



Thai herbal extracts from garlic and turmeric suppress the type 3 secretion system of *Salmonella* Typhimurium

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ABSTRACT

Introduction: In Thailand, garlic and turmeric have been used widely as basic spices in Thai food and traditional medicine. Previous studies reported that both of them had antibacterial activities. A few data have shown that extracts from both herbs are type 3 secretion system (T3SS) inhibitors. Therefore, this study aimed to investigate anti-T3SS in garlic and turmeric extracts and identify their specific mechanisms.

Methods: *Salmonella* Typhimurium containing chromosomally infusion between the gene encoding SipA effector protein and strep-tag epitope was used for the determination of anti-T3SS in garlic and turmeric extracts. The mechanism of inhibition was identified by the determination of mRNA expression of T3SS genes with semiquantitative RT-PCR.

Results: Garlic and turmeric extracts contained T3SS inhibitory activity at the concentrations of 100 µg/mL and 75 µg/mL, respectively. These extracts reduced the ability of bacterial invasion into epithelial cell cultures. However, the effective dose of both extracts did not affect bacterial growth or toxic effects on HeLa cells. Moreover, the results from RNA transcriptional levels illustrated that these extracts suppressed the transcription of the T3SS regulation genes.

Conclusion: It may conclude that garlic and turmeric extracts blocked T3S activity for the secretion of effector proteins and bacterial invasion by interfering the expression of the T3SS regulatory cascade. Therefore, the extracts from garlic and turmeric might be potential sources for the development of new anti-T3SS therapeutic agents.

Implication for health policy/practice/research/medical education:

Garlic and turmeric extracts exhibited anti-T3SS in *Salmonella* Typhimurium by interruption of SPI-1 T3SS regulatory genes expression. Hence, these herbs have promising values to develop novel anti-T3SS therapeutic agents.

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Introduction

Antibiotic-resistant bacteria are a major problem in human health worldwide. They cause more complications, mortality, and cost of treatment (1,2). World Health Organization (WHO) predicted that in 2050 about 28.3 million people would be pushed into extreme poverty by drug resistance and 10 million people would be died from drug-resistant infection (3,4). The development of new agents for the treatment of drug-resistant organisms is important for the solution of this problem (5). Hence, research and development of novel antibiotics have become compulsory. This antimicrobial treatment might lead bacteria to emerge drug resistance. Therefore, several studies have been conducted to find new targets for the inhibition of bacterial pathogenesis or virulence factors

without direct killing of bacteria, for example, toxin, pili, and secretion systems (6).

Type 3 secretion system (T3SS) is one of attractive targets for new anti-bacterial drugs, because T3SS is a primitive bacterial virulent factor secretion system. Moreover, T3SS has been highly conserved in many pathogenic gram-negative bacteria including *Shigella* spp., Enteropathogenic *Escherichia coli* (EPEC), *Yersinia* spp., *Chlamydia* spp., and *Salmonella* spp. The structure of T3SS is a needle-like shape made up of more than 20 proteins (7).

Salmonella enterica serovar Typhimurium causes disease in humans as intestinal diseases ranging from gastroenteritis, usually lasting 3-5 days, to more debilitating enteritis lasting 2-3 weeks (8). These bacteria have two

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distinct T3SSs encoded by *Salmonella* pathogenicity island 1 and 2 (SPI-1 and SPI-2). The SPI-1 T3SS is expressed during the initial interaction of bacteria with intestinal cells. This T3SS delivers effector proteins from bacteria cells to host cells for bacterial invasion, whereas the SPI-2 T3SS is required for systemic infection. Inside the host cells, the bacterial SPI-2 T3SS mediated effector proteins for bacterial survival and replication within host cells (9).

