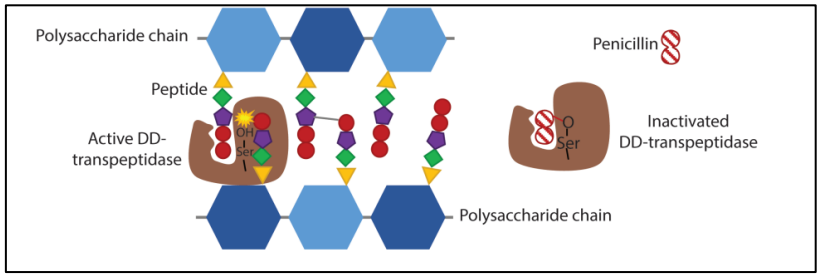


Figure 2 Schematic representation of the mechanism of Penicillin action

Peptidoglycan is composed of polysaccharide chains made of N-acetylglucosamin (GlcNAc) and N-acetylmuramic acid (MurNAc) which in turn have small peptides attached to them. The transpeptidase enzyme (PBP) (in brown) catalyzes the formation of cross-linkages between these peptides, by specifically binding the last two D-alanine residues of one peptide (red circles). Penicillin mimics the structure of these residues and inactivates the PBP by forming an irreversible covalent bond to the catalytic serine residue of the enzyme (26).

The second mechanism is breakdown of the cell membrane. The antibiotics destroy cell membrane of bacteria for dysfunction and lead the bacteria to be died, for example polymyxin B. Polymyxin B binds to the outer and cell membranes of gram-negative bacteria and creates pores that cause leakage and lysis (22). Detail of polymyxin B action is shown in figure 3.

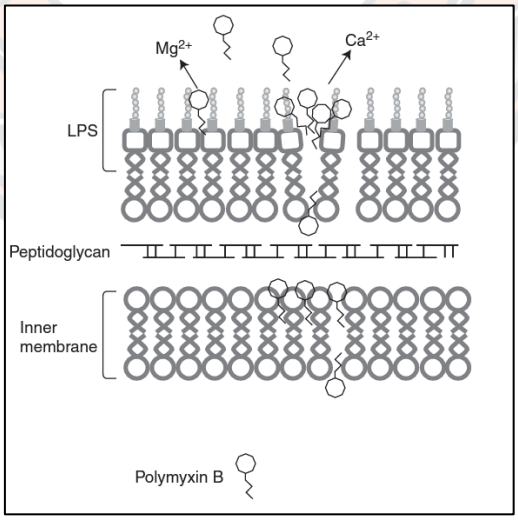


Figure 3 Schematic representation of the mechanism of Polymyxin B action

Polymyxin B interacts with the lipid A portion of the lipopolysaccharide (LPS) outer membrane. The peptides cross the outer membrane through a “self-promoted uptake” mechanism and then interact with the cytoplasmic membrane to inhibit cellular energization, and possibly cause inhibition of cell division and/or cytoplasmic membrane permeabilization and subsequent cell death (27).

The third mechanism is interference of nucleic acid synthesis. The antibiotics interfere with nucleic acid synthesis by blocking synthesis of nucleotides, inhibiting replication, or stopping transcription. For example, Rifamycins. Rifamycins bind highly affinity to β-subunit of DNA-dependent RNA polymerase. It blocks the initiation of mRNA transcription and then leads to cell death (28). Detail of Aminoglycosides action is shown in figure 4.

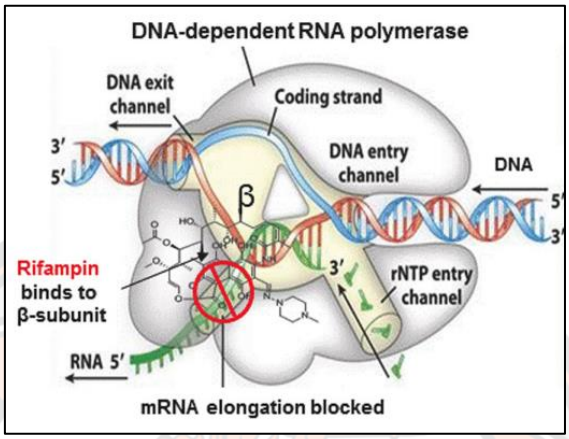


Figure 4 Schematic representation of the mechanism of Rifampin action

DNA-dependent RNA polymerase composes of an α-subunit dimer, a β-subunit, a β′-subunit, and a ω-subunit. However, the target of rifampin is the β-subunit. It interferes the binding between the β-subunit and DNA for coupling the production of mRNA. Finally, it leads to death of bacterial cell (29).

The fourth mechanism is interference of protein synthesis. The antibiotics of this mechanism preclude ribosome of bacteria either 30s or 50s ribosome of 70s ribosome and lead the bacteria to be unable production of important proteins for living. For example, Aminoglycosides. They bind to the 30S subunit of ribosome of 70s ribosome. They cause the misreading of the mRNA and lead to abnormal proteins and misfold proteins. The misfold proteins that involve membrane proteins lead to increased drug uptake. High uptake of the drug increase binding between drug and ribosome and it conduce to cell death (28). Detail of Aminoglycosides action is shown in figure 5.

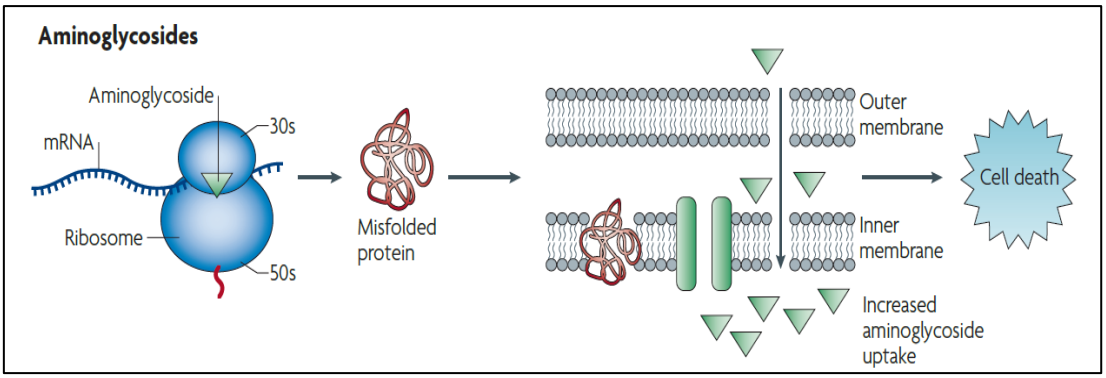


Figure 5 Schematic representation of the mechanism of Aminoglycosides action
Aminoglycosides bind to the 30S subunit of the ribosome and cause misincorporation of amino acids into elongating peptides. These mistranslated proteins can misfold, and incorporation of misfolded membrane proteins into the cell envelope leads to increased drug uptake. This, together with an increase in ribosome binding, has been associated with cell death (28)

The last mechanism is blockage of metabolic pathways. The structure of the antibiotics is similar to substrates of the metabolic pathways that lead to competitive inhibition and affect to process of the metabolic pathways. Sulfonamides is one of antibiotics of this mechanism. Sulfonamides involves folate metabolism pathway. A structure of this drug is similar to p-aminobenzoic acid (PABA) that is a substrate of dihydropteroate synthetase for synthesis of tetrahydrofolic acid (THFA). Therefore, Sulfonamides block activity of dihydropteroate synthetase to generate folic acid. Lack of folic acid impact bacteria for synthesis of methionine, purine and pyrimidine and thus for the bacterial growth and reproduction (22). Detail of Sulfonamides action is shown in figure 6.

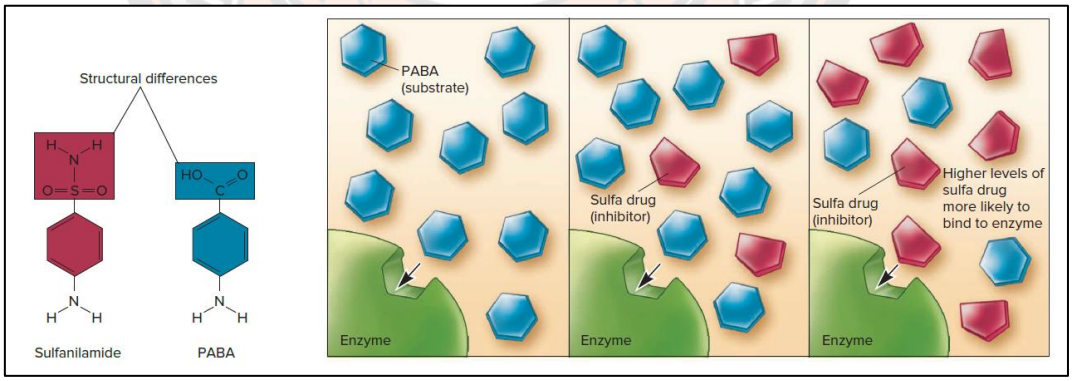


Figure 6 Schematic representation of the mechanism of Sulfonamides action
Sulfonamides can insert into the active site in the enzyme instead of PABA. Sulfonamides can outcompete with the PABA and keep the enzyme sites filled. Since little or no PABA can bind, the synthesis of folic acid will be inhibited (22).

Therefore, the mechanisms as mentioned above make the antibiotics have potential to destroy the bacteria. Moreover, the antibiotic is able to decrease illness and mortality from infectious diseases.

Even though the antibiotic is extremely important and effective for cure of the infectious diseases, scientists have been found that some bacteria are not response to the antibiotics and call the bacteria as antibiotic resistant bacteria. For example, the first antibiotic resistance was found in penicillin 3 years before the penicillin was approved for public use in 1943 (30). Causes of antibiotic resistance bacteria include over-prescribing of antibiotics, patients not finishing their treatment, over-use of antibiotic in livestock and fish farming, poor infection control in hospitals and clinics, lack of hygiene and poor sanitation and lack of new antibiotics being developed (23, 31). All the causes as mentioned above possibly lead to genetic versatility and adaptability of bacterial populations that affect the activity of the antibiotic and preserve bacterial survival in the presence of the antibiotic. This event is called intrinsic resistance. Nowadays, the antibiotic resistant bacteria are classified mechanisms for bacterial survival in the presence of the antibiotic that compose of 5 mechanisms as mentioned below (22, 32).

1. Decrease permeability: the bacteria modified themselves to inhibit and reduce uptake of antibiotic. For example, *Escherichia coli* contains *omp* gene that encodes porin protein for uptake of antibiotics. If the *omp* gene is mutant, it leads the porin to dysfunction or decrease and the bacteria are tolerant and survive in the presence of the antibiotic (33).

2. Production of antibiotic inactive enzyme: the bacteria produce enzymes to inhibit function of antibiotic. For example, *bla* gene encodes β-lactamase to hydrolyze β-lactam ring in antibiotic and then the antibiotic is not able to function (34).

3. Target modification: the target of antibiotic is modified that leads the antibiotic to be unable to destroy the bacteria. For example, *van* gene encodes D-Ala-D-Lac-Ala peptidoglycan instead of D-Ala-D-Ala-Ala peptidoglycan and then the bacteria exhibit vancomycin resistance (35).

4. Metabolic bypass: the bacteria alter from usual metabolic pathway to another pathway to avoid antibiotic. For example, *mecA* gene encodes penicillin binding protein 2a (PBP2a) instead of PBP. Therefore, β-lactam ring antibiotics are not capable binding PBP to destroy the bacteria (36).

5. Efflux pump: the bacteria construct pumping protein to take the antibiotic out from themselves. For example, *tet* gene encode protein that eject Tetracycline from the bacteria (37).

Furthermore, the antibiotic resistant bacteria are able to transfer antibiotic resistant genes to other bacteria. Then, the antibiotic resistant gene received bacteria become to antibiotic resistant bacteria and it is called acquired resistance. The bacterium that donates the resistant gene is called a donor cell and the bacterium that receive the resistant gene is called a recipient cell. The mechanisms of transferring antibiotic resistant genes compose of conjugation, transformation and transduction that are shown in figure 7 (22, 32).

Conjugation is a genetic recombination which a plasmid DNA or fragment DNA is transferred from a donor cell to a recipient cell via a direct connection. Briefly, the donor cell synthesize pilus to attach to receptor on the recipient cell. Then, a plasmid DNA or fragment DNA from donor cell is transferred to recipient cell. Finally, the recipient cell contains a plasmid DNA or fragment DNA that is similar to the donor cells (22).

Transformation is facilitated by special DNA-binding proteins on the cell wall that capture free DNA fragment from environment. The free DNA fragment is passed through a DNA uptake system from cell wall to cytoplasm, the fragment is inserted into the chromosome of the recipient cell, Finally, the recipient cell contains the DNA fragment in its chromosome. The cell that is able to accept genetic material through itself is called a competent cell (22).

Transduction is transferring DNA from the donor cell to the recipient cell through the Bacteriophages (bacterial viruses). First of all, bacteriophages infect the donor cell. During replication and assembly in the donor cells, a phage particle incorporates a segment of the donor cells by mistake. Then, the donor cell is lysed and the mature bacteriophages are released. After that, the mature bacteriophages penetrate the recipient cell and inject the DNA from the donor cells instead of viral nucleic acid. Finally, the recipient cell receives the DNA from the donor cells and recombines with its own DNA (22).

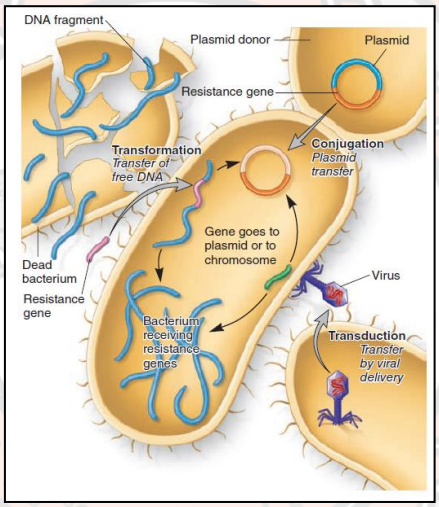


Figure 7 Mechanisms of transferring antibiotic resistant genes
The mechanisms compose of conjugation, transduction, and transformation (22)

After the bacteria adapt themselves to resist the antibiotics, it can make all antibiotics to be ineffective and untreatable infections. Unfortunately, antibiotic resistant bacteria can transfer antibiotic resistant gene to other bacteria that have not been exposed to antibiotics. It leads to spreading of the antibiotic resistant genes between bacteria and increasing of antibiotic resistant bacteria. Moreover, antibiotic resistant bacteria can spread between people, animals, and the environment. They can cause infections in patient receiving care, make people sick through common activities in community, contaminate in the environment and food supply for people, and finally spread between continents around the world (2).

World Health Organization (WHO) and Centers for Disease control and Prevention (CDC) currently report that antibiotic resistant bacteria are not only the one of major problem for human health worldwide but also the one of the top 10 global public health threats facing humanity (1, 2). Moreover, the antibiotic resistant bacteria are known as superbugs which cause infectious diseases without treatable condition. They also made infectious patients die approximately 1.2 million every year. In 2050, about 28.3 million people could be pushed into extreme poverty by drug resistance and 10

million people would be died by drug resistance. Moreover, they increase cost of treatment for hospital infections that is about USD 13.5 billion in annual financial losses (3, 4). There are a lot of current antibiotic resistant bacteria which are a major concern for public health and have been identified in all regions of the world, for example, Extended-spectrum β-lactamase (ESBLs) Enterobacteriaceae and Carbapenem-Resistant Enterobacteriaceae (CRE). The ESBLs-bacteria are serious threat that require prompt and sustained action. These bacteria generate enzymes (ESBLs) to break down commonly used antibiotics, such as penicillins and cephalosporins, and then make the antibiotics be ineffective. They can spread rapidly in the hospital and the community and cause infectious complication in healthy people. In USA, ESBLs-bacteria have increased since 2012. They infect 197,400 cases in hospitalized patients, cause 9,100 patients' death and More than USD 1.2 billion are spent for treatment of infection (38). On the other hand, The CRE-bacteria are urgent threat that require urgent and aggressive action. Some bacteria in this group are resistant to almost all the current antibiotics and lead to less effective treatment options These bacteria can generate an enzyme (carbapenemase) to makes carbapenem antibiotics ineffective. They can rapidly spread resistance between bacteria for destroying carbapenem antibiotics. In USA, CRE-bacteria have increased since 2012. They infect 13,100 cases in hospitalized patients, cause 1,100 patients' death and More than USD 130 million are spent for treatment of infection (38). For Thailand, a preliminary study reported that antibiotic resistant bacteria made 88,000 cases of infectious patients each year. Approximately 38,000 of infectious patients were dead. And economic impact is approximately THB 42 billion (39). There are a lot of current antibiotic resistant bacteria which are a major problem for public health and have been identified in all regions of Thailand, for example, Carbapenem-Resistant Acinetobacter. These bacteria are a major problem in nosocomial infection and mainly causes of mortality in hospitals. During 2011-2020, Survey research report these bacteria were identified from samples in 51 hospitals around Thailand and they increase every year for 10 years. Moreover, these bacteria found to be 71.3% among the hospitals across Thailand in 2021 that are the highest incidence along with other antibiotic resistant bacteria (40). As mentioned above, incidence of antibiotic resistant bacteria increases infectious diseases that lead to increasing complication, hospital length of stay, mortality and health-care costs. Furthermore, increasing antibiotic resistance bacteria seriously affect worldwide public health for protection of infection and fighting with the resistant bacteria. Therefore, there is strategy for fighting with drug-resistant infection. The first step is protection by yourself, such as cleaning hands, getting vaccine and using appropriate antibiotics, that can reduce risk of bacterial infection. The second step is protection by healthcare providers that play an essential role for patients in preventing infections and stopping the spread of the antibiotic resistant bacteria. The third step is protection by health departments that can rapidly detect resistance threats, help improve antibiotic use and lead local responses for stopping the spread of resistant bacteria. The fourth step is protection by veterinarians that are leaders and stewards in preserving antibiotics for animals and people. And the last step is protection by livestock and poultry producers that are practices to protect the health of animals and people who work on farms, the community, and food supply (2). Although the strategy is used worldwide public health for fighting with drug-resistant infections, the antibiotic resistant bacteria still

increase and spread every year around the world. Moreover, some last-resort treatments for infections are resisted by antibiotic resistant bacteria (1, 2), for example colistin. The colistin is the only last resort treatment for bacterial infections that caused by carbapenem resistant Enterobacteriaceae and multi-drug resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. These bacteria modify lipid A for reduction of colistin affinity and lead to colistin resistance (41, 42). In addition, the study in 2015 reported that colistin resistant bacteria are expressed and control by mcr-1 gene and the gene can transfer to other bacteria by conjugation (43). And then colistin resistant bacteria have also been detected in several countries since 2015. The colistin resistance around the world lead to no effective antibiotic treatment at present (1, 41). Therefore, the strategy for protection of the antibiotic resistant bacteria may not be enough to prevent the drug-resistant infections and stop the spread of the antibiotic resistant bacteria. It leads to the last choice for controlling the drug-resistant infections which is development of new treatments for drug-resistant infections (1).

2. Development of new treatments for the drug-resistant infections

There are mainly 2 strategies that scientists attempt to use for solving the antibiotic resistance bacteria problem. The strategies include finding novel antibiotics for inhibition of bacterial and anti-virulence drug for opposition of bacterial infection

2.1. Novel antibiotics

In recent years, there has been several studies that attempt to develop and discover the novel antibiotics for struggle with antibiotic resistant bacteria. The source of antibiotics is mostly from natural products including prokaryotic bacteria, eukaryotic microorganisms, plants and various animal organisms (5). To develop the novel drugs or novel antibiotics, there are 4 steps from FDA's guideline for development of the drugs (44).

The first step is discovery and development. Typically, researchers try to identify compounds from natural products, high-throughput screens of large compound libraries or synthesized analogs of compounds that are known to be effective against the interested target. After identifying a potential lead compound, the compound is studied its mechanism for action at the interested target and safety tests, such as, the best dosage, the best way to give the drug and side effects.

The second step is preclinical research that consists of *in vitro* (cellular model) and *in vivo* (animal model) assays. Both of them are used to detect the compound in laboratory experiments, such as activity, safety, dosage, and toxicity of the compound in cellular and living system, that are safe and suitable before testing in humans.

The third step is clinical research that studies the efficient drug in humans. This step consists of 4 phases including Phase 1, Phase 2 Phase 3 and Phase 4. Firstly, phase 1 is testing safety and appropriate dosage of the drug in 20 -80 healthy people. Next, 100 hundred people with the disease are tested with efficacy and side effects of the drug in phase 2. After that, the drug in phase 3 is tested efficacy and monitoring of adverse reactions. Purpose of this phase demonstrate whether or not a product offers a treatment benefit to a specific population and involves 300 to 3,000 people with the

disease during 1-4 years. And phase 4 is testing safety and efficacy of the drug in several thousand people with the disease.

The fourth step is the last step for FDA drug review and FDA Post-Market Drug Safety Monitoring. The FDA team firstly examines all data of the drug from its early tests, preclinical research and clinical research. Next, they decide whether or not to approve the drug. After the drug is approved and used for treatment, the FDA team continues to monitor the safety and side effects of the drug. If the drug has problems with prescription and over-the-counter drugs, the FDA team can decide to add cautions to the dosage or usage information, as well as other measures for more serious issues.

The FDA team has approved the new antibiotics for treatment of the infectious diseases from bacteria since 2015 that new antibiotics are shown in table 1. However, there are no antibiotics that have been approved by the FDA since 2020 (45). Although Winlevi (Clascoterone) is a drug for topical treatment of acne vulgaris which was approved by the FDA in 2020, this drug is a novel acne cream for inhibition of topical androgen receptors in the skin but it is not an antibiotic for treatment of infectious diseases from antibiotic resistant bacteria (46). Actually, the latest antibiotic is Fetroja (Cefiderocol) which was approved by the FDA in November 2019. Cefiderocol is a cephalosporin antibiotic which the structure of it is shown in figure 8. This drug is used for injection in patients 18 years of age or older for the treatment of complicated urinary tract infections (cUTI). And this antibiotic was indicated later for hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia (HABP/VABP) in 2020. Cefiderocol functions as a siderophore and binds to extracellular free (ferric) iron. In addition to passive diffusion via porin channels, cefiderocol is actively transported across the outer cell membrane of bacteria into the periplasmic space using the bacterial siderophore iron uptake mechanism. Cefiderocol exerts bactericidal action by inhibiting cell wall biosynthesis through binding to penicillin-binding proteins (PBPs). Dosage and administration of Fetroja are 2 grams of Fetroja for injection every 8 hours by intravenous infusion (47).

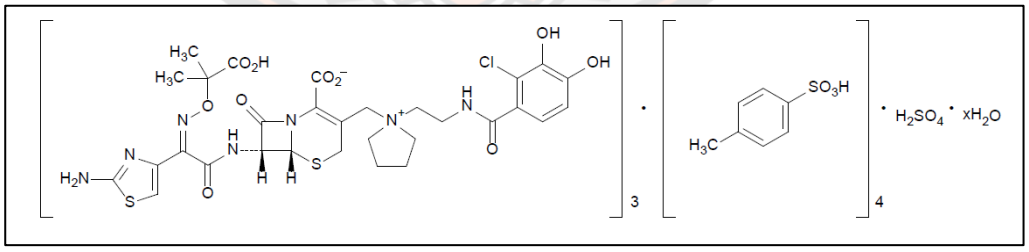


Figure 8 Chemical structure of Cefiderocol Sulfate Tosylate (46)

Table 1 Antibiotics have been approved by FDA since 2015 (45)

Trade name/ Active Ingredient	Treatment	Approval year
Ceftazidime-avibactam/ Avycaz	Complicated intra-abdominal infections and complicated urinary tract infections	2015
Delafloxacin/Baxdela	Acute bacterial skin and skin structure infections	2017
Vabomere/Vaborbactam and meropenem	Adults with complicated urinary tract infections	2017
Ozenoxacin/Xepi	Impetigo	2017
Plazomicin/Zemdri	Complicated urinary tract infections	2018
Eravacycline/Xerava	Complicated intra-abdominal infections	2018
Sarecycline/Seysara	Severe acne vulgaris	2018
Omadacycline/Nuzyra	Community-acquired bacterial pneumonia and acute bacterial skin and skin structure infections	2018
Rifamycin/Aemcolo	Travelers' diarrhea	2018
Imipenem, cilastatin, relebactam/ Recarbrio	Complicated urinary tract infections and complicated intra-abdominal infections	2019
Pretomanid	Treatment-resistant forms of tuberculosis	2019
Lefamulin/Xenleta	Community-acquired bacterial pneumonia	2019
Cefiderocol/Fetroja	Complicated urinary tract infection	2019

Because of the COVID-19 pandemic since 2020, it has refocused attention worldwide on the dangers of infectious diseases and affected worldwide health and especially worldwide economy. All countries spend their money for the covid-19 pandemic instead of infectious diseases, it leads to lack of economic support for development of new antibiotics and no new antibiotics in 2 years (48). Unfortunately, antibiotic resistant bacteria still increase in all regions of the world every year. Lack of new antibiotics may affect fighting with the rising antibiotic resistance worldwide.

2.2. New strategies for opposition of bacterial infection

Although antibiotics are highly effective for disruption of bacterial growth or survival such as inhibition of cell wall synthesis and protein synthesis, the stress from the antibiotics is able to induce some population of bacteria to resist the antibiotics in spite of killed some population of bacteria. After some population is killed by the antibiotic, the resistant population may quickly grow and become the dominant of the population to resist the antibiotic. For example, Vancomycin-resistant *Enterococcus* species (VRE) and Methicillin-resistant *Staphylococcus aureus* (MRSA) that oppose the antibiotic in the short period after using inappropriate and indiscriminate the antibiotic (7). Therefore, there are a lot of studies that have investigated natural or chemical substances for inhibition of virulence or pathogenesis from bacteria instead of bacterial growth (7, 8). The substances that inhibit virulence are called anti-virulence drug or virulence blocker. In addition, since the anti-virulence drug does not kill the bacteria, the drug evolves fewer resistant bacteria when compared with antibiotic. However, the drug should be used combination with other antibiotic in synergistic manner to extend the lifespan of the drug (7).

The anti-virulence drugs inhibit virulence factors of the bacteria that are critical for initiation of infection and cause disease. The factors which have anti-virulence drugs for inhibition of their factors include adhesins and biofilm for adhesion on host cell; toxins for destroying or changing signal transduction in mammalian cells; secretion systems for transportation of effector proteins from bacteria to host cells. Furthermore, virulence gene expression also has the anti-virulence drug for inhibition of virulence. Therefore, the anti-virulence drugs have 4 targets to inhibit virulence of bacteria as shown in figure 9 (7, 8).

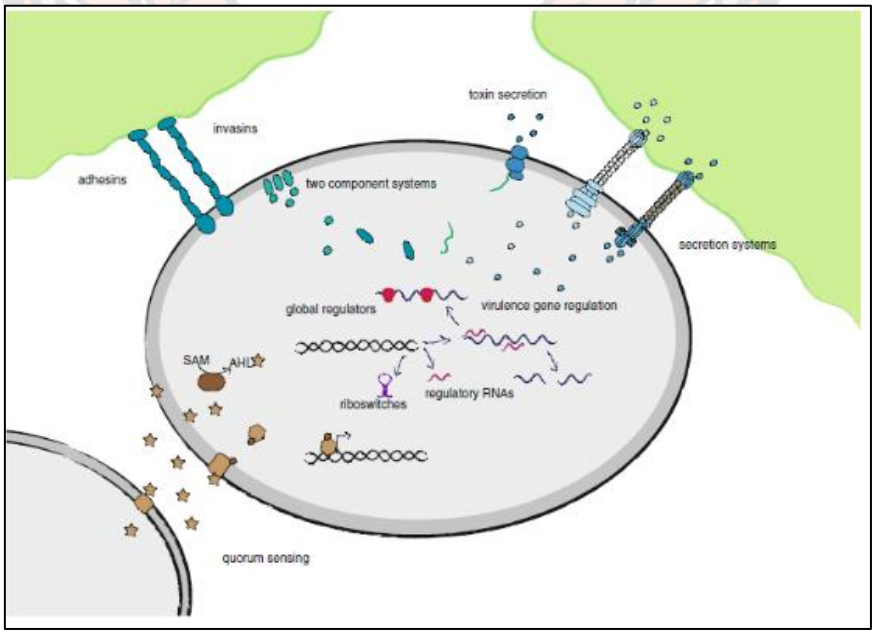


Figure 9 Overview of anti-virulence targets

The anti-virulence drugs have 4 targets to inhibit virulence of bacteria. They include cell adhesion, toxins, bacterial secretory systems and regulation of virulence gene expression (8)

1. Inhibition of Adhesion

Before invading into host cells and cause disease, bacteria have to adhere the host cells. The adhesion composes of using adhesin and biofilm that have different anti-virulence drugs for inhibition.

- Adhesin is a protein at the terminal of pili or fimbriae on bacterial surface that found both of gram-positive and negative bacteria. The adhesin function is to interact with specific host receptors for adhesion on host cells. However, the adhesin found on bacteria has many types that depend on individual bacteria. Therefore, each bacterium interacts with different specific receptors and host cells (49). For example, uropathogenic *Escherichia coli* (UPEC) which cause urinary tract infection. UPEC encodes the P pilli and type 1 pili. The adhesin of P pilli is PapG that interact with Galα-1,4 Galβ receptors on uroepithelial cells (50). On the other hands, the adhesin of type 1 pilli is FimH that interact with both of Galα-1,4 Galβ receptors on uroepithelial cells and tubular epithelial cells (51). The both of adhesins prevent the UPEC for washing out of the normal flow of urine and lead UPEC to invasion into epithelial cells for cause of urinary tract infection later (52). For inhibition of adhesion via adhesins, there are anti-virulence that divides 2 mechanisms. The first mechanism is to inhibit pili formation or biogenesis (53). The anti-virulence in the group, such as pilicides, mimics the normal pilin subunit for being competitive inhibition that prevent elongation and formation of pili structure (54). Therefore, bacteria presented with the anti-virulence are unable to generate completely pili structures that lead bacteria to no adhesion and cause of disease (55). On the other hand, the second mechanism is to inhibit interaction between pili and host cell receptors. The anti-virulence in this group, for example biphenyl mannosides, is mimics of host cell receptors for being competitive inhibition (56, 57). Thereby, the pili of bacteria interact with the anti-virulence instead of host cell receptors and the bacteria are not adhesion on the host cell to cause disease (55).

- Biofilms are communities of bacterial adhesion on host cell surface by using extracellular polymeric substances such as polysaccharides or glycoproteins. For biofilm formation bacteria firstly move and attach on surface of host cells. Then, bacteria grow on host cells for colonization and synthesize extracellular matrix. After that, biofilm formation is matured to be thick layered deposits containing sessile cells. Finally, biofilms protect bacteria inside from immune system and antibiotic for cause of disease (22, 58). For example, *Pseudomonas aeruginosa* are capable forming biofilms and lead to cause of nosocomial infection and cystic fibrosis (59). For inhibition of adhesion *via* biofilm, the anti-virulence, such as D-amino acid and norspermidine, destroy biofilm formation. Then, the inside bacteria are released from biofilm and are not able to continuously cause the disease (60, 61).

2. Inhibition of toxins

The toxins system is divided into 2 system including endotoxins and exotoxins. The endotoxin is lipopolysaccharides (LPS) on gram-negative bacterial surface that is not secreted from the bacteria. Structure of LPS is similar in every bacterium that composes of lipid A for being poison part; oligosaccharide for being core part; and o antigen which is able to activate immune response (49, 62). After bacterial disruption, LPS are released to active macrophages for induction of cytokine

secretion such as IL-1 and TNF-α (63, 64). Therefore, the high level of cytokines leads infected patients to fever and septic shock syndrome (65). In contrast, the exotoxin is able to be secreted by bacteria to alter or change host cells for developing cause of disease. Structures of endotoxin mostly are A-B structure. The function of A subunit is active part for cause of disease but B subunit functions binding the host cell receptors. For cause of disease, the B subunit binds to host cell receptors firstly. Then, the A subunit is released from the B subunit and go into host cells for developing disease. For example, cholera toxin containing 5 B subunit and 1 A subunit. This toxin is secreted from *Vibrio cholera*. The B subunits of this toxin bind to the ganglioside GM1 on intestine cells and then the toxin go into host cells. After that, the A subunit is removed from the B subunits and affects to G protein of host cells for causing an electrolyte imbalance (49).

For inhibition of toxin activity in the present, the endotoxin or LPS has anti-LPS to inhibit interaction between LPS and TLR-4 (66). For inhibition of exotoxin, the mechanisms of the anti-virulence are divided 2 mechanisms including inhibition of interaction between toxin and target and inhibition of toxin synthesis. First of all, the anti-virulence for inhibition of interaction is small compounds or development of antibody to bind with toxin that are competitive inhibition. For example, anti-PA subunit of anthrax toxin which is secreted from *Bacillus anthracis* or Synsorb-Pk that mimic Gb3 receptor for being competitive inhibition of shiga toxin from *Shigella* spp. (67, 68). On the other hands, the anti-virulence for inhibition of toxin synthesis have barely investigated such as Virstatin. The Virstatin interferes homodimeization of transcription factor ToxT which active expression of cholera toxin. Therefore, cholera toxin is not able to be encoded from *Vibrio cholera* (69, 70).

3. Inhibition of bacterial secretory systems

Secretion systems are an important part of bacteria for secretion of proteins or toxins from inside bacteria to outside bacteria. However, there are some secretion systems that pathogenic bacteria use to cause disease including Type III secretion system (T3SS) and Type IV secretion system (T4SS). These systems form syringe-like structure to secrete toxins or effector proteins from bacteria into host cells. Then, the effector proteins mimic functions of host cell proteins and lead to alter signaling pathway in host cell for cause of disease (7, 8). To inhibit secretory systems, the mechanisms of anti-virulence are composed of prevention of secretory systems assembly; inhibition of interaction between secretory systems and host cells; and inhibition of secretion of effector proteins. For example, Sallicylidene Acylhydrazides (SAMs) and B8I-2. The SAMs being small-molecule are the first anti-virulence for inhibition of T3SS. It is able to inhibit secretion of effector proteins in many pathogenic bacteria including *Yersinia* spp., *Salmonella* spp., *Chlamydia* spp., *Shigella* spp. and Enteropathogenic *Escherichia coli*. However, the SAMs doesn't affect to bacterial growth (12). While, The B8I-2 is a derivative molecule of salicylidene acylhydrazide that inhibits T4SS in Brucella abortus. It interferes dimerization of VirB8 for formation of T4SS and lead the T4SS are not function. Moreover, B8I-2 also reduces *virB* transcription for encode of VirB proteins that affect to formation of T4SS (71, 72).

4. Regulation of virulence gene expression

Bacteria regulate strictly expression of virulence traits to avoid using unnecessary energy. The regulation requires specific signals from environment, such as temperature, pH or chemical compound from other bacteria, to express virulence factors. The expression of virulence factors has many control circuits and networks that have been found similarity in many bacteria. Therefore, the regulation of the expression of virulence factors is interesting for developing the anti-virulence for inhibition of virulence in bacteria (7, 8). For example, quorum-sensing system. The quorum-sensing system is a cell-cell communication system that synchronizes the production of virulence factors such as biofilm formation of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The steps of the system compose of 3 steps. First of all, the bacteria can produce small molecules called autoinducers that are secreted from bacterial cells and detected by receptors at membrane or cytoplasm. Then, when the bacteria are low and cannot contact together, the produced autoinducers cannot interact with receptors of other bacteria that lead to be unable to induce expression of virulence factors. Finally, when the bacteria are high and contact together, the produced autoinducers interact with receptors of other bacteria to induce expression of virulence factors (73). The anti-virulence for inhibition of the quorum-sensing system include the blockage of signal production, degradation of signal molecules and disruption of the interaction between signal and receptor (8, 74-76).

