

Inhibition of *Salmonella enterica* serovar Typhimurium Type Δ Secretion System and Infection Using Small Molecule Quercitrin

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Abstract

Aims

This study was conducted to screen the type III secretion system (T3SS) inhibitors of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) from natural compounds. Through systemic analysis the pharmacological activity and action mechanism of candidate compounds *in vivo* and *in vitro*.

Methods and results

Using an effector- β -lactamase fusion reporter system in *S. Typhimurium*, we discovered that quercitrin could block effector SipA translocation into eukaryotic host cell without affecting bacterial growth, and inhibit invasion or epithelial cells damage. Using β -galactosidase activity and Western blot assay, it was found that quercitrin significantly inhibits the expression of SPI-1 genes (*hilA* and *sopA*) and effectors (SipA and SipC). The animal experiment results indicated that quercitrin reduces mortality, pathological damages and colony colonization of infected mice.

Conclusions

Small-molecule inhibitor quercitrin directly inhibits the function of T3SS in *S. Typhimurium*, and provides a potential alternative antimicrobial against *Salmonella* infection.

Significance and impact of the study

Natural compounds have become valuable resources for antibacterials discovery due to their widely structures and biological activities. However, the potential targets and molecular action mechanisms of candidate compounds responsible for anti-infections remain elusive. The T3SS plays a crucial role in bacterial invasion and pathogenesis process in *S. Typhimurium*. Compared with traditional antibiotics, small molecular compounds can inhibit the T3SS of *Salmonella* and achieve the effect of anti-infection. They have less pressure on bacterial survival and are not easy to produce drug resistance. This provides strong evidence for development novel anti-virulence drugs against *Salmonella* infection.

Introduction

Salmonella is a Gram-negative enterobacter commonly found in external environment, that also exists in the intestinal tract of humans and animals¹. *Salmonella* has a wide range of hosts, mainly cause animal diseases such as chicken, pigs, horses, cattle and sheep, and cause contamination of animal food. It mainly causes human diseases such as typhoid, paratyphoid and gastroenteritis through the faecal-oral pathway². Salmonellosis is one of the most common food-borne diseases in humans³. Typhoid fever caused by *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) remains a significant human health problem, especially in developing countries³. In short, *Salmonella* not only hinders the development of the livestock industry and endangers food safety, but also causes panic about public health safety issues.

Antibiotics are still the first choice against *Salmonella .spp* infections. However, antibiotic resistance leads to a huge challenge to preventing the spreading of infection disease of *Salmonella* ⁴. New anti-infection therapies are urgently needed, such as using alternative drugs to prevent or treat pathogens infections. *S. Typhimurium* induces inflammatory diarrhoea and invades non-phagocytic epithelial cells using the type III secretion system (T3SS) encoded by SPI-1 ⁵, which is critical to bacteria infection. The T3SS is a needle-like structure that injects several effectors into eukaryotic host cytoplasm ⁶. Structural genes for the functional T3SS device assembling and effectors are encoded in the SPI-1 *prg/ org, inv/ spa*, and *sic/ sip* operons, meanwhile three AraC-like regulators, *hilD*, *hilC*, and *rtsA*, control the expression of *hilA* in SPI-1 signal cascade pathway activation ⁷. HilD is the dominant regulator, while HilC and RtsA act as the signal amplifiers ⁸. SipA could bind to host cells actin and promote bacterial internalization ⁹. The killing effect of macrophages is caused by *Salmonella*-induced apoptosis and occurs after caspase-1 is activated by SipB ¹⁰. SipC is responsible for the translocation of effectors and regulation of actin ¹¹. Thus, T3SS has become an attractive drug screening target to reduce the risk of antimicrobial resistance by indirect sterilization or antimicrobial dependence manner while treating bacterial infection.

In this study, quercitrin, a glycosylated flavonoid isolated from whole grass of *Hypericum perforatum*, which could significantly inhibit the biological function of *Salmonella* T3SS. In addition, its pharmacological activity *in vitro* was evaluated by eukaryotic cell adhesion, invasion and cell damage assay, and the protective effect was confirmed *in vivo*. In conclusion, quercitrin could be used as a potential drug for prevention and treatment of *Salmonella* infection.