In recent years SPI-encoded T3SS of *S. Typhimurium* was used as a target for the development of therapeutic drugs (10). Some natural products have been identified as SPI-1 T3SS inhibitors, such as Myricanol and Thymol. Myricanol is isolated from medicinal plant *Myrica nagi* in South and East Asia. The Myricanol was able to inhibit the activity of SPI-1 T3SS for the secretion of effector proteins and bacterial invasion. Moreover, in this study it was found that the mechanism of myricanol for the inhibition of SPI-1 T3SS is interfering with the DNA binding activity of HilD to the promoters of the *hilA* and *invF* genes (11). While Thymol is a compound from traditional Chinese medicine (TCM), this compound inhibits SPI-1 T3SS for bacterial invasion and protects mice from infection (12). In addition, several SPI-1 T3SS inhibitors, such as salicylidene, Acyl hydrazides, and cytosporone B have been identified from natural or chemical synthesis (10,13).

Herbs have been used widely in Thailand as basic spices in Thai foods, ingredients in cosmetics, and health supplements (14). Previous studies have shown that herbs usually possess several phytochemicals, including polyphenols, flavonoids, flavanols, tannins, and terpenoids (15). Many herbs were identified as antimicrobial agents or T3SS inhibitors (13,16). We selected garlic and turmeric, common kitchen spices and health supplements in Thailand. These herbs showed inhibition activity for bacterial growth such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (17,18). Moreover, the purified curcumin from turmeric showed the inhibitory effect of the secretion of SPI-1 T3SS effector protein, a SopD in *S. Typhimurium* (19). This study aims to investigate biological properties of garlic and turmeric extracts as anti-T3SS and the mechanisms in *S. Typhimurium*.

Materials and Methods

Cell line and bacterial cell culture condition

Cell lines and bacterial strains used in this study are listed in Table 1. The human cervical carcinoma Hela cells were grown at 37°C with 5% CO₂ in the DMEM containing 10%

fetal bovine serum (FBS).

Salmonella Typhimurium isogenic mutants were grown in Luria-Bertani (LB) medium overnight and diluted 1:100 into fresh medium with 0.3M NaCl for the induction of SPI-1 expression condition. Bacterial cells were grown for 4 hours at 37°C, and 200 rpm.

Preparation of herbal extracts

Garlic tablets (*Allium sativum*) were purchased from the commercial vendor Khaolaor Laboratories Co., Ltd., Thailand. Each tablet of garlic contained 200 mg of garlic extract powder, while the turmeric (*Curcuma longa*) extract capsules were purchased from Chao Phraya Abhaibhubejhr Hospital Foundation, Thailand. Each capsule contained 500 mg of turmeric extract powder. Both herbal extracts were grounded and dissolved in DMSO at a concentration of 100 mg/mL for stock solution. The newly prepared stock solutions were stored at -80°C.

Western blot analysis

Salmonella Typhimurium SA strain encoding strep-tag epitope on C-terminus of SipA effector protein was grown in SPI-1 induction condition. Various concentrations of the herbal extract were added to the culture medium. The negative control was added DMSO without any herbal extracts. After incubation for 4 hours at 37°C, culture supernatants and bacterial cells were separated by centrifugation at 14000 × g for 10 minutes. The proteins in culture supernatant were precipitated with acetone and 10% TCA after filtering the supernatant with 0.22 μm pore size. The precipitated proteins were separated on SDS-PAGE and analysed by Western blotting with polyclonal antibody against strep-tag (IBA Lifesciences, Germany) and goat anti-mouse horseradish peroxidase-labeled secondary antibody (Sigma-Aldrich, USA) for the detection of SipA strep-tag fusion proteins. Then, detecting was carried out using a Immobilon Classico Western HRP substrate kit (Merck, USA). The chemiluminescent signal was detected by ChemiDoc™ MP Imaging System (Bio-Rad, USA).

Measurement of bacterial growth

Salmonella Typhimurium SL1344 was cultured overnight in LB broth at 37°C. An overnight culture was diluted 100-fold in the wells of a 96-well microtiter plate containing fresh LB broth supplemented with herbal extract. The plates were incubated in a plate reader (SpectraMax

Table 1. Cell lines and bacterial strains used in this study

Cell line or bacterial strain	Properties	Reference or source
Hela cells	Human cervical carcinoma cell line	American Type Culture Collection
<i>Salmonella</i> Typhimurium strains		
SL1344	Wild-type	<i>Salmonella</i> Genetic Stock Center
SA	<i>sipA</i> ::strep-tag	Boonyom et al (20)
SW003	<i>invA</i> ::kan ^R	Boonyom et al (21)

iD3; Molecular Devices, USA) for 24 hours at 37°C. The measurement of optical density at 600 nm (OD_{600}) was carried out every hour. Bacterial cells were grown in LB medium with DMSO as negative control.