The steps for development of the anti-virulence drug compose of 4 steps which are similar to the steps for development of the antibiotic as mentioned above. In the present, there are only 5 anti-virulence drug that have been approved by FDA since 2003. However, they do not indicate for solving antibiotic resistance. The 5 anti-virulence drugs are shown in Table 2 and a target of them is toxin. These are two immunoglobulins which include BabyBIG and BAT for *Clostridium botulinum*. *Clostridium botulinum* is a Gram-positive bacterium which secrete Botulinum (BoNT) neurotoxin to interfere with the neurons at the neuromuscular junction which leads to weakness and a possible paralytic condition in the patients calling botulism. Both of immunoglobulins as anti-virulence drug bind to botulinum neurotoxin for prevention of releasing neurotransmitters at the synaptic cleft. Botulism immunoglobulin intravenous (BabyBIG) binds to botulinum neurotoxin types A and B for neutralization and has been used for the treatment of infant botulism (types A and B). While Equine-derived botulism antitoxin heptavalent (BAT) binds to botulinum neurotoxin types A, B, C, D, E, F, and G for neutralization and has been used for the treatment of botulism in children and adults. Other drugs are monoclonal antibodies which include raxibacumab and obiltoxaximab for *Bacillus anthracis* and bezlotoxumab for *Clostridium difficile*. *B. anthracis* is a Gram-positive bacterium which secretes anthrax toxin containing protective antigen (PA) to bind to the host cell receptors. After that, B. anthracis transport lethal factor and the edema factor into cytosol of the cells for pathogenesis of anthrax. Raxibacumab and Obiltoxaximab are monoclonal antibody which bind free PA for inhibition of binding between PA and cellular receptors. They lead to preventing the intracellular entry of the anthrax lethal factor and edema factor for pathogenesis of anthrax and have been used for the treatment of antrax in children and adults. And the last anti-virulence drug is bezlotoxumab for *Clostridium difficile*. *C. difficile* is a Gram-positive bacterium which secretes Toxin A and Toxin B for disruption of cytoskeleton structure. They

lead to weakness, abdominal pain, and diarrhea in infections. Bezlotoxumab is monoclonal antibody which binds only toxin B for reducing recurrence of C. difficile infection. It has been used for the treatment of C. difficile infection in patients 18 years of age or older. However, Bezlotoxumab does not bind to Toxin A (77, 78).

Table 2 Anti-virulence drugs have been approved by FDA since 2003 (79-83)

Trade name	Treatment	Approval year
BabyBIG	Treatment of infant botulism caused by toxin types A or B in patients below one year of age	2003
Raxibacumab	Treatment of adult and pediatric patients with inhalational anthrax	2012
BAT	Treatment of symptomatic botulism following documented or suspected exposure to botulinum neurotoxin serotypes A, B, C, D, E, F, or G in adults and pediatric patients.	2013
Obiltoxaximab	Treatment of inhalational anthrax for in adult and pediatric patients	2016
Bezlotoxumab	Reducing recurrence of *C. difficile* infection (CDI) in patients 18 years of age or older	2016

In conclusion, all new strategies for opposition of bacterial infection as mentioned above will be effective enough to solve the antibiotic resistance problem in future. The type three secretion systems (T3SS) have been gotten more attention to study identification of T3SS-inhibitor molecules for inhibition of virulence from T3SS instead of other virulence factors. Since the T3SS is able to be found in pathogenic gram-negative bacteria for cause of disease in animals and plants and it has high similarity in both of structures and functions. Therefore, the T3SS-inhibitors that are identified from one bacterium may be able to be used for decrease of virulence in other bacteria. Moreover, some studies found that inhibition of T3SS *via* the T3SS-inhibitors affect the virulence of bacteria rather than the bacterial growth (11, 12, 84). Therefore, the T3SS is interesting to study and identify T3SS-inhibitors as well as in this study focused on the T3SS only.

3. Type Three Secretion System (T3SS) inhibitor

3.1. Type Three Secretion Systems (T3SS)

The T3SS is a virulence factor that has been found in abundant gram negative bacteria for example *Salmonella* spp., *Shigella* spp., *Escherichia coli*., *Yersinia* spp. and *Chlamydia* spp (9). The function of T3SS involves mainly secretion

of effector proteins from bacterial cells into host cells to module a variety of cellular functions. The structure of the T3SS is similar to needle shape or called injectisome on the bacterial surface that there are a lot of proteins to assemble formation of the T3SS. It includes 6 components for formation of the structure as shown in figure 10 (85-87).

- Basal structure: it includes export apparatus, inner rod, ATPase complex, Inner membrane ring and outer membrane ring. The function of this part translocates several effector proteins into needle complex.

- Needle: it is next to basal structure that morphology is similar to needle shape. Length and width of the needle are approximately 30 -70 nm and 10 - 30 nm, respectively. It's passage way of effector proteins.

- Needle tip: it interacts directly with the host cell surface to facilitate the insertion of translocons for occurring pore within membrane of the host cell. If the needle tip has not reached the host cells surface, it doesn't open itself to translocate translocons. Therefore, the needle tip is important for beginning mechanism of T3SS.

- Translocon: after needle tip interacts with the host cells surface, the translocon is translocated into cell membrane of the host cell. Then, it forms pore about 20-30 Ă of width in the cell membrane for preparation of secreting effector proteins later.

- Chaperones: they are low molecular that bind with the specific effector proteins. The function of them keep the effector proteins for unfolded or partially folded structures before secretion through T3SS. Moreover, they encourage delivery of specific effector proteins through T3SS (88).

- Effector proteins: these proteins are delivered through T3SS to modulate a variety of cellular functions. Each effector protein is individual function for pathogenesis.

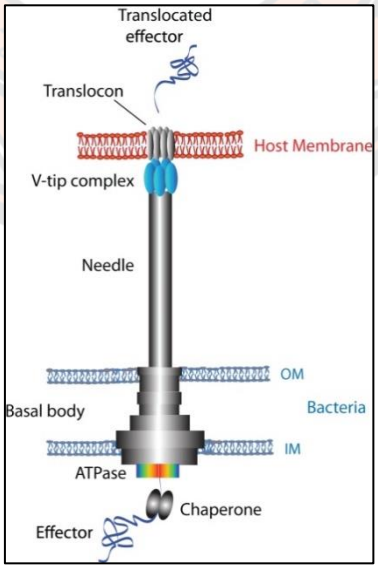


Figure 10 Structure and components of T3SS

Structure of T3SS is similar to needle shape or called injectisome that composed of 6 components, including basal structure, needle, needle tip, translocon, chaperones and effector proteins (89).

3.2. T3SS of *Salmonella* Typhimurium as a model

Although the T3SS are found and has high-conservation in both of structure and function among many gram-negative bacteria, we selected only the T3SS of *Salmonella* Typhimurium for doing the experiment in this study that there are 2 reasons for selection. The first reason, the illness from *Salmonella* Typhimurium infection, including diarrhea, fever, and abdominal cramps, mostly is able to recover the diseases without treatment (90). It is good and safe for us if laboratory-acquired infection occurs. And the second reason, *Salmonella* Typhimurium requires Biosafety level-2 (BSL-2) for performance that are appropriate for our laboratory and laboratory-personnel to do the experiment with it (91).

3.2.1. *Salmonella enterica* subspecies serovar Typhimurium

Salmonella enterica subspecies serovar Typhimurium or *S.* Typhimurium is gram-negative bacilli bacteria ranging in diameter from around 0.7 to 1.5 µm with a length of 2 to 5 µm that belongs to the Enterobacteriaceae family. Moreover, it is facultative anaerobic and facultative intracellular bacteria that can be motile by using peritrichous flagella. However, it cannot form spore to cause disease. *S.* Typhimurium can be grown appropriately in temperature around 35 to 40 ºC with pH condition around 4.5 to 9 (92, 93).

S. Typhimurium contaminates in food and water for infection. After uptake and ingestion of *S.* Typhimurium contaminated food and water, *S.* Typhimurium enters the lower gastrointestinal tract for beginning process of infection. It leads to inflammation and bacterial dissemination in the infected area and then causes diseases including diarrhea, fever and abdominal cramps. Furthermore, Salmonella infection may spread from the intestines to the bloodstream in some persons including older adults, infants, and impaired immune systems. It can cause death unless the persons are treated immediately (94).

3.2.2. SPI-1 T3SS of *S.* Typhimurium as a model

The T3SS of *S.* Typhimurium is encoded by 2 gene clusters including *Salmonella* pathogenicity island-1 (SPI-1) and *Salmonella* pathogenicity island-2 (SPI-2) that the structure of the both are almost similar. Even though, the function of T3SS is secretion of effector proteins. The aim of SPI-1 and SPI-2 T3SS is different. The function of SPI-1 T3SS is secretion of effector proteins for invasion of *S.* Typhimurium into the host cells. While, SPI-2 T3SS involves secretion of effector proteins for regulation of survival and replication in *Salmonella*-containing vacuole (SCV) within the host cells. The functions of both T3SS are shown in figure 11. Therefore, the SPI-1 and SPI-2 T3SS are necessary to *S.* Typhimurium for pathogenesis (95-97).

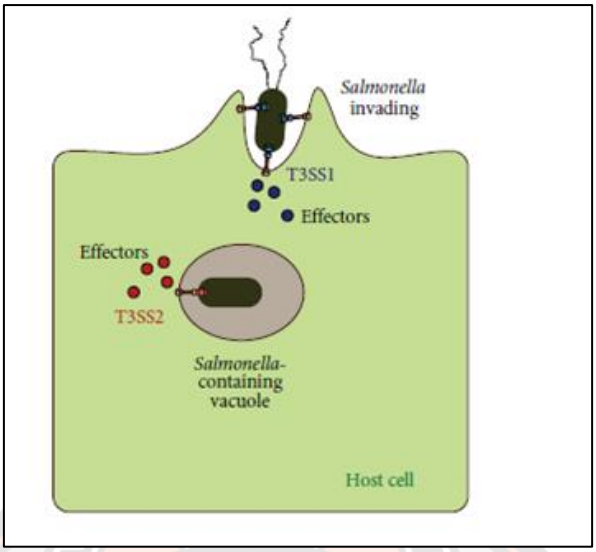
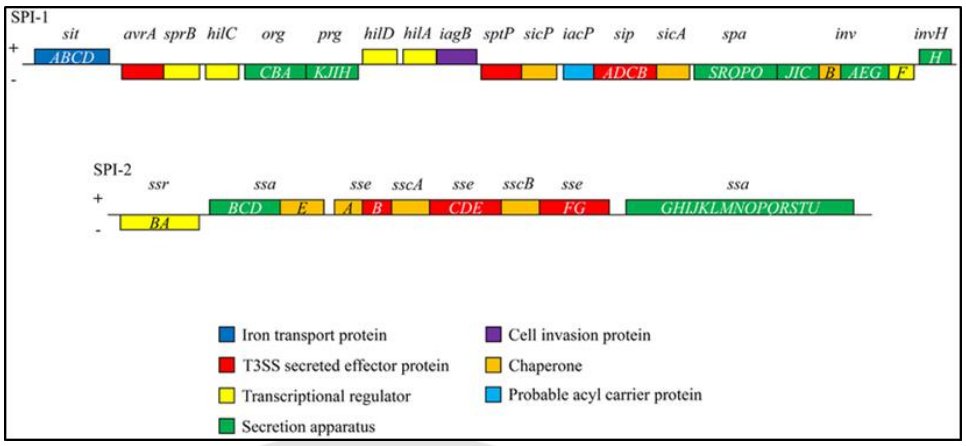


Figure 11 Functions of SPI-1 and SPI-2 T3SS
SPI-1 T3SS involve *Salmonella* invasion while SPI-2 T3SS involve *Salmonella*-containing vacuole (95)

Salmonella pathogenicity island-1 is the gene cluster that encodes proteins for construction of SPI-1 T3SS and bacterial invasion into the host cells. The gene cluster of SPI-1 and structure of SPI-1 T3SS are shown in the figure 12A. This cluster composes of 40.3 kb that has 41 genes for encoding proteins involving SPI-1 T3SS. The basal body includes PrgH, PrgK (inner membrane ring), SpaP, SpaO, SpaR, SpaS, InvA (inner membrane protein), InvC (ATPase) and InvG (outer membrane secretin). The needle is PrgJ that is controlled length by InvJ (it's celled molecular ruler). The translocons are SipB, SipC and SipD. And chaperons are SicA, SicP and SpaB. While, Salmonella pathogenicity island-2 encodes proteins for construction of SPI-2 T3SS which regulate bacterial survival and replication in SCV within the host cells. The gene cluster of SPI-2 and structure of SPI-2 T3SS are shown in the figure 12B. This cluster composes of 39.8 kb that has 44 genes for encoding proteins involving SPI-2 T3SS. The basal body include SsaD, SsaJ, SsaQ, SsaR, SsaS, SsaT, SsaU, SsaV (inner membrane ring and inner membrane protein), SsaN (ATPase) and SsaC (outer membrane secretin). The needle is SsaG controlling length by InvJ (Molecular ruler). And chaperons are SicA, SicP and SpaB (95-97).

(A)



(B)

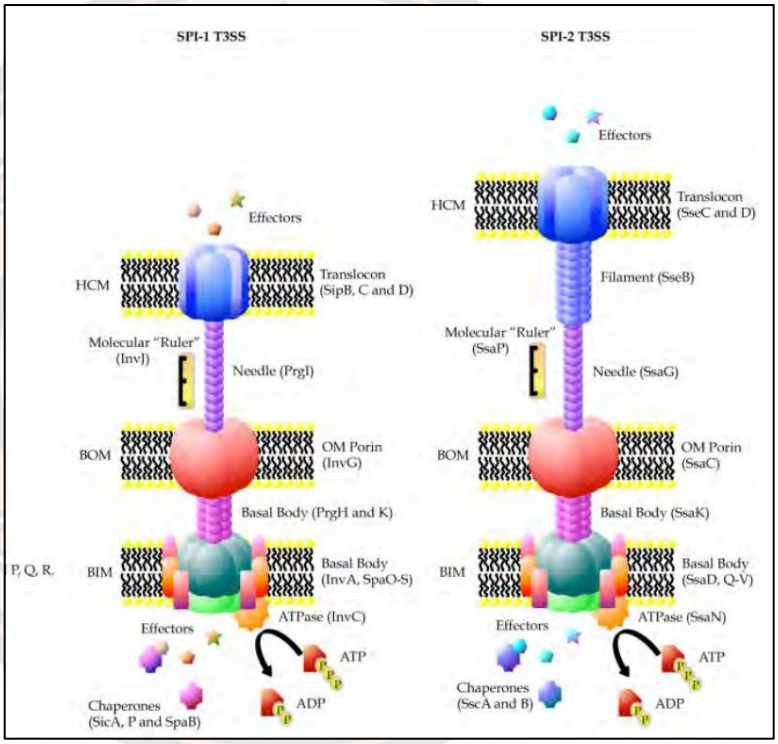


Figure 12 SPI-1 and SPI-2 T3SS
(A) The encoded genes of SPI-1 and SPI-2 of *Salmonella* indicate their functions (97)
(B) Schematics represent structures of SPI-1 and SPI-2 T3SS of *Salmonella* (96)

Expression of SPI-1 T3SS is regulated by environmental and genetic signals. The environmental signals involving inducing expression of SPI-1 T3SS include pH, osmolarity, the presence of bile, divalent cations concentration, and the presence of short chain fatty acids. The appropriate environment for inducing SPI-1 expression is at near neutral pH (pH 7-8), high osmolarity, high divalent cations and iron, low oxygen tension, nutrient rich and absence of bile and short chain fatty acids that found at the distal small intestine. After S. Typhimurium enters at the appropriate environment in the distal small intestine, the expression of SPI-1 T3SS then is activated by genetic signals called regulatory system. The system has several regulators, for example HilA, EnvZ/OmpR, HilC, HilS and InvF, to construct the

structure of SPI-1 T3SS and generate effector proteins. After HilA is expressed by HiD, HilC or RtsA activation, the HilA leads to transcription of several proteins in *inv/spa* and *prg/org* operon. The translated proteins for these operons involve the structure of SPI-1 T3SS. Moreover, InvF from *inv/spa* then activate transcription of sic/sip operon for generation of chaperon and effector proteins (94, 96, 98). Whereas, expression of SPI-2 T3SS is regulated by environmental and genetic signals. The appropriate environment for inducing SPI-2 expression is within the SCV that include an acidic pH (pH 4-5), low osmolarity, low divalent cations and nutrient poor. The SPI-2 T3SS inducing condition is opposite extremely from SPI-1 T3SS inducing condition. Therefore, the SPI-1 T3SS isn't expressed while the SPI-2 T3SS is expressed within SCV. The expression of SPI-2 T3SS is depend on two-component regulatory systems *phoP/phoQ* and *envZ/ompR*. These regulators lead to transcription of *Ssr* and *Ssa/Sse* operon for construction of SPI-2 T3SS and generation of chaperon and effector proteins (96). The expression of SPI-1 and SPI-2 T3SS is shown in figure 13.

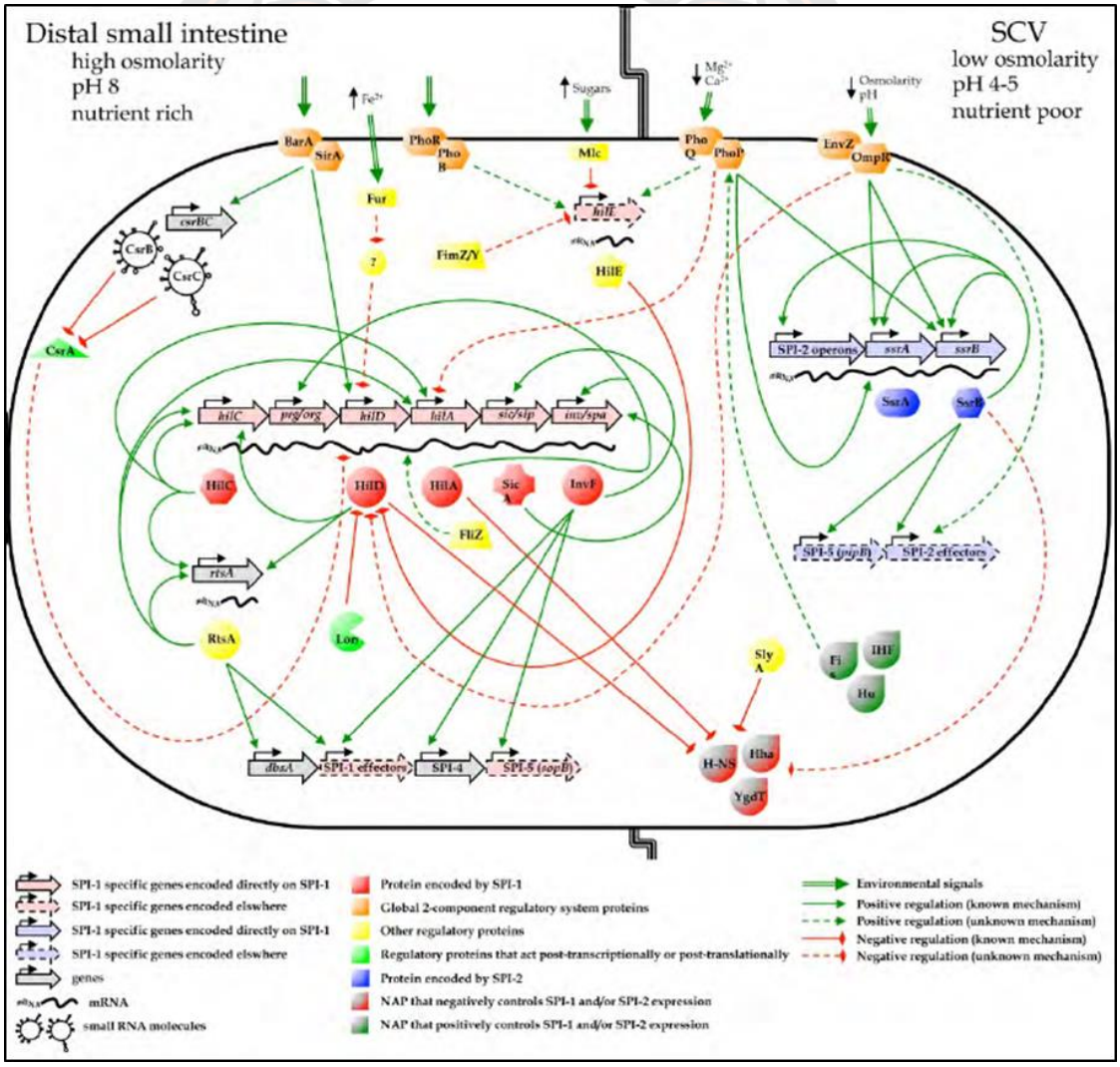


Figure 13 Regulation of SPI-1 and SPI-2 T3SS
(96)

The function of SPI-1 T3SS is secretion of effector proteins for invasion of *S.* Typhimurium into the host cells. After *S.* Typhimurium enters to the appropriate environment in the distal small intestine. The SPI-1 T3SS then is expressed to ready for bacterial invasion. First of all, needle tip of the T3SS contacts with the host cells membrane including M-cells or intestinal epithelial cells. The contact signal leads to dislodging of the gatekeeper (InvE) for secretion of effector. Then, translocons are released from the chaperones by catalyzation of ATPase and secrete for formation of pore within cell membrane. After that, other effector proteins are released from the bacteria into the host cell for bacterial invasion. Mechanism of bacterial invasion: 1.) For actin polymerization, SipA (The first secreted effect proteins) and SipC bind actin directly while SopE, SopE2 and SopB stimulate Rho GTPase activity though N-WASP, WAVE, WASH for activation of Arp2/3. 2.) For occurring Filopodial and Lamellipodial, SopB also encourages actomyosin contractility and changes phosphoinositide concentrations. It facilitates the dissociation between actin cytoskeleton and membrane at the entry site. Moreover, SopB may trigger VAMP-8 to deliver vesicles to the bacterial entry site. 3.) For formation of *Salmonella*-containing vacuole (SCV), SopD contributes to the sealing and the formation of the SCV. 4.) For regaining, SptP inhibit the activity of RhoGTPases and permit the host cell for regaining its initial shape. Finally, *S.* Typhimurium is take up in SCV within the host cells (95, 98). The summary of SPI-1 T3SS functions is shown in figure 14.

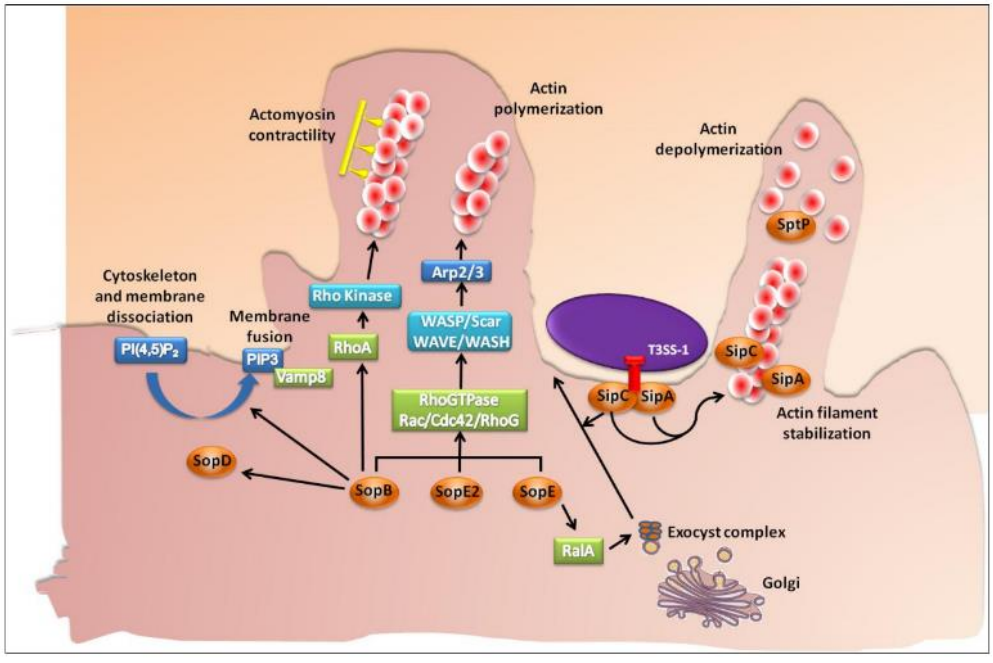


Figure 14 Membrane ruffling's occurred by effector proteins of SPI-1 T3SS
This figure shows function of SPI-1 T3SS for invasion into host cell (98)

The functions of SPI-2 T3SS involve secretion of effector proteins for regulation of survival and replication in SCV within the host cells. The SPI-2 T3SS functions maintenance of Salmonella in the SCV via the action of the effectors SifA, SopD2, SseJ and PipB2. The effectors SseF and SseG involve localization of

the SCV near the Golgi of host cells. Moreover, the effectors SpvC, SspH1 and SseL mediate in modulation of host immune signaling and the effectors SteC, SpvB, SspH2 and SrfH proteins involve target the host cytoskeleton (99). The summary of SPI-2 T3SS functions is shown in figure 15.

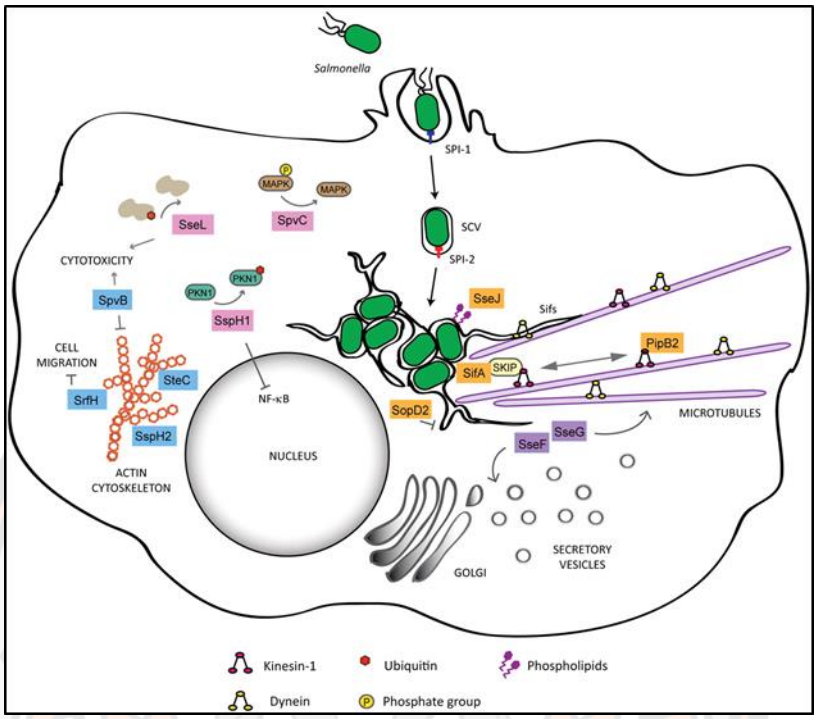


Figure 15 Function of effector proteins of the SPI-2 T3SS
This figure shows function of SPI-2 T3SS for maintain into host cell (99)

Although both of T3SS in *S.* Typhimurium function secretion of effector proteins from themselves and also can be potential for being a model to identify T3SS inhibitors, SPI-1 T3SS in *S.* Typhimurium have been spotlighted mostly from the scientists to apply it for being a model and identification of T3SS inhibitors. Because if the SPI-1 T3SS of *S.* Typhimurium is inhibited by T3SS inhibitors, *S.* Typhimurium is not able to invade into the host cells and is also decreased its pathogenesis (11, 12, 84). Therefore, SPI-1 T3SS of *S.* Typhimurium is used in this study to discover and develop new anti-virulence drugs as T3SS inhibitors that can inhibit functions or expression of SPI-1 T3SS.

3.3. SipA protein as a target for monitoring in SPI-1 T3SS activity

SPI-1 T3SS of *S.* Typhimurium secrete a lot of effector proteins and various functions in invasion which are shown in Table 3. However, we selected only SipA protein as a target for monitoring SPI-1 T3SS activity and identification of T3SS inhibitors.

Table 3 *Salmonella* secreted effectors and their role in disease (100)

Effector	Function cellular
AvrA	Regulates Salmonella induced inflammation
SipA	Mediates Salmonella invasion of epithelial cells by inducing actin bundling and polymerization, promotes formation and maintenance of SCV, induces neutrophil transmigration and also processes and activates caspase-3
SipB	Translocon
SipC	Promotes bacteria entry (invasion) by mediating actin bundling, component of the translocon
SipD	promotes the secretion of effectors and functions at the post-transcriptional and post-translational levels
SopA	Ubiquitin E3 ligase, promotes bacteria escape from SCV and is required for invasion, promotes neutrophil transmigration
SopB	Phosphatidyl inositol phosphatase, promotes bacteria entry, macropinocytosis, regulates SCV localization and formation and promotes fluid secretion during infection, Akt activation
SptP	Contains GTPase activating protein (GAP) and tyrosine activities, reverses Salmonella-induced pro-inflammatory responses
SopD	Promotes bacteria invasion and fluid secretion/accumulation
SspH	E3 ubiquitin ligase
SteB	Putative piconate reductase, required for Salmonella biofilm formation

Salmonella invasion proteins A or SipA is encoded by *sipA* gene which compose of 2055 nucleotides and molecular weight of it is 89 kDa (101). SipA protein is the first effector protein which deliver from SPI-1 T3SS into host intestinal epithelial cells (102). The SipA is an actin-binding protein which enhances the efficiency of the entry process. The SipA leads to *Salmonella* invasion into host cells via inducing different stages in the formation of membrane ruffles and rearrangement of the actin cytoskeleton. Moreover, the SipA controls actin molecules at the site of bacterial entry for the concentration, polymerization and stability and induces the bundling activity of host cell fimbrin. After Salmonella is invaded into the host cell and contained in SCV, the SipA also is secreted on the cytoplasmic face of the SCV

in both non-phagocytic cells and macrophages for regulation of phagosome maturation and intracellular *Salmonella* replication. The action of invasion involves the C-terminal domain of SipA while the N-terminal domain of SipA induces polymorphonuclear leukocyte transmigration. Furthermore, the SipA also activate and release caspase-3 which plays multiple roles in the immune response of host cells, including, apoptosis, differentiation, proliferation, immunomodulation, immune cell migration, and signal transduction (103). The summary of SipA functions is shown in figure 16.

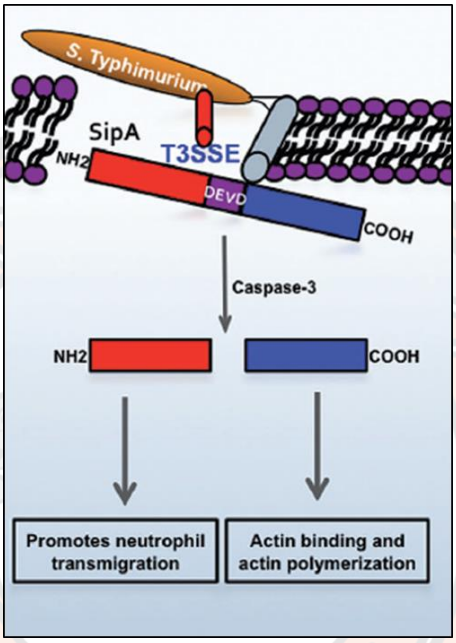


Figure 16 Summary of SipA functions

SipA can induce activation of caspase 3 which lead to cleavage of SipA. The C terminal of SipA involves actin binding and actin polymerization. While, the N terminal of SipA promotes neutrophil transmigration (100)

Therefore, we focused only the SipA as the target for monitoring SPI-1 T3SS activity and identification of T3SS inhibitors in this study. Because SipA protein is the first effector protein which deliver from SPI-1 T3SS into host intestinal epithelial cells. Moreover, this effector is an important virulence factor for invasion. It induces actin polymerization that leads to membrane ruffling and then bacterial uptake into the intestinal epithelium of infection. If the T3SS inhibitors can inhibit SPI-1 T3SS activity, we expect that quantity of secreted-SipA is decreased or absent and *Salmonella* cannot invade into the host cells.

3.4. Type Three Secretion Systems (T3SS) inhibitors

Since 2002, the natural or chemical substances have been determined the property for inhibition of SPI-1 T3SS; no effect to bacterial growth; and no effect to eukaryotic cells by using SPI-1T3SS model of different bacteria such as *Salmonella* and *Yersinia.* Therefore, there are a lot of T3SS inhibitors that have been found for 20 years. Moreover, some T3SS inhibitors have already identified target in mechanisms of T3SS for interruption of T3SS functions which are shown in Table 4. Almost of

compounds which inhibit SPI-1 T3SS is extracted by natural products and chemical products. The mechanisms of T3SS inhibitors are composed of prevention of T3SS assembly, inhibition of secretion of effector proteins and inhibition of interaction between T3SS and host cells (11, 12, 84).

Table 4 T3SS inhibitors and their targets
(12, 104, 105)

Compound	Model T3SS (Bacteria)	Target
(−)-Hopeaphenol	*Yersinia, Pseudomonas, Chlamydia*	Unknown
1-butyl-4-nitromethyl-3-quinolin-2-yl-4H-quinoline	*Shigella*	VirF (transcription factor)
2,2′-thiobis-(4-methylphenol)	*Yersinia*	YopD (translocon)
8-hydroxyquinolines	*Yersinia, Chlamydia*	ATPase
Aurodox	*EPEC, Citrobacter*	Downregulation of the master regulator Ler.
Benzimidazole	*Yersinia* *Pseudomonas*	LcrF (transcription factor) ExsA (transcription factor)
Caminoside A	*EPEC*	Unknown
Cytosporone B	*Salmonella*	Hha–H-NS–HilD–HilC–RtsA–HilA regulatory pathway
Fusaric acid	*Salmonella*	SicA/InvF (Regulator)
Licoflavonol	*Salmonella*	sicA/invF (Regulator)
Myricanol	*Salmonella*	HilD
Omeprazole	*Salmonella*	ATPase
Phenoxyacetamide	*Pseudomonas*	PscF (needle protein)
Piericidin A1	*Yersinia*	Formation of Ysc-type needle units
Pseudolipasin A	*Pseudomonas*	ExoU (effector)
Salicylidene Acylhydrazides	*Chlamydia* *Salmonella* *Shigella*	HemG (heme metabolism) FlhA (flagellar inner membrane protein) Needle assembly
Sanguinarine chloride	*Salmonella*	HilA
Small molecule compound (C23H18N4O3S2)	*Yersinia* *Burkholderia*	YscN (ATPase) BsaS (ATPase)
Thymol	*Salmonella*	Lon protease

Beside natural and chemical substances, there is antibody called antibody KB001-A to interrupt T3SS functions of *P. aeruginosa*. It is reported by Kalobios in 2014. Moreover, it is in phase II clinical trials currently for treating patients who have respiratory tract inflammation and cystic fibrosis (CF) caused by chronic *P. aeruginosa* infection (11, 106).

Even through the T3SS inhibitors have potential for not only inhibition T3SS functions and also decrease of bacterial infection, but they don't damage bacteria and lead the bacteria to still be within the host cell or body. Therefore, they may need to be combined with other antibiotics for helping clearance the bacteria (12).