Materials And Methods

Bacterial strains, growth conditions, and natural compounds

The wild-type *S. Typhimurium* SL1344 was donated by Professor Xiaoyun Liu of Peking University. In accordance with previous study, InvA is a prominent inner-membrane component of the T3SS apparatus, which is responsible for regulating virulence protein export in pathogenic bacteria ¹². We used *invA* mutant as negative control involved in encoding key structural proteins of T3SS-1. The *hilA::lacZ* (JS749) and *sopA::lacZ* (JS751) strains were provided by Dr James Slauch from University of Illinois. *S. Typhimurium* SL1344-SipA-3×Flag is produced by inserting PKS 3×Flag into chromosome ¹³. Bacteria were stored at -80°C in Luria-Bertani (LB) broth containing 40% glycerol and overnight cultures grown in LB broth at 37°C with aeration. All used natural compounds including quercitrin are from the candidate compounds libraries constructed and preserved in our laboratory, which are purchased from Heibpurify (Chengdu, China). Unless otherwise specified, it is generally prepared at 40 mg/ml of dimethyl sulfoxide (DMSO) solution and stored at 4°C.

High-throughput screening for natural compounds T3SS inhibitors by effector-β-lactamase fusion reporter system

HeLa cells were plated into 96-well plates at a density of 1.2×10^4 cells/well and incubated overnight before infection. *S. Typhimurium* SL1344 containing SipA- β -lactamase fusion plasmid and the *invA* mutant grown overnight at 37°C in LB (0.3 M NaCl to activate T3SS) and diluted by 1:20 in the presence of quercitrin with DMSO as controls. After incubation for 4 h at 37°C with shaking, the bacterial suspensions were adjusted to 3×10^6 CFUs/ml. 200 μ l of each sample was transferred into a 96-well plate, and the monolayers were infected at a multiplicity of infection (MOI) of ~ 50 for 1 h. Non-internalized bacteria were washed three times with PBS, and incubated with 100 μ l PBS containing 6 \times CCF4/AM reagent (Life Technologies, USA) for 45 min at room temperature. Fluorescence micrographs were captured and analyzed by fluorescence microscope (model: Olympus IX-81, Japan).

Determination of bacterial viability following quercitrin exposure

In order to identify the potential bactericidal or bacteriostatic effects of quercitrin, bacterial viability following quercitrin exposure determination was performed. Overnight *S. Typhimurium* SL1344 cultures were diluted 1:100 in LB for 2 h at 37°C until the optical density of 600 nm (OD_{600nm}) reached logarithmic growth phase. The culture contained different concentrations of quercitrin, and the group without quercitrin was the positive control. The OD_{600nm} were measured with a UV-2100 spectrophotometer (Unico, China) every 30 min until the stationary phase.

Cytotoxicity of quercitrin indicated by lactate dehydrogenase release

The HeLa cells were cultured in Dulbecco's Modified Eagle's Medium/high glucose (DMEM, HyClone, USA) containing 10% foetal calf serum (Biological Industries, Israel), and 1% penicillin-streptomycin (MRC, USA) at 37°C and containing 5% CO₂ for more than 24 h. The cells were inoculated into 96-well plates at a concentration of 1×10^5 cells/ml and allowed to adhere overnight. Adherent HeLa cells were subjected to 8 h of quercitrin exposure (at concentration of 0~64 μ g/ml) in triplicate. DMSO (0.1% of final concentration) and 0.2% Triton X-100 were set as the negative or positive controls. The lactate dehydrogenase (LDH) release measured by the LDH cytotoxicity detection kit (Roche, Germany) and microplate spectrophotometer (Tecan, Austria) at a wavelength of 490 nm¹⁴.

Adherence assay

In brief, HeLa cells were infected with *S. Typhimurium* at an MOI of 50. After incubation at 37°C for 20 min, the wells were washed three times to remove unattached bacteria. Then, the cells were lysed with 0.2% (v/v) Triton X-100 for 10 min and plated on LB agar plates.

Gentamicin protection and immunofluorescence internalization

The effect of quercitrin on bacterial invasion was determined by gentamicin protection with some modifications as described previously¹⁵. HeLa cells (4×10^4 cells/well) were plated in 24-well plates, and

overnight cultures of *S. Typhimurium* SL1344 were added to fresh LB containing different final concentrations of quercitrin for 4 h. HeLa cells were infected at an MOI of ~100 at 37°C in 5% CO₂ for 1 h. HeLa cells were washed three times with PBS to remove unattached bacteria, and then incubated in DMEM containing gentamicin (100 µg/ml) at 37°C for 5 min. After washing three times with PBS, cells were lysed in 0.2% (v/v) Triton X-100 to enumerate viable intracellular bacteria through a series of dilutions on LB agar plates.