Cytotoxicity assay

The HeLa cells (approximately 5000 cells) were seeded in 96-well culture plate, supplemented with DMEM medium, and incubated with 5% CO_2 at 37°C overnight. Culture mediums were removed and replaced with DMEM medium with various concentrations of herbal extracts. The treated cells were incubated with 5% CO_2 at 37°C for 24 hours. The viability of treated cells was measured with MTT assay. The cells treated with DMSO were served as a negative control.

Bacterial invasion assay

This protocol was modified from Steele-Mortimer, O's protocol (22). The HeLa cells (approximately 50 000 cells) were seeded in 24-well culture plate containing DMEM medium and incubated overnight with 5% CO_2 at 37°C. HeLa cells were infected by bacteria pretreated with herbal extract at a multiplicity of infection (MOI) of 10 at 37°C, 5% CO_2 . After incubation for 30 minutes for bacterial invasion into cells, the cells were washed with PBS and DMEM containing 100 μ g/mL of gentamicin to kill extracellular bacteria. Then, the infected cells were washed twice with PBS and lysed by solubilizing buffer. The intracellular bacteria were counted by plating on LB agar. *S. Typhimurium* strain SW003 was used as a positive control, while DMSO-treated *S. Typhimurium* SL1344 was conducted as a negative control.

Real-time PCR

Total RNA of bacteria treated with herbal extracts or DMSO at 37°C for 4 hours was isolated with the Total RNA Extraction Kit Mini (RBC Bioscience Corp., Taiwan). For real-time PCR analysis, 1 μ g of DNase-treated bacterial RNA was synthesized to cDNA by the Tetro cDNA Synthesis Kit (Meridian Bioscience Inc., USA) according to the manufacturer protocol. The SYBR green real-time PCR assay was performed in a reaction volume of 25 μ L containing 12.5 μ L of SYBR premix (SensiFAST™, Meridian Bioscience Inc., USA), 0.5 μ M of each specific primer set (Table 2), and 2 μ L of cDNA. The LineGene 9600 Plus cycler (Hangzhou Bioer Technology Co., Ltd., China) was programmed for 45 cycles at 95°C for 5 seconds, 57°C for 10 seconds, and 72°C for 35 seconds. The comparative cycle threshold (CT) method was used for the analysis of relative changes in transcriptional levels. The mRNA expression level of *spi-1* genes was normalized to the 16S rRNA expression level.

Statistical analysis

The experiments were performed in triplicate and the data were analyzed using GraphPad Prism 5 (GraphPad, CA).

Table 2. Primers for qRT-PCR in this study

Gene name	Primer sequence (5'-3')	Product size (bp)
<i>hilD</i>	TCTGCTGTTCTGCTTACTG TTGCTGCCTGATTCATTCTTG	248
<i>hilC</i>	ATGTCCACGGGTTTGTAGTAAT GATTCATACGAGCATCCAGGTA	296
<i>hilA</i>	GCTGTGGATTACCTATTATCATAACC AAGGCACGCTCACTATTCTG	243
<i>invF</i>	GCGAGAGTTACTGGTTGGTT TCTACACTATTGAGCAGCGATT	190
<i>invH</i>	GTGTCAGTCTCTCCGATATGT ACGCATGATTGTGGATGTTTC	181
<i>prgI</i>	CTTGCTCAGGCTATCTGGATGACG AAAGCTTACGGAAGTTCTGAATAATGGCAGC	238
<i>sicA</i>	ATGGATTATCAAAATAATGTCAGCG TCTGTCTCCGCCGTTTTAGCGCC	470
<i>sipA</i>	AGAGGCTGTGACCAACGCTTCAT CGTGTCTGATTGTAAGGTATCGGTTT	268
16S	GTTGGTGAGGTAACGGCTCACCAAGG CTCCACCGCTTGTGCGGGCCCCCGTC	697

The data were presented as mean and standard deviation (SD). The one-way and two-way ANOVA methods were used to consider the significance at a *P* value of <0.01.