4. Natural products

There are a lot of natural products, including bacteria, fungus and plants, which are used for discovery of the antibiotics and T3SS inhibitors (13-15). However, this study focusses on only plant, especially herbs.

4.1. Definition of the herbs

The terms of the herbs have more than one definition nowadays. For example, Peter, K. V. defines that "herbs are plants that some parts of them contain essential oils useful in food, medicine and/or cosmetics and they usually grow in temperate regions which are both in the wild and under cultivation" or "herbs are the dried leaves of aromatic plants used to impart flavour and odour to foods" (107).

4.2. History of herbs

The herbs have a long history for using and distribute around the world. The first region is Egypt in African. Egyptians found several herbs for themselves Before Common Era (BCE) and used as ingredients in the meal for health effects. For example, garlics and mints. They found Garlics in their tombs and King Tutankhamen around 1500 BCE. They used garlics with radishes and onions to feed their slaves for keeping healthy. Whereas, mints were found in Egyptian pyramids around 1000 BCE (108, 109). The second region is Greece and Rome in Europe. Hippocrates that is Greek physician in 460 to 377 BCE used herbs for 300 remedies. He reportedly used garlic to treat uterine cancer. He also used liquorice for anti-inflammatory, asthma, chest problems and mouth ulcers. Even though, the liquorice was used as a sweet, Moreover, he used rosemary to improve and harden memory. While, Galen who lived in Rome from 131 to 200 CE developed herbal remedies by using complicated mixtures that are more than 100 ingredients (109). The third region is China in East Asia. The Chinese traditionally used herbs as ingredient of food such as soups for nourishment and health benefits. For example, Ginseng and Ginkgo biloba are reportedly used to improve stamina and cognitive performance, respectively. While, galangal, nutmeg and cinnamon are used for abdominal pain, diarrhea, and flu, respectively (109). The four region is India in South Asia. It has traditional medicine that is called Ayurveda more than 5000 years ago. The Ayurveda uses herbs for health effects such as turmeric for jaundice, basil for protection of a heart, mace for stomach infections, cinnamon for stimulation of circulation, and ginger for relieving nausea and indigestion. In addition, the herbs are also ingredients of several Indian meals

(110, 111). And the last region is in Australia. Indigenous population used and developed herbs for being herbal medicines. For example, mint is used for coughs and colds. While, wattle and eucalyptus are used for diarrhea, fever and headache (112). In summary, the herbs in each region around the world are not exactly similar. However, an aim of using the herbs is only for being ingredient of cooking and medicine (16).

4.3. Using herbs as traditional medicine (TM)

The traditional medicine is "the sum total of the knowledge, skill, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness". It has the WHO Traditional Medicine Strategy 2014-2023 by WHO to support harnessing the potential contribution of Traditional medicine to health, wellness and people-centred health care. Moreover, it also promotes the safe and effective use of TM by regulating, researching and integrating TM products, practitioners and practice into health systems (113).

In the present, using herbs for protection and therapy of the diseases, called herbal medicine, is categorized the one of traditional medicines. Because the herbs in local regions are still used for protection and treatment of the diseases as same as the past. Moreover, there are a lot of scientists that attempt to study their local herbs for protection and treatment of the diseases. According to WHO, medicinal plants would be the best source to obtain a variety of drugs (17). They are also used herbs as an ingredient mixing other ingredients to generate novel drugs for treatment of the diseases. As these reasons lead the herbal medicine to being the traditional medicine. The herbal medicine includes several herbs that are applied for protection and treatment of the diseases. For example, garlic and assum tea. Consumption of a clove of garlic daily is able to reduce cholesterol, in blood pressure and be anticlotting that reduce risk of cardiovascular disease (16). So, it's kind of protection of the diseases. Whereas, the assum tea leaves are capable of inhibiting growth of antibiotic resistant bacteria (114). It shows that the assum tea leaves may be used for treatment of infectious disease in the future.

4.4. Herbs for the bacterial infection

Because the herbs contain a variety of phytochemicals such as tannins, alkaloids and flavonoids, which have been found *in vitro* to have antimicrobial activity (18). Moreover, they are fewer side effects and reduced toxicity (19). Therefore, a lot of various herbs are screened and applied for treatment of infectious diseases (15, 115). In the present, there are still several studies that screen the antimicrobial activity in different herbal extracts. For example, *Aquilaria crassna* and *Ocimum gratissimum* L. *A. crassna* or agarwood leaves were extracted by using aqueous and ethanol and consequently the leaves extracts were screened antimicrobial activities by Disk diffusion, MIC or MBC. The results showed that the extracts are an antimicrobial agent against *Staphylococcus epidermidis* and probably other gram-positive bacteria for growth (20, 116). It summarizes *A. crassna* may be applied for treatment of the infectious diseases. While, the *O. gratissimum* L or tree basil leaves were subjected to essential oil by hydro-distillation. The essential oil showed activity

against Gram-negative bacteria and Gram-positive bacteria. Moreover, it was used for treatment of pneumonia, diarrhea and conjunctivitis (117, 118). Although all of the herbs have almost antimicrobial activity and are used for inhibition of bacterial growth in the infectious disease, some of them have been found interruption of T3SS activity instead of bacterial growth. They are able to interfere secretion of effector proteins from T3SS. However, they don't inhibit bacterial growth. For example, *Scutellaria baicalensis* or chinese skullcap. *S. baicalensis* is used as Traditional Chinese Medicines (TCMs). It has been found that its baicalein, a specific flavonoid, is able to inhibit SPI-1 T3SS activity of *S.* Typhimurium. It leads *S.* Typhimurium to unable secretion of effecter proteins and invasion into the target cells (21). The results demonstrate that the herbs have a variety of phytochemicals for being not only the antibacterial agent, but also antivirulent factor (T3SS inhibitor). From the previous studies of the herbs about treatment of infectious, the herbs are potential as both antibacterial activity for inhibition of bacterial growth and anti-T3SS activity for interruption of bacterial pathogenesis. Therefore, this study interested whether Thai herbs as the antibacterial agents could also inhibit T3SS activity as the T3SS inhibitors. The Thai herbs include Garlic, Chantaleela, Turmeric, and King of bitters.

Garlic or *Allium sativum* L is an herbaceous plant that belongs to the Amaryllidaceae family (119). It has an approximately 1 m long fragrant stem divided into 6 to 12 bulblets (garlic cloves) joined by a thin shell, which forms the garlic head or bulb of garlic. Height of garlic head with leaves and roots is about 70-100 cm. Leaf number is 13-15 leaves. Weight of garlic head is about 50 grams which composed of 12-13 cloves and diameter of the bulb is about 5 cm (120). The bulb of garlic is used as a basic spice for many types of Thai foods in the all regions of Thailand such as Thai crispy spicy garlic fried chicken, Papaya salad and Thai Basil Minced Pork. Moreover, it is used as Traditional Medicine for treatment of diseases for example hypertension, cancer, cardiovascular diseases and microbial infections especially bacterial infection (121). Bulbs of garlic contains a lot of constituents which are shown in Table 5. However, the main phytochemical which plays a lot of activities is Thiosulfinates or allicin. When garlic is cut or chewed, Allicin is converted from alliin by allinase enzyme. But allicin is unstable and quickly oxidized to yield sulfur compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), ajoene, and hydrogen sulfide. Diallyl-thiosulfinate is the major thiosulfinate which is found in garlic for 70-80% of sulfur compound (119). The garlic extract is reported that inhibit a variety of antibiotic resistant bacteria such as *Shigella, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Salmonella* Typhimurium (122). Although garlic extract is able to inhibit bacterial growth, there are no reports about garlic extracts for interruption of bacterial pathogenesis through T3SS as T3SS inhibitors. Therefore, we interest to study the garlic extract whether it is potential for being the T3SS inhibitor.

Turmeric or *Curcuma longa* L is herbaceous perennial plant that belongs to the Zingiberaceae family. Height of turmeric with leaves and rhizomes is about 90-150 cm. The rhizomes of turmeric are made yellow-colored curry paste and then used as a basic spice for many types of Thai foods in the southern regions of Thailand such as Turmeric Chicken Soup Recipe, Fried Mackerel with Turmeric and Roasted Chicken with Turmeric. Moreover, it is used as Traditional Medicine for treatment of diseases for example dyslipidemia, stomach disorders, arthritis, hepatic diseases and

microbial infections (123). The rhizomes of turmeric contain a lot of constituents which are shown in Table 5. However, the main phytochemical which plays a lot of activities is curcuminoids. The curcuminoids are polyphenol from the rhizomes of the turmeric. The curcuminoids compose of curcumin (60−70%), demethoxycurcumin (20−27%), and bisdemethoxycurcumin (10−15%) (124). The turmeric extract are reported that inhibit a variety of bacteria such as *Bacillus subtilis, Staphylococcus aureus, Salmonella* Typhimurium and *Pseudomonas aeruginosa* (125). Although turmeric extract is able to inhibit bacterial growth, there are no reports about turmeric extracts for interruption of bacterial pathogenesis through T3SS as T3SS initiators. Therefore, we interest to study the turmeric extract whether it is potential for being the T3SS inhibitor.

Chantaleela is alternative medicine for relieving fever in Thailand. It is comprised of 9 Thai herbs including *Gymnopetalum chinense* (Lour.) Merr, *Atractylodes lancea* (Thunb.) DC, *Artemisia annua* L, *Angelica dahurica, Santalum album* L, *Dracaena cochinchinensis* (Lour.), *Tinospora crispa* (L.), *Eurycoma longifolia* and *Pogostemon cablin* (126). Chantaleela is reported that inhibit a variety of antibiotic resistant bacteria such as Methicillin resistant *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (127). However, there are no reports about chantaleela for interruption of bacterial pathogenesis through T3SS as T3SS inhibitors. Therefore, we interest to study chantaleela whether it is potential for being the T3SS inhibitor.

King of bitters or Fah talai chon in Thailand or *Andrographis paniculata* (Burm. f.) Wall. ex Nees is herbaceous plant that belongs to the Acanthaceae family. Plant height is about 30-100 cm. Morphology of it compose of stem, leaves, roots and flowers (128). The aerial parts of *Andrographis paniculate* are extracted and then formulated in tablet or capsule as alternative medicine for common cold in Thailand. The *A. paniculata* extract contains a lot of constituents which are shown in Table 5. However, the main phytochemical which plays a lot of activities is andrographolide. The andrographolide is diterpene lactone which contain in any part of *A. paniculata* especially leaves. The *A. paniculata* extract is reported that inhibit a variety of antibiotic resistant bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (129). Although *A. paniculata* extract is able to inhibit bacterial growth, there are no reports about *A. paniculate* extracts for interruption of bacterial pathogenesis through T3SS as T3SS inhibitors. Therefore, we interest to study the *A. paniculata* extract whether it is potential for being the T3SS inhibitor.

Table 5 Constituents in blubs of garlic, rhizomes of turmeric and leaves of king of bitters.

Constituents	Garlic (130)	Turmeric (124)	King of bitters (131)
Water	~65%	6-13%	73%
Carbohydrate	~28%	60-70%	12%
Protein	~3.2%	6-8%	3%
Fiber	~1.5	2-7%	1.3%
Fatty acid	<0.1%	5-10%	1%
Sulfur compound	~2.2%	-	-
Curcuminoids	-	1-6%	-
Andrographolide	-	-	2%
Ash	-	-	4%
Others (Phenols, Flavonoid, Trance elements and Vitamins)	<0.1%	6-14%	3.7%

From the previous studies of garlic, turmeric, chantaleela and king of bitters about treatment of infectious disease, the 4 herbs are able to inhibit bacterial growth. However, there are no reports about the herbs for interruption of T3SS activity as T3SS inhibitors. Therefore, this study interested to study garlic, turmeric, chantaleela and king of bitters whether they were potential for being the T3SS inhibitors. The objectives of this study are to screen garlic, turmeric, chantaleela and king of bitters as the antibacterial agents whether they are potential for being the T3SS inhibitors and to detect mechanisms of Thai herbs for inhibition of T3SS, if they are able to inhibit T3SS. And SPI-1 T3SS of *S.* Typhimurium was used as a model for monitoring activity of T3SS. Briefly, Thai herbal extracts were prepared for screening anti-T3SS activity. Secondly, the minimal concentration of the Thai herbal extracts for inhibition of SPI-1 T3SS was measured by Western blotting. Next, antibacterial activity of Thai herbal extracts against *S.* Typhimurium was evaluated by Time-kill assay. Then, cytotoxicity of Thai herbal extracts to Hela cells was measured by MTT assay. After that, Thai herbal extracts for inhibiting bacterial invasion of *S.* Typhimurium were detected by invasion assay. Finally, mechanisms of Thai herbal extracts for inhibition of SPI-1 T3SS were detected by measurement of transcription level of genes regulating the SPI-1 T3SS. Therefore, the results of this study demonstrated which Thai herbs could inhibit SPI-1 T3SS of *S.* Typhimurium for being T3SS inhibitors and mechanisms of Thai herbs for inhibition of SPI-1 T3SS.

CHAPTER III

RESEARCH METHODOLOGY

Summary of the methods

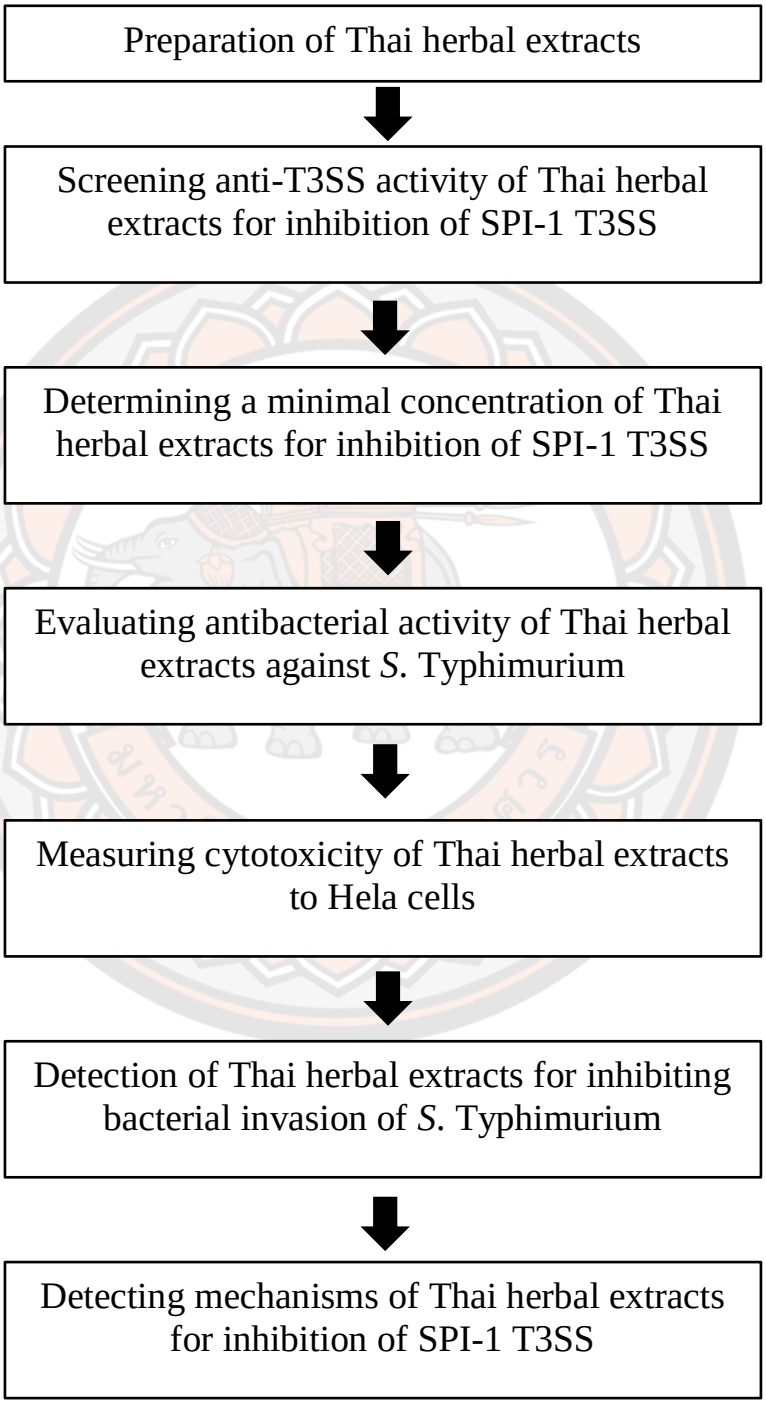


Figure 17 Summary of the methods.

Materials

1. Chemicals and reagents

1.) 2x RBC Blue Taq Mastermix	RBC Bioscience Corp., Taiwan
2.) 2x OnePCR Ultra	BIO-HELIX CO., LTD., Taiwan
3.) Agarose	Research Orgranics, Inc., USA
4.) Ethidium bromide (EtBr)	Vivantis Technologies Sdn. Bhd., Malaysia
5.) GeneRuler 1 Kb plus 100 bp DNA E Ladder Marker	LPIS BIOTECH. INC., Korea
6.) GeneRuler 100 bp DNA Ladder Marker	BIO-HELIX CO., LTD., Taiwan
7.) Tris	AMRESCO LLC, USA
8.) Ethylene diamine tetraacetic acid (EDTA)	Ajax Finechem Pty Ltd., Australia
9.) Glacial acetic acid	CARLO ERBA Reagent S.A.S., France
10.) Ethanol (EtOH)	Merck KGaA, Germany
11.) Methanol (MeOH)	Labsolv Scientific Pvt Ltd., India
12.) Glycine	Thermo Fisher Scientific, USA
13.) NaCl	Ajax Finechem Pty Ltd., Australia
14.) Yeast extract	Bio Basic Inc., USA
15.) Agar powder	HiMedia Laboratories Pvt Ltd., India
16.) Ampicillin	Bio Basic Inc., USA
17.) Kanamycin	Bio Basic Inc., USA
18.) Trichloroacetic acid (TCA)	Merck KGaA, Germany
19.) Acetone	Merck KGaA, Germany
20.) Acrylamide/BisTM 29:1 40%(W/V) solution	AMRESCO LIC, USA
21.) Ammomium persulfate	Ajax Finechem Pty Ltd., Australia
22.) Sodium dodecyl sulfate (SDS)	LOBA CHEMIE Pvt Ltd., India
23.) Tetramethyl ethylenediamine (TEMED)	Bio Basic Inc., USA
24.) β-mercaptoethanol	Bio Basic Inc., USA
25.) Smart Color Pre-stained protein Marker	ELPIS BIOTECH. INC., Korea
26.) Coomassie brilliant blue G-250	Thermo Fisher Scientific, USA
27.) LuminataTM Western HRP Chemiluminescence Substrates	Merck KGaA, Germany
28.) Dimethyl sulfoxide (DMSO)	Thermo Fisher Scientific, USA
29.)Triton-X 100	Merck KGaA, Germany
30.) 5x Reverse transcription Buffer	Meridian Bioscience Inc., USA
31.) 2x SensiFAST™ SYBR® No-ROX mix	Meridian Bioscience Inc., USA

2. Enzymes and Restriction endonuclease enzymes

1.) Taq DNA polymerase	RBC Bioscience Corp., Taiwan
2.) T4 DNA ligase	Thermo Fisher Scientific, USA
3.) *EcoRI*	SibEnzyme Ltd., Russia
4.) *HindIII*	SibEnzyme Ltd., Russia
5.) *XmnI*	Thermo Fisher Scientific, USA
*6.) Dpn*I	Fermentas Life Sciences, Canada
7.) Tetro Reverse Transcriptase	Meridian Bioscience Inc., USA
8.) *DNase* I	Thermo Fisher Scientific, USA

3. Instruments

1.) Biological safety cabinet class II Type A2	NuAire Inc., USA
2.) Temperature controlled Megafuge 1.0R	Kendro Laboratory, Germany
3.) Heraeus Biofuge Fresco Refrigerated Centrifuge	Thermo Fisher Scientific, USA
4.) TDB-U-400 Dry block thermostat	Talron Biotech Ltd., Israel
5.) Shaking bacterial incubator	Amerex Instruments Inc., USA
6.) Major Cycler	Major Science, USA
7.) Gene Pulser Xcells^{TM}	Bio-rad Laboratories, Inc., USA
8.) Electroporation Systems	
9.) Mini-PROTEAN 3 cell	Bio-rad Laboratories, Inc., USA
10.) EletroBLOT-Mini blotter	Cleaver Scientific Ltd., UK
11.) CO_2 incubator	MEMMERT GmbH & Co. KG, Germany
12.) Platinum D55 gel documentation	Topac Inc., UK
13.) ChemiDocTMXRS+ Imager	Bio-rad Laboratories, Inc., USA
14.) LineGene 9600 Plus Fluorescent Quantitative Detection System	Hangzhou Bioer Technology Co., Ltd, China
15.) MR-1 Mini-Rocker Shaker	Biosan, Ltd., Latvia
16.) Enspire® plate reader	PerkinElmer InC., USA
17.) SpectraMax iD3 Multi-Mode Microplate Reader	Molecular Devices, LLC., USA

4. Bacterial strains

Table 6 *E. coli* and *S.* Typhimurium strains used in this study

Names	Properties	References or sources
E. coli strain		
BL21 (DE3)	F-, *omp*T. *hsdS*$_\beta$ (r^β-, m^β-), *gal*, *dcm* (DE3)	Novagen
S. Typhimurium strain		
SL1344	Wild Type	*Salmonella* Genetic Stock Centre (SGSC)
SA	*SipA::strep-tag*	Boonyom, R., *et al.* (132) and this study
Δ*invA*	*invA::Kan*^{R}	Boonyom, R., *et al.* (133)

5. Cell line

Hela cell (ATCC No. CCL-2) was purchased from the American Type Culture Collection (ATCC).

6. Plasmids

Table 7 Plasmids used in this study

Plasmids	Properties	References or sources
pKD13	Template of Kanamycin resistance gene (*Kan*^{R})	Datsenko, K. A., *et al.* (134)
pKD46	Lambda-red recombination expression plasmid; *Amp*^{R}	Datsenko, K. A., *et al.* (134)
pCP20	FLP recombinase expression plasmid; *Amp*^{R}	Datsenko, K. A., *et al.* (134)
pJBT	pBAD24 derivatives, encoding C terminal step epitope tag; *Amp*^{R}	Karavolos, M. H., *et al.* (135)
pJWA	pJBT encoding *SipA* with C terminal step epitope tag; *Amp*^{R}	This study
pJWAK	pJBT encoding *SipA*, *strep tag*, *Kan*^{R}	This study

7. Primers

Table 8 Primers used in this study

Primer sets	Sequences (5'→ 3')	Size (Base pairs, bp)
SA	FP : AGGAATTCACCATGGTTACAAGTGTAAGGA CTCAGCC RP : TCGAAAGCTTACGCTGCATGTGCAAGCCAT CAAC	2,055
CSA	FP : AGGAATTCACCATGGTTACAAGTGTAAGGA CTCAGCC RP : CAAGCTTTTATTTTTCAAACTGTGGGTGCG	2086
KFRT	FP : GAGAGAAGATTTTCAGATTGCAGCATTACA CGTCTTGAGC RP : TATGAGAATTAATTCCGGGGATCCGTCGAC CTGCAGTTCG	1,330
CSAK	FP : GCAGAGCGTTTGACGGCTT RP : TATGAGAATTAATTCCGGGGATCCGTCGAC CTGCAGTTCG	1,731
AK	FP : GCAGAGCGTTTGACGGCTT RP : TGACTCTTGCTTCAATATCCATATTCATCGC ATCTTTCCCTCAAACATGAGAATTAATTC	1,776
CCUT	FP : GCAGAGCGTTTGACGGCTT RP : CCAATGAGTCAGCGTAAAGATCC	501
CHiD	FP : TCTGCTGTTCCTGCTTACTG RP : TTGCTGCCTGATTCATTCTTG	248
CHiC	FP : ATGTCCACGGGTTTGTAGTAAT RP : GATTCATACGAGCATCCAGGTA	296
CHiA	FP : GCTGTGGATTACCTATTATCATACC RP : AAGGCACGCTCACTATTCTG	243
CInF	FP : GCGAGAGTTACTGGTTGGTT RP : TCTACACTATTCAGCAGCGATT	190
CInH	FP : GTGTCAGTCTCTTCCGTATGT RP : ACGCATGTATTGTGGATGTTC	181
CPrI	FP : CTTGGTCAGGCTATCTGGATGACG RP : AAAGCTTACGGAAGTTCTGAATAATGGCAGC	238
CScA	FP : ATGGATTATCAAAATAATGTCAGCG RP : TCTGTCTCCGCCGTTTTTAGCGCC	470
CSpA	FP : AGAGGCTGTGACCAACGCTTCAT RP : CGTGTCTGATTGTAAGGTATCGGTTT	268
C16S	FP : GTTGGTGAGGTAACGGCTCACCAAGG RP : CTCCACCGCTTGTGCGGGCCCCCGTC	697

8. Ethical approval

This study was approved by Naresuan University Institutional Biosafety Committee (review protocol No. NUIBC MI 61-04-18) for laboratory biosafety. Bacteria and genetic material in the study were performed with reference to the legal requirements of the committee.

Methods

1. Molecular biology methods

1.1. Genomic DNA Purification

Genomic DNA of *S.* Typhimurium strain SL1344 was extracted and purified by using TIANamp Genomic DNA kit (TIANGEN Biotech (Beijing) Co., Ltd., China). Firstly, *S.* Typhimurium strain SL1344 was grown overnight in LB broth at 37ºC with 200 rpm shaking. Bacterial cells were collected by centrifugation at 4000g for 15 min. The cells were resuspended with 200 µl of buffer GA. Twenty µl of Proteinase K were added to the suspension and mixed by vortexing. The mixture was added 200 µl of buffer GB, mixed by vortexing and incubated at 70ºC for 10 min. Next, the mixture was added 200 µl of ethanol (96-100%) and mixed by vortexing for 15s. The mixture was pipetted into the TIANamp Spin Column CB3 and the spin column was centrifuged at 12,000 rpm for 30s. Flow-through solution was discarded and the spin column was placed into the collection tube. The spin column was washed with 500 µl of buffer GD, 700 µl of Buffer PW and 500 µl of Buffer PW, respectively. Then, the spin column was centrifuged at 12,000 rpm for 2 min to dry the membrane completely. After that, the spin column was placed in a new clean 1.5 ml microcentrifuge tube and added 50 µl of distilled water. Finally, the spin column was centrifuged to elute purified genomic DNA.

1.2. Plasmid DNA purification

Plasmid DNA of *E. coli* strain BL21 (DE3) and *S.* Typhimurium strain SL1344 were extracted and purified by using Plasmid *mini* PREP kit (BIO-HELIX CO., LTD, Taiwan). Firstly, *E. coli* or *S.* Typhimurium was grown in LB broth at 37ºC for 16 -18 hours with 200 rpm shaking. Bacterial cells were collected by centrifugation at 4000 g for 15 min. The cells were resuspended with 200 µl of Buffer S1. Two hundred µl of Buffer S2 were added to the suspension and mixed by inverting the tube 10 times. After the mixture stand at the room temperature for 2 minutes, the mixture was added 300 µl of the Buffer S3 and mixed immediately by inverting the tube 10 times. The mixture was centrifuged at 13,000 rpm for 3 min and the supernatant was pipetted into a PM column. Next, the PM column was centrifuged at 13,000 rpm for 30s. The PM column was washed with 400 µl of the Buffer W1 and 600 µl of the Buffer W2, respectively. Then, the PM column was centrifuged at 13,000 rpm for 2 min to dry the membrane completely. After that, the PM column was placed in a new clean 1.5 ml microcentrifuge tube and added 50 µl of distilled water. Finally, the RB column was centrifuged to elute purified plasmid DNA.

1.3. Amplification of DNA with Polymerase Chain Reaction (PCR)

RBC Blue Taq Mastermix (RBC Bioscience Corp., Taiwan) was used for PCR reaction. The PCR solution composed of template DNA, 1X Blue Taq Mastermix (including 0.075U/µl RBC Taq DNA polymerase, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH8.3), 0.1 mg/ml BSA, 10mM $(NH_4)_2SO_4$, 0.2 mM dNTP mix, stabilizers and blue dye), 0.4 µM forward primer, 0.4 µM reverse primer and sterile nuclease-free water. The description of PCR reaction was demonstrated in table 9.

Table 9 Description of PCR reaction

Step	Temperature	Time	Cycle
Pre-denaturation	95ºC	5 min	1
Denature	95ºC	30 sec	30
Annealing	56ºC	30 sec	
Extension	72ºC	30 sec/ 1 Kb	
Final extension	72ºC	5 min	1

1.4. Agarose gel electrophoresis

PCR products were detected by Agarose gel electrophoresis. Firstly, ethidium bromide was added into 0.8% agarose gel concentration for preparation of the agarose gel. Next, PCR products were mixed with 1X DNA loading dye. Then, the mixtures were loaded into channel of agarose gel. After that, the electrophoresis run at constant voltage,80 V, for 30 minutes. Finally, product bands in the agarose gel were visualized by Platinum D55 Gel documentation (Topac Inc., UK) for checking and comparison with standard DNA marker.

1.5. Purification of PCR fragment

PCR products were purified by using Hi Yield™ Gel/PCR DNA Fragment Extraction kit (RBC Bioscience Corp., Taiwan). Firstly, PCR products were transferred to a microcentrifuge tube for adding 5 volume of DF Buffer to 1 volume of the PCR products and mixing by vortexing. The mixture was pipetted into a DF column and centrifuged at 13,000 rpm for 30s. Flow-through solution was discarded and DF column was washed with 600 µl of Wash Buffer. Next, the DF column was centrifuged at 13,000 rpm for 30s and flow-through solution was discarded. Then, the DF column was centrifuged at 13,000 rpm for 2 min to dry the membrane completely. After that, the DF column was placed in a new clean 1.5 ml microcentrifuge tube and added 50 µl of distilled water. Finally, the DF column was centrifuged to elute purified DNA.

1.6. Purification of PCR fragment from agarose gel

PCR products from agarose gel were purified by using Hi Yield™ Gel/PCR DNA Fragment Extraction kit (RBC Bioscience Corp., Taiwan). After

running agarose gel electrophoresis, DNA product at expected band from agarose gel was cut and removed to minimize gel slice. Next, the gel slice was transfer to a microcentrifuge tube. The tube was added 500 µl of DF Buffer, mixed by vortex and incubated at 55°C for 10-15 minutes or until the gel slice dissolved completely. The dissolved mixture was pipetted into a DF column and centrifuged at 13,000 rpm for 30s. Flow-through solution was discarded and DF column was washed with 600µl of Wash Buffer. The DF column was centrifuged at 13,000 rpm for 30s and flow-through solution was discarded. Then, the DF column was centrifuged at 13,000 rpm for 2 min to dry the membrane completely. After that, the DF column was placed in a new clean 1.5 ml microcentrifuge tube and added 50 µl of distilled water. Finally, the DF column was centrifuged to elute purified DNA.

1.7. Restriction digestion

Digestion of DNA by restriction endonuclease enzyme was operated base on protocol of molecular cloning by Molecular Cloning: A Laboratory Manual (136). Reaction mixture composed of DNA fragment or plasmid DNA, 1X restriction enzyme buffer and restriction endonuclease enzyme in a total volume of 20 µl. Restriction enzymes in this study include *Eco*RI, *Hind*III, *Xmn*I and *Dpn*I (Elimination of methylated template DNA). All of restriction enzyme was activated at 37ºC for 2 hours. *Eco*RI and *Xmn*I were inactivated at 65ºC for 20 min, whereas *Hind*III and *Dpn*I were inactivated at 80 ºC for 20 min. Moreover, digested plasmid was treated with FastAP Thermosensitive alkaline phosphatase to prevent recirculation. The FastAP Thermosensitive alkaline phosphatase was activated at 37ºC for 30 min and inactivated 75ºC for 10 min. After all of processes, the digested DNA fragment and plasmid is ready for next process.

1.8. DNA ligation

DNA ligation by T4 DNA Ligase enzyme was operated base on protocol of molecular cloning by Molecular Cloning: A Laboratory Manual (136). Reaction mixture composed of digested plasmid DNA, digested inserted DNA, 1X ligation buffer (including 40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP), nuclease-free water and 1 Weiss Unit of T4 DNA Ligase enzyme in a total volume of 20 µl. This study used molar ratio 1:3 vecter to inserted DNA. The ligation mixture was mixed by pipetting, spun briefly and incubated at 16ºC for 16 hours. Finally, the ligase enzyme was inactivated at 65ºC for 10 min and the ligation mixture is ready for transformation process.

1.9. Preparation of *E. coli* competent cells

Preparation of competent cells was operated base on Inoue, H., *et al*.'s protocol (137). *E. coli* strain BL21 (DE3) was inoculated overnight in LB broth at 37ºC with 200 rpm shaking. The next day, bacterial culture was subcultured 1:100 in LB broth and incubated at 37ºC with 200 rpm shaking until optical density (OD) at 600 nm was approximately 0.4 – 0.6. Bacterial cells were collected by centrifugation at 4000 g for 15 min at 4ºC. Cell pellets were resuspended in ice-cold TB buffer (including 15 mM $CaCl_2$, 250 mM KCl and 55 mM $MnCl_2$) and placed on ice for 10 min. The cell suspension was centrifuged at 4000 g for 15 min at 4ºC and the cell

pellets were resuspended in ice-cold TB buffer additional with 7%(v/v) Dimethyl sulfoxide (DMSO). The cell resuspension was incubated on ice at least 10 min. Finally, 100 µl of the cell suspension was pipetted in a microcentrifuge tube to use for transformation process or stored immediately at -80 ºC before using.

1.10. Bacterial transformation

Heat shock method was used for bacterial transformation in this study which based on Molecular Cloning: A Laboratory Manual's protocol (136). Briefly, the competent cells were incubated with plasmid DNA on ice for 30 min. Next, the mixture was incubated at 42ºC for 90s for heat processing. Then, the mixture was shocked by incubation on ice for 2 min. For recovery cells from heat shock processing, the cells were added with 1 ml of pre-warmed LB broth and incubated at 37ºC for 60 min with gentle shaking. After that, the cell pellets were collected by centrifugation at 6000 rpm for 2 min and spreaded on LB agar with appropriated antibiotic at 37ºC overnight. Finally, colony formation on plate was investigated on the next day.

1.11. Preparation of *S.* Typhimurium electrocompetent cells

Preparation of electrocompetent cells was operated base on Molecular Cloning: A Laboratory Manual's protocol (136). The *S.* Typhimurium strain SL1344 was inoculated overnight in LB broth at 37ºC with 200 rpm shaking. The next day the *S.* Typhimurium were diluted at 1:100 in LB broth and incubated at 37ºC with 200 rpm shaking until optical density (OD) at 600 nm was approximately 0.4 - 0.6. The bacterial culture was centrifuged at 4,000 g for 15 min at 4ºC. Cell pellets were collected and resuspended with sterile ice-cold distilled water. The pellets were washed twice with sterile ice-cold distilled water. Finally, *S.* Typhimurium's pellets were centrifuged at 4,000 g for 15 minutes at 4ºC and resuspended with sterile ice-cold distilled water for being electrocompetent cell.

1.12. Electroporation

The electrocompetent cells were mixed with plasmid DNA or DNA fragment cassette on ice. The mixture was added in a pre-chilled 1 mm gap electroporation cuvette and the cuvette was immediately pulsed at 1.8 kV, 200 Ω and 25 µF. Next, 1 ml of pre-warmed LB broth was added into cuvette and mixed by pipetting. Then, the mixture was transferred into a microcentrifuge tube for incubation at 37ºC for 60 min with gentle shaking. After that, the cell pellets were harvested by centrifugation at 6000 rpm for 2 min and spreaded on LB agar with appropriated antibiotic at 37ºC overnight. Finally, colony formation on plate was investigated on the next day.