For immunofluorescence analysis, infected cells on coverslips were fixed with 4% paraformaldehyde for 10 min at room temperature. After blocking for 1h, the cells were incubated with 1000-fold diluted primary antibody of *S. Typhimurium* for 1 h, and 1000-fold dilution of Alexa Fluor 488-conjugated secondary antibody (Abcam, USA) for 30 min to stain extracellular bacteria. Then, 0.3% (v/v) Triton X-100 was used to permeabilize cells for 10 min, and incubated in Alexa Fluor 594-conjugated secondary antibody (Abcam, USA) diluted 500-fold for 30 min to stain the bacteria in the cells. Finally, coverslips were mounted on slides with DAPI (Abcam, USA) for 10 min. Cell coverslips were visualized using a fluorescence microscope (Olympus IX-81, Japan).

Antibodies and western blot

Western blot analysis was used to further validate the effect of quercitrin on expression of T3SS genes and effector. Briefly, the overnight cultures of SL1344, SipA-3×Flag-SL1344 and SipB-3×Flag-SL1344 were added to fresh LB containing different concentrations of quercitrin. Each group was centrifuged at 12000 rpm for 10 min and the precipitates were collected. Resuspend the bacteria in 100µl 1×loading buffer, and boil them at 95°C for 6 min. Protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA).

The antibodies and their dilutions were as follows: anti-SipC rabbit polyclonal antibody (1:2000, laboratory preservation), anti-Flag mouse monoclonal antibody (1:2000, CW Biotech, China), anti-rabbit HRP or goat anti-mouse conjugated secondary antibodies (1:2000, Proteintech, USA), and anti-isocitrate dehydrogenase rabbit polyclonal antibody (ICDH, 1:2000), a kind gift from Pro. Zhaoqing Luo from Purdue University served as an internal control. The blots were quantified using NIH Image J software (GE Healthcare Life Sciences, UK).

β-Galactosidase activity assay

β-Galactosidase activity was determined using a previously described method¹⁶. Briefly, overnight cultures of JS749 and JS751 bacterial were added to fresh LB with or without quercitrin. Centrifuge the culture at 12000 rpm for 10 min and resuspend it in Z-buffer. Then added 20 µl 0.1% SDS and 40 µl chloroform to bacteria suspension, shake and lyse for 30 s.

Add 100 µl mixture into a 96-well plate with 3 replicates in each group. Add 20µl ONPG to initiate the reaction, incubated at room temperature for 10 min, and finally 50µl 1 M Na₂CO₃ to terminate the

reaction. The absorbance was detected at wavelength of 450 nm with a microplate reader (Tecan, Austria).

Animal experiment

All experimental protocols were reviewed and approved by the Animal Welfare and Research Ethics Committee at Jilin University (2021052625M). All animal experiments were performed in strict compliance with the guide-lines of the Animal Welfare Council of China. All efforts were made to minimize the suffering of animals, and daily health checks were performed throughout the experiments. And all the procedures were carried out in accordance with **ARRIVE guidelines**.

Female Balb/c mice (6-8-weeks old) were purchased from Changsheng BioTechnology (Liaoning, China). In order to reduce the intestinal flora, we added 5 g/L streptomycin to the drinking water three days before the experiment¹⁶. The model of *S. Typhimurium* infection was established by intragastric inoculation. The mice were divided into three groups with 10 mice in each group, including 50 mg/ml quercitrin treatment group, PBS control group and DMSO challenge group. Briefly, fasting and banning water for 8 h before infection, mice with 5×10^7 or 10^7 CFU, test survival rate and other indicators, gavage once every 12 h for 4 days. The treatment group was given 50 mg/kg quercitrin, the control group was given the same amount of PBS. Mice in each group were killed by cervical dislocation. Liver, spleen and cecum tissues were removed by aseptic operation. The organ was weighed, ground evenly with 0.2% Triton X-100, were serially diluted and plated on LB plates containing streptomycin, cultured overnight at 37°C and colonies were counted the next day. Pathological tissue sections were prepared with hematoxylin and eosin (H&E) and histopathology was performed by capturing images using an optical microscope.

Data analysis

All experiments data were conducted with least three biological replicates for analysis and statistical significance testing by using GraphPad Prism 6.0 software (La Jolla, CA). Results from treated and control samples were expressed as the mean \pm SEM and analyzed using Student's *t* tests analysis. * $P < 0.05$; ** $P < 0.01$; NS, $P > 0.05$, not significant.