Results

Extracts from garlic and turmeric inhibited the activity of SPI-1 T3SS but did not affect bacterial growth

Firstly, the inhibition of SPI-1 T3SS of garlic and turmeric extracts was investigated. *S. Typhimurium* SA strain was grown in media in presence of garlic or turmeric extract at final concentrations of 25, 50, 75, and 100 μ g/mL for 4 hours. Proteins from supernatants were detected for SipA strep-tag protein by strep-tag specific antibody. Results from Western blotting showed that the signal of SipA strep-tag protein was absent in bacterial culture medium treated with garlic extract at a concentration of 100 μ g/mL (Figure 1A), while the turmeric extract with final concentration of 75 μ g/mL inhibited SipA strep-tag secretion through SPI-1 T3SS (Figure 1B). Additionally, we studied the effect of garlic extract (100 μ g/mL) and turmeric extract (75 μ g/mL) on bacterial growth. Bacteria were grown in media supplement with herbal extract for 24 hours. The results showed no apparent differences between bacterial growth rates in herbal treatment groups and DMSO control (Figure 1C). It indicated that garlic and turmeric extracts had anti-T3SS activity but not antimicrobial activity.

Garlic and turmeric extracts exhibited anti-invasion activities

The effector protein, SipA, has been shown to potentiate SipC for the regulation of membrane ruffling and bacterial entry into the host cells (23). The above results demonstrated that the extract of garlic and turmeric were able to abolish function of SPI-1 T3SS. We expected that these herbal extracts might affect on bacterial invasion of

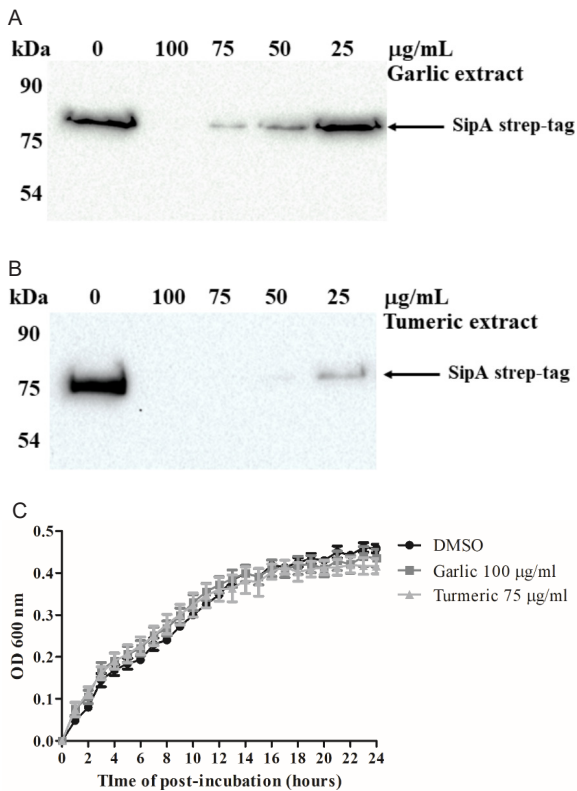


Figure 1. Garlic and turmeric extracts interrupted SPI-1 T3S but did not interfere bacterial growth. The results from Western blotting indicated that (A) Garlic (100 µg/mL) and (B) Turmeric (75 µg/mL) extracts inhibited T3SS. (C) Both of garlic and turmeric extracts had no inhibitory effect on bacterial growth. The control was bacterial culture with DMSO.

host cells. Before bacterial invasion assay, we determined the toxicity of garlic and turmeric extracts against mammalian cell cultures at the concentrations of 100 and 75 µg/mL, respectively. The results from cytotoxicity assay showed that both extracts did not interfere to cell viability after 24 hours of treatment in Hela cells (Figure 2A). Result from bacterial invasion assay demonstrated that *S. Typhimurium* treated with garlic (100 µg/mL) or turmeric (75 µg/mL) extract showed significantly reduced capacity to invade cultured epithelial cells compared with the control group (approximately 50% reduction at 30 minutes after inoculation; Figure 2B). It indicated that disruption of bacterial SipA secretion with garlic and turmeric extracts causes loss of bacterial invasion.