1.13. Colony PCR screening

OnePCR Ultra Mastermix (BIO-HELIX CO., LTD., Taiwan) was used for PCR reaction. Bacterial colonies on the plate from Bacterial transformation and Electroporation were selected and placed at the bottom of PCR tube. The PCR solution compose of a bacterial colony as template DNA, 1X One PCR Ultra Master

mix (Thermostable DNA polymerase, PCR buffer, dNTPs, gel loading dyes, enhancer, and fluorescence dye), 0.4 µM forward primer, 0.4 µM reverse primer and sterile nuclease-free water. The description of PCR reaction is demonstrated in table 10.

Table 10 Description of Colony PCR reaction

Step	Temperature	Time	Cycle
Pre-denaturation	94ºC	5 min	1
Denature	94ºC	30 sec	30
Annealing	55ºC	45 sec	
Extension	72ºC	30 sec/ 1 Kb	
Final extension	72ºC	5 min	1

1.14. Total RNA Purification

Total RNA of *S*. Typhimurium SL1344 was extracted by using Total RNA Extraction Kit Mini (RBC Bioscience Corp., Taiwan). Firstly, *S*. Typhimurium was grown overnight in LB broth at 37ºC with 200 rpm shaking. Bacterial cells were collected at 4000 g for 15 min and resuspended with 200 µl of RT buffer. Cell suspension was incubated at room temperature for 5 min. Next, the cell suspension was added 400µl of RB Buffer to the sample lysate, mixed by vortex and incubated at room temperature for 5 min. The mixture was pipetted into a Filter Column Set and centrifuged at 13,000 rpm for 2 min. Clarified filtrate was transfer to a new microcentrifuge tube for adding 400µl of 70% ethanol and mixing by vortex. Ethanol-added mixture was pipetted into RB column and it was centrifuged at 13,000 rpm for 2 min. The RB column was washed with 400 µl of R-W1 Buffer and 600 µl of R-Wash Buffer, respectively. Then, the RB column was centrifuged at full speed for 3 min to dry the membrane completely. After that, the RB column was placed in a new clean 1.5 ml microcentrifuge tube and added 50 µl of RNase free water. Finally, the RB column was centrifuged to elute the extracted genomic RNA.

To purify the genomic RNA, Deoxyribonuclease I (DNase I) was used. Firstly, a microcentrifuge tube on ice was added 1 µg of the extracted genomic, 1 µl of 10X DNase I Reaction Buffer, 1 µl of DNase I and DEPC-treated water to 10 µl. The microcentrifuge tube was incubated at room temperature for 15 min. at 65°C for 10 min. Finally, the genomic RNA is pure and ready to use in reverse transcription.

1.15. Reverse transcription PCR (RT-PCR)

Tetro cDNA Synthesis Kit (Meridian Bioscience Inc., USA) was used to synthesize cDNA from genomic RNA. The RT-PCR solution composed of 5 g genomic RNA, 40 µM Random Hexamer, 10mM dNTP mix, 5x RT Buffer, RiboSafe RNase Inhibitor, Tetro Reverse Transcriptase (200 U/ µL) and DEPC-treated water. Mixture was mixed by pipetting and incubated at 45 °C for 30 min, 10 min at 25 °C

and 45 °C for 30 min, respectively. Terminate reaction by incubating at 85 °C for 5 min, chill on ice. Finally, cDNA proceeds to PCR immediately or store at -20 °C.

1.16. Amplification of cDNA with Real-time PCR

SensiFAST™ SYBR® No-ROX Kit (Meridian Bioscience Inc., USA) was used for Real-time PCR reaction. The Real-time PCR solution consist of cDNA, 1x SensiFAST SYBR® No-ROX Mix, 0.4 µM forward primer, 0.4 µM reverse primer and distilled water. The description of PCR reaction is demonstrated in table 11.

Table 11 Description of Real-time PCR reaction

Step	Temperature	Time	Cycle
Pre-denaturation	95ºC	2 min	1
Denature	95ºC	5 sec	45
Annealing	57ºC	10 sec	
Extension and SYBR detection	72ºC	35 sec	

2. Chromosome integration by λ red recombination

Datsenko, K.A., *et al.*'s protocol was used to modify the targeted chromosome DNA of *S.* Typhimurium (134). This step is replacement of precise targeted chromosome DNA of *S.* Typhimurium with generated DNA cassette by using Homologous recombination system required λ red recombination enzyme. Eventually, the chromosome of *S.* Typhimurium has generated DNA cassette instead of former chromosome.

2.1. DNA cassette preparation

The *partial SipA-strep tag-KanR-downstream sipA locus* cassette which *partial SipA* and *downstream sipA locus* were homologous sequences for integration into the chromosome was amplified by PCR and using pJBT plasmid as a template. Then, the amplified cassette was treated with *DpnI* at 37ºC for 2 hours for elimination of methylated (unamplified) template DNA. After that, the cassette was purified by Hi YieldTM Gel/PCR DNA Fragment Extraction kit. Therefore, the cassette was ready for electroporation.

2.2. Electroporation and chromosomal integration detection

Electrocompetent cells in this step used *S.* Typhimurium strain SL1344 containing pKD46 plasmid which was incubated in LB broth containing 100 µg/ml ampicillin at 30ºC for 3 hour and then added 1 mM arabinose at 30ºC for 1 hour to express λ red recombinase. After electroporation between the cells and the cassettes, the electroporated cells were recovered and then spreaded on LB agar containing 50 µg/ml kanamycin at 37ºC overnight. The colonies on the LB agar were

picked to perform the colony PCR for detection of correct chromosome in *SipA-strep tag-kan*^{R} inserted *S.* Typhimurium.

2.3. pKD46 plasmid elimination

Because the pKD46 plasmid contains temperature sensitive replicon (repA101ts), the plasmid is able to be eliminated by elevation of temperature approximately 42 - 44ºC. A colony of *SipA-strep tag-kan*^{R} inserted *S.* Typhimurium containing pKD46 was inoculated LB broth at 43ºC overnight. The next day, bacterial suspension was streaked 4 planes on LB agar and then incubated at 43ºC overnight. To analyze removal of pKD46 plasmid from the *SipA-strep tag-kan*^{R} inserted *S.* Typhimurium, a selected single colony was patched on 3 plates including LB agar, LB agar containing ampicillin and LB agar containing kanamycin and incubated at 37ºC overnight. If the selected colony is able to grow on LB agar and LB agar containing kanamycin except LB agar containing ampicillin, it demonstrated that pKD46 was removed from *SipA-strep tag-kan*^{R} cassette inserted *S.* Typhimurium.

2.4. Kanamycin resistant gene elimination by FLP recombination system

The kanamycin resistance gene in the cassette was removed by FLP recombination system. The FLP recombinase was expressed from pCP20 plasmid to bound two homologous short flippase recognition target (FRT) site for reverse orientation. Therefore, a selective antibiotic sequence in a specific position was eliminated. First of all, *SipA-strep tag-kan*^{R} inserted *S.* Typhimurium was prepared to be electrocompetent cells. Next, pCP20 plasmids were transferred into the prepared electrocompetent cells by electroporation. Then, *SipA-strep tag-kan*^{R} inserted *S.* Typhimurium containing pCP20 plasmids was recovered, spreaded and incubated on LB agar containing ampicillin at 30ºC overnight for cutting both FRT flanked *kan*^{R} site in the *partial SipA-strep tag-Kan*^{R}*-downstream sipA locus* cassette. To analyze removal of the *kan*^{R} from chromosome of the *SipA-strep tag* integrated *S.* Typhimurium, the colonies on the LB agar were picked to perform the colony PCR for detection of *kan*^{R} elimination. Finally, the pCP20 plasmid was removed from the *S.* Typhimurium by incubation at 43ºC that is similar to removal of pKD46 plasmid method. On the other hands, if the selected colony grew on only LB agar, it demonstrated that pCP20 plasmid was removed from the *SipA-strep tag* integrated *S.* Typhimurium. Therefore, *SipA-strep tag* integrated *S.* Typhimurium was constructed and called *S.* Typhimurium strain SA.

3. Preparation of Thai herbal extracts

The Thai herbal extracts were obtained from the Faculty of Pharmacy in Mahidol University. The extracts compose of garlic (*Allium sativum*), turmeric (*Curcuma longa*), chantaleela (*Gymnopetalum cochinchinense*, *Myristica fragrans*, *Dracaena loureiri*, *Tinospora crispa*, *Eurycoma longifolia*, *Atractylodes lancea*, *Angelica sylvestris*, *Artemisia vulgaris Linn*), and king of bitters (*Andrographis paniculata*). The garlic and chantaleela were formulated in tablet while the turmeric and king of bitters were formulated in capsule. To prepare a stock solution of the extracts, the herbal powder in the capsules were ready to use. Whereas, the herbal tablets were grinded into herbal powder by mortar and pestle before use. All powders

were dissolved in dimethyl sulfoxide (DMSO) for concentration of 100 mg/ml (w/v) as stock solution and sterilized by filtration through 0.22 µm pore size of Nylon filter membrane. Finally, the stocks were stored at -80ºC before use.

4. Screening anti-T3SS activity of Thai herbal extracts for inhibition of SPI-1 T3SS

This experiment was modified from Condry, D. L., *et al.*'s protocol (138).

4.1. Recombinant protein expression in SPI-1 inducing condition

S. Typhimurium strain SA was induced SPI-1 expression by salt, such as NaCl, that enhance virulence gene expression. This step was referred by Hideaki, M., *et al.*'s protocol (139). Briefly, a colony of *S.* Typhimurium strain SA was inoculated in LB broth at 37ºC overnight. The next day, the bacterial culture was subcultured 1:100 in LB broth with 0.3 M NaCl, for SPI-1 inducing, and the herbal extracts at a final concentration of 100 µg/ml. DMSO at final concentration of 0.001% (v/v) was used as negative control. Next, the bacterial culture was grown at 37°C for 4 hours with 150 rpm. The culture was centrifuged to collect the supernatant. Then, the proteins in the supernatant were sterilized by filtration with a 0.22 µm pore size hydrophilic PVDF membrane and precipitated by 10% (w/v) trichloroacetic acid (TCA) at 4˚C for 10 minutes. The pellets were collected by centrifuge at 13,000 rpm for 10 min and washed twice by ice-cold acetone. Dry pellets were resuspended with 1X SDS loading dye containing 5% (w/v) β-Mercaptoethanol. The protein mixture was boiled at 95˚C for 10 min and briefly spinned.

4.2. Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE)

SDS-PAGE was based on Laemmli UK's protocol (140). This study used 4% of stacking gel and 10% of separating gel. Firstly, the samples were mixed by pipetting and loaded into channel of the SDS-PAGE instrument, Mini-PROTEAN 3 (Bio-Rad Laboratories, Inc., USA). Electrical process began at 120 Volt until bromophenol blue tracking dye on the top of separating gel and then finished at 150 Volt until the tracking dye in the end of separating gel. SDS-PAGE gel was ready to perform Coomassie blue staining or Western blotting.

4.3. Coomassie blue staining

Proteins in SDS-PAGE gel was stained and detected by Coomassie blue staining. The gel was soaked and stained by Coomassie blue solution at room temperature for 60 min with horizontal shaking. To clear the background in the gel, the gel was soaked by destaining solution until the background is nearly clear to visualize protein bands.

4.4. Western blotting

The separated proteins from SDS-PAGE were transferred to polyvinylidene fluoride (PVDF) membrane by using EletroBLOT-Mini blotter (Cleaver Scientific Ltd., UK) at constant 100 Volt for 1 hour. After transferring

proteins onto PVDF membrane, the membrane was blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBS-T) for 60 min. Next, the membrane was incubated and shake with primary antibody in TBS-T buffer at 4ºC overnight. Then, the membrane was washed 3 times with TBS-T and subsequently incubated with horseradish peroxidase-conjugated secondary antibody in TBS at room temperature for 1 hour. After that, the membrane was washed 3 times with TBS-T. Finally, chemiluminescent substrates were added onto the membrane and the chemiluminescent signal of reaction was detected by Chemidoc^{TM} XRS+ Imager (Bio-Rad Laboratories, Inc., USA).

5. Determining a minimal concentration of the Thai herbal extracts for inhibition of SPI-1 T3SS

Briefly, a colony of *S.* Typhimurium strain SA was inoculated in LB broth at 37ºC overnight. The overnight bacterial culture was subcultured 1:100 in LB broth with 0.3 M NaCl and the herbal extracts at various concentration. Garlic and turmeric were varied at a final concentration of 100, 75, 50 and 25 µg/ml and DMSO at final concentration of 0.001% (v/v) was used as negative control for treatment of garlic and turmeric. While, chantaleela was varied at a final concentration of 200, 175, 150, 125 and 100 µg/ml and DMSO at final concentration of 0.002% (v/v) was used as negative control for treatment of chantaleela. Next, the bacterial culture was grown at 37°C for 4 hours with 150 rpm. The culture was centrifuged to collect the supernatant. Then, the proteins in the supernatant were sterilized by filtration with a 0.22 µm pore size hydrophilic PVDF membrane and precipitated by 10% (w/v) trichloroacetic acid (TCA) at 4˚C for 10 minutes. The pellets were collected and prepared for detection in SDS-PAGE with Coomassie blue staining and Western blotting as mentioned which similar to step 4.

6. Evaluating antibacterial activity of Thai herbal extracts against *S.* Typhimurium

Time-kill kinetic assay was used in this experiment. This step detected bacterial growth that treated with the herbal extracts using SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, LLC., USA). The reader was able to automatically incubate bacteria and automatically measure the absorbance of bacterial culture. A colony of *S.* Typhimurium strain SL1344 was cultured in LB broth at 37˚C overnight. The overnight culture was seeded in 96 well plates containing LB broth with the herbal extracts or DMSO at final concentration of 0.002% (v/v) as negative control. The total volume of culture was 100 µl/well and the final concentration of garlic, turmeric and chataleela was 100, 75, 150 µg/ml, respectively. The plate was incubated in SpectraMax iD3 reader for 24 hours at 37 ˚C and the instrument measured the absorbance of bacterial culture at 600 nm every hour. The experiment was performed in triplicate.

7. Hela cells culture

Hela cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 µl/ml penicillin and 100 µl/ml

streptomycin at 37ºC in 5% CO2. When the cells were 80% confluent in a flask, they were subcultured for passaging themselves. Culture medium was removed and the cells were washed by Phosphate-buffered saline (PBS). Then, the cells were incubated with 0.25% (w/v) Trypsin-EDTA solution for detachment of adherent cells. After that, culture medium was added and cell suspension was centrifuged at 1500 rpm for 5 min. Finally, the cells were resuspended by pre-warm DMEM to divide in ratio 1:10 for subculturing or prepare for next process.

8. Measuring cytotoxicity of Thai herbal extracts to Hela cells

8.1. Cell culture preparation

The Hela cells with 80% confluent in a flask were subcultured and calculated for preparation. The cells at 5,000 cells were seeded into 96-well plate and incubated with DMEM including fetal bovine serum and penicillin-streptomycin at 37ºC, 5% CO2 for 16-18 hours. The culture medium was replaced with culture medium containing the herbal extracts or DMSO at final concentration of 0.002% (v/v) as negative control. The final concentration of garlic, turmeric and chataleela was 100, 75, 150 µg/ml, respectively. Then, the cell culture with 4 treatments was incubated at 37˚C, 5% CO_2 for 24 hours.

8.2. Cytotoxicity assay

This assay measured cellular metabolic activity as an indicator of cell viability or cytotoxicity by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. It is based on the reduction of a yellow tetrazolium salt to purple formazan crystals by metabolically active cells. The viable cells contain NAD(P)H-dependent oxidoreductase enzymes which reduce the MTT to formazan (141). The insoluble formazan crystals are dissolved using a solubilization solution and the resulting colored solution is quantified by measuring the absorbance at 570 nm.

Therefore, the prepared cells were washed with PBS and added MTT solution (0.5 mg/ml) 50 µl each well. After incubation for 3 hours at 37 ˚C, the MTT solution was discharged and DMSO 200 µl was added into the well. Finally, the mixture was mixed by pipetting and the absorbance at 570 nm was measured using Enspire® plate reader (PerkinElmer InC., USA). The percentage of cell viability was calculated using the following formula: (%) = [OD of treated cells/ OD of untreated cells] × 100. The experiment was performed in triplicate.

9. Detection of Thai herbal extracts for inhibiting bacterial invasion of

S. Typhimurium

9.1. Cell culture preparation

The Hela cells with 80% confluent in a flask were subcultured and calculated for preparation. The cells at 50,000 cells were seeded into in a 24 wells plate and incubated with DMEM including fetal bovine serum and penicillin-streptomycin at 37ºC, 5% CO2 for 16-18 hours.

9.2. Bacterial preparation

A colony of *S.* Typhimurium strain SL1344 and a colony of *S.* Typhimurium strain Δ*invA* (SPI-1 T3SS isn't functional) were cultured in LB broth at 37ºC overnight. The overnight bacterial culture of SL1344 was subcultured 1:100 in LB broth with 0.3 M NaCl and the herbal extracts or DMSO at final concentration of 0.002% (v/v) as negative control, while Δ*invA* was subcultured 1:100 in only LB broth with 0.3 M NaCl. The final concentration of garlic, turmeric and chataleela was 100, 75, 150 µg/ml, respectively. After that, the bacterial cultures were grown at 37°C for 4 hours. Finally, the absorbance of bacterial culture at 600 nm was measured and then the bacterial cultures were diluted to a concentration of 1 x 10^5 CFU/ml in fresh DMEM cell culture medium without antibiotics.

9.3. Bacterial invasion

This technique was modified from Steele-Mortimer, O.'s protocol (142). Gentamycin was used to kill extracellular bacteria without killing bacterial invasion into the cells and then quantity of intracellular bacteria was measured.

To detect bacterial invasion, the prepared Hela cells were infected with the prepared *S.* Typhimurium at a multiplicity of infection (MOI) of 10 at 37°C with 5% CO_2 for 30 min. Then, the culture medium was discarded and the cells were washed with PBS. The fresh DMEM medium containing 50 µg/ml gentamycin was added to each well to kill noninvasive bacteria at 37°C with 5% CO_2 for 40 min. After that, the cells were washed 3 times with PBS and lysed with 1% Triton X-100 solution for 10 minutes to release the bacteria. The bacteria were harvested and performed a ten-fold serial dilution. Finally, 10 µl of diluted bacteria were on LB gar for counting and calculation of the number of bacteria. The experiment was performed in triplicate.

10. Detecting mechanisms of Thai herbal extracts for inhibition of SPI-1 T3SS

10.1. Bacterial cDNA preparation

A colony of *S.* Typhimurium strain SL1344 was cultured in LB broth at 37ºC overnight. The overnight bacterial culture was subcultured 1:100 in LB broth with 0.3 M NaCl and the herbal extracts or DMSO at final concentration of 0.002% (v/v) as negative control. The final concentration of garlic, turmeric and chataleela was 100, 75, 150 µg/ml, respectively. Then, the bacterial cultures were incubated at 37ºC for 4 hours. After that, total RNA was extracted from bacteria by using Total RNA Extraction Kit Mini and then reversed to cDNA by using Tetro cDNA Synthesis Kit Protocol. Therefore, bacterial cDNA samples were ready to perform Real-time PCR.

10.2. Detection of the SPI-1 T3SS-related genes in gene expression by Real-time PCR

The prepared cDNA was mixed with primers of CHiD, CHiC, CHiA, CInF, CInH, CPrI, CScA, CSpA and C16S in SensiFAST SYBR® No-ROX Mix buffer. Then, the mixtures were put in LineGene 9600 Plus Fluorescent

Quantitative Detection System (Hangzhou Bioer Technology Co., Ltd, China). Finally, the comparative CT (Cycle threshold) method was used for analysis of relative changes in transcriptional levels. The change in gene transcription was calculated using the formula sample/control = $2^{-\Delta\Delta CT}$ which $\Delta\Delta CT$ is ΔCTsample - ΔCTcontrol and $\Delta CT = CT_{\text{target gene}} - CT_{\text{16S rRNA (internal reference)}}$ (143). The experiment was performed in triplicate.

11. Statistical analysis

Means and standard deviations were calculated by using Graph Pad Prism 5.0 (GraphPad Software, San Diego, CA). The statistical significance was determined by using the one-way and two-way ANOVA method and statistically significance was considered a P-value of < 0.05.

CHAPTER IV

RESULTS

All of results in this chapter were shown respectively. First of all, anti-T3SS activity of Thai herbal extracts were screened by SDS-PAGE with Coomassie blue staining and Western blotting. The *SipA-strep* tag integrated *S.* Typhimurium as *S.* Typhimurium strain SA was constructed and used to monitor the activity of SPI-1 T3SS. The results of SDS-PAGE with Coomassie blue staining and Western blotting showed secretion of SipA-strap tag from the SPI-1 T3SS of *S.* Typhimurium strain SA. If Thai herbal extracts inhibit activity of SPI-1 T3SS, the SipA-strep tag protein cannot be secreted from SPI-1 T3SS and absent in SDS-PAGE with Coomassie blue staining and Western blotting. Secondly, the minimal concentration of Thai herbal extracts for inhibition of SPI-1 T3SS was measured by detecting the absence of the SipA-strep tag protein on SDS-PAGE with Coomassie blue staining and Western blotting. Next, antibacterial activity of Thai herbal extracts against *S.* Typhimurium strain SL1344 was evaluated by Time-kill assay. This step detected the absence of SipA-strep tag proteins whether Thai herbal extracts inhibit activity of SPI-1 T3SS for secretion of the protein or kill *S.* Typhimurium. If Thai herbal extracts kill *S.* Typhimurium, they lead reduction of the bacterial growth curve. Then, cytotoxicity of Thai herbal extracts to Hela cells was measured by MTT assay. This step assured Thai herbal extracts that did not affect the Hela cells resulting in the result of invasion assay. After that, Thai herbal extracts for inhibiting bacterial invasion of *S.* Typhimurium SL1344 were detected by invasion assay. The result of invasion assay showed the number of bacteria that invaded the Hela cells. If Thai herbal extracts truly inhibit SPI-1 T3SS for secretion of effector proteins, they cause *S.* Typhimurium to reduce invasion. Finally, measuring transcription level of genes regulating the SPI-1 T3SS using Real-time PCR was used for detecting mechanisms of Thai herbal extracts for inhibition of SPI-1 T3SS. If an upper gene in the cascade of SPI-1 T3SS is a target of Thai herbal extracts for inhibition of SPI-1 T3SS, the expression of lower genes in the cascade is reduced and the SPI-1 T3SS cannot be functional. Therefore, all results can demonstrate which Thai herbal extracts are inhibit activity of SPI-1 T3SS in *S.* Typhimurium. Moreover, they also demonstrate mechanisms of Thai herbal extracts for inhibition of SPI-1 T3SS in *S.* Typhimurium.

1. Screening anti-T3SS activity of Thai herbal extracts for inhibition of SPI-1 T3SS

SipA-strep tag integrated *S.* Typhimurium was constructed for secretion of SipA strep tag protein from SPI-1 T3SS. Moreover, The SipA-strep tag proteins were detected by SDS-PAGE with Coomassie blue staining and Western blotting with anti-strep tag antibody. Therefore, *SipA-strep tag* integrated *S.* Typhimurium was applied to monitor the activity of SPI-1 T3SS and screen anti-T3SS activity of Thai herbal extracts.

1.1. Construction of *SipA-strep tag* integrated *S.* Typhimurium as *S.* Typhimurium strain SA

SipA protein was selected for tagging with strep-tag and monitoring the activity of SPI-1 T3SS in this study. The SipA protein is the first effector protein which deliver from SPI-1 T3SS into host intestinal epithelial cells. Moreover, this effector is an important virulence factor for invasion. It induces actin polymerization that leads to membrane ruffling and then bacterial uptake into the intestinal epithelium of infection.

Briefly, pJWAK plasmid containing *SipA-strep tag-Kan*^{R} cassette was constructed which the processes of generating the plasmid were shown in figure 18. Next, partial *SipA-strep tag-Kan*^{R}-downstream *SipA gene* locus cassette was amplified from the plasmid. Then, the cassette was electroporated into the *S.* Typhimurium SL1344 for exchanging between the cassette and chromosome of *S.* Typhimurium using lambda red recombination system. After that, the *kan*^{R} in the chromosome of *SipA-strep tag-Kan*^{R} integrated *S.* Typhimurium was removed by FLP recombination technique. Finally, the SipA in the chromosome of *SipA-strep tag* integrated *S.* Typhimurium was replaced by *SipA-strep tag* gene for production and secretion of SipA-strep tag protein from SPI-1 3SS. The construction process of *SipA-strep* tag integrated *S.* Typhimurium was shown in figure 19.

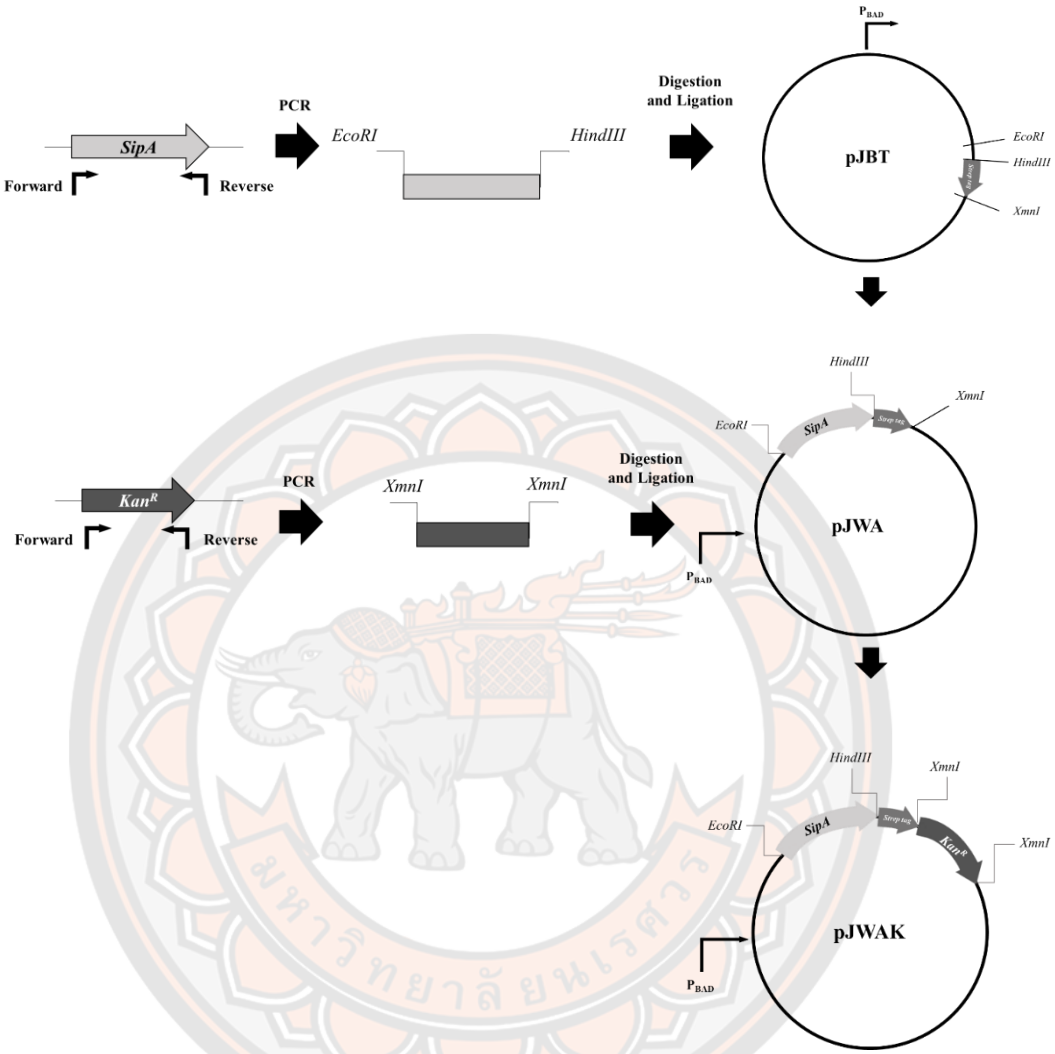


Figure 18 The schematic construction of pJWAK plasmid.
SipA and Kan^{R} genes were amplified and ligated into pJBT plasmid

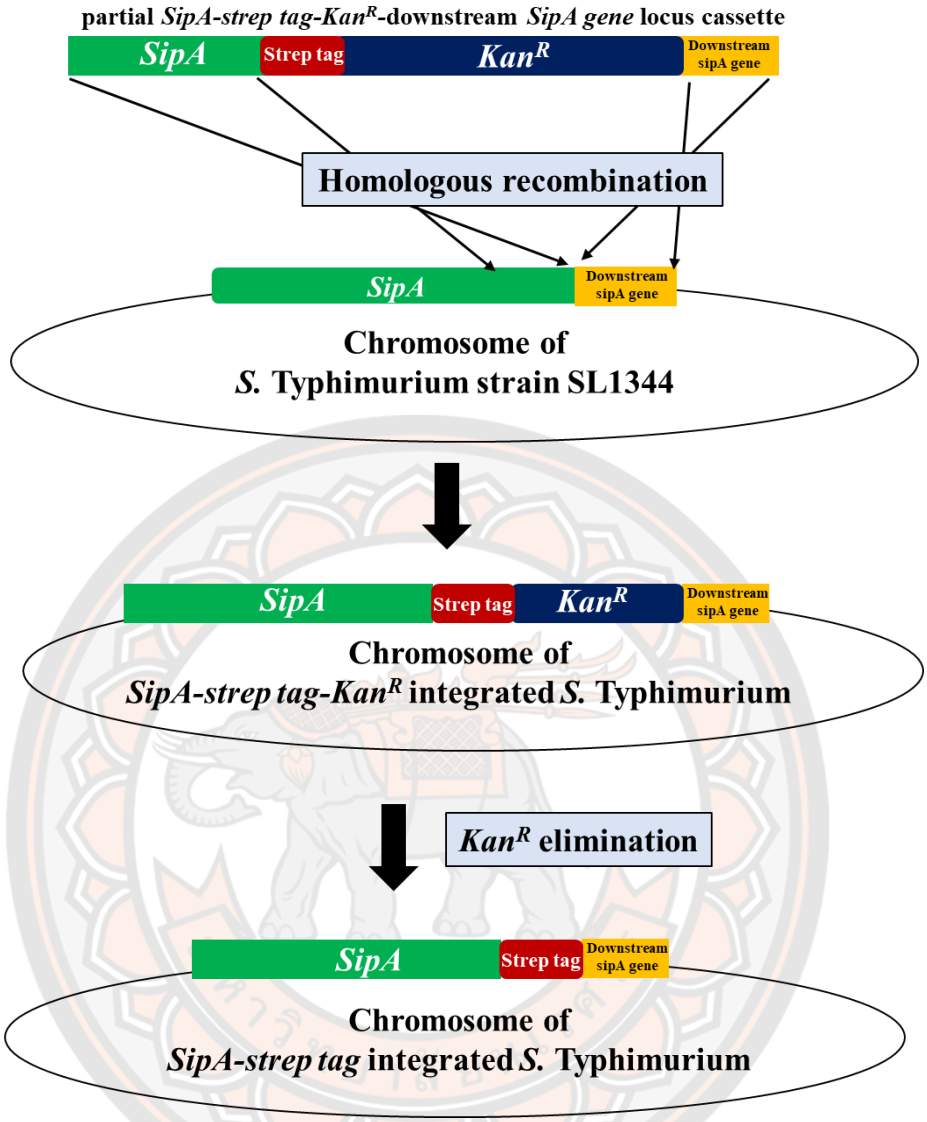


Figure 19 The schematic construction of *SipA-strep tag* integrated *S.* Typhimurium using lambda red recombination and *Kan*^{R} elimination.

Firstly, genomic DNA of *S.* Typhimurium strain SL1344 was used as a template to amplify full-*SipA* gene. The *SipA* gene was amplified by using SA primer set in PCR processing and then PCR products of the full-*SipA* gene was analyzed by agarose gel electrophoresis and visualized by Platinum D55 gel documentation. Expected band size being approximately 2,076 bp was shown in figure 20.

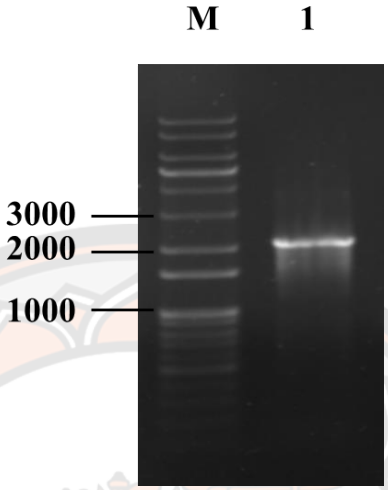


Figure 20 PCR product of full-*SipA* gene was analyzed by agarose gel electrophoresis.
Lane M : Molecular weight marker
Lane 1 : PCR product of full-*SipA* DNA fragment

The full-*SipA* gene and pJBT plasmid were digested by *EcoRI* and *HindIII* restriction enzymes and then were ligated by T4 DNA Ligase enzyme. The ligation between full-*SipA* and pJBT plasmid was transformed into *E coli* strain BL21. After that, 3 colonies after bacterial transformation were analyzed by colony PCR using CSA primer set. The result of ligation between full-*SipA* and pJBT plasmid was shown in figure 21. There was only 1 colony that had expected size being approximately 2,097 bp. This result indicated that the full-*SipA* was already ligated in pJBT plasmid resulting in pJWA plasmid. The colony containing pJWA plasmid was used to purify pJWA plasmid for next procedure.

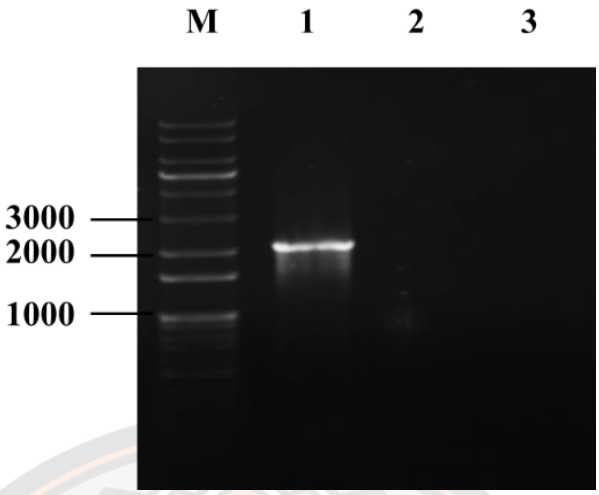


Figure 21 PCR product of full-*SipA*-strep tag was analyzed by agarose gel electrophoresis.
Lane M : Molecular weight marker
Lane 1 : PCR product of full-*SipA-strep tag* from colony 1
Lane 2 : PCR product of full-*SipA-strep tag* from colony 2
Lane 3 : PCR product of full-*SipA-strep tag* from colony 3

The pKD13 plasmid was used as a template to amplify Kan^R gene. The Kan^R gene was amplified by using KFRT primer set in PCR processing and then PCR products of the Kan^R gene was analyzed by agarose gel electrophoresis and visualized by Platinum D55 gel documentation. Expected band size being approximately 1,359 bp was shown in figure 22.