Results

Screening natural compound inhibitors of *S. Typhimurium* T3SS

Seven inhibitors of the virulence factor T3SS were identified from 354 natural compounds by using the effector-lactamase fusion reporter system in *Salmonella typhimurium*, as in previous studies¹⁷. At the same time, we monitored the LDH release rate in HeLa cells after 6 hours of incubation, eliminating one compound that had a negative impact on cell viability. Then, Western Blot and β -lactamase colorimetry were used to screen quercitrin inhibiting T3SS(Figure 1).

Quercetin blocks the translocation of T3SS effector sipA

Quercitrin was screened to effectively block the translocation of SipA, the T3SS effector of *S. Typhimurium*(Figure 2A). In order to verify that the mechanism of the inhibitory activity without bactericidal or bacteriostatic, the growth curve showed that quercitrin did not inhibit the activity of bacteria in the concentration range of 4 µg/ml~32 µg/ml(Figure 2B). Our screen of natural compounds and fluorescence images showed that 16 µg/ml quercitrin significantly inhibited the translocation of T3SS effector SipA into eukaryotic host cells compared with the control group(Figure 2C).

Quercitrin inhibits *S. Typhimurium* adhesion of eukaryotic host cells in a dose-dependent manner

We then tested the inhibition ability of quercitrin to inhibit *S. Typhimurium* adhesion to cultured eukaryotic host cells¹⁸. Before the adhesion assay, the cytotoxicity of quercitrin to HeLa cells were determined by Lactate dehydrogenase (LDH) release assay. HeLa cells were co-incubated with different concentrations of quercitrin, but no cytotoxicity was observed in the concentration range of 2 µg/ml~64 µg/ml(Figure 3A). Next, bacteria cultured in the presence of quercitrin showed significant reduction in adhesion of HeLa cells in a dose-dependent manner. The adhesion inhibitory effect of quercitrin at 32 µg/ml was reduced to 15% compared to untreated group(Figure 3B).

Quercitrin inhibits *S. Typhimurium* invasion of eukaryotic host cells in a dose-dependent manner

As an intracellular bacterium, adhesion and invasion are prerequisites for bacteria-mediated eukaryotic host cell damage. 32µg/ml of quercitrin reduced *S. Typhimurium*-mediated cell damage by more than 80%(Figure 4A). The protective effect of quercitrin in HeLa cells damage was reduced up to 50% compared with untreated group(Figure 4B). As expected, the inhibition of *S. Typhimurium* invasion could be readily observed by immunofluorescence analysis of intracellular and extracellular bacteria(Figure 4C). These results demonstrated that quercitrin inhibit the T3SS-dependent *S. Typhimurium* adhesion and invasion of eukaryotic host cells without cytotoxicity.

Quercitrin inhibits expression of T3SS related genes and effectors in *S. Typhimurium*

According to the above results, quercitrin inhibited the translocation of T3SS effector SipA. Using Western Blot with chromosomal insertions of 3×Flag and wild-type SL1344 strain to prove whether quercitrin inhibited the expression of effectors. Indeed, we found that quercitrin significantly inhibited the expression of SipA and SipC, it was dose-dependent within a certain range compared with internal control ICDH(Figure 5A). Compared with the untreated group, the inhibition rates of 16 µg/ml quercitrin to SipA-3×Flag was more than 50%, and the expression of SipC, another effector responsible for translocation and actin regulation¹¹, was reduced more than 30% after co-culture with 16 µg/ml quercitrin(Figure 5B). The western blot results showed that quercitrin inhibited the T3SS effectors expression of SipA and SipC. Other important effectors were further detected by β-galactosidase activity assay. The result shown that quercitrin significantly reduced the expression of *hilA* and *sopA*, which are mainly responsible for the regulation and function of *S. Typhimurium* T3SS(Figure 5C~D).