Garlic and turmeric extracts suppressed mRNA levels of SPI-1 T3SS genes

To further identify the inhibition mechanism of garlic and turmeric extracts on SPI-1 T3SS, we analyzed the mRNA levels of the related regulatory, structural, effector proteins, and chaperone of SPI-1 T3SS genes by the quantitative real-time PCR (qRT-PCR). The relative transcriptional levels of T3SS structural (*invH* and *prgI*), chaperone (*sicA*), and effector (*sipA*) genes in herbal extracts-treated groups were lower than the levels in the

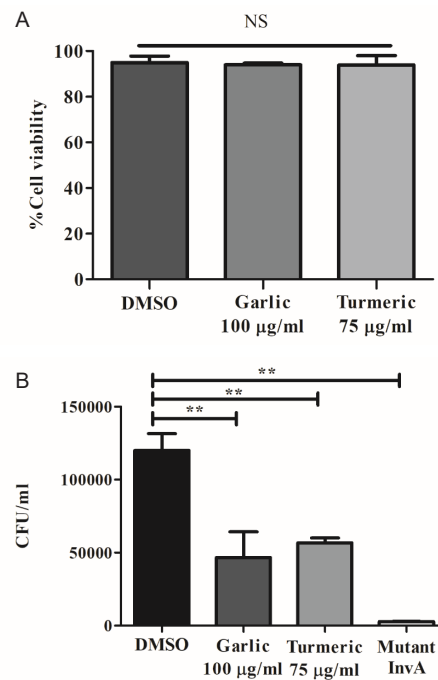


Figure 2. The extracts from garlic and turmeric reduced bacterial invasion and were non-toxic to cells. Garlic (100 µg/mL) and turmeric (75 µg/mL) extracts had no cytotoxic effects on mammalian cells (A). However, these extracts decreased the ability of bacteria to enter cells (B). The standard deviations from three individual experiments are indicated. The P values were compared and calculated between samples and wild-type of *Salmonella* treatment with or without herbal extract. The level of statistical significance is shown as: ** $P < 0.01$ and ^{NS} $P > 0.01$. The SPI-1 T3SS negative strain (*invA::kan^R*) was used as control.

DMSO-treated group. Moreover, the relative levels of mRNA encoding for regulatory genes (*hilD*, *hilA*, *hilC*, and *invF*) were suppressed by both herbal extracts (Figure 3A). From the above results it can be confirmed that garlic and turmeric extracts appear to repress the transcription of the feed-forward regulatory loop complex of T3SS (Figure 3B). This repression affects the expression of SPI-1-T3SS hierarchy through the reduction of *hilA* regulation.

Discussion

At the present time, antibiotic-resistant bacteria are the major problem of human health worldwide (1, 2). Therefore, there are alternative substances for the inhibition of bacterial virulence instead of killing the bacteria. This study focused on the inhibition of T3SS. This system is required for bacteria to invade the host cells. In this study, we selected common 2 Thai herbs, including garlic and turmeric for determining their efficiencies to inhibit T3SS activity.

This study demonstrated that the lowest concentrations for SPI1-T3SS inhibition of garlic and turmeric extracts were 100 and 75 µg/mL, respectively. In addition, at these concentrations did not affect the bacterial growth of *S. Typhimurium*. It showed that garlic and turmeric extracts were identified as T3SS inhibitors. Moreover, garlic and