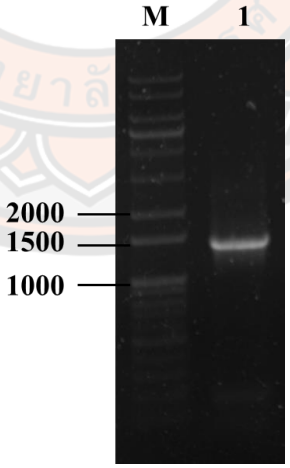


Figure 22 PCR product of Kan^R gene was analyzed by agarose gel electrophoresis.
Lane M : Molecular weight marker
Lane 1 : PCR product of Kan^R gene

The *Kan*^{R} gene and pJWA plasmid were digested by *XmnI* restriction enzyme and then were ligated by T4 DNA Ligase enzyme. The ligation between *Kan*^{R} gene and pJWA plasmid was transformed into *E coli* strain BL21. After that, 3 colonies after bacterial transformation were analyzed by colony PCR using CSAK primer set. The result of ligation between *Kan*^{R} gene and pJWA plasmid was shown in figure 23. There was only 1 colony that had expected size being approximately 1,731 bp. This result indicated that *Kan*^{R} gene was ligated in pJWA plasmid resulting in pJWAK plasmid. The colony containing pJWAK plasmid was used to purify pJWAK plasmid for next procedure.

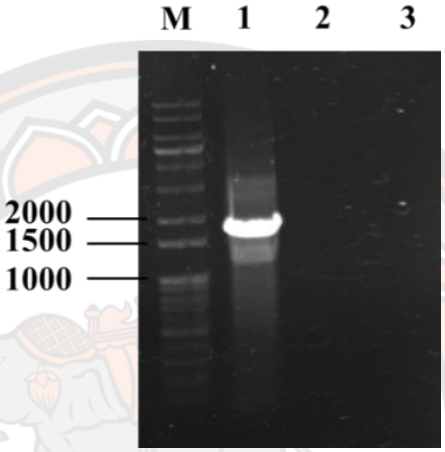
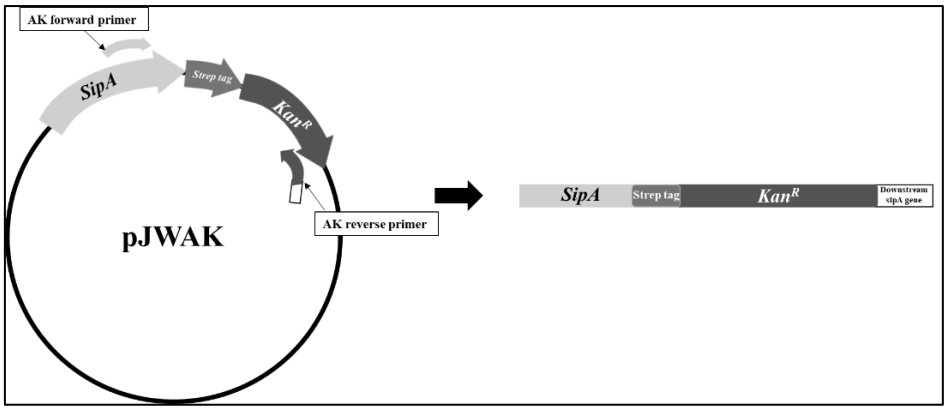


Figure 23 PCR product of partial *SipA*-strep tag-*Kan*^{R} was analyzed by agarose gel electrophoresis.
Lane M : Molecular weight marker
Lane 1 : PCR product of partial *SipA-strep tag-Kan*^{R} from colony 1
Lane 2 : PCR product of partial *SipA-strep tag-Kan*^{R} from colony 2
Lane 3 : PCR product of partial *SipA-strep tag-Kan*^{R} from colony 3

The pJWAK plasmid was used as a template to amplify partial *SipA-strep tag-Kan*^{R}-downstream *sipA* gene locus cassette. The cassette was amplified by PCR using AK primer set which shown in figure 24A. Forward primer attached to the partial *sipA* gene and reverse primer attached to the terminal *Kan*^{R} gene with tagging 40 nucleotide sequences downstream *SipA* gene locus. PCR products of the cassette was analyzed by agarose gel electrophoresis and visualized by Platinum D55 gel documentation. Expected band size being approximately 1,786 bp was shown in figure 24B.

(A)



(B)

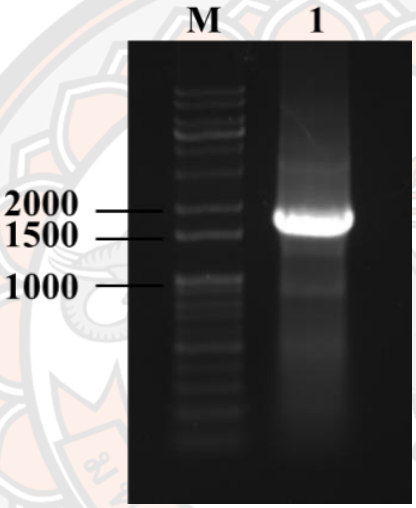


Figure 24 The attachment of the AK primer set on pJWAK plasmid and PCR product using AK primer set.
(A) The cassette was amplified by PCR using AK primer set.
(B) PCR product of partial *SipA-strep tag-Kan*^{R}-downstream *SipA* gene loci was visualized by agarose gel electrophoresis
Lane M : Molecular weight marker
Lane 1 : PCR product of partial *SipA-strep tag-Kan*^{R}-downstream *SipA* gene locus

The partial *SipA-strep tag-Kan*^{R}-downstream *sipA* gene locus cassette were electroporated into *S.* Typhimurium strain SL1344 containing pKD46 plasmid. The pKD46 exchanged the cassette and chromosome of *S.* Typhimurium strain SL1344 using homologous recombination system required λ red recombination enzyme. After exchanging the cassette and chromosome of *S.* Typhimurium, 3 colonies were analyzed the exchanging by colony PCR using CCUT primer set. The attachment of the primers was shown in figure 25A. The CCUT-forward and reverse primers attached to the partial *SipA* gene and nucleotide sequences downstream *SipA* gene locus, respectively. If the chromosome of wild type is amplified by the CCUT-forward and reverse primers, the PCR product is approximately 501 bp. On the other hand, if the chromosome of wild type is replaced by the cassette resulting in adding nucleotides from *Kan*^{R} gene, the PCR product is approximately 1876 bp. The result of

colony PCR was shown in figure 25B. The PCR product of wild type was approximately 501 bp while the PCR products of 3 colonies are approximately 1,876 bp. This result indicated that *SipA* gene of 3 colonies was replaced by partial *SipA-strep tag-Kan*^{R}-downstream *SipA* gene locus cassette. Therefore, *SipA-strep tag-Kan*^{R} integrated *S.* Typhimurium was constructed and 1 colony of them was selected for Kanamycin elimination.

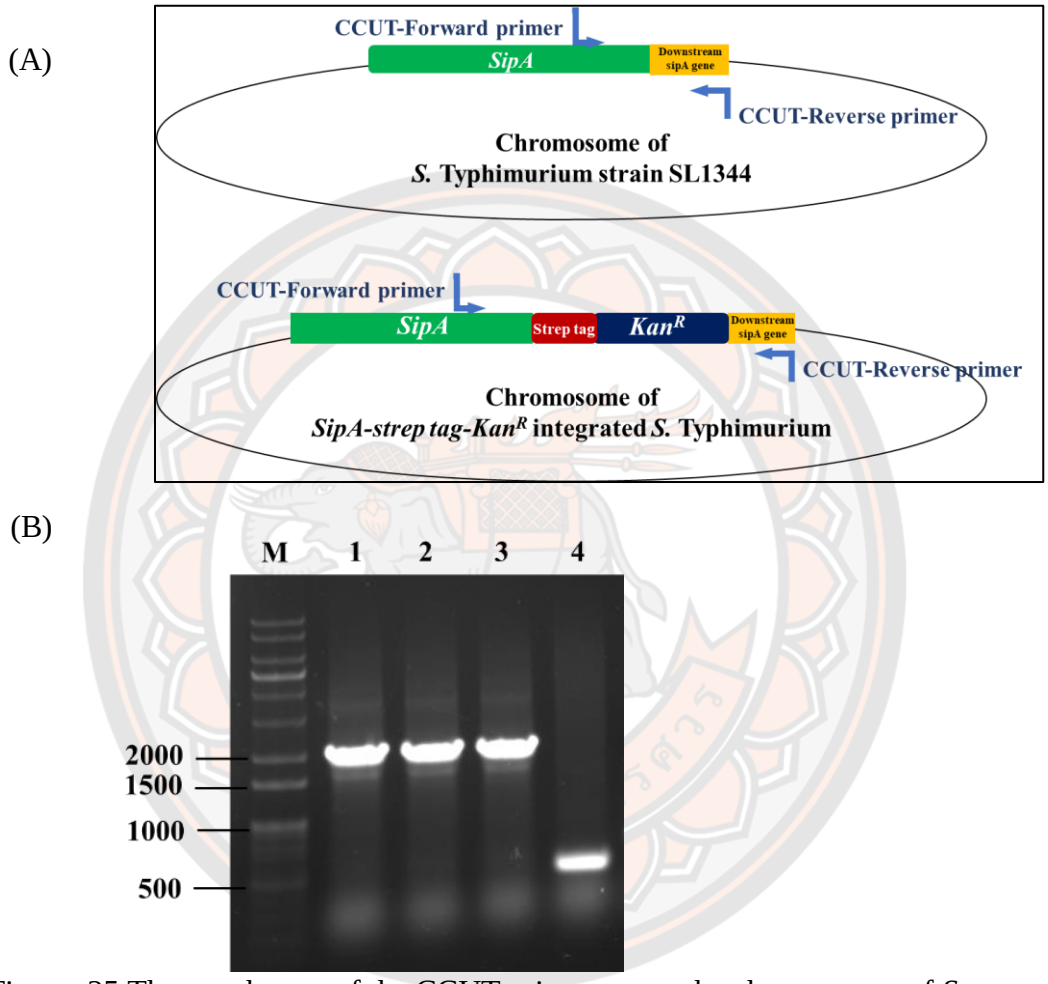
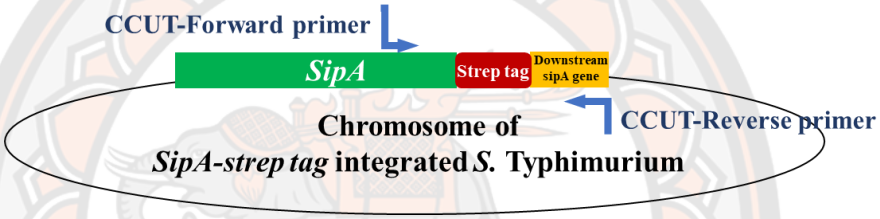


Figure 25 The attachment of the CCUT primer set on the chromosome of *S.* Typhimurium after exchanging the cassette and chromosome, and PCR product using CCUT primer set.
(A) The attachment of the CCUT primers on the chromosome of *S.* Typhimurium strain SL1344 and *SipA-strep tag-Kan*^{R} integrated *S.* Typhimurium
(B) PCR products using CCUT primer set were analyzed by agarose gel electrophoresis
Lane M : Molecular weight marker
Lane 1 : PCR product of colony 1
Lane 2 : PCR product of colony 2
Lane 3 : PCR product of colony 3
Lane 4 : PCR product of wild type

The *Kan*^{R} gene was eliminated from the chromosome of *SipA-strep tag-Kan*^{R} integrated *S.* Typhimurium by pCP20 plasmid. The pCP20 plasmid was electroporated into *SipA-strep tag-Kan*^{R} integrated *S.* Typhimurium and then eliminated *Kan*^{R} gene from chromosome of *SipA-strep tag-Kan*^{R} integrated *S.* Typhimurium. After that, 3 colonies were analyzed the *Kan*^{R} gene elimination by colony PCR using CCUT primer set and shown in figure 26A. If *Kan*^{R} gene is eliminated from the chromosome of *SipA-strep tag-Kan*^{R} integrated *S.* Typhimurium, the PCR product from the chromosome without *Kan*^{R} gene is less than 1,876 bp. The result of colony PCR was shown in figure 26B. The PCR products of 3 colonies after *Kan*^{R} gene elimination was approximately 669 bp while the PCR products of *SipA-strep tag-Kan*^{R} integrated *S.* Typhimurium and wild type were approximately 1,876 and 501 bp, respectively. This result indicated that *kan*^{R} gene of 3 colonies was eliminated by pCP20 plasmid. Therefore, *SipA-strep tag* integrated *S.* Typhimurium was constructed and called *S.* Typhimurium strain SA.

(A)



(B)

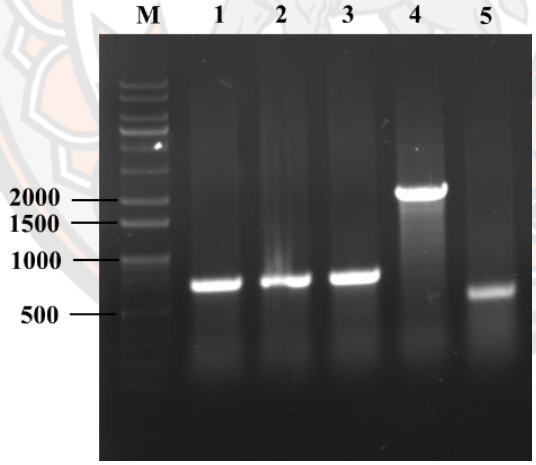


Figure 26 The attachment of the CCUT primer set on the chromosome of *S.* Typhimurium after *Kan*^{R} gene elimination, and PCR product using CCUT primer set.
(A) The attachment of the CCUT primers on the chromosome of *SipA-strep tag* integrated *S.* Typhimurium
(B) PCR product*s* using CCUT primer set *were* analyzed by agarose gel *e*lectrophoresis
Lane M : Molecular weight marker
Lane 1 : PCR product of colony 1
Lane 2 : PCR product of colony 2
Lane 3 : PCR product of colony 3
Lane 4 : PCR product of *SipA-strep tag-Kan*^{R} integrated *S.* Typhimurium
Lane 5 : PCR product of wild type

Before using *S.* Typhimurium strain SA for detecting anti-T3SS activity of Thai herbal extracts in Western blotting, secretion of SipA-strep tag protein from SPI-1 T3SS of *S.* Typhimurium strain SA was detected. The 3 colonies of *S.* Typhimurium strain SA were incubated in SPI-1 inducing condition and then proteins in supernatants were precipitated and detected secretion of SipA-strep tag protein by Western blotting with Anti-strep tag as primary antibody. The result of Western blotting was shown in figure 27. The band protein being approximately 89 kDa from all colonies were detected. This result indicated that all colonies of *S.* Typhimurium strain SA, especially colony 1, were able to secrete SipA-strep tag protein through SPI-1 T3SS into the supernatant. Therefore, *S.* Typhimurium strain SA could secrete SipA-strep tag protein through SPI-1 T3SS and the SipA-strep tag protein was detected by Western blotting. Moreover, *S.* Typhimurium strain SA was used to detect anti-T3SS activity of Thai herbal extracts for inhibition of SPI-1 T3SS.

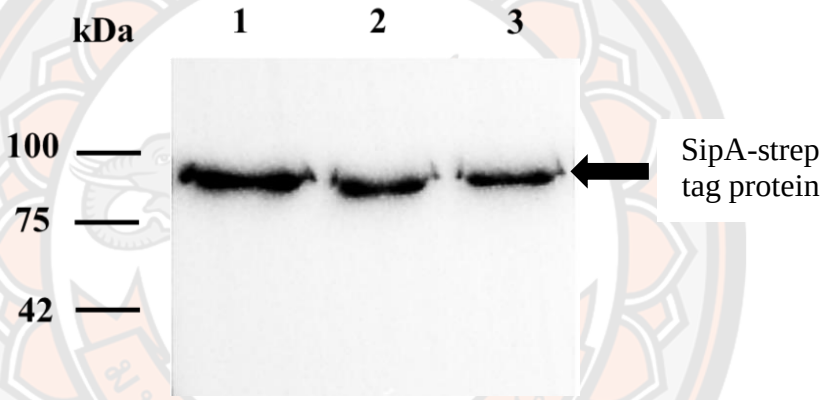


Figure 27 SipA-strep tag protein expression and secretion from *S.* Typhimurium strain SA in SPI-1 inducing condition.
Lane 1 : Supernatant fraction of colony 1
Lane 2 : Supernatant fraction of colony 2
Lane 3 : Supernatant fraction of colony 3
Arrow indicated protein expression at about 89 kDa

1.2. Detecting anti-T3SS activity of Thai herbal extracts for inhibition of SPI-1 T3SS

Thai herbal extracts including garlic, turmeric, chantaleela and king of bitters were screened the inhibition of SPI-1 T3SS of *S.* Typhimurium. If Thai herbal extracts inhibit activity of SPI-1 T3SS, SipA-strep tag protein cannot be secreted by SPI-1 T3SS and is absent in SDS-PAGE with Coomassie blue staining and Western blotting. The final concentration of Thai herbal extracts for screening was modified from study of Ma, Y.-N., *et al*. They screened the final concentration of compounds ranging from 200 µg/ml to 25 µg/ml (144). Therefore, we chose at final concentration of 100 µg/ml for screening the inhibition of SPI-1 T3SS of *S.* Typhimurium that was half of the maximum final concentration from study of Ma, Y.-N., *et al*. Briefly, overnight culture of *S.* Typhimurium strain SA was incubated in SPI-1 inducing condition with garlic, turmeric, chantaleela and king of bitters at the final

concentration of 100 µg/ml for 4 hours. The DMSO at final concentration of 0.001% (v/v) was used as negative control. The SipA-strep tag protein in supernatant of all treatments was collected and detected by SDS-PAGE with Coomassie blue staining and Western blotting. The results of SDS-PAGE with Coomassie blue staining and Western blotting were shown in figure 28 and figure 29, respectively. The SipA-strep tag band protein was at 89 kDa. The SipA-strep tag band protein from treatment with garlic or turmeric at the final concentration of 100 µg/ml was absent in SDS-PAGE with Coomassie blue staining and Western blotting. While, the SipA-strep tag band protein from treatment with chantaleela at the final concentration of 100 µg/ml was slightly detected in SDS-PAGE with Coomassie blue staining and Western blotting. However, the SipA-strep tag band protein from treatment with king of bitters at the final concentration of 100 µg/ml was completely appeared in SDS-PAGE with Coomassie blue staining and Western blotting and similar to negative control. Therefore, treatment of garlic or turmeric at the final concentration of 100 µg/ml was able to inhibit secretion of SipA-strep tag protein from SPI-1 T3SS indicating that garlic and turmeric had anti-T3SS activity. Furthermore, treatment of chantaleela at a final concentration of 100 µg/ml slightly decreased secretion of SipA-strep tag protein from SPI-1 T3SS which indicating that chantaleela had anti-T3SS activity and should be added more concentration for complete inhibition of SPI-1 T3SS. On the other hand, treatment of king of bitters at a final concentration of 100 µg/ml did not reduce secretion of the SipA-strep tag band protein from SPI-1 T3SS indicating that king of bitters did not have anti-T3SS activity.

Therefore, only 3 Thai herbal extracts including garlic, turmeric and chantaleela were able to decrease secretion of SipA-strep tag protein from SPI-1 T3SS and then were determined a minimal concentration for inhibition of SPI-1 T3SS as T3SS inhibitors.

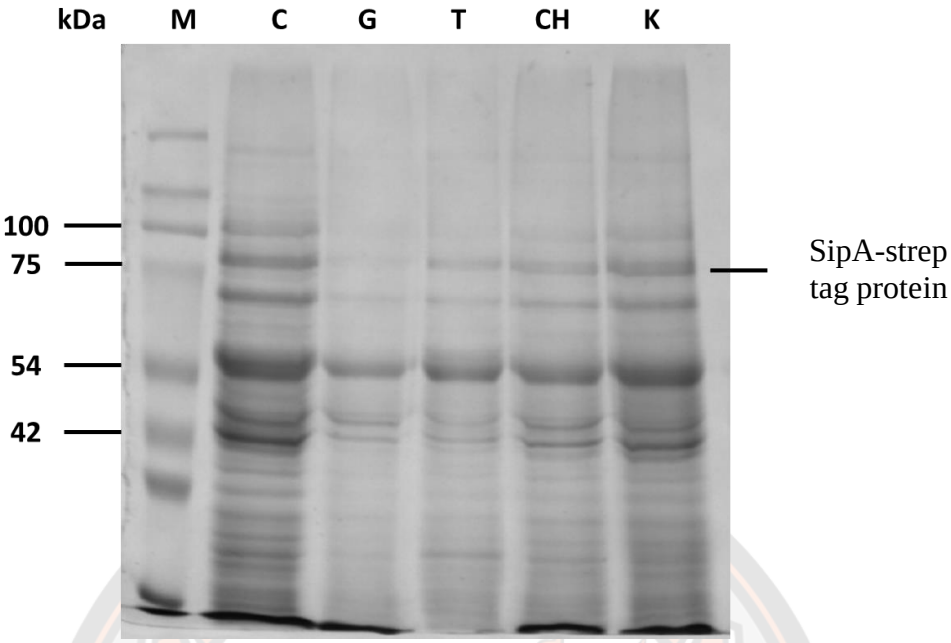


Figure 28 Detection of secreting SipA-strep tag protein from *S*. Typhimurium strain SA after treatment with garlic, turmeric, chantaleela and king of bitter at a final concentration of 100 µg/ml by SDS-PAGE with Coomassie blue staining.
Lane M : Molecular weight marker
Lane C : Treatment with DMSO as negative control
Lane G : Treatment with garlic
Lane T : Treatment with turmeric
Lane CH : Treatment with chantaleela
Lane K : Treatment with king of bitter
Black line indicated SipA-strep tag protein expression at about 89 kDa

Figure 29 Detection of secreting SipA-strep tag protein from *S*. Typhimurium strain SA after treatment with garlic, turmeric, chantaleela and king of bitter at a final concentration 100 µg/ml by Western blotting.
Lane C : Treatment with DMSO as negative control
Lane G : Treatment with garlic
Lane T : Treatment with turmeric
Lane CH : Treatment with chantaleela
Lane K : Treatment with king of bitter
Arrow indicated SipA-strep tag protein expression at about 89 kDa

2. Determining a minimal concentration of Thai herbal extracts for inhibition of SPI-1 T3SS

After screening garlic, turmeric and chantaleela at the final concentration of 100 µg/ml, the minimal concentration of garlic, turmeric and chantaleela for the inhibition of SPI-1 T3SS were detected by SDS-PAGE with Coomassie blue staining and Western blotting.

Treatment of *S.* Typhimurium with garlic at a final concentration of 100 µg/ml resulted in absence of the SipA-strep tag protein in SDS-PAGE with Coomassie blue staining and Western blotting. To determine a minimal concentration of garlic for the inhibition of SPI-1 T3SS, garlic was varied the final concentration of 100, 75, 50 and 25 µg/ml, respectively. Therefore, the overnight culture of *S.* Typhimurium strain SA was incubated with garlic at the various final concentrations for 4 hours and DMSO was used as negative control. After that, the SipA-strep tag protein in supernatant of all treatments was collected and detected by SDS-PAGE with Coomassie blue staining and Western blotting. The results of SDS-PAGE with Coomassie blue staining and Western blotting were shown in figure 30 and figure 31, respectively, and the SipA-strep tag band protein was at 89 kDa. The SipA-strep tag band protein from treatment with garlic at the final concentration of 75, 50 and 25 µg/ml was detected by SDS-PAGE with Coomassie blue staining and Western blotting. On the other hand, the SipA-strep tag band protein from treatment with garlic at a final concentration of 100 µg/ml was absent in SDS-PAGE with Coomassie blue staining and Western blotting. Therefore, the garlic at final concentration of 100 µg/ml was the minimal concentration for inhibiting secretion of SipA-strep tag protein from SPI-1 T3SS of *S.* Typhimurium strain SA. Moreover, the result showed that inhibition of SipA-strep tag band protein in higher concentration of garlic was better than the lower concentration. This demonstrated that inhibition of the SipA-strep tag band protein depends on the concentration of the garlic and consequently the garlic inhibited secretion of SipA-strep tag protein from SPI-1 T3SS of *S.* Typhimurium in a dose-dependent manner.

Additionally, flagellar protein which involve in construction of bacterial flagellum for motility was affected by treatment with garlic. The result of SDS-PAGE with Coomassie blue staining showed that FliC band protein at 55 kDa which is flagellar filament (flagellin) were decreased by the garlic at the concentration of 100 and 75 µg/ml when compared to negative control. This indicated that garlic had influence on not only SPI-1 T3SS but also the flagella of the bacteria.

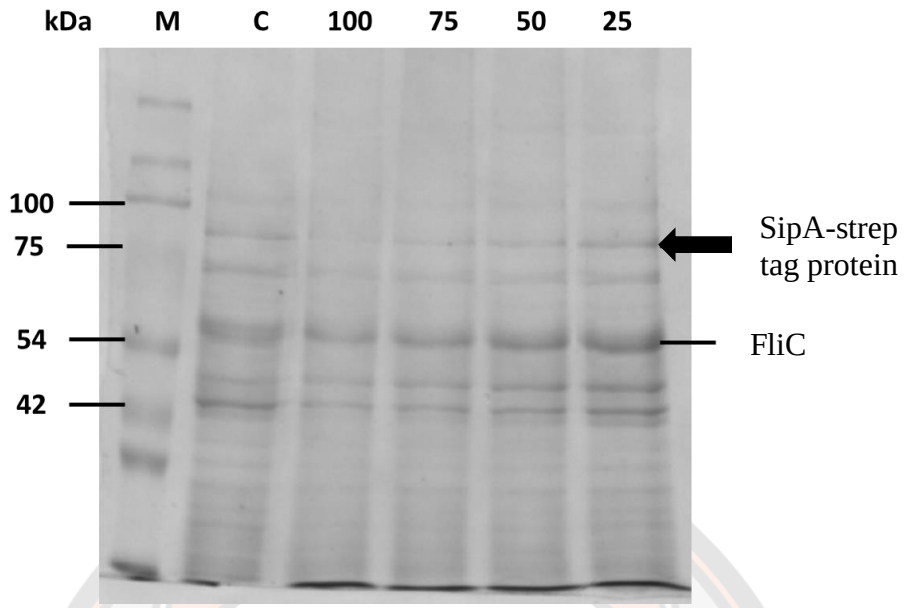


Figure 30 Detection of secreting SipA-strep tag protein from *S*. Typhimurium strain SA after treatment with garlic at various concentration by SDS-PAGE with Coomassie blue staining.
Lane M : Molecular weight marker
Lane C : Treatment with DMSO as negative control
Lane 100 : Treatment with garlic at a final concentration of 100 µg/ml
Lane 75 : Treatment with garlic at a final concentration of 75 µg/ml
Lane 50 : Treatment with garlic at a final concentration of 50 µg/ml
Lane 25 : Treatment with garlic at a final concentration of 25 µg/ml
Arrow indicated SipA-strep tag protein expression at about 89 kDa
Black line indicated FliC protein expression at about 55 kDa

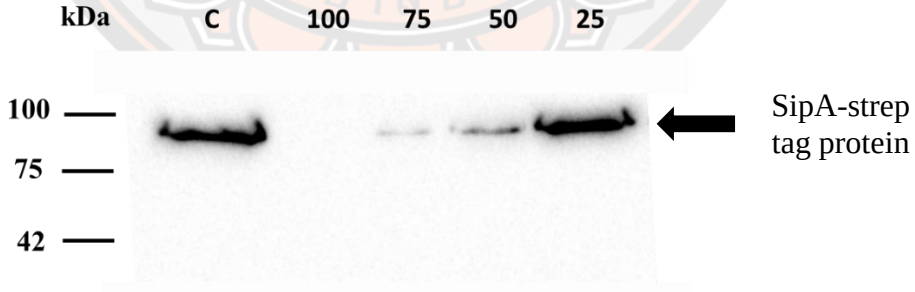


Figure 31 Detection of secreting SipA-strep tag protein from *S*. Typhimurium strain SA after treatment with garlic at various concentration by Western blotting.
Lane C : Treatment of DMSO as negative control
Lane 100 : Treatment with garlic at a final concentration of 100 µg/ml
Lane 75 : Treatment with garlic at a final concentration of 75 µg/ml
Lane 50 : Treatment with garlic at a final concentration of 50 µg/ml
Lane 25: Treatment with garlic at a final concentration of 25 µg/ml
Arrow indicated SipA-strep tag protein expression at about 89 kDa

To determine a minimal concentration of turmeric for the inhibition of SPI-1 T3SS, treatment of *S.* Typhimurium with turmeric at a final concentration of 100 µg/ml resulted in absence of the SipA-strep tag protein in SDS-PAGE with Coomassie blue staining and Western blotting. Therefore, turmeric for determination was varied the final concentration of 100, 75, 50 and 25 µg/ml, respectively. The overnight culture of *S.* Typhimurium strain SA was incubated with turmeric at the various final concentrations or DMSO as negative control for 4 hours. After that, the SipA-strep tag protein in supernatant of all treatments was collected and detected by SDS-PAGE with Coomassie blue staining and Western blotting. The results of SDS-PAGE with Coomassie blue staining and Western blotting were shown in figure 32 and figure 33, respectively, and the SipA-strep tag band protein was at 89 kDa. The SipA-strep tag band protein from treatment with turmeric at a final concentration of 25 µg/ml was detected by SDS-PAGE with Coomassie blue staining and Western blotting. While, treatment with turmeric at a final concentration of 50 µg/ml was slightly detected after 150s exposure by Western blotting. On the other hand, the SipA-strep tag band protein from treatment with turmeric at the final concentration of 100 and 75 µg/ml disappeared in SDS-PAGE with Coomassie blue staining and Western blotting. Therefore, the minimal concentration of turmeric for inhibiting secretion of SipA-strep tag protein from SPI-1 T3SS of *S.* Typhimurium strain SA was 75 µg/ml. Moreover, the result demonstrated that inhibition of the SipA-strep tag band protein depends on the concentration of the turmeric. It summarized that the turmeric inhibited secretion of SipA-strep tag protein from SPI-1 T3SS of *S.* Typhimurium in a dose-dependent manner.

For flagellar protein, it had no an evident effect from treatment of turmeric. The result of SDS-PAGE with Coomassie blue staining showed that FliC band proteins of all concentrations of turmeric were similar to negative control. It showed that turmeric had influence on only SPI-1 T3SS.

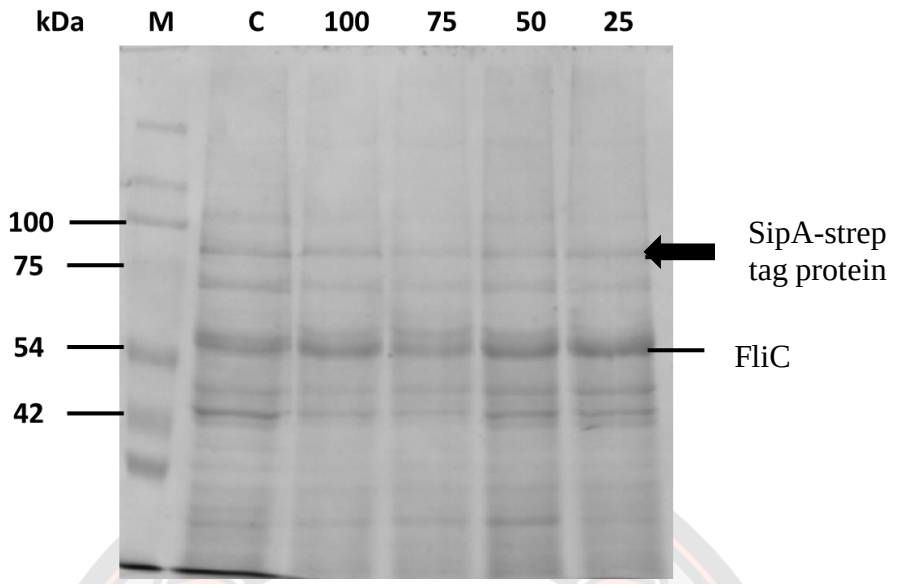


Figure 32 Detection of secreting SipA-strep tag protein from *S.* Typhimurium strain SA after treatment with turmeric at various concentration by SDS-PAGE with Coomassie blue staining.
Lane M : Molecular weight marker
Lane C : Treatment with DMSO as negative control
Lane 100 : Treatment with turmeric at a final concentration of 100 µg/ml
Lane 75 : Treatment with turmeric at a final concentration of 75 µg/ml
Lane 50 : Treatment with turmeric at a final concentration of 50 µg/ml
Lane 25 : Treatment with turmeric at a final concentration of 25 µg/ml
Arrow indicated SipA-strep tag protein expression at about 89 kDa
Black line indicated FliC protein expression at about 55 kDa

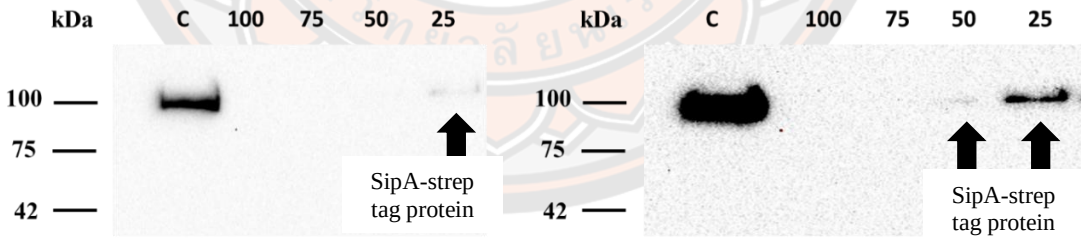


Figure 33 Detection of secreting SipA-strep tag protein from *S.* Typhimurium strain SA after treatment with turmeric at various concentration by Western blotting.
Left figure : Result after 30s exposure
Right figure : Result after 150s exposure
Lane C : Treatment of DMSO as negative control
Lane 100 : Treatment with turmeric at a final concentration of 100 µg/ml
Lane 75 : Treatment with turmeric at a final concentration of 75 µg/ml
Lane 50 : Treatment with turmeric at a final concentration of 50 µg/ml
Lane 25 : Treatment with turmeric at a final concentration of 25 µg/ml
Arrow indicated SipA-strep tag protein expression at about 89 kDa

Because the SipA-strep tag protein in SDS-PAGE with Coomassie blue staining and Western blotting slightly appeared after treatment of *S.* Typhimurium with chantaleela at a final concentration of 100 µg/ml. Therefore, chantaleela was varied the final concentration of 200, 175, 150, 125 and 100 µg/ml, respectively, to determine a minimal concentration of chantaleela for the inhibition of SPI-1 T3SS. The overnight culture of *S.* Typhimurium strain SA was incubated with chantaleela at the various final concentrations or DMSO as negative control for 4 hours. After that, the SipA-strep tag protein in supernatant of all treatments was collected and detected by SDS-PAGE with Coomassie blue staining and Western blotting. The results of SDS-PAGE with Coomassie blue staining and Western blotting were shown in figure 34 and figure 35, respectively, and the SipA-strep tag band protein was at 89 kDa. Although the result of SDS-PAGE with Coomassie blue staining showed that SipA-strep tag band protein was detected all concentrations of chantaleela, the result of Western blotting was different from SDS-PAGE with Coomassie blue staining and then used to summarize the minimal concentration of chataleela. It showed that the SipA-strep tag band protein from treatment with chantaleela at the final concentration of 125 and 100 µg/ml was detected by Western blotting. However, the SipA-strep tag band protein disappeared in treatment with chantaleela at the final concentration of 200, 175 and 150 µg/ml. Therefore, the minimal concentration of chantaleela was 150 µg/ml for inhibiting secretion of SipA-strep tag protein in SPI-1 T3SS of *S.* Typhimurium. Moreover, inhibition of SipA-strep tag band protein depended on the concentration of the chantaleela indicating that chataleela inhibited secretion of SipA-strep tag protein from SPI-1 T3SS of *S.* Typhimurium in a dose-dependent manner.

For flagellar protein, there was no effect in the treatment of chantaleela. The result of SDS-PAGE with Coomassie blue staining showed that FliC band proteins of all concentrations of turmeric were similar to negative control. It showed that chantaleela had influence on only SPI-1 T3SS.

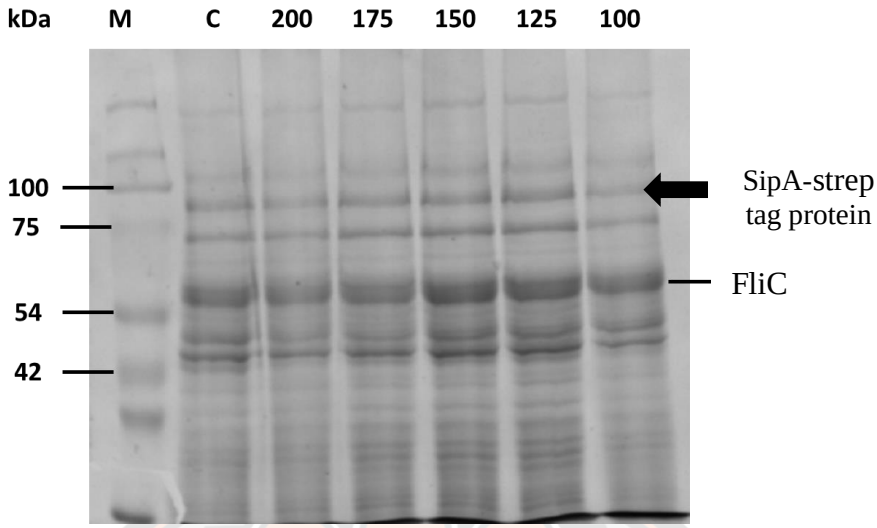


Figure 34 Detection of secreting SipA-strep tag protein from *S.* Typhimurium strain SA after treatment with chantaleela at various concentration by SDS-PAGE with Coomassie blue staining
Lane M : Molecular weight marker
Lane C : Treatment with DMSO as negative control
Lane 200 : Treatment with chantaleela at a final concentration of 200 µg/ml
Lane 175 : Treatment with chantaleela at a final concentration of 175 µg/ml
Lane 150 : Treatment with chantaleela at a final concentration of 150 µg/ml
Lane 125 : Treatment with chantaleela at a final concentration of 125 µg/ml
Lane 100 : Treatment with chantaleela at a final concentration of 100 µg/ml
Arrow indicated SipA-strep tag protein expression at about 89 kDa
Black line indicated FliC protein expression at about 55 kDa

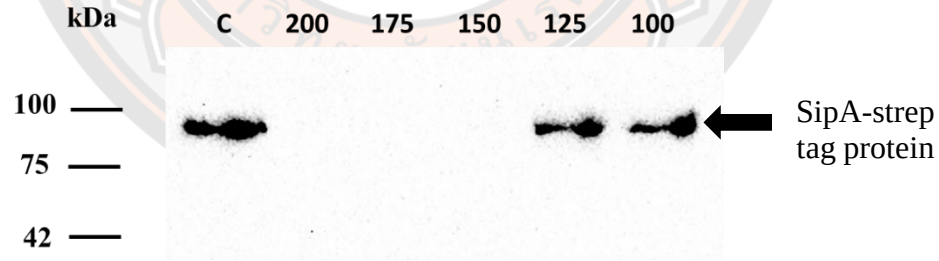


Figure 35 Detection of secreting SipA-strep tag protein from *S.* Typhimurium strain SA after treatment with chantaleela at various concentration by Western blotting
Lane C : Treatment with DMSO as negative control
Lane 200 : Treatment with chantaleela at a final concentration of 200 µg/ml
Lane 175 : Treatment with chantaleela at a final concentration of 175 µg/ml
Lane 150 : Treatment with chantaleela at a final concentration of 150 µg/ml
Lane 125 : Treatment with chantaleela at a final concentration of 125 µg/ml
Lane 100 : Treatment with chantaleela at a final concentration of 100 µg/ml
Arrow indicated SipA-strep tag protein expression at about 89 kDa

Therefore, the results of SDS-PAGE with Coomassie blue staining and Western blotting demonstrated that garlic, turmeric and chantaleela inhibit the secretion of SipA-strep tag protein in a dose-dependent manner. Moreover, the minimal concentrations of garlic, turmeric and chantaleela for inhibition of SPI-1 T3SS in *S.* Typhimurium are 100 µg/ml, 75 µg/ml and 150 µg/ml, respectively. However, the only garlic has influence on the flagella of the bacteria.

3. Evaluating antibacterial activity of Thai herbal extracts against *S.* Typhimurium

This experiment used time-kill kinetic assay to detect absence of SipA-strep tag protein in supernatant whether Thai herbal extracts including garlic, turmeric and chantaleela at minimal concentration inhibited activity of SPI-1 T3SS for secretion of the protein or killed the *S.* Typhimurium.

To evaluate antibacterial activity of garlic against *S.* Typhimurium, the overnight culture of *S.* Typhimurium strain SL1344 was treated with garlic at a final concentration 100 µg/ml or DMSO as negative control. The bacterial cultures were incubated and measured bacterial growth every hour for 24 hours by using SpectraMax iD3 Multi-Mode Microplate Reader. The result of bacterial growth curve was showed in figure 36. The bacterial growth of treatment with garlic was not different when compared to the DMSO condition. It demonstrated that garlic at minimal concentration of 100 µg/ml certainly inhibited secretion of the SipA-strep tag protein from SPI-1 T3SS of *S.* Typhimurium instead of killing *S.* Typhimurium.

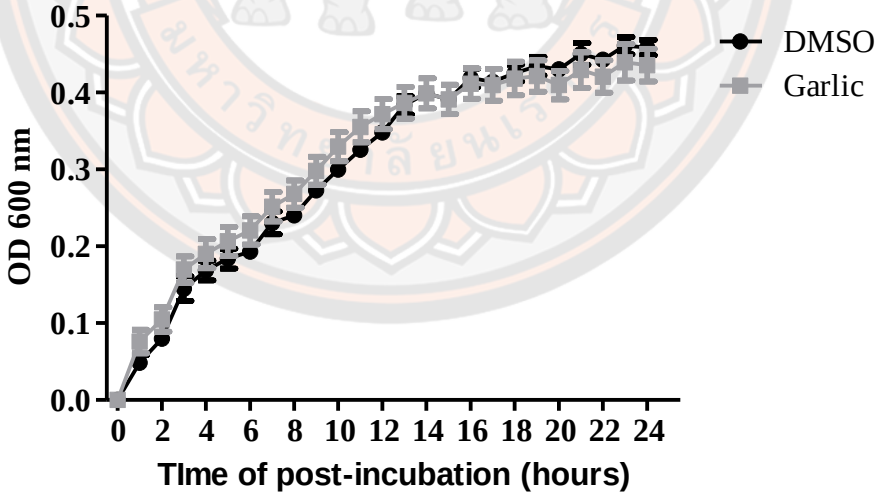


Figure 36 Bacterial growth curve of *S.* Typhimurium SL1344 when treated with garlic and DMSO.
Black line : Bacterial growth curve which treated with DMSO
Gray line : Bacterial growth curve which treated with garlic
The samples were performed in triplicate and showed in the standard error of the mean (SEM).

To evaluate antibacterial activity of turmeric against *S.* Typhimurium, the overnight culture of *S.* Typhimurium strain SL1344 was incubated with turmeric at a final concentration 75 µg/ml and DMSO as negative control for 24 hours. The bacterial growth was measured every hour. The bacterial growth curve of treatment with turmeric showed in figure 37 that was similar to negative control. This result demonstrated that turmeric at minimal concentration of 75 µg/ml certainly inhibited secretion of the SipA-strep tag protein from SPI-1 T3SS of *S.* Typhimurium instead of killing *S.* Typhimurium.

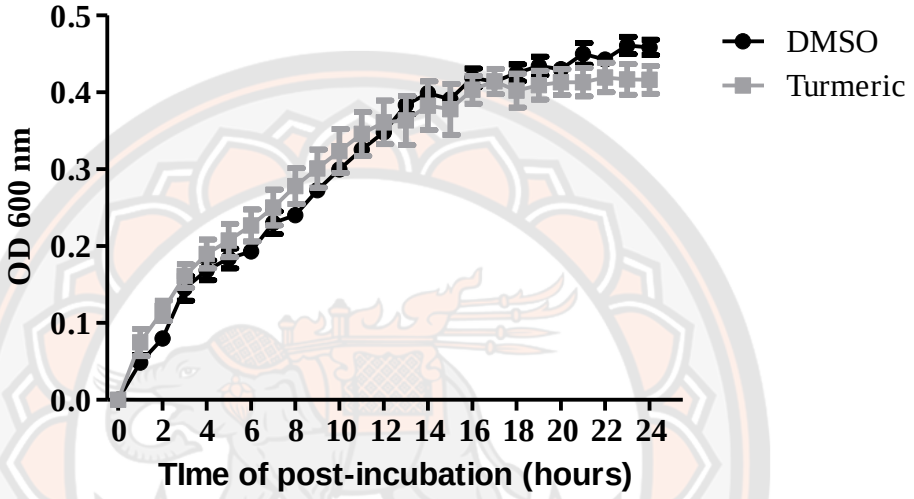


Figure 37 Bacterial growth curve of *S.* Typhimurium strain SL1344 when treated with turmeric and DMSO.
Black line : Bacterial growth curve which treated with DMSO
Gray line : Bacterial growth curve which treated with turmeric
The samples were performed in triplicate and showed in the standard error of the mean (SEM)

Finally, the overnight culture of *S.* Typhimurium strain SL1344 was treated with chantaleela at a final concentration 150 µg/ml and DMSO as negative control for evaluating antibacterial activity of turmeric against *S.* Typhimurium. The bacterial growth was measured every hour for 24 hours. The result of bacterial growth curve was showed in figure 38. The bacterial growth of treatment with chantaleela was as same as the DMSO condition. This demonstrated that chantaleela at minimal concentration of 150 µg/ml certainly inhibited secretion of the SipA-strep tag protein from SPI-1 T3SS of *S.* Typhimurium instead of killing *S.* Typhimurium.

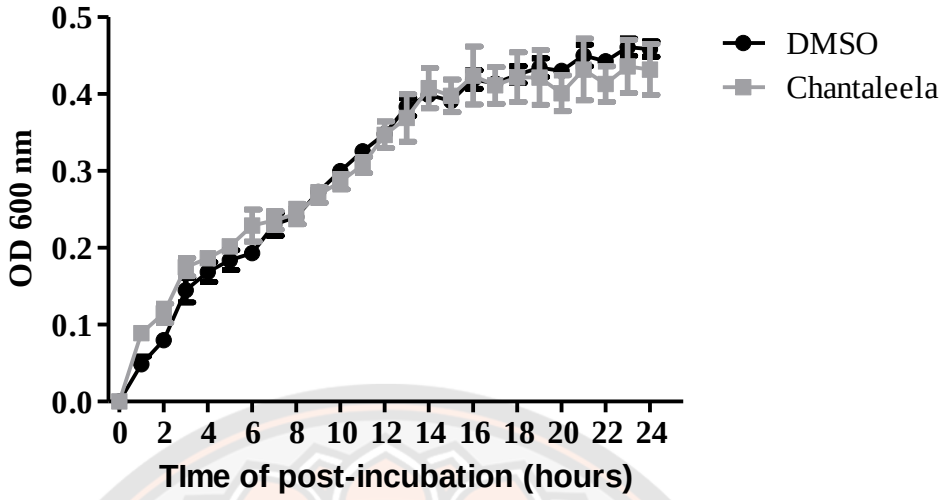


Figure 38 Bacterial growth curve of *S.* Typhimurium strain SL1344 when treated with chantaleela and DMSO.
Black line : Bacterial growth curve which treated with DMSO
Gray line : Bacterial growth curve which treated with chantaleela
The samples were performed in triplicate and showed in the standard error of the mean (SEM).

Therefore, the results of time-kill kinetic assay demonstrated that garlic, turmeric and chantaleela at the minimal concentration definitely inhibit the SPI-1 T3SS of *S.* Typhimurium resulting in reduction of secreting the SipA-strep tag protein from SPI-1 T3SS. However, garlic, turmeric and chantaleela at the minimal concentration do not kill *S.* Typhimurium.

4. Measuring cytotoxicity of Thai herbal extracts to Hela cells

Because the bacterial culture containing the Thai herbal extracts was directly added in the Hela cell during processing of the invasion assay. Therefore, the cytotoxicity of the Thai herbal extracts to the Hela cell was measured by MTT assay. This experiment assured that garlic, turmeric and chantaleela at minimal concentration did not affect the Hela cell resulting in interfering the results of invasion assay.

To measure cytotoxicity of garlic to Hela cells, the Hela cells at 5,000 cells were incubated with garlic at a final concentration 100 µg/ml or DMSO as negative control for 24 hours. After that, cell viability was measured by MTT assay and the percentage of cell viability was calculated. The result of the percentage of cell viability was showed in figure 39. The percentage of cell viability from treatment with DMSO was 94.96 while the percentage of cell viability from treatment of garlic was 94.08. The percentage of cell viability of the treating garlic was not significantly different when compared to the DMSO condition. This result demonstrated that garlic at minimal concentration of 100 µg/ml did not affect cell viability of Hela cells for 24 hours. Moreover, garlic could not interfere the result of invasion assay.

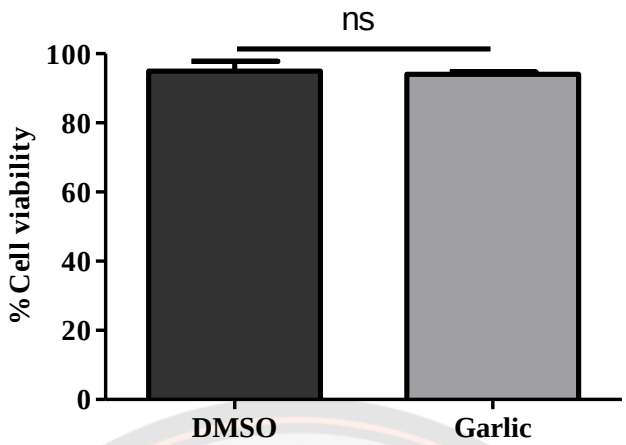


Figure 39 Percentage of cell viability treated with garlic and DMSO.
Black column : Percentage of cell viability which treated with DMSO
Gray column : Percentage of cell viability which treated with garlic
The samples were performed in triplicate and showed in the standard error of the mean (SEM). NS: not significantly.

To measure cytotoxicity of turmeric to Hela cells, the Hela cells at 5,000 cells were incubated with turmeric at a final concentration 75 µg/ml for 24 hours. After that, cell viability was measured by MTT assay and the percentage of cell viability were calculated. The result of the percentage of cell viability was showed in figure 40. The percentage of cell viability from treatment with turmeric was 93.86 which was not significantly different when compared to the DMSO condition. This result demonstrated that turmeric at minimal concentration of 75 µg/ml did not affect cell viability of Hela cells for 24 hours. Moreover, turmeric could not interfere the result of invasion assay.

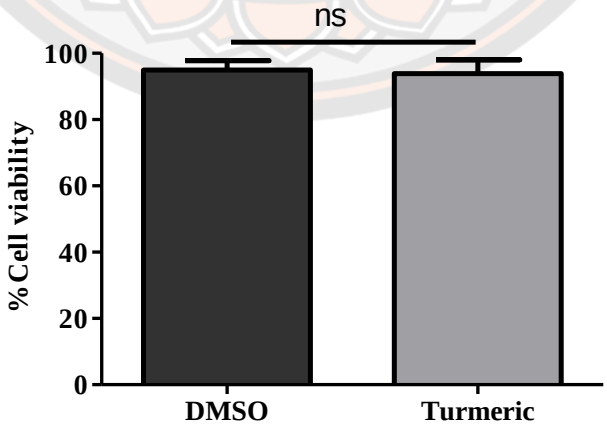


Figure 40 Percentage of cell viability treated with turmeric and DMSO.
Black column : Percentage of cell viability which treated with DMSO
Gray column : Percentage of cell viability which treated with turmeric
The samples were performed in triplicate and showed in the standard error of the mean (SEM). NS: not significantly.

To measure cytotoxicity of chantaleela to Hela cells, the Hela cells at 5,000 cells were incubated with chantaleela at a final concentration 150 µg/ml for 24 hours. After that, cell viability was measured by MTT assay and the percentage of cell viability were calculated. The result of the percentage of cell viability was showed in figure 41. The percentage of cell viability from treatment with chantaleela was 89.91. It was not significantly different when compared to the DMSO condition. It demonstrated that chantaleela at minimal concentration of 150 µg/ml did not affect cell viability of Hela cells for 24 hours. Moreover, chantaleela could not interfere the result of invasion assay.

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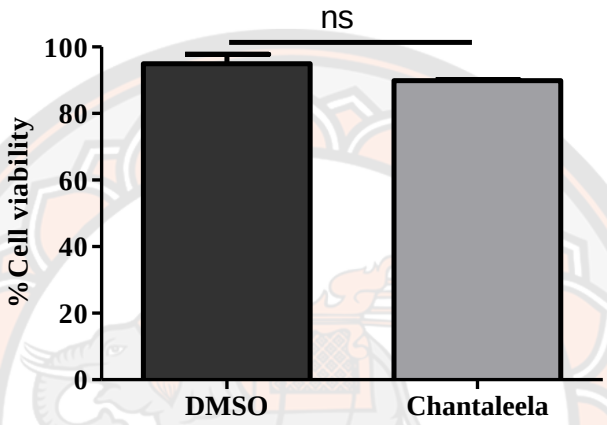

Figure 41 Percentage of cell viability treated with chantaleela and DMSO.
Black column : Percentage of cell viability which treated with DMSO
Gray column : Percentage of cell viability which treated with chantaleela
The samples were performed in triplicate and showed in the standard error of the mean (SEM). NS: not significantly.

Therefore, garlic, turmeric and chantaleela at the minimal concentration do not affect cell viability of Hela cells. Moreover, the 3 herbal extracts cannot interfere the results of invasion assay

5. Detection of Thai herbal extracts for inhibiting bacterial invasion of *S*. Typhimurium

This experiment detected ability of garlic, turmeric and chantaleela for inhibiting bacterial invasion of *S*. Typhimurium by using invasion assay. If the 3 Thai herbal extracts inhibit activity of SPI-1 T3SS of *S*. Typhimurium, *S*. Typhimurium cannot invade the Hela cells resulting in decreasing the number of *S*. Typhimurium inside the cell.

To detect garlic for inhibition of bacterial invasion, the overnight culture of *S.* Typhimurium strain SL1344 was incubated with the garlic at a final concentration of 100 µg/ml or DMSO as negative control for 4 hours. While, *S.* Typhimurium strain Δ*invA* was used as positive control. Then, the Hela cells were infected by *S.* Typhimurium strain Δ*invA* and *S.* Typhimurium treated with garlic or DMSO for 30 min. After that, extracellular bacteria were killed by gentamycin and finally the intracellular bacteria in the Hela cells were measured. The result of invasion assay was showed in figure 42. The number of *S.* Typhimurium strain Δ*invA* as positive control significantly decreased when compared to the DMSO condition. The result of intracellular bacteria from treatment with garlic was similar to the positive control which significantly decreased when compared to the DMSO condition. This result demonstrated that the garlic at a minimal concentration of 100 µg/ml was able to inhibit bacterial invasion of *S.* Typhimurium. Moreover, the result of invasion assay correlated with the result of Western blotting that garlic at minimal concentration of 100 µg/ml could inhibit activity of SPI-1 T3SS for secretion of effector proteins resulting in decreasing bacterial invasion of *S.* Typhimurium. Therefore, garlic was certainly the T3SS inhibitor.

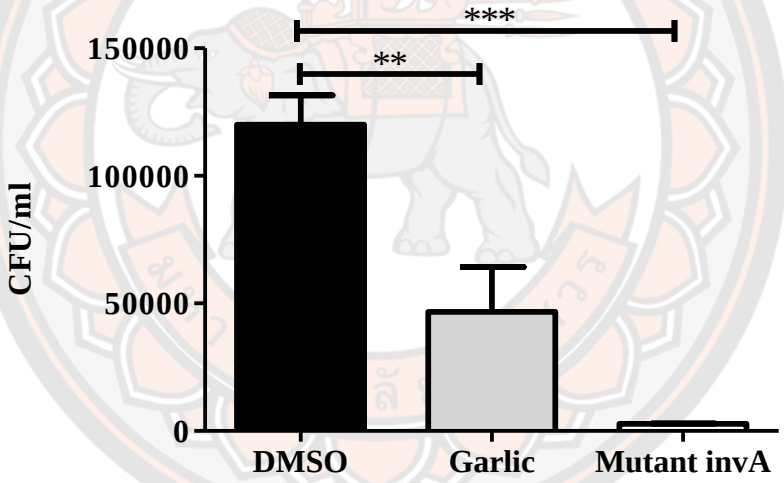

Figure 42 The number of *S.* Typhimurium strain SL1344 treated with garlic and DMSO as negative control.
Black column : The number of *S.* Typhimurium SL1344 treated with DMSO
Gray column : The number of *S.* Typhimurium SL1344 treated with garlic
White column : The number of *S.* Typhimurium strain Δ*invA*
The number of *S.* Typhimurium strain Δ*invA* as positive control. The samples were performed in triplicate and showed in the standard error of the mean (SEM). The P value was compared and calculated between the samples and DMSO as negative control which levels of statistical significance was showed as: **, $P \leq 0.01$; ***, $P \leq 0.001$.

To detect turmeric for inhibition of bacterial invasion, the overnight culture of *S.* Typhimurium strain SL1344 was incubated with the turmeric at a final concentration of 75 µg/ml for 4 hours. Then, *S.* Typhimurium strain Δ*invA* and *S.* Typhimurium treated with turmeric or DMSO infected the Hela cells for 30 min. After that, the intracellular bacteria in Hela cells were measured. The result of invasion assay was showed in figure 43. The number of intracellular bacteria from treatment with turmeric significantly decreased compared to the DMSO condition which was similar to positive control. This result demonstrated that the turmeric at a minimal concentration of 75 µg/ml could reduce bacterial invasion of *S.* Typhimurium. Moreover, the result of invasion assay correlated with the result of Western blotting that turmeric at minimal concentration of 75 µg/ml could inhibit activity of SPI-1 T3SS for secretion of effector proteins resulting in decreasing bacterial invasion of *S.* Typhimurium. Therefore, turmeric was certainly the T3SS inhibitor.

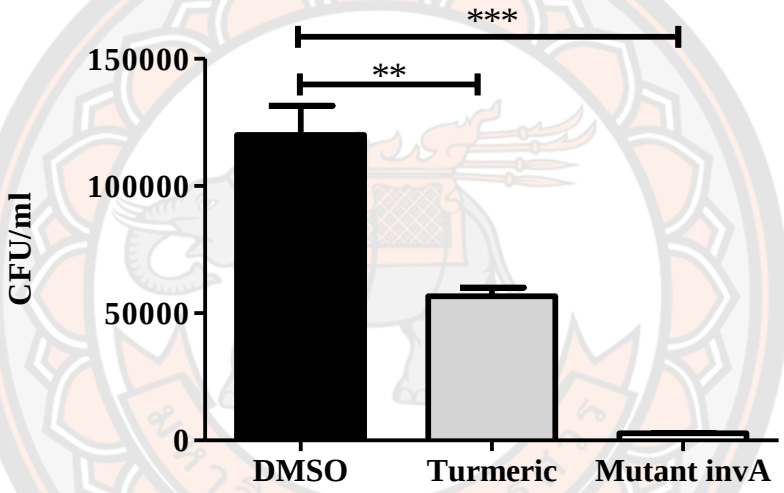

Figure 43 The quantity of *S.* Typhimurium strain SL1344 treated with turmeric and DMSO as negative control
Black column : The number of *S.* Typhimurium SL1344 treated with DMSO
Gray column : The number of *S.* Typhimurium SL1344 treated with turmeric
White column : The number of *S.* Typhimurium strain Δ*invA*
The number of *S.* Typhimurium strain Δ*invA* as positive control. The samples were performed in triplicate and showed in the standard error of the mean (SEM). The P value was compared and calculated between the samples and DMSO as negative control which levels of statistical significance was showed as: **, $P \leq 0.01$; ***, $P \leq 0.001$.

To detect chantaleela for inhibition of bacterial invasion, the overnight culture of *S.* Typhimurium strain SL1344 was incubated with the Chantaleela at a final concentration of 150 µg/ml for 4 hours. *S.* Typhimurium strain Δ*invA* and *S.* Typhimurium treated with chantaleela or DMSO infected the Hela cells for 30 min. After that, the intracellular bacteria in Hela cells were measured. The result of invasion assay was showed in figure 44. The number of intracellular bacteria from treatment with chantaleela was lower than the intracellular bacteria from treatment with DMSO. This demonstrated that the chantaleela at a minimal concentration of 150 µg/ml was able to inhibit bacterial invasion of *S.* Typhimurium. Moreover, the result of invasion assay correlated with the result of Western blotting that chantaleela at minimal concentration of 150 µg/ml could inhibit activity of SPI-1 T3SS for secretion of effector proteins resulting in decreasing bacterial invasion of *S.* Typhimurium. Therefore, chantaleela was certainly the T3SS inhibitor.

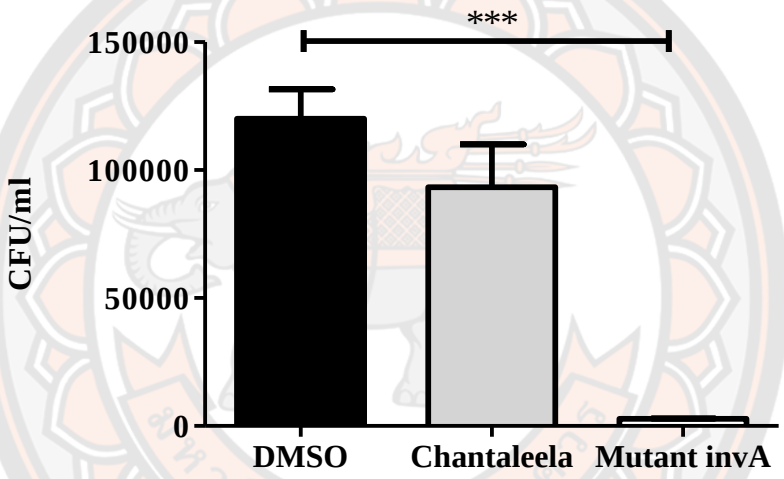

Figure 44 The quantity of *S.* Typhimurium strain SL1344 treated with chantaleela and DMSO as negative control.
Black column : The number of *S.* Typhimurium SL1344 treated with DMSO
Gray column : The number of *S.* Typhimurium SL1344 treated with chantaleela
White column : The number of *S.* Typhimurium SL1344 strain Δ*invA*
The *number* of S. Typhimurium strain Δ*invA* as positive control. The samples were performed in triplicate and showed in the standard error of the mean (SEM). The P value was compared and calculated between the samples and DMSO as negative control which levels of statistical significance *was* showed as: **, $P \leq 0.01$; ***, $P \leq 0.001$.

Therefore, garlic, turmeric and chantaleela at the minimal concentration of 100 µg/ml, 75 µg/ml and 150 µg/ml, respectively, are able to reduce bacterial invasion of S. Typhimurium. Moreover, the result of invasion assay correlated with the result of Western blotting that garlic, turmeric and chantaleela at minimal concentration are able to inhibit activity of SPI-1 T3SS for secretion of effector proteins resulting in decreasing bacterial invasion of *S.* Typhimurium. Both the results can conclude that garlic, turmeric and chantaleela are definitely the T3SS inhibitors

6. Detecting mechanisms of Thai herbal extracts for inhibition of SPI-1 T3SS

This experiment measured transcription level of genes regulating the SPI-1 T3SS by Real-time PCR. The genes regulating the SPI-1 T3SS included transcriptional regulators (*HilD*, *HilC*, *HilA* and *InvF*), secretion apparatus (*InvH* and *PrgI*), chaperone (*SicA*) and effector protein (*SipA*). Moreover, 16s rRNA gene being housekeeping gene of bacteria was used as an internal reference.

Before measuring transcription level of genes using Real-time PCR, the appropriate condition for performing Real-time PCR with the primers was optimized by using PCR. Briefly, the genomic DNA of *S.* Typhimurium strain SL1344 was used to amplify the genes regulating the SPI-1 T3SS by using PCR with the primer sets. The PCR profile consisted 45 cycles of denaturation at 95ºC for 5s, annealing at 57ºC for 10s and extension at 72ºC for 35s. The result of the PCR using the primer sets was showed in figure 45. The result showed that all PCR products from the optimizing condition were expected size. The products included 248 bp for CHiD primer set, 296 bp for CHiC primer set, 243 bp for CHiA primer set, 190 bp for CInF primer set, 181 bp for CInH primer set, 238 bp for CPrI primer set, 470 bp for CScA primer set, 268 bp for CSpA primer set and 697 bp for C16S primer set. This result demonstrated that the PCR condition with the primer sets was appropriate for performance in Real-time PCR. Therefore, the PCR condition with the primer sets was used to perform in the Real-time PCR.

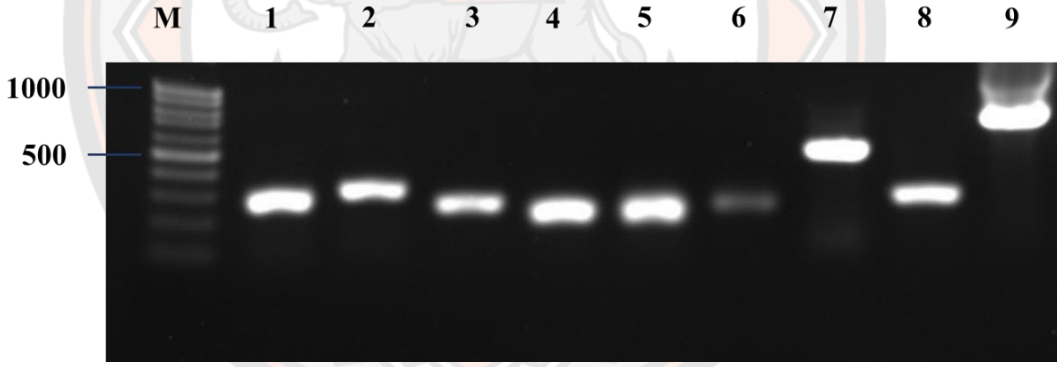

Figure 45 PCR products using the specific primer sets were analyzed by agarose gel electrophoresis.
Lane M : Molecular weight marker
Lane 1 : PCR product of CHiD primer set
Lane 2 : PCR product of CHiC primer set
Lane 3 : PCR product of CHiA primer set
Lane 4 : PCR product of CInF primer set
Lane 5 : PCR product of CInH primer set
Lane 6 : PCR product of CPrI primer set
Lane 7 : PCR product of CScA primer set
Lane 8 : PCR product of CspA primer set
Lane 9 : PCR product of C16S primer set

After optimizing condition for performing Real-time PCR with the specific primer sets, the transcription level of genes regulating the SPI-1 T3SS were measured. To detect mechanism of garlic for inhibition of SPI-1 T3SS, total RNA of *S.* Typhimurium strain SL1344 which treated with garlic at final concentration of 100 µg/ml or DMSO as negative control were extracted and conversed to cDNA. After that, cDNA was used to measure the transcription level of genes regulating the SPI-1 T3SS using LineGene 9600 Plus cycler for Real-time PCR. Finally, the Cycle thresholds (CT) of all treatments were calculated as $2^{-\Delta\Delta CT}$ for analysis of relative changes in transcriptional level. The results of comparison between treatment with garlic and DMSO were showed in figure 46. The result showed that transcriptional levels of all genes from treatment with garlic significantly decreased when compared to treatment of DMSO. This result demonstrated that *HilD* gene may be the target of garlic resulting in down regulation of other genes including transcriptional regulators, secretion apparatus, chaperone and effector proteins, respectively.

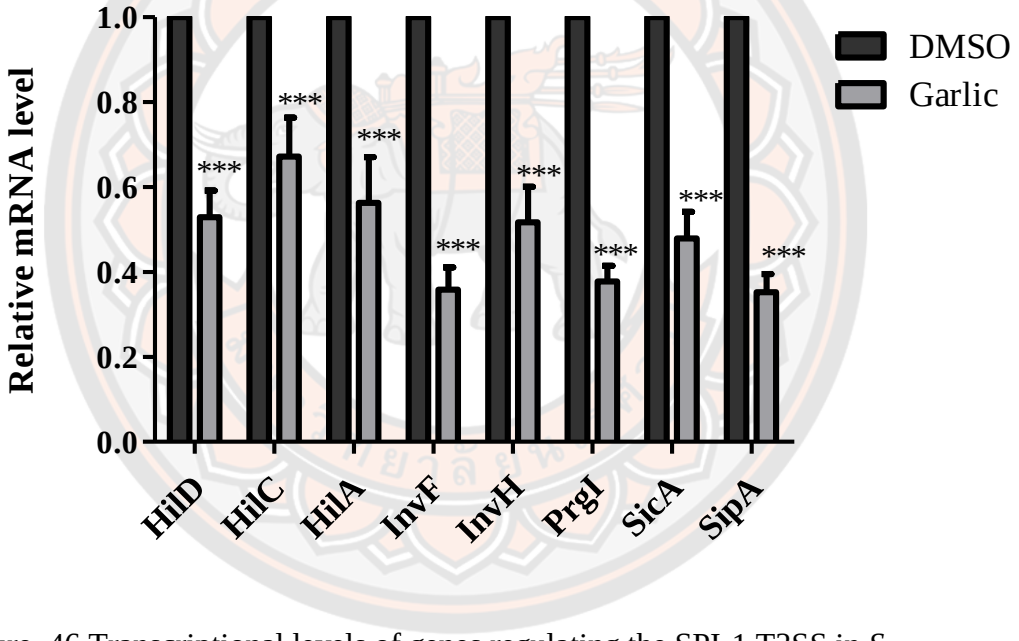

Figure 46 Transcriptional levels of genes regulating the SPI-1 T3SS in *S.* Typhimurium strain SL1344 treated with garlic and DMSO as negative control.
Black column : Transcriptional level of *S.* Typhimurium treated with DMSO
Gray column : Transcriptional level of *S.* Typhimurium treated with garlic
The samples were performed in triplicate and showed in the standard error of the mean (SEM). The P value was compared and calculated between the samples and DMSO as negative control which levels of statistical significance was showed as : ***, $P \leq 0.001$.

To detect mechanism of turmeric for inhibition of SPI-1 T3SS, total RNA of *S.* Typhimurium strain SL1344 which treated with turmeric at final concentration of 75 µg/ml or DMSO as negative control were extracted and conversed to cDNA. After that, the transcription level of genes regulating the SPI-1 T3SS was measured and the CT was calculated for analysis of relative changes in transcriptional level. The result was showed in figure 47. The result showed that transcriptional levels of all genes from treatment with turmeric significantly decreased when compared to treatment of DMSO. This result demonstrated that *HilD* gene may be the target of turmeric resulting in down regulation of other genes including transcriptional regulators, secretion apparatus, chaperone and effector proteins, respectively.

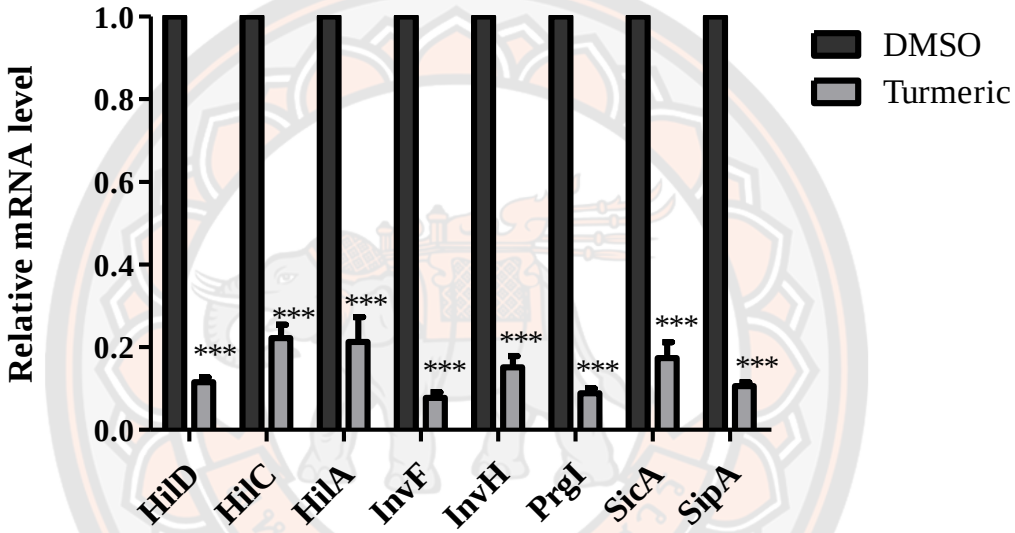

Figure 47 Transcriptional levels of genes regulating the SPI-1 T3SS in *S.* Typhimurium strain SL1344 treated with turmeric and DMSO as negative control.
Black column : Transcriptional level of *S.* Typhimurium treated with DMSO
Gray column : Transcriptional level of *S.* Typhimurium treated with turmeric
The samples were performed in triplicate and showed in the standard error of the mean (SEM). The P value was compared and calculated between the samples and DMSO as negative control which levels of statistical significance was showed as : ***, $P \leq 0.001$.

To detect mechanism of chantaleela for inhibition of SPI-1 T3SS, total RNA of *S.* Typhimurium strain SL1344 which treated with chantaleela at final concentration of 150 µg/ml or DMSO as negative control were extracted and conversed to cDNA. After that, the transcription level of genes regulating the SPI-1 T3SS was measured and the CT was calculated for analysis of relative changes in transcriptional level. The result was showed in figure 48. The result showed that transcriptional levels of all genes from treatment with chantaleela significantly decreased when compared to treatment of DMSO. This result demonstrated that *HilD* gene may be the target of chantaleela resulting in down regulation of other genes including transcriptional regulators, secretion apparatus, chaperone and effector proteins, respectively.

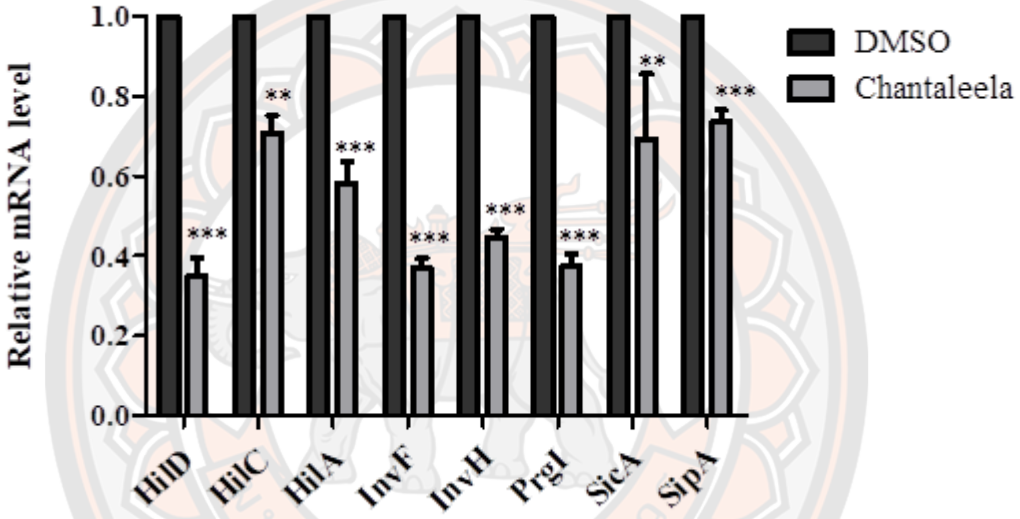

Figure 48 Transcriptional levels of genes regulating the SPI-1 T3SS in *S.* Typhimurium strain SL1344 treated with chantaleela and DMSO as negative control.
Black column : Transcriptional level of *S.* Typhimurium treated with DMSO
Gray column : Transcriptional level of *S.* Typhimurium treated with chantaleela
The samples were performed in triplicate and showed in the standard error of the mean (SEM). The P value was compared and calculated between the samples and DMSO as negative control which levels of statistical significance was showed as : ***, $P \leq 0.001$.

Therefore, the target of garlic, turmeric and chantaleela may be the *HilD* gene which is the top of regulatory cascade in SPI-1 T3SS. Moreover, reducing of *HilD* gene led down regulation of lower genes in SPI-1 T3SS including transcriptional regulators, secretion apparatus, chaperone and effector proteins.

CHAPTER V

DISCUSSION

Nowadays antibiotic resistant bacteria are the major problem of human health worldwide. If the patient is infected with the antibiotic resistant bacteria, it may become more complicated, prolonged stays in hospital, increase health care cost and untreated the infection (1, 2). Hence, there have been several studies that attempt to develop the novel antibiotics for struggle of antibiotic resistant bacteria. However, the developed novel antibiotics in the present are not sufficient to flight with the rising antimicrobial resistance worldwide. Therefore, there are another strategy that has investigated natural or chemical substances for inhibition of virulence or pathogenesis from bacteria instead of bacterial growth. In this study, we focused on the type three secretion system which is virulence factor for helping bacterial invasion into the host cell for pathogenesis. Since the T3SS has high similarity in both of structures and functions and is able to be found in pathogenic gram-negative bacteria such as *Shigella* spp., *Escherichia coli*., *Yersinia* spp. and *Chlamydia* spp. for cause of disease in animals and plants (9). Therefore, we studied and identified 4 Thai herbs including garlic, turmeric, chantaleela and king of bitters for inhibition of T3SS of *S.* Typhimurium as T3SS inhibitors. If some Thai herbs are able to inhibit T3SS of *S.* Typhimurium, they will be able to be used as alternative medicine for decreasing virulence of T3SS in other bacteria.

For identification of the Thai herbs as T3SS inhibitors, *S.* Typhimurium secreting SipA protein with strep tag as *S.* Typhimurium strain SA was constructed using lambda red recombination system and FLP recombination technique for monitoring SPI-1 T3SS activity of *S.* Typhimurium. Because the SipA protein is the first effector protein which deliver from SPI-1 T3SS into host intestinal epithelial cells and is important virulence factor for invasion (102). Moreover, there are a lot of studies which select SipA protein to monitor activity of SPI-1 T3SS in *S.* Typhimurium such as Liu, Y., *et. al*.'s study and Lv, Q., *et. al*.'s study (145, 146). These studies constructed *S.* Typhimurium secreting SipA protein with 3xFlag for monitoring SPI-1 T3SS activity of *S.* Typhimurium. Liu, Y., *et. al*. identified cinnamaldehyde as a T3SS inhibitor *via* monitoring secretion of SipA protein with 3xFlag from *S.* Typhimurium. While, Lv, Q., *et. al*. identified syringaldehyde as a T3SS inhibitor *via* monitoring secretion of SipA protein with 3xFlag from *S.* Typhimurium. The results of these studies showed that SipA protein was an appropriated protein for using monitoring SPI-1 T3SS activity of *S.* Typhimurium. Therefore, this study selected the SipA protein for tagging with strep tag and then constructed *S.* Typhimurium secreting SipA protein with strep tag for monitoring SPI-1 T3SS activity of *S.* Typhimurium.

The 4 Thai herbal extracts including garlic, turmeric, chantaleela and king of bitters at the final concentration of 100 µg/ml were firstly screened the ability for inhibition of secreting SipA-strep tag protein from *S.* Typhimurium strain SA by SDS-PAGE with Coomassie blue staining and Western blotting. The garlic and turmeric at the concentration of 100 µg/ml completely inhibited secretion of SipA-strep tag proteins and chantaleela at the concentration of 100 µg/ml slightly inhibited secretion of SipA-strep tag proteins. However, king of bitters at the concentration of 100 µg/ml did not inhibit secretion of SipA-strep tag protein. Therefore, this study

interested and focused on only 3 Thai herbs including garlic, turmeric and chantaleela. The minimal concentration of these Thai herbs for inhibition of SPI-1 T3SS were determined as T3SS inhibitors. It was similar to Li, J., *et. al*.'s study. They chose and focused only compounds which could inhibit secretion of SipA and SipC proteins from *S*. Typhimurium. The compounds subsequently determined the minimal concentration of them for inhibition of secreting SipA and SipC proteins (147). The minimal concentration of the 3 Thai herbs were determined for inhibition of secreting SipA-strep tag proteins from *S*. Typhimurium strain SA by SDS-PAGE with Coomassie blue staining and Western blotting. The minimal concentration of garlic, turmeric and chantaleela for inhibition of secreting SipA-strep tag was 100, 75 and 150 µg/ml, respectively. The Thai herbal extracts including garlic, turmeric and chantaleela at the minimal concentration were used for other experiments.

The minimal concentration of garlic, turmeric and chantaleela for inhibition of secreting SipA-strep tag protein was evaluated by time-kill kinetic assay whether the minimal concentration of the 3 Thai herbal extracts could inhibit SPI-1 T3SS of *S*. Typhimurium for secretion of the protein or could kill *S*. Typhimurium. The result of time-kill kinetic assay showed that the minimal concentration of the 3 Thai herbal extracts could not kill *S*. Typhimurium for 24 hours post-treatment and could not lead to absence of the effector protein. Therefore, the Thai herbal extracts truly inhibit SPI-1 T3SS of *S*. Typhimurium for secretion of effect proteins. This process was similar to Li, T., *et. al*.'s study. They measured bacterial growth after treatment with different herbal extracts from Myanmar for 12 hours. The results showed that all the herbal extracts could not interrupt bacterial growth and the extracts actually inhibited only T3SS for secretion of effect proteins (148). Therefore, this process ensured ability of the 3 That herbal extracts including garlic, turmeric and chantaleela for inhibition of SPI-1 T3SS of *S*. Typhimurium instead of bacterial growth.

Because the bacterial culture containing the Thai herbal extracts was directly added in the Hela cell during processing of the invasion assay. Therefore, the cytotoxicity of the 3 Thai herbal extracts at the minimal concentration was measured by MTT assay before invasion assay. It assured that the Thai herbal extracts did not damage and interrupt the Hela cell for the results of invasion assay. The results of cytotoxicity showed that the 3 Thai herbal extracts did not affect cell viability of Hela cells for 24 hours post-treatment as well as did not interfere the results of invasion assay. The study of Li, J., *et. al*. also measured the cytotoxicity of fusaric acid to the Hela cell by using MTT assay before detection of the compound *via* invasion assay (149). They treated Hela cells with fusaric acid for 72 hours. The result showed that fusaric acid at different concentrations had no toxic effect on the viability of HeLa cell and then continued detection of the Thai herbal extracts for inhibition of bacterial invasion of *S*. Typhimurium.

To recheck the efficiency of the 3 Thai herbal extracts for inhibition of T3SS, bacterial invasion being a phenotype predominantly mediated by T3SS was detected by invasion assay. Therefore, Ability of the Thai 3 herbal extracts at minimal concentration for inhibition of bacterial invasion were detected by invasion assay. The results of invasion assay showed that all the herbal extracts were able to inhibit bacterial invasion of *S*. Typhimurium. Moreover, the result of invasion assay correlated with the result of Western blotting. Both studies demonstrated that After *S*. Typhimurium treated with Garlic, turmeric and chantaleela at minimal concentration,

the herbs could inhibit SPI-1 T3SS of *S.* Typhimurium for secretion of effector proteins showing in Western blotting results and then lead *S.* Typhimurium to reducing invasion showing in invasion results. Therefore, garlic, turmeric and chantaleela are definitely the T3SS inhibitors. The study of Li, J., *et. al*. also detected ability of 3 compounds for inhibition of bacterial invasion by invasion assay (147). They found that ability of 3 compounds including secocurvularin, C5 and cytosporone B for inhibition of bacterial invasion was not the same. Cytosporone B compounds inhibited extremely T3SS and lead to strong inhibitory effect on bacterial invasion. They chose only cytosporone B for other experiments. However, our study interested all the 3 Thai herbal extracts for doing another experiment.

The last experiment was detection of mechanisms of Thai herbal extracts for inhibition of SPI-1 T3SS by Real-time PCR. This process detected transcriptional levels of genes regulating the SPI-1 T3SS. The genes regulating the SPI-1 T3SS include transcriptional regulators (*HilD, HilC, HilA* and *InvF*), secretion apparatus (*InvH* and *PrgI*), chaperone (*SicA*) and effector protein (*SipA*). Moreover, 16s rRNA was used as housekeeping gene of bacteria. Briefly, the cascade of SPI-1 T3SS begin in appropriate environment including pH 8, high osmolarity, the presence of bile, divalent cations concentration, and the presence of short chain fatty acids which increase the expression of *HilD*. The *HilD* leads to expression of *HilC* which can also activate expression of themselves. Both *HilD* and *HilC* can bind to the *HilA* promoter to activate the expression of *HilA*. After that, *HilA* is then free to activate transcription of the *prg/org, inv/spa*. The *prg/org* operon expresses secretion apparatuses, such as *PrgI* while the *inv/spa* operon expresses secretion apparatuses, such as I*nvH*, and only a transcriptional regulator, *InvF*. Moreover, *HilA* and *InvF* activate the expression of the *sic/sip* operon which are mainly the effectors, such as *SipA*, and chaperone, *SicA* (94, 96, 103). The results of Real-time PCR in this study showed that the transcriptional levels of all genes with treatment with garlic, turmeric or chantaleela decreased significantly when compared to treatment of DMSO. It demonstrated that *HilD* gene may be the first target of the 3 Thai herbal extracts for inhibition of SPI-1 T3SS and leads to down regulation of other genes including transcriptional regulators, secretion apparatus, chaperone and effector proteins, respectively. Therefore, the target of the 3 Thai herbal extracts for inhibition of SPI-1 T3SS is *HilD* gene which leads to down regulation of other genes for activity of SPI-1 T3SS. Th study of Lv, Q., *et. al*. also detected transcriptional level of several SPI-1 T3SS genes including *HilD, HilC, RstA, InvF, HilA, SipA, SipB* and *SipC* after treatment with syringaldehyde (146). The result showed that the transcription level of these genes decreased when treated with syringaldehyde. The syringaldehyde had effects to suppress the transcription of the related key gene through the *HilD, HilC, RstA* and *HilA* to reduce the expression of the SPI-1 genes encoding downstream effector proteins (*SipA, SipB* and *SipC*). Therefore, garlic, turmeric and chantaleela affect genes regulating the SPI-1 T3SS through the *HilD* to reduce the expression of the SPI-1 genes encoding downstream proteins including HilC, HilA, InvF, PrgI, InvH, SicA and SipA which the proposed mechanisms of them are showed in figure 49.

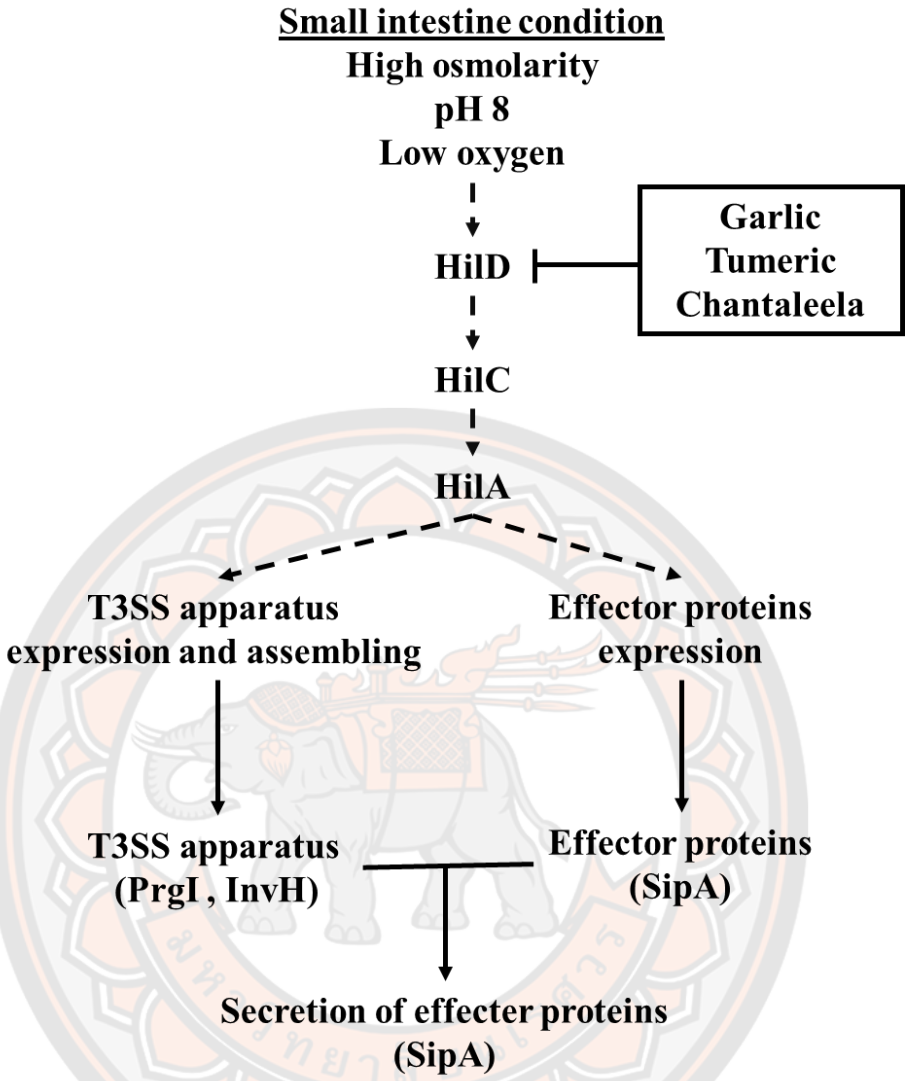

Figure 49 The proposed mechanism of garlic, turmeric and chantaleela for inhibition of SPI-1 T3SS through reduction of *HilD* expression.
The dotted line arrow indicated transcription activation
The blunt ended line indicated inhibition

In summary of study, garlic, turmeric and Chantaleela at minimal concentration of 100, 75 and 150 µg/ml, respectively, were identified as novel T3SS inhibitors against *S*. Typhimurium. These herbs at the concentration are able to inhibit the capability of T3SS in *S*. Typhimurium for secretion of effector proteins and bacterial invasion. But these herbs at the concentration have no effect the bacterial growth of *S*. Typhimurium. Moreover, these herbs repress the transcription of *HilD* gene which relates a key gene in regulatory cascade in SPi-1 T3SS. Then, reducing the *HilD* gene decreases the expression of the other genes in SPI-1 T3SS which encode a lot of downstream proteins including other transcriptional regulators (HilC, HilA and InvF), secretion apparatus (InvH and PrgI), chaperone (SicA) and effector protein (SipA).

In addition, garlic, turmeric and chantaleela have been reported that they have antibacterial activity. This study found that garlic, turmeric and chantaleela have anit-T3SS activity. From previous study and this study showed that the 3 Thai herbs including garlic, turmeric and chantaleela seem to be both antibacterial activity and anti-T3SS activity. The study of Zhang, Y., *et al.* also found that Thymol have not only antibacterial activity but also anti-T3SS activity. Thymol at MICs ranging from 1 to 5mM have been shown to have antimicrobial activity for the different bacterial strains. However, thymol at concentration of 0.4mM in their study did not detectably inhibit the growth of *S.* Typhimurium in and damage to the membranes of mammalian cells. Moreover, thymol at least 0.2 mM could inhibit activity of SPI-1 T3SS in *S.* Typhimurium for effector translocation and bacterial invasion. Their study demonstrated that thymol could have antibacterial activity at high concentration and anti-T3SS activity at lower concentration (150). Therefore, garlic, turmeric and chataleela could have both antibacterial activity and anti-T3SS activity in themselves. Firstly, the garlic extract at a final concentration of 100 µg/ml in this study was showed that it could inhibit SPI-1 T3SS of *S.* Typhimurium for secretion of effector proteins and bacterial invasion. While, Ross, Z. M., *et al*. studied ability of garlic extracts for inhibition of bacterial growth in *S.* Typhimurium. They showed that garlic extract at least concentration of 340 µg/ml was minimal inhibitory concentration (MIC) to inhibit bacterial growth of *S.* Typhimurium (151). This study and previous study demonstrated that the garlic extract at the concentration of 100 µg/ml can inhibit activity of T3SS as a T3SS inhibitor and does not affect the bacterial growth of *S.* typhimurium. Whereas, the garlic extract at the concentration of 340 µg/ml can inhibit bacterial growth of *S.* Typhimurium as an antibiotic. Secondly, this study showed that the turmeric extract at a final concentration of 75 µg/ml could inhibit SPI-1 T3SS of *S.* Typhimurium for secretion of effector proteins and bacterial invasion. While, Okiki, A., *et al*. studied the effect of turmeric extract against *S.* Typhimurium. The turmeric extract at the concentration of 560 µg/ml was minimal inhibitory concentration (MIC) to inhibit bacterial growth of *S.* Typhimurium (152). This study and previous study demonstrated that the turmeric extract at the concentration of 75 µg/ml can inhibit activity of T3SS as a T3SS inhibitor and does not affect the bacterial growth of *S.* typhimurium. Whereas, the turmeric extract at the concentration of 560 µg/ml can inhibit bacterial growth of *S.* Typhimurium as an antibiotic. Thirdly, the chantaleela extract at a final concentration of 150 µg/ml in this study could inhibit SPI-1 T3SS of *S.* Typhimurium for secretion of effector proteins and bacterial invasion. While, Chusri, S., *et. al.*'s studied the antibacterial activity of chantaleela for inhibition of both positive and negative bacteria. They showed that at least 1000 µg/ml of chantaleela could inhibit bacterial growth of Enterobacteriaceae (127). This study and previous study demonstrated that chantaleela at the concentration of 150 µg/ml can inhibit activity of T3SS as a T3SS inhibitor and does not affect the bacterial growth of *S.* typhimurium. Whereas, at least 1000 µg/ml of chantaleela can inhibit bacterial growth of *S.* Typhimurium as an antibiotic. Therefore, we conclude that garlic, turmeric and chantaleela have both antibacterial activity and anti-T3SS activity for *S.* Typhimurium but the activities of these herbs depend on the concentration of them. On the other hand, this study found that king of bitters at a final concentration of 100 µg/ml could not inhibit SPI-1 T3SS of *S.* Typhimurium for secretion of effector proteins. Moreover, Mishra, U. S., *et al*. found

that MIC of this herb at the concentration of 100 µg/ml could not create inhibition zone against *S.* Typhimurium and inhibit bacterial growth of *S.* Typhimurium (153). But Singh, A., *et al*. found that king of bitters at the concentration of 120 mg/ml could create inhibition zone against *S.* Typhimurium and inhibit bacterial growth of *S.* Typhimurium (154). This study and the previous studies showed that king of bitters has only antibacterial activity for *S.* Typhimurium. Therefore, king of bitters at the concentration of 120 mg/ml can inhibit bacterial growth of *S.* Typhimurium as an antibiotic. While, this herb at the concentration of 100 µg/ml cannot inhibit activity of T3SS as a T3SS inhibitor.

Although this study has already known that garlic, turmeric and chantaleela extracts can inhibit activity of SPI-1 T3SS in *S.* Typhimurium as the T3SS inhibitors, it has not studied detail of the compounds in the 3 Thai herbal extracts for action. Normally, the herbal extracts which have antibacterial activity or antioxidant activity are isolated and identified bioactive compounds. Then, the compounds are detected to explore the best compound for action on the activities (155) for example, study of Ramadwa, T. E., *et al*. They studied the antibacterial and antimycobacterial activity of sneezewood or *Ptaeroxylon obliquum*. They found that sneezewood extract could inhibit bacterial growth of *Mycobacterium smegmatis, M. bovis, M. aurum, M. fortuitum, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa* and *Escherichia coli*. After that, they isolated and identified several compounds from the sneezewood. Finally, they concluded that obliquumol compound had antimicrobial and antimycobacterial activities (156). Therefore, we recommend that the garlic, turmeric and chantaleela extracts should be isolated and identified bioactive compounds from themselves in the future. Then, the compounds from each the herbal extract should be detected to explore the best compound for action on the T3SS activity. At last we will know which compounds of each extract can be inhibit the T3SS activity as the T3SS inhibitors. However, we may predict the bioactive compound from the turmeric extract. Because, there is a study about the curcumin compound which is the main natural polyphenol found in the rhizome of *Curcuma longa* (turmeric) and in others *Curcuma* spp. (157). The curcumin can be found about 1-6% of turmeric extract (124). Marathe, S. A., *et. al.* studied ability of the curcumin compound for action in SPI-1 and SPI-2 T3SS activity. They reported that treating the curcumin decrease bacterial invasion of *S.* Typhimurium but it increases number of bacteria reaching the intestinal epithelium. Moreover, they found that curcumin can repress expression of *HilA* gene resulting in down regulation of *SopD* gene (158). Their results correlate with the results of our study. The turmeric extracts in this study can inhibit bacterial invasion of *S.* Typhimurium which is similar to curcumin compound's study. Furthermore, both turmeric extract and curcumin can repress expression of *HilA* and lead down regulation of other genes in regulatory cascade of SPI-1 T3SS. Therefore, the main compound in turmeric extract which acts on anti-T3SS activity may be the curcumin compound. This compound can be found about 1-6% of turmeric extract and may also lead to turmeric extract for being the T3SS inhibitors. Although turmeric has a study about bioactive compound from itself, there are no studies about compounds in garlic and chantaleela for inhibition of SPI-1 T3SS in *S.* Typhimurium. However, the bioactive compound which is found most in garlic and chantaleela may be a major compound for action on the SPI-1 T3SS as T3SS inhibitors. For the bioactive compounds in garlic, there are 2 compounds in garlic

which may be major compounds for inhibition of SPI-1 T3SS in *S.* Typhimurium as T3SS inhibitors. The first bioactive compound is diallyl-thiosulfinate or allicin. It is the major thiosulfinate and found 70-80% of sulfur compound in garlic (119, 130). The allicin which is found most in garlic may be the bioactive compound as the T3SS inhibitor and lead to inhibition of SPI-1 T3SS in *S.* Typhimurium. And the second bioactive compound is quercetin. It is the major flavonoid isolated from garlic (119, 159). This compound is reported by Tsou, L. K., *et al.* that the quercetin from Chinese medicinal plants is able to inhibit SPI-1 T3SS of *S.* Typhimurium and bacterial invasion of *S.* Typhimurium (21). Therefore, either one of them or both of them may be the bioactive compound as the T3SS inhibitor which make garlic can inhibit SPI-1 T3SS of *S.* Typhimurium and lead garlic as the T3SS inhibitor. For the bioactive compounds in chantaleela, it is quite difficult to expect what the major compound in chantaleela is the bioactive compound as the T3SS inhibitor and lead chantaleela for inhibition of SPI-1 T3SS in *S.* Typhimurium. Because chantaleela composed of 9 herbs including *G. chinense, A. lancea, A. annua, A. dahurica, S. album, D. cochinchinensis, T. crispa, E. longifolia* and *P. cablin* which each herb in chantaleela has a major bioactive compound for example, Atractylenolide III of *A. lancea* and Ferulic acid of *A. sinensis* (126, 160, 161). Therefore, one or more than one of the bioactive compounds in chantaleela may be the major bioactive compound as the T3SS inhibitor which make chantaleela can inhibit SPI-1 T3SS of *S.* Typhimurium and lead chantaleela as the T3SS inhibitor. However, we have just discussed and expected which the compound in garlic, turmeric and chantaleela may play inhibition of SPI-1 T3SS as T3SS inhibitors. For recommendation, garlic, turmeric and chataleela should be isolated, identified and explored the best compound for action on the T3SS activity in the future. We will know which the compound from garlic, turmeric and chantaleela is the T3SS inhibitor.

CHAPTER VI

CONCLUSION

From this study, garlic turmeric and chantaleela are the novel T3SS inhibitors against *S.* Typhimurium. The garlic, turmeric and chnataleela at minimal concentration of 100, 75 and 150 µg/ml, respectively, are able to inhibit secretion of effector proteins from SPI-1 T3SS of *S.* Typhimurium as well as inhibit bacterial invasion of *S.* Typhimurium. However, these herbal extracts at the minimal concentration have no effect the bacterial growth of *S.* Typhimurium. Moreover, the herbal extracts repress the transcription of *HilD* gene which relates a key gene in regulatory cascades activating the expression of SPI-1 T3SS cascade. They result in down regulation of other genes in the cascade including transcriptional regulators, secretion apparatus, chaperone and effector proteins. On the other hand, king of bitters is not the T3SS inhibitor. It is not able to inhibit secretion of effector proteins from SPI-1 T3SS of *S.* Typhimurium. The knowledges of study demonstrated alternative ability of garlic, turmeric and chantaleela instead of antibacterial activity for fight with infectious diseases. In additional, the knowledges of study may be used to explore bioactive compound for developing therapeutic drugs or alternative medicine against antibiotic resistant bacteria in the future.

REFERENCES

1. World Health Organization (WHO). Antimicrobial resistance 2021 [cited 2022 Jan 22]. Available from: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance.
2. Centers for Disease Control and Prevention (CDC). Antibiotic / Antimicrobial Resistance (AR / AMR) 2021 [cited 2022 Mar 1]. Available from: https://www.cdc.gov/drugresistance/.
3. The Review on Antimicrobial Resistance chaired by Jim O'Neill. Tackling Drug-Resistant Infections Globally: final report and recommendations 2016 [cited 2017 Aug 3]. Available from: https://amr-review.org/sites/default/files/160525_Final%20paper_with%20cover.pdf.
4. Global Antibiotic Research and Development Partnership (GARDP). Accelerating the development of treatments for drug-resistant infections, for every person who needs them 2022 [cited 2022 Mar 26]. Available from: http://gardp.org/.
5. Balouiri M, Sadiki M, Ibnsouda KS. Methods for *in vitro* evaluating antimicrobial activity. J Pharm Anal. 2015;6.
6. Boucher HW, Talbot GH, Benjamin DK, Jr., Bradley J, Guidos RJ, Jones RN, et al. 10 x '20 Progress--development of new drugs active against gram-negative bacilli: an update from the Infectious Diseases Society of America. Clin Infect Dis. 2013;56(2):1685 - 94.
7. Rasko DA, Sperandio V. Anti-virulence strategies to combat bacteria-mediated disease. Nature reviews Drug discovery. 2010;9(2):117-28.
8. Muhlen S, Dersch P. Anti-virulence Strategies to Target Bacterial Infections. Current topics in microbiology and immunology. 2016;398:147-83.
9. Coburn B, Sekirov I, Finlay BB. Type III secretion systems and disease. Clinical microbiology reviews. 2007;20(4):535-49.
10. Gong L, Lai SC, Treerat P, Prescott M, Adler B, Boyce JD, et al. *Burkholderia pseudomallei* type III secretion system cluster 3 ATPase BsaS, a chemotherapeutic target for small-molecule ATPase inhibitors. Infection and immunity. 2015;83(4) :1276-85.
11. Gu L, Zhou S, Zhu L, Liang C, Chen X. Small-Molecule Inhibitors of the Type III Secretion System. Molecules. 2015;20(9):17659-74.
12. McShan AC, De Guzman RN. The bacterial type III secretion system as a target for developing new antibiotics. Chemical biology & drug design. 2015;85(1) :30-42.
13. Khokhani D, Zhang C, Li Y, Wang Q, Zeng Q, Yamazaki A, et al. Discovery of plant phenolic compounds that act as type III secretion system inhibitors or inducers of the fire blight pathogen, *Erwinia amylovora*. Applied and environmental microbiology. 2013;79(18):5424-36.
14. Mahajan GB, Balachandran L. Sources of antibiotics: Hot springs. Biochemi- cal pharmacology. 2017;134:35-41.
15. Khan R, Islam B, Akram M, Shakil S, Ahmad A, Ali SM, et al. Antimicrobial activity of five herbal extracts against multi drug resistant (MDR) strains of bacteria and fungus of clinical origin. Molecules. 2009;14(2):586-97.
16. Tapsell LC, Hemphill I, Cobiac L, Patch CS, Sullivan DR, Fenech M, et al. Health benefits of herbs and spices: the past, the present, the future. Med J Aust. 2006;185(4 Suppl):S4-24.