Quercitrin reduces *S. Typhimurium* infection in mice

The streptomycin-pretreated of *S. Typhimurium* infection mouse model was established according to the previously research¹⁹. Mice were infected with the *S. Typhimurium* SL1344 by oral gavage at the dose of 1×10^7 CFUs, and control group was set without treatment. The results shown that the mortality was 80% on the 5 day, until all mice died on the 7 day. In contrast, the mortality of quercitrin treatment group was 20% until the 7 day (Figure 6A). Quercitrin not only prolonged the survival time of mice infected with *S. Typhimurium*, but also improves the survival rate of infected mice. At the same time, the macropathological changes and analysis of HE staining in cecal tissues were performed as previously described²⁰. Compared with the infected group, colony colonization in liver, spleen and cecum in quercitrin treatment group was significantly reduced (Figure 6B). The *S. Typhimurium* challenged group had severe cecal bleeding, and the blind end was significantly atrophied. There was no significant difference between the control group and the quercitrin treatment group, suggesting that quercitrin significantly reduced the intestinal damage of *S. Typhimurium* infection (Figure 6C). The histopathological analysis revealed severe submucosal edema, a loss of goblet cells, impaired epithelial integrity, and the infiltration of polymorphonuclear cells (PMN) in the lamina propria of the wild-type infection group. In contrast, the quercitrin-treated group showed mild intestinal damage and inflammation (Figure 6D). In summary, quercitrin effectively protects mice from *S. Typhimurium* infection.

Discussion

Although antibodies and vaccines against pathogens infections are further along in the pipeline, long development cycles and high economic costs prevent them from reaching the market. In recent years, antibiotic alternative strategies targeting pathogen virulence factors have shown promise. Because virulence factors, but not the growth, of many Gram-negative bacterial pathogens depends on the multi-component type three secretion system injectisome (T3SSi), the T3SSi has been an attractive target for identifying novel drugs that inhibit its function to render the pathogen avirulent.

T3SS is widely distributed in Gram-negative bacteria, and its main function is to secrete and transport pathogenic bacteria virulence factors²¹. Inhibition of *Salmonella* T3SS can achieve anti-infection effect. Previous studies have reported that screening inhibitors targeting *Salmonella* T3SS have achieved significant progress. For example, salicylidene acylhydrazides (SAHs) are the first identified and most widely studied class of synthetic small molecule inhibitor that target the T3SSi across many bacterial species²². Licoflavonol exerts a strong inhibitory effect on the secretion of the *S. Typhimurium* SPI-1 effectors by regulating the transcription of the *SicA/InvF* genes and the transport of the SipC²³. According to Zhang et al. study, thymol was capable of blocking the activity of T3SS-1 at concentrations that did not affect either bacterial viability or the integrity of mammalian cell membranes. Although the detection methods are constantly updated, the scarcity of natural product origin inhibitors and the unclear mechanism of action still limit the discovery and clinical application of natural compounds or derivatives¹⁷.

Compared with conventional antibacterial drugs, antibodies and probiotics, most of these candidate drugs inhibit virulence factors that are not essential for bacteria, and the possibility of inducing bacterial resistance is significantly reduced²⁴. Yet importantly, study of such a molecule could help elucidate structure-function relations of the pathogen T3SS and serve as a highly efficient molecular platform to develop active molecules against homologous components in other T3SS²⁵.

Quercitrin is a natural glycosylated flavonoid obtained from the whole grass of *Hypericum perforatum* and the bark of different species of *oak* trees with anti-bacterial, anti-inflammatory and hypidemia effects²⁶. In this study, quercitrin, a T3SS inhibitor, was screened from a series of natural compounds by SipA-TEM- β -lactamase fusion reporter system. As the results shown that quercitrin inhibited *S. Typhimurium* invasion of host cells and host cell damage without affecting bacterial growth. Animal experiments showed that quercitrin reduced the mortality and pathological damage caused by *S. Typhimurium* infection. In conclusion, quercitrin is an ideal leading compound for treatment of *S. typhimurium* infection and has broad application prospects in the future.

Declarations

Acknowledgments

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Conflicts of interest

The authors have no conflicts of interest to declare.

Author contributions

Lv Q and Deng X contributed to the concepts and design of the study. Li Q, Wang L, Liu S, Xu J, Song Z and Chen T participated in the project operation, acquisition, analysis, and interpretation of the data. Li Q and Wang L drafted the manuscript. All authors reviewed the manuscript.

Data Availability Statements

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

References

- 1 Knodler, L. A. & Effenbein, J. R. Salmonella enterica. *Trends Microbiol* **27**, 964-965, doi:10.1016/j.tim.2019.05.002 (2019).