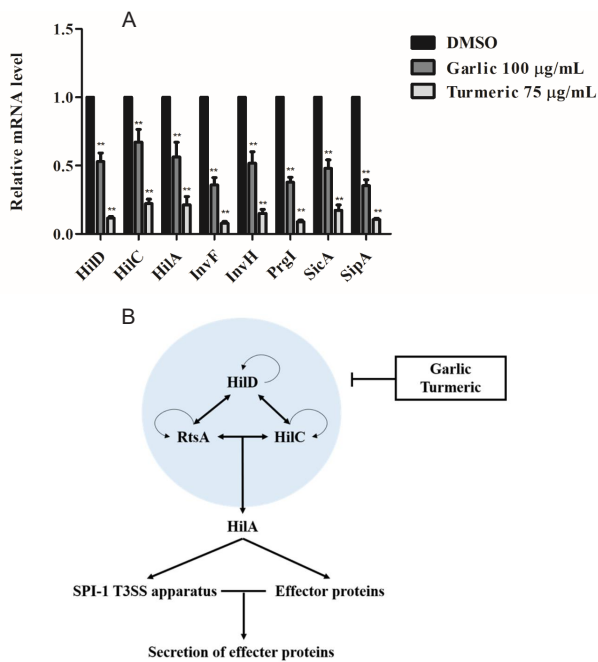


Figure 3. The extracts from garlic and turmeric repressed transcription of SPI-1 T3SS genes. (A) The transcriptional levels of T3SS regulators, apparatuses, chaperones, and effector genes were reduced in bacteria culture with garlic or Turmeric extract. Asterisks indicate significantly different ($P < 0.01$) levels between *Salmonella* treatment with or without extracts. (B) The proposed inhibition pathways illustrated that garlic and turmeric extracts down-regulated SPI-1 T3SS expression by interference of forward regulatory loop.

turmeric extracts suppressed the activity of SPI-1 T3SS through the inhibition of activity in the feed-forward regulatory loop of HilC–RtsA–HilD. This loop is the main part of regulatory networks to control the transcription of *hilA* gene whereas HilA is the central regulator of SPI-1 (24,25). From this reason, decreasing the expression of *hilD* in the loop reduced *hilA* expression. A fall in the level of HilA reduced the assembly of SPI-1 T3SS apparatus through repression of *prg/org* genes expression. Moreover, it reduced the secretion of effector proteins through the repression of *inv/spa* genes expression (Figure 3B). Therefore, T3SS of the bacteria can not completely function for secretion of effector proteins and pathogenesis. The mechanism of garlic and turmeric extracts for the inhibition of SPI-1 T3SS is similar to the study of Shi et al. They showed that fraxetin isolated from *Fraxinus spp.* affected the transcription and expression of the feed-forward regulatory loop of T3SS (26).

Although this study showed that garlic and turmeric did not affect the bacterial growth of *S. Typhimurium*, the previous studies found that garlic and turmeric had the ability to inhibit microbial growth. For example, garlic extracts at a final concentration of 340 µg/mL was minimal inhibitory concentration (MIC) to inhibit bacterial growth of *S. Typhimurium* (27), while turmeric extract had MIC at 560 µg/mL for killing *S. Typhimurium*

(28). Therefore, garlic and turmeric extracts have both antibacterial and anti-T3SS activities for *S. Typhimurium*. These activities of both herbs depend on the concentration of them. It is similar to the study of Zhang et al. They found that thymol had not only antibacterial activity but also anti-T3SS activity. Thymol at MICs ranging from 1 to 5 mM had antimicrobial activity for the different bacterial strains. However, thymol at 0.2 mM showed the inhibition activity of SPI-1 T3SS in *S. Typhimurium* for effector translocation and bacterial invasion but had not antibacterial activity (12).

Garlic and turmeric extracts inhibited activity of SPI-1 T3SS in *S. Typhimurium* as the T3SS inhibitors. It means that bioactive compounds in both herbs showed the ability of anti-T3SS. We recommend that garlic and turmeric extracts should be isolated and identified bioactive compounds for action on the T3SS activity.

Conclusion

The findings of this study suggest that the extracts from garlic and turmeric were new sources to develop anti-T3SS therapeutic agents. However, further studies are needed to determine the bioactive compounds for T3SS inhibitors.

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Authors' contribution

PT contributed to the practical laboratory work, interpretation, and original article drafting. NS contributed to the practical laboratory work, and RB contributed to the conceptualization, research design, and article editing. The authors have read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Ethical considerations

Ethical issues regarding authorship, review, and data analysis have been carefully observed by the authors. All experiment protocols were approved by Naresuan University Institutional Biosafety Committee (review protocol No. NUIBC MI 61-04-18) for laboratory biosafety.

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