17. Eloff JN. Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of ethnopharmacology. 1998;60(1):1-8.
18. Lewis K, Ausubel FM. Prospects of plant derived antibacterials. Nature biotechnology. 2006;24(12):1504-7.
19. Lee SB, Cha KH, Kim SN, Altantsetseg S, Shatar S, Sarangerel O, et al. The Antimicrobial Activity of Essential Oil from *Dracocephalum foetidum* Against Pathogenic Microorganisms. Journal of microbiology (Seoul, Korea). 2007;45(1):53-7.
20. Kamonwannasit S, Nantapong N, Kumkrai P, Luecha P, Kupittayanant S, Chudapongse N. Antibacterial activity of *Aquilaria crassna* leaf extract against *Staphylococcus epidermidis* by disruption of cell wall. Annals of clinical microbiology and antimicrobials. 2013;12:20.
21. Tsou LK, Lara-Tejero M, RoseFigura J, Zhang ZJ, Wang YC, Yount JS, et al. Antibacterial Flavonoids from Medicinal Plants Covalently Inactivate Type III Protein Secretion Substrates. J Am Chem Soc. 2016;138(7):2209-18.
22. Talaro KP, Chess B. Foundations in microbiology : basic principles. 9th ed. New York, N.Y.: McGraw-Hill; 2015 c2015.
23. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P & T : a peer-reviewed journal for formulary management. 2015;40(4):277-83.
24. International Historic Chemical Landmark. Discovery and Development of Penicillin: American Chemical Society; 1999 [cited 2017 Aug 1]. Available from: https://www.acs.org/content/dam/acsorg/education/whatischemistry/landmarks/flemingpenicillin/the-discovery-and-development-of-penicillin-commemorative-booklet.pdf.
25. Sengupta S, Chattopadhyay MK, Grossart HP. The multifaceted roles of antibiotics and antibiotic resistance in nature. Frontiers in microbiology. 2013;4:47.
26. Lobanovska M, Pilla G. Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future? The Yale journal of biology and medicine. 2017;90(1):135-45.
27. Trimble MJ, Mlynárčik P, Kolář M, Hancock RE. Polymyxin: Alternative Mechanisms of Action and Resistance. Cold Spring Harbor perspectives in medicine. 2016;6(10).
28. Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. Nature reviews Microbiology. 2010;8(6):423-35.
29. Cambau E, Williams D. Anti-leprosy drugs: Modes of action and mechanisms of resistance in *Mycobacterium leprae* 2019 [cited 2022 May 14]. Available from: https://www.internationaltextbookofleprosy.org/.
30. Centers for Disease Control and Prevention (CDC). Antibiotic resistance threats in the United States, 2013 2013 [cited 2017 Aug 3]. Available from: https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf.
31. World Health Organization (WHO). World Antibiotic Awareness Week, 16-22 November 2015 2015 [cited 2017 Aug 2]. Available from: http://www.who.int/mediacentre/events/2015/world-antibiotic-awareness-week/infographics/en/.
32. Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. Microbiology spectrum. 2016;4(2).
33. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiology and molecular biology reviews : MMBR. 2003;67(4):593-656.
34. Bush K, Jacoby GA. Updated functional classification of beta-lactamases. Antimicrobial agents and chemotherapy. 2010;54(3):969-76.
35. Miller WR, Munita JM, Arias CA. Mechanisms of antibiotic resistance in

enterococci. Expert review of anti-infective therapy. 2014;12(10):1221-36.
36. Hiramatsu K, Ito T, Tsubakishita S, Sasaki T, Takeuchi F, Morimoto Y, et al. Genomic Basis for Methicillin Resistance in *Staphylococcus aureus*. Infection & chemotherapy. 2013;45(2):117-36.
37. Roberts MC. Update on acquired tetracycline resistance genes. FEMS microbiology letters. 2005;245(2):195-203.
38. Centers for Disease control and Prevention (CDC). Antibiotic resistance threats in the United States in in 2019 2019 [updated November 23, 2021; cited 2021 Mar 28]. Available from: https://www.cdc.gov/drugresistance//biggest-threats.html.
39. Pumart P, Phodha T, Thamlikitkul V, Riewpaiboon A, Prakongsai P, Limwattananon S. Health and economic impacts of antimicrobial resistance in Thailand. J Health Systems Res. 2012;6:352-60.
40. National Surveillance System for Antimicrobial Resistance. Global and national antimicrobial resistant situation 2022 [cited 2022 Mar 27]. Available from: http://narst.dmsc.moph.go.th/.
41. Global Antimicrobial Resistance and Use Surveillance System (GLASS). The detection and reporting of colistin resistance 2021 [cited 2022 Mar 28]. 2nd Edition:[Available from: https://www.who.int/publications/i/item/glass-the-detection-and-reporting-of-colistin-resistance-2nd-ed.
42. Centers for Disease Control and Prevention (CDC). Newly Reported Gene, *mcr-1*, Threatens Last-Resort Antibiotics 2021 [cited 2021 Nov 20]. Available from: https://www.cdc.gov/drugresistance/solutions-initiative/stories/gene-reported-mcr.html.
43. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. The Lancet Infectious diseases. 2016;16(2):161-8.
44. The U.S.Food and Drug Administration (FDA). The Drug Development Process 2018 [cited 2022 31 March]. Available from: https://www.fda.gov/patients/ learn-about-drug-and-device-approvals/drug-development-process.
45. The U.S.Food and Drug Administration (FDA). New Drugs at FDA: CDER's New Molecular Entities and New Therapeutic Biological Products 2022 [Available from: https://www.fda.gov/drugs/development-approval-process-drugs/new-drugs-fda-cders-new-molecular-entities-and-new-therapeutic-biological-products.
46. The U.S. Food and Drug Administration (FDA). WINLEVI (clascoterone) cream, for topical use Initial U.S. Approval: 2019. 2020 [cited 2022 Mar 31]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/213433s000lbl.pdf.
47. The U.S. Food and Drug Administration (FDA). FETROJA (cefiderocol) for injection, for intravenous use Initial U.S. Approval: 2019 2021 [cited 2022 Mar 31]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2021/209445s004lbl.pdf.
48. Al-Tawfiq JA, Momattin H, Al-Ali AY, Eljaaly K, Tirupathi R, Haradwala MB, et al. Antibiotics in the pipeline: a literature review (2017-2020). Infection. 2022;50(3):553-64.
49. Slonczewski J, Watkins FJ. Microbiology : an evolving science. New York: W.W. Norton & Co.; 2009.
50. Zav'yalov V, Zavialov A, Zav'yalova G, Korpela T. Adhesive organelles of

Gram-negative pathogens assembled with the classical chaperone/usher machinery: structure and function from a clinical standpoint. FEMS microbiology reviews. 2010;34(3):317-78.
51. Allen WJ, Phan G, Waksman G. Pilus biogenesis at the outer membrane of Gram-negative bacterial pathogens. Current opinion in structural biology. 2012;22(4):500-6.
52. Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nature reviews Microbiology. 2015;13(5):269-84.
53. Pinkner JS, Remaut H, Buelens F, Miller E, Aberg V, Pemberton N, et al. Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(47):17897-902.
54. Svensson A, Larsson A, Emtenas H, Hedenstrom M, Fex T, Hultgren SJ, et al. Design and evaluation of pilicides: potential novel antibacterial agents directed against uropathogenic *Escherichia coli*. Chembiochem : a European journal of chemical biology. 2001;2(12):915-8.
55. Krachler AM, Orth K. Targeting the bacteria-host interface: strategies in anti-adhesion therapy. Virulence. 2013;4(4):284-94.
56. Han Z, Pinkner JS, Ford B, Chorell E, Crowley JM, Cusumano CK, et al. Lead optimization studies on FimH antagonists: discovery of potent and orally bioavailable ortho-substituted biphenyl mannosides. Journal of medicinal chemistry. 2012;55(8):3945-59.
57. Hartmann M, Papavlassopoulos H, Chandrasekaran V, Grabosch C, Beiroth F, Lindhorst TK, et al. Inhibition of bacterial adhesion to live human cells: activity and cytotoxicity of synthetic mannosides. FEBS letters. 2012;586(10):1459-65.
58. Duan Q, Zhou M, Zhu L, Zhu G. Flagella and bacterial pathogenicity. Journal of basic microbiology. 2013;53(1):1-8.
59. Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. Journal of bacteriology. 2002;184(4):1140-54.
60. Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R. D-amino acids trigger biofilm disassembly. Science. 2010;328(5978):627-9.
61. Kolodkin-Gal I, Cao S, Chai L, Bottcher T, Kolter R, Clardy J, et al. A self-produced trigger for biofilm disassembly that targets exopolysaccharide. Cell. 2012;149(3):684-92.
62. Burton GRW, Engelkirk PG. Microbiology for the health sciences. 6th ed. Philadelphia, Pa.: Lippincott Williams & Wilkins; 2000 c2000.
63. Hajishengallis G, Martin M, Schifferle RE, Genco RJ. Counteracting interactions between lipopolysaccharide molecules with differential activation of toll-like receptors. Infection and immunity. 2002;70(12):6658-64.
64. Takasawa K, Kano R, Maruyama H, Hasegawa A, Kamata H. The antagonist activity of lipid IVa on the stimulation by lipid A of TNF-alpha production from canine blood mononuclear cells. Veterinary immunology and immunopathology. 2011;143(1-2):167-9.
65. Chaudhry H, Zhou J, Zhong Y, Ali MM, McGuire F, Nagarkatti PS, et al. Role of cytokines as a double-edged sword in sepsis. In vivo. 2013;27(6):669-84.

66. Cohen TS, Pelletier M, Cheng L, Pennini ME, Bonnell J, Cvitkovic R, et al. Anti-LPS antibodies protect against *Klebsiella pneumoniae* by empowering neutrophil-mediated clearance without neutralizing TLR4. JCI insight. 2017;2(9).
67. Chen Z, Moayeri M, Zhou YH, Leppla S, Emerson S, Sebrell A, et al. Efficient neutralization of anthrax toxin by chimpanzee monoclonal antibodies against protective antigen. The Journal of infectious diseases. 2006;193(5):625-33.
68. Armstrong GD, Rowe PC, Goodyer P, Orrbine E, Klassen TP, Wells G, et al. A phase I study of chemically synthesized verotoxin (Shiga-like toxin) Pk-trisaccharide receptors attached to chromosorb for preventing hemolytic-uremic syndrome. The Journal of infectious diseases. 1995;171(4):1042-5.
69. Hung DT, Shakhnovich EA, Pierson E, Mekalanos JJ. Small-molecule inhibitor of *Vibrio cholerae* virulence and intestinal colonization. Science. 2005;310(5748):670-4.
70. Shakhnovich EA, Hung DT, Pierson E, Lee K, Mekalanos JJ. Virstatin inhibits dimerization of the transcriptional activator ToxT. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(7):2372-7.
71. Paschos A, den Hartigh A, Smith MA, Atluri VL, Sivanesan D, Tsolis RM, et al. An *in vivo* high-throughput screening approach targeting the type IV secretion system component VirB8 identified inhibitors of *Brucella abortus* 2308 proliferation. Infection and immunity. 2011;79(3):1033-43.
72. Smith MA, Coincon M, Paschos A, Jolicoeur B, Lavallee P, Sygusch J, et al. Identification of the binding site of *Brucella* VirB8 interaction inhibitors. Chemistry & biology. 2012;19(8):1041-8.
73. Ng WL, Bassler BL. Bacterial quorum-sensing network architectures. Annual review of genetics. 2009;43:197-222.
74. Hentzer M, Givskov M. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. The Journal of clinical investigation. 2003;112(9):1300-7.
75. LaSarre B, Federle MJ. Exploiting quorum sensing to confuse bacterial pathogens. Microbiology and molecular biology reviews : MMBR. 2013;77(1):73-111.
76. Lu C, Maurer CK, Kirsch B, Steinbach A, Hartmann RW. Overcoming the unexpected functional inversion of a PqsR antagonist in *Pseudomonas aeruginosa*: an *in vivo* potent antivirulence agent targeting pqs quorum sensing. Angewandte Chemie. 2014;53(4):1109-12.
77. Ghazaei C. Anti-virulence Therapy Against Bacterial Infections: Mechanisms of Action and Challenges. J Kermanshah Univ Med Sci. 2021;25.
78. Ogawara H. Possible drugs for the treatment of bacterial infections in the future: anti-virulence drugs. The Journal of antibiotics. 2021;74(1):24-41.
79. The U.S Food & Drug Administration (FDA). BabyBIG [Botulism Immune Globulin Intravenous (Human) (BIG-IV)] 2003 [cited 2022 Apr 2]. Available from: https://www.fda.gov/media/150406/download.
80. The U.S Food & Drug Administration (FDA). RAXIBACUMAB injection 2012 [cited 2022 Apr 3]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2012/125349s000lbl.pdf.
81. The U.S Food & Drug Administration (FDA). BAT® [Botulism Antitoxin Heptavalent (A, B, C, D, E, F, G) - (Equine)] 2013 [cited 2022 Apr 3].
82. The U.S Food & Drug Administration (FDA). ANTHIM® (obiltoxaximab)

injection 2016 [cited 2022 Apr 3]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/125509s001lbl.pdf.
83. The U.S Food & Drug Administration (FDA). ZINPLAVA™ (bezlotoxumab) injection 2016 [cited 2022 Apr 3]. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/761046s000lbl.pdf.
84. Duncan MC, Linington RG, Auerbuch V. Chemical inhibitors of the type three secretion system: disarming bacterial pathogens. Antimicrobial agents and chemotherapy. 2012;56(11):5433-41.
85. Chatterjee S, Chaudhury S, McShan AC, Kaur K, De Guzman RN. Structure and biophysics of type III secretion in bacteria. Biochemistry. 2013;52(15):2508-17.
86. Galan JE, Lara-Tejero M, Marlovits TC, Wagner S. Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. Annual review of microbiology. 2014;68:415-38.
87. Notti RQ, Stebbins CE. The Structure and Function of Type III Secretion Systems. Microbiology spectrum. 2016;4(1).
88. Feldman MF, Cornelis GR. The multitalented type III chaperones: all you can do with 15 kDa. FEMS microbiology letters. 2003;219(2):151-8.
89. Sato H, Frank DW. Multi-Functional Characteristics of the *Pseudomonas aeruginosa* Type III Needle-Tip Protein, PcrV; Comparison to Orthologs in other Gram-negative Bacteria. Frontiers in microbiology. 2011;2:142.
90. Centers for Disease Control and Prevention (CDC). Typhimurium and Newport Infections Linked to Cantaloupe 2012 [cited 2017 Sept 15]. Available from: https://www.cdc.gov/salmonella/typhimurium-cantaloupe-08-12/signs-symptoms.
html.
91. Centers for Disease Control and Prevention (CDC). Biosafety in Microbiological and Biomedical Laboratories 2009 [cited 2017 Sept 17]. 5th Edition:[Available from: https://www.cdc.gov/biosafety/publications/bmbl5/BMBL.
pdf.
92. Murray PR, Baron EJ. Manual of clinical microbiology. 7th ed. Washington, D.C: ASM Press; 1999 c1999.
93. Coburn B, Grassl GA, Finlay BB. *Salmonella*, the host and disease: a brief review. Immunology and cell biology. 2007;85(2):112-8.
94. Fabrega A, Vila J. *Salmonella enterica* serovar Typhimurium skills to succeed in the host: virulence and regulation. Clinical microbiology reviews. 2013;26(2):308-41.
95. Ramos-Morales F. Impact of *Salmonella enterica* Type III Secretion System Effectors on the Eukaryotic Host Cell. ISRN Cell Biol. 2012;2012:Article ID 787934.
96. Amanda W, Taseen D, Aaron W, Andrew P, Wolfgang K. The *Salmonella* Pathogenicity Island-1 and -2 Encoded Type III Secretion Systems 2012 [cited 2017 Sept 20]. Available from: https://www.researchgate.net/publication/221922741_The_Salmonella_Pathogenicity_Island-1_and_-2_Encoded_Type_III_Secretion_Systems.
97. Hurley D, McCusker MP, Fanning S, Martins M. *Salmonella*-host interactions - modulation of the host innate immune system. Frontiers in immunology. 2014;5:481.
98. Manon R, Nadia A, Fatemeh N, Virlogeux-Payant I, Velge P, Wiedemann A. The Different Strategies Used by *Salmonella* to Invade Host Cells. 2012. p. 339-50.
99. Figueira R, Holden DW. Functions of the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system effectors. Microbiology. 2012;158(Pt 5):1147-61.
100. Agbor TA, McCormick BA. *Salmonella* effectors: important players modulating

host cell function during infection Cellular microbiology. 2011;13(12):1858-69.
101. The National Center for Biotechnology Information (NCBI). *Salmonella* Pathogenicity Island 1 effector protein : SipA [*Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2] 2022 [updated 16-May-2022; cited 2022 Apr 1]. Available from: https://www.ncbi.nlm.nih.gov/gene/1254405.
102. Lilic M, Galkin VE, Orlova A, VanLoock MS, Egelman EH, Stebbins CE. *Salmonella* SipA polymerizes actin by stapling filaments with nonglobular protein arms. Science. 2003;301(5641):1918-21.
103. Lou L, Zhang P, Piao R, Wang Y. *Salmonella* Pathogenicity Island 1 (SPI-1) and Its Complex Regulatory Network. Frontiers in cellular and infection microbiology. 2019;9:270.
104. Pendergrass HA, May AE. Natural Product Type III Secretion System Inhibitors. Antibiotics (Basel, Switzerland). 2019;8(4).
105. Hotinger JA, Pendergrass HA, May AE. Molecular Targets and Strategies for Inhibition of the Bacterial Type III Secretion System (T3SS); Inhibitors Directly Binding to T3SS Components. Biomolecules. 2021;11(2).
106. Milla CE, Chmiel JF, Accurso FJ, VanDevanter DR, Konstan MW, Yarranton G, et al. Anti-PcrV antibody in cystic fibrosis: a novel approach targeting Pseudomonas aeruginosa airway infection. Pediatric pulmonology. 2014;49(7):650-8.
107. Peter KV. Handbook of Herbs and Spices. 2nd Edition ed. Philadelphia, Pa: Woodhead Publishing; 2012.
108. Block E. Antithrombotic agent of garlic: a lesson from 5000 years of folk medicine. . In: Steiner RP e, editor. Washington DC: American Chemical Society; 1986.
109. Bellamy D, Pfister A. World medicine: plants, patients and people. Oxford: Blackwell Publishers; 1992.
110. Govindarajan R, Vijayakumar M, Pushpangadan P. Antioxidant approach to disease management and the role of ‘Rasayana’ herbs of Ayurveda. Journal of ethnopharmacology. 2005;99(2):165-78.
111. Thatte UM, Dahanukar SA. Ayurveda and contemporary scientific thought. Trends Pharmacol Sc. 1986;7:247-51.
112. Steiner RP. Folk medicine, the art and the science. Washington DC:: American Chemical Society; 1985.
113. World Health Organization (WHO). WHO traditional medicine strategy: 2014-2023. 2013 [cited 2022 Apr 1]. Available from: http://apps.who.int/iris/ bitstream/10665/92455/1/9789241506090_eng.pdf.
114. Mehrotra S, Srivastava AK, Nandi SP. Comparative antimicrobial activities of Neem, Amla, Aloe, Assam Tea and Clove extracts against *Vibrio cholerae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. J Med Plants Res. 2010;4(18):2473-8.
115. Betoni JE, Mantovani RP, Barbosa LN, Di Stasi LC, Fernandes Junior A. Synergism between plant extract and antimicrobial drugs used on *Staphylococcus aureus* diseases. Mem Inst Oswaldo Cruz. 2006;101(4):387-90.
116. Dash M, Patra JK, Panda PP. Phytochemical and antimicrobial screening of extracts of *Aquilaria agallocha* Roxb. Afr J Biotechnol. 2008;7.
117. Matasyoh LG, Matasyoh JC, Wachira FN, Kinyua MG, Muigai AW, Mukiama TK. Antimicrobial activity of essential oils of *Ocimum gratissimum* L. From different populations of Kenya. Afr J Tradit Complement Altern Med. 2008;5(2):187-93.

118. Nakamura CV, Ueda-Nakamura T, Bando E, Melo AF, Cortez DA, Dias Filho BP. Antibacterial Activity of *Ocimum gratissimum* L. Essential Oil. Mem Inst Oswaldo Cruz. 1999;94(5):675-8.
119. El-Saber Batiha G, Magdy Beshbishy A, L GW, Elewa YHA, A AA-S, Abd El-Hack ME, et al. Chemical Constituents and Pharmacological Activities of Garlic (*Allium sativum* L.): A Review. Nutrients. 2020;12(3).
120. Wu C, Wang M, Dong Y, Cheng Z, Meng H. Growth, bolting and yield of garlic (*Allium sativum* L.) in response to clove chilling treatment. Sci Hortic. 2015;194:43-52.
121. Ansary J, Forbes-Hernández TY, Gil E, Cianciosi D, Zhang J, Elexpuru-Zabaleta M, et al. Potential Health Benefit of Garlic Based on Human Intervention Studies: A Brief Overview. Antioxidants (Basel, Switzerland). 2020;9(7).
122. Bhatwalkar SB, Mondal R, Krishna SBN, Adam JK, Govender P, Anupam R. Antibacterial Properties of Organosulfur Compounds of Garlic (*Allium sativum*). Frontiers in microbiology. 2021;12:613077.
123. Ahmad RS, Hussain MB, Sultan MT, Arshad MS, Waheed M, Shariati MA, et al. Biochemistry, Safety, Pharmacological Activities, and Clinical Applications of Turmeric: A Mechanistic Review. Evidence-based complementary and alternative medicine : eCAM. 2020;2020:7656919.
124. Nelson KM, Dahlin JL, Bisson J, Graham J, Pauli GF, Walters MA. The Essential Medicinal Chemistry of Curcumin. Journal of medicinal chemistry. 2017;60(5):1620-37.
125. Moghadamtousi SZ, Kadir HA, Hassandarvish P, Tajik H, Abubakar S, Zandi K. A Review on Antibacterial, Antiviral, and Antifungal Activity of Curcumin. Biomed Res Int. 2014;2014:186864.
126. Food and drug administration in Thailand. Chantaleela recipe [cited 2022 Apr 20]. Available from: https://www.fda.moph.go.th/.
127. Chusri S, Sinvaraphan N, Chaipak P, Luxsananuwong A, Voravuthikunchai SP. Evaluation of antibacterial activity, phytochemical constituents, and cytotoxicity effects of Thai household ancient remedies. Journal of alternative and complementary medicine (New York, NY). 2014;20(12):909-18.
128. Hossain MS, Urbi Z, Sule A, Hafizur Rahman KM. *Andrographis paniculata* (Burm. f.) Wall. ex Nees: a review of ethnobotany, phytochemistry, and pharmacology. TheScientificWorldJournal. 2014;2014:274905.
129. Hossain S, Urbi Z, Karuniawati H, Mohiuddin RB, Moh Qrimida A, Allzrag AMM, et al. *Andrographis paniculata* (Burm. f.) Wall. ex Nees: An Updated Review of Phytochemistry, Antimicrobial Pharmacology, and Clinical Safety and Efficacy. Life (Basel, Switzerland). 2021;11(4).
130. Zhang Y, Liu X, Ruan J, Zhuang X, Zhang X, Li Z. Phytochemicals of garlic: Promising candidates for cancer therapy. Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie. 2020;123:109730.
131. Chauhan DK, Puranik V, Mishra V. Analysis of stem of *Tinospora cordifolia*, leaves of *Andrographis paniculata* and root and leaves of *Boerhaavia diffusa* for nutritional and phytochemical composition. Int J Food Sci Nutr. 2014;3(4).
132. Boonyom R, Roytrakul S, Thinwang P. A small molecule, $C_{24}H_{17}ClN_4O_2S$, inhibits the function of the type III secretion system in *Salmonella* Typhimurium. Journal, genetic engineering & biotechnology. 2022;20(1):54.
133. Boonyom R, Karavolos MH, Bulmer DM, Khan CMA. *Salmonella*

pathogenicity island 1 (SPI-1) type III secretion of SopD involves N- and C-terminal signals and direct binding to the InvC ATPase. Microbiology. 2010;156 (Pt 6):1805-14.
134. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(12):6640-5.
135. Karavolos MH, Roe AJ, Wilson M, Henderson J, Lee JJ, Gally DL, et al. Type III secretion of the *Salmonella* effector protein SopE is mediated via an N-terminal amino acid signal and not an mRNA sequence. Journal of bacteriology. 2005;187(5):1559-67.
136. Sambrook J, Green MR. Molecular Cloning: A Laboratory Manual. Fourth ed. Cold Spring Harbor, New york: Cold Spring Harbor Laboratory Press; 2012.
137. Inoue H, Nojima H, Okayama H. High efficiency transformation of *Escherichia coli* with plasmids. Gene. 1990;96(1):23-8.
138. Condry DL, Nilles ML. Introduction to Type III Secretion Systems. Methods in molecular biology (Clifton, NJ). 2017;1531:1-10.
139. Mizusaki H, Takaya A, Yamamoto T, Aizawa S. Signal pathway in salt-activated expression of the *Salmonella* pathogenicity island 1 type III secretion system in *Salmonella enterica* serovar Typhimurium. Journal of bacteriology. 2008;190(13):4624 -31.
140. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227(5259):680-5.
141. Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Archives of biochemistry and biophysics. 1993;303(2):474-82.
142. Steele-Mortimer O. Infection of epithelial cells with *Salmonella enterica*. Methods in molecular biology (Clifton, NJ). 2008;431:201-11.
143. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature protocols. 2008;3(6):1101-8.
144. Ma Y-N, Chen L, Si N-G, Jiang W-J, Zhou Z-G, Liu J-L, et al. Identification of Benzyloxy Carbonimidoyl Dicyanide Derivatives as Novel Type III Secretion System Inhibitors *via* High-Throughput Screening. Frontiers in plant science. 2019;10.
145. Liu Y, Zhang Y, Zhou Y, Wang T, Deng X, Chu X, et al. Cinnamaldehyde inhibits type three secretion system in *Salmonella enterica* serovar Typhimurium by affecting the expression of key effector proteins. Vet Microbiol. 2019;239:108463.
146. Lv Q, Chu X, Yao X, Ma K, Zhang Y, Deng X. Inhibition of the type III secretion system by syringaldehyde protects mice from *Salmonella enterica* serovar Typhimurium. Journal of cellular and molecular medicine. 2019;23(7):4679-88.
147. Li J, Lv C, Sun W, Li Z, Han X, Li Y, et al. Cytosporone B, an inhibitor of the type III secretion system of *Salmonella enterica* serovar Typhimurium. Antimicrobial agents and chemotherapy. 2013;57(5):2191-8.
148. Li T, Zhang D, Oo TN, San MM, Mon AM, Hein PP, et al. Investigation on the Antibacterial and Anti-T3SS Activity of Traditional Myanmar Medicinal Plants. Evidence-based complementary and alternative medicine : eCAM. 2018;2018:2812908.
149. Li J, Sun W, Guo Z, Lu C, Shen Y. Fusaric acid modulates Type Three Secretion System of *Salmonella enterica* serovar Typhimurium. . Biochemical and biophysical research communications. 2014;449(4):455-9.

150. Zhang Y, Liu Y, Qiu J, Luo ZQ, Deng X. The Herbal Compound Thymol Protects Mice From Lethal Infection by *Salmonella* Typhimurium. Frontiers in microbiology. 2018;9:1022.
151. Ross ZM, O'Gara EA, Hill DJ, Sleightholme HV, Maslin DJ. Antimicrobial properties of garlic oil against human enteric bacteria: evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. Applied and environmental microbiology. 2001;67(1):475-80.
152. Okiki A, Muhammad A, Ogunmefun O. Anti-inflammatory actions and *Salmonella* Typhimurium-bacteraemia clearance by methanol extract of *Curcuma longa* Linn. (Turmeric). Malays J Microbiol. 2019;51:24-33.
153. Mishra US, Mishra A, Kumari R, Murthy PN, Naik BS. Antibacterial Activity of Ethanol Extract of *Andrographis paniculata*. Indian journal of pharmaceutical sciences. 2009;71(4):436-8.
154. Singh A, Maqsood Ahamad Khan M, Sahu D, Vishwakarma N, Yadav A, Singh DAN. Pharmacological and Anti-bacterial Activities of the leaves of *Andrographis paniculata* Nees.. J Pharmacogn Phytochem. 2017;6(418-420).
155. Altemimi A, Lakhssassi N, Baharlouei A, Watson DG, Lightfoot DA. Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. Plants (Basel, Switzerland). 2017;6(4).
156. Ramadwa TE, Awouafack MD, Sonopo MS, Eloff JN. Antibacterial and Antimycobacterial Activity of Crude Extracts, Fractions, and Isolated Compounds From Leaves of Sneezewood, *Ptaeroxylon obliquum* (Rutaceae). Nat Prod Commun. 2019;14(11):1934578X19872927.
157. Hewlings SJ, Kalman DS. Curcumin: A Review of Its Effects on Human Health. Foods (Basel, Switzerland). 2017;6(10).
158. Marathe SA, Ray S, Chakravortty D. Curcumin increases the pathogenicity of *Salmonella enterica* serovar Typhimurium in murine model. PloS one. 2010;5(7):e11511.
159. Gorinstein S, Leontowicz H, Leontowicz M, Namiesnik J, Najman K, Drzewiecki J, et al. Comparison of the main bioactive compounds and antioxidant activities in garlic and white and red onions after treatment protocols. Journal of agricultural and food chemistry. 2008;56(12):4418-26.
160. Jun X, Fu P, Lei Y, Cheng P. Pharmacological effects of medicinal components of *Atractylodes lancea* (Thunb.) DC. Chinese medicine. 2018;13:59.
161. Chao WW, Lin BF. Bioactivities of major constituents isolated from *Angelica sinensis* (Danggui). Chinese medicine. 2011;6:29.

Appendix

Appendix A Culture media and reagents for bacterial growth

1. Laria-Bertani (LB) broth

Tryptone	10.00 g
Sodium Chloride (NaCl)	10.00 g
Yeast Extract	5.00 g
Deionized H_2O to	1.00 L

2. Laria-Bertani (LB) agar

Tryptone	10.00 g
Sodium Chloride (NaCl)	10.00 g
Yeast Extract	5.00 g
Bacteriological agar	15.00 g
Deionized H_2O to	1.00 L

Stream sterilization by 121ºC, 15 pounds per square inch of pressure for at least 15 min. Sterile medium was allowed to cool to 50ºC. Stock antibiotic was added as working antibiotic that showed in table 12.

Table 12 Stock and working concentration of antibiotic in bacterial culture.

Antibiotics	Stock concentration	Working concentration
Ampicillin	100 mg/ml	100 µg/ml
Kanamycin	50 mg/ml	50 µg/ml

3. Laria-Bertani (LB) broth for SPI-1 inducing

Tryptone	10.00 g
NaCl (0.3 M)	17.53 g
Yeast Extract	5.00 g
Deionized H_2O to	1.00 L

4. TB buffer, pH 6.7

$CaCl_2$	242.00 g
KCl	18.61 g
$MnCl_2$	10.00 µL
Deionized H_2O to	1.00 L

$CaCl_2$ and KCl were dissolved in 500 ml of deionized H_2O and then adjust pH to 6.7. After that, $MnCl_2$ was added into the solution. Finally, deionized H_2O was added to 1 L and then sterilized by filtration through 0.22 µm pore size of filter membrane.

Appendix B Reagents for molecular biological techniques

1. 50x TAE buffer

Tris-free base	242.00 g
EDTA	18.61 g
Acetic acid	57.10 mL
Deionized H_2O to	1.00 L

2. Ethidium Bromide (EtBr) solution

10 mg/mL EtBr stock solution	1.00 mL
Deionized H_2O to	100.00 mL

3. 6x DNA loading buffer

6x DNA loading buffer	1.00 µL
DNA sample	5.00 µL

4. 10%(w/v) trichloroacetic acid (TCA) solution

TCA	10.00 g
Protein sample up to	90.00 mL

5. 10%(w/v) Sodium dodecyl sulfate (SDS) solution (100 ml)

SDS	10.00 mL
Deionized H_2O to	100.00 mL

6. 1 M Tris-HCl, pH 7.6 (100 ml)

Tris-free base	12.11 g
Deionized H_2O to	80.00 mL

The solution was mixed and then adjust pH to 7.6. Finally, deionized H_2O was added to 100 mL.

7. 0.5 M Tris-HCl, pH 6.8 (100 ml)

Tris-free base	6.06 g
Deionized H_2O to	80.00 mL

The solution was mixed and then adjust pH to 6.8. Finally, deionized H_2O was added to 100 mL

8. 10% (w/v) Ammonium persulfate [$(NH_4)_2S_2O_8$] solution

[$(NH_4)_2S_2O_8$]	0.10 g
Deionized H_2O to	1.00 mL

9. 2x SDS gel loading buffer

0.5 M Tris-HCl, pH 6.8	1.25 mL
10 % (w/v) SDS solution	2.00 mL
0.5 % (w/v) Bromophenol blue solution	0.20 mL
Glycerol	2.50 mL
Deionized H_2O to	10.00 mL

10. 1X SDS running buffer Tris

Tris	3.30 g
Glycine	4.40 g
SDS	1.00 g
Deionized H_2O to	1.00 L

11. Towbin transfer buffer

Tris	3.30 g
Glycine	14.40 g
Methanol	200.00 mL
Deionized H_2O to	1.00 L

12. 4% Stacking SDS-polyacrylamide gel

40% Acrylamide & Bis-acrylamide solution	0.50 mL
0.5 M Tris-HCl solution, pH 6.8	0.50 mL
10% (w/v) SDS solution	40.00 µL
10% (w/v) ammonium persulfate solution	40.00 µL
Deionized H_2O to	5.00 mL
TEMED	5.00 µL

13. 10% Separating SDS-polyacrylamide gel

40% Acrylamide & Bis-acrylamide solution	2.50 mL
0.5 M Tris-HCl solution, pH 8.8	2.50 mL
10% (w/v) SDS solution	100.00 µL
10% (w/v) ammonium persulfate solution	100.00 µL
Deionized H_2O to	5.00 mL
TEMED	10.00 µL

14. Tris-buffered saline-0.1 % Tween-20 (TBS-T), pH 7.6

Tris	6.07 g
NaCl	8.76 g
Deionized H_2O to	800.00 mL

The solution was mixed and adjust pH to 7.6. Then, deionized H_2O was added to 1000 mL. After that, Tweet-20 was add to the solution 1 mL (0.1%). Finally, the solution was sterilized by autoclave at 121 °C, 15 pounds per square inch of pressure for at least 15 minutes.

15. 5% (w/v) Skim-milk blocking solution

Skim-milk	5.00 g
TBS-T to	100.00 mL

16. Coomassie brilliant blue staining solution

Coomassie brilliant blue R-250	1.00 g
Methanol	400.00 mL
Glacial acetic acid	50.00 mL
Deionized H_2O to	1.00 L

17. Destaining solution

Methanol	400.00 mL
Glacial acetic acid	100.00 mL
Deionized H_2O to	1.00 L

Appendix C Culture media and reagents for cell culture assay

1. Completed Dulbecco's Modified Eagle Medium (DMEM), pH 7.4

powdered medium	1 package
Sodium Bicarbonate (NaHCO3)	3.70 g
Heated-Fetal Bovine Albumin (FBS)	100.00 mL
Deionized H_2O to	1.00 L

The solution was mixed and then adjust pH to 7.4. Finally, the solution was sterilized by filtration through a 0.22 μm pore size of filter membrane. Stock antibiotic was added as working antibiotic that showed in table 13.

Table 13 Stock and working concentration of antibiotic in cell culture.

Antibiotics	Stock concentration	Working concentration
Penicillin	10,000 I.U./mL Penicillin	100 I.U./mL Penicillin
Gentamycin	100 mg/ml	50 μg/ml

2. Phosphate Buffered Saline (PBS), pH 7.4

Sodium chloride (NaCl)	8.00 g
Potassium chloride (KCl)	0.20 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.44 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.24 g
Deionized H_2O to	800.00 mL

The solution was mixed and adjust pH to 7.4. Then, deionized H_2O was added to 1000 mL. Finally, the solution was sterilized by autoclave at 121 °C, 15 pounds per square inch of pressure for at least 15 minutes.

3. Bacterial lysis buffer

Triton-X 100	1.00 mL
10% (w/v) SDS solution	0.01 mL
Deionized H_2O to	100.00 mL

Appendix D Thai herbal extracts

1. Garlic and chantaleela extract tablets

Garlic and chantaleela extract tablets in figure 50 are manufactured and distributed by Khaolaor Laboratoies CO., TD. Each tablet of garlic contains 200 mg of garlic extract powder which include 2,000 µg of allicin corresponding to 1% T.A.P (Total Allicin Potency). While, each tablet of chantaleela contains 400 mg of 9 extract powders including *Artemisia vulgaris* L., *Atractylodes lyrata*, Sieb.et Zucc, *Tinospora tuberculate* Beumee, *Dracaena loureiri* Gagnep, etc.

Figure 50 Garlic and chantaleela extract tablets
Left figure : Garlic extract tablet and Right figure : Chantaleela extract tablet

2. Turmeric and king of bitters extract capsules

Turmeric extract capsules in figure 51 are manufactured and distributed by Chao Phya Abhaibhubejhr Foundation. Each capsule contains 500 mg of turmeric extract powder. While, king of bitters extract capsules in figure 51 are manufactured and distributed by Khaolaor Laboratoies CO., TD. Each capsule contains 9 mg of Andrographolides in the extract powder.

Figure 51 Turmeric and king of bitters extract capsules
Left figure : Turmeric extract tablet and Right figure : King of bitters extract tablet