- 2 Johnson, R., Mylona, E. & Frankel, G. Typhoidal Salmonella: Distinctive virulence factors and pathogenesis. *Cell Microbiol* **20**, e12939, doi:10.1111/cmi.12939 (2018).
- 3 Deiwick, J. *et al.* The translocated Salmonella effector proteins SseF and SseG interact and are required to establish an intracellular replication niche. *Infect Immun* **74**, 6965-6972 (2006).
- 4 Threlfall, E. J. Antimicrobial drug resistance in Salmonella: problems and perspectives in food- and water-borne infections. *FEMS Microbiol Rev* **26**, 141-148 (2002).
- 5 Galán, J. E. & Curtiss, R. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. *Proc Natl Acad Sci U S A* **86**, 6383-6387 (1989).
- 6 Wallis, T. S. & Galyov, E. E. Molecular basis of Salmonella-induced enteritis. *Mol Microbiol* **36** (2000).
- 7 Lostroh, C. P. & Lee, C. A. The Salmonella pathogenicity island-1 type III secretion system. *Microbes Infect* **3**, 1281-1291 (2001).
- 8 Lou, L., Zhang, P., Piao, R. & Wang, Y. Pathogenicity Island 1 (SPI-1) and Its Complex Regulatory Network. *Front Cell Infect Microbiol* **9**, 270, doi:10.3389/fcimb.2019.00270 (2019).
- 9 Zhou, D., Mooseker, M. S. & Galán, J. E. An invasion-associated Salmonella protein modulates the actin-bundling activity of plastin. *Proc Natl Acad Sci U S A* **96**, 10176-10181 (1999).
- 10 Hersh, D. *et al.* The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc Natl Acad Sci U S A* **96**, 2396-2401 (1999).
- 11 Kaniga, K., Tucker, S., Trollinger, D. & Galán, J. E. Homologs of the Shigella IpaB and IpaC invasins are required for Salmonella typhimurium entry into cultured epithelial cells. *J Bacteriol* **177**, 3965-3971 (1995).
- 12 Worrall, L. J., Vuckovic, M. & Strynadka, N. C. J. Crystal structure of the C-terminal domain of the Salmonella type III secretion system export apparatus protein InvA. *Protein Sci* **19**, 1091-1096, doi:10.1002/pro.382 (2010).
- 13 Luo, Z.-Q. & Isberg, R. R. Multiple substrates of the Legionella pneumophila Dot/Icm system identified by interbacterial protein transfer. *Proc Natl Acad Sci U S A* **101**, 841-846 (2004).
- 14 Smedowski, A. *et al.* FluoroGold-Labeled Organotypic Retinal Explant Culture for Neurotoxicity Screening Studies. *Oxid Med Cell Longev* **2018**, 2487473, doi:10.1155/2018/2487473 (2018).
- 15 Tsou, L. K. *et al.* Antibacterial Flavonoids from Medicinal Plants Covalently Inactivate Type III Protein Secretion Substrates. *Journal of the American Chemical Society* **138**, 2209-2218, doi:10.1021/jacs.5b11575 (2016).

- 16 Zhang, Y. *et al.* The Herbal Compound Thymol Targets Multiple Salmonella Typhimurium Virulence Factors for Lon Protease Degradation. *Front Pharmacol* **12**, 674955, doi:10.3389/fphar.2021.674955 (2021).
- 17 Zhang, Y., Liu, Y., Qiu, J., Luo, Z.-Q. & Deng, X. The Herbal Compound Thymol Protects Mice From Lethal Infection by Typhimurium. *Front Microbiol* **9**, 1022, doi:10.3389/fmicb.2018.01022 (2018).
- 18 Deng, W. *et al.* Assembly, structure, function and regulation of type III secretion systems. *Nature reviews. Microbiology* **15**, 323-337, doi:10.1038/nrmicro.2017.20 (2017).
- 19 Kaiser, P., Diard, M., Stecher, B. & Hardt, W.-D. The streptomycin mouse model for Salmonella diarrhea: functional analysis of the microbiota, the pathogen's virulence factors, and the host's mucosal immune response. *Immunol Rev* **245**, 56-83, doi:10.1111/j.1600-065X.2011.01070.x (2012).
- 20 Tournier, I., Bernuau, D., Poliard, A., Schoevaert, D. & Feldmann, G. Detection of albumin mRNAs in rat liver by in situ hybridization: usefulness of paraffin embedding and comparison of various fixation procedures. *J Histochem Cytochem* **35**, 453-459 (1987).
- 21 Pinaud, L., Sansonetti, P. J. & Phalipon, A. Host Cell Targeting by Enteropathogenic Bacteria T3SS Effectors. *Trends Microbiol* **26**, 266-283, doi:10.1016/j.tim.2018.01.010 (2018).
- 22 Negrea, A. *et al.* Salicylidene acylhydrazides that affect type III protein secretion in Salmonella enterica serovar typhimurium. *Antimicrob Agents Chemother* **51**, 2867-2876 (2007).
- 23 Guo, Z. *et al.* Licoflavonol is an inhibitor of the type three secretion system of Salmonella enterica serovar Typhimurium. *Biochem Biophys Res Commun* **477**, doi:10.1016/j.bbrc.2016.07.018 (2016).
- 24 Rasko, D. A. & Sperandio, V. Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Discov* **9**, 117-128, doi:10.1038/nrd3013 (2010).
- 25 Hussain, S. *et al.* Type 3 secretion system 1 of Salmonella typhimurium and its inhibitors: a novel strategy to combat salmonellosis. *Environ Sci Pollut Res Int* **28**, 34154-34166, doi:10.1007/s11356-021-13986-4 (2021).
- 26 Córdoba, A. *et al.* Quercitrin Nanocoated Implant Surfaces Reduce Osteoclast Activity In Vitro and In Vivo. *Int J Mol Sci* **19**, doi:10.3390/ijms19113319 (2018).

Figures

354 Natural compounds

Primary screen



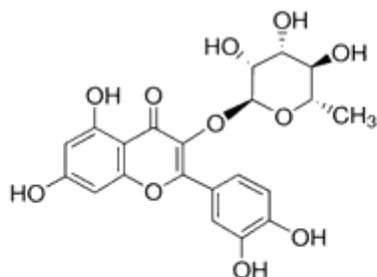
7 Candidate inhibitors

Potency analysis



T3SS inhibitors

Action mechanism



Quercitrin

Figure 1

Flowchart for high-throughput screening of *S. Typhimurium* T3SS inhibitors.

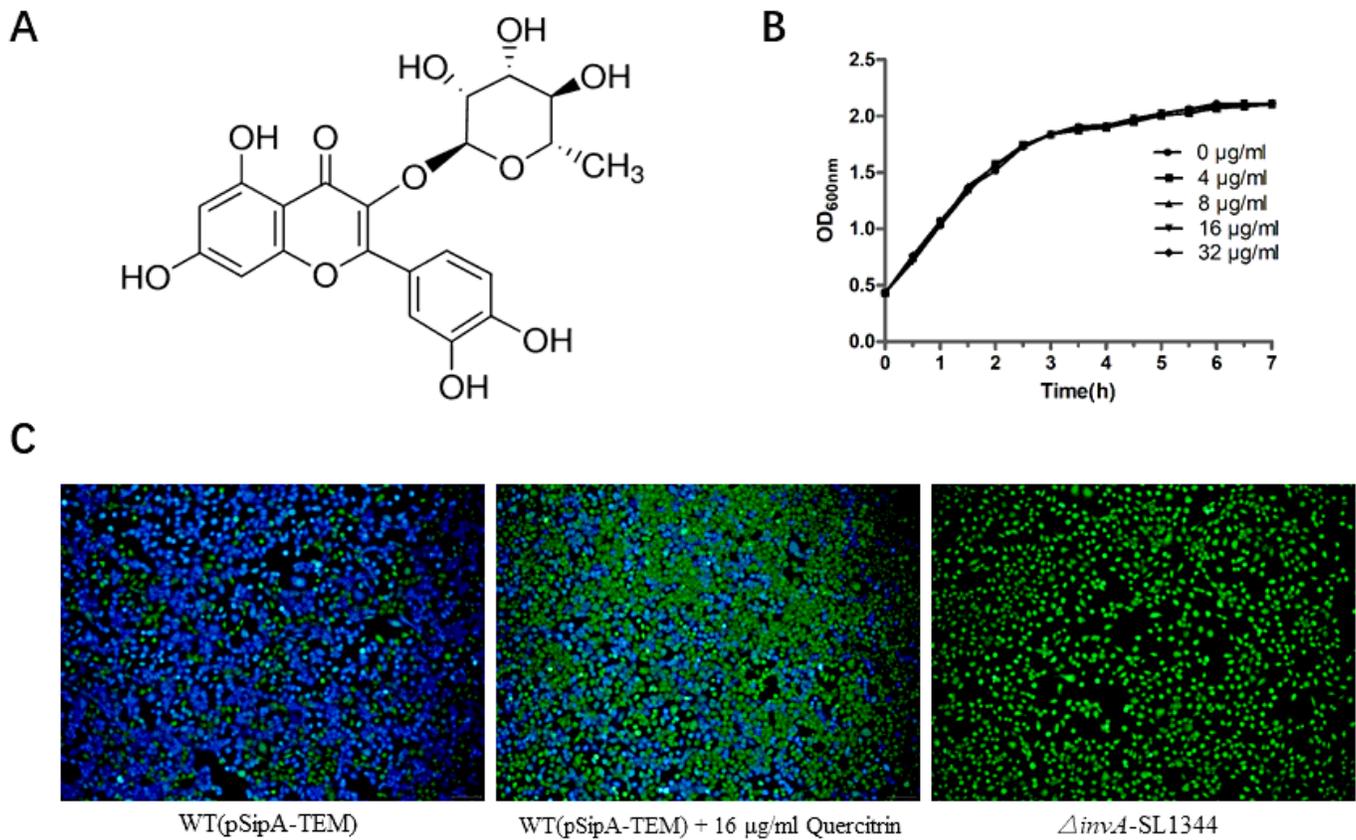


Figure 2

Quercitrin inhibits T3SS function by blocking the translocation of effector protein SipA. **(A)** Chemical structure of quercitrin. **(B)** Quercitrin does not inhibit the growth of *S. Typhimurium* SL1344. Data at each point are from three independent experiments. **(C)** Quercitrin inhibits the translocation of *S. Typhimurium* effector SipA to eukaryotic host cells (Magnification 200 \times). The untreated group was set as 100%. $\Delta invA$ -SL1344 treatment was used as negative control. Blue fluorescence represents normal effector translocation, and green fluorescence represents impaired translocation.

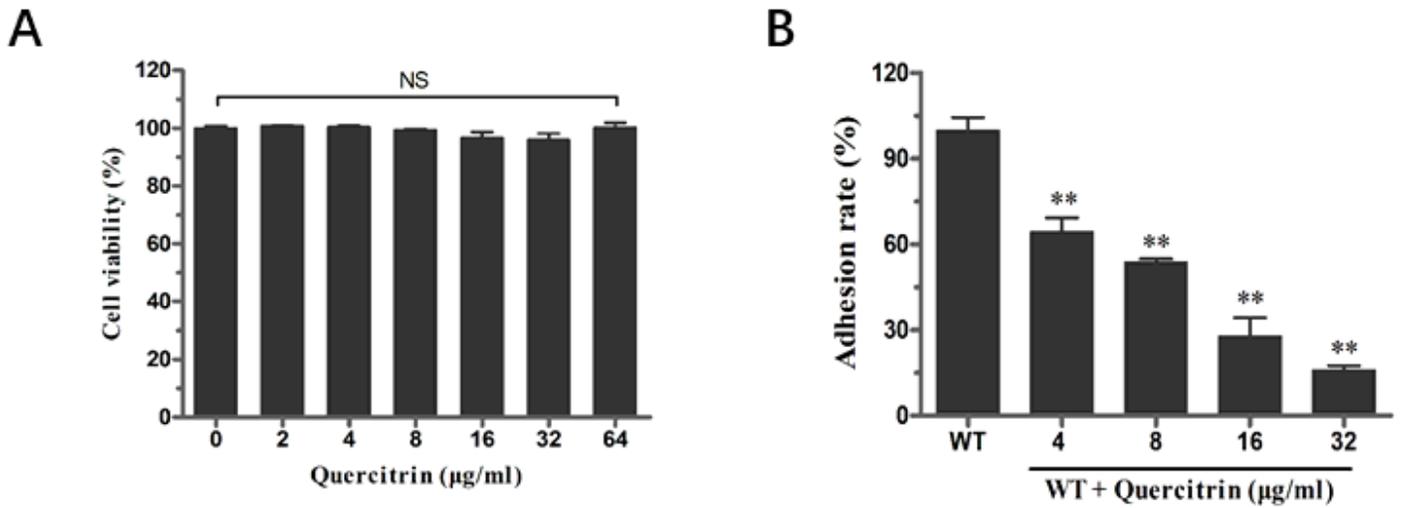


Figure 3

Quercitrin inhibits the adhesion of *S. Typhimurium* to HeLa cells without cytotoxicity. **(A)** The cytotoxicity of quercitrin on HeLa cells at different concentrations. **(B)** Inhibitory effect of quercitrin on the adhesion of *S. Typhimurium*. HeLa cells were infected with *S. Typhimurium* SL1344 pretreated with quercitrin. All data are shown as the mean \pm SEM from three independent experiments. * $p < 0.05$; ** $p < 0.01$ compared to the control group. NS, $P > 0.05$, not significant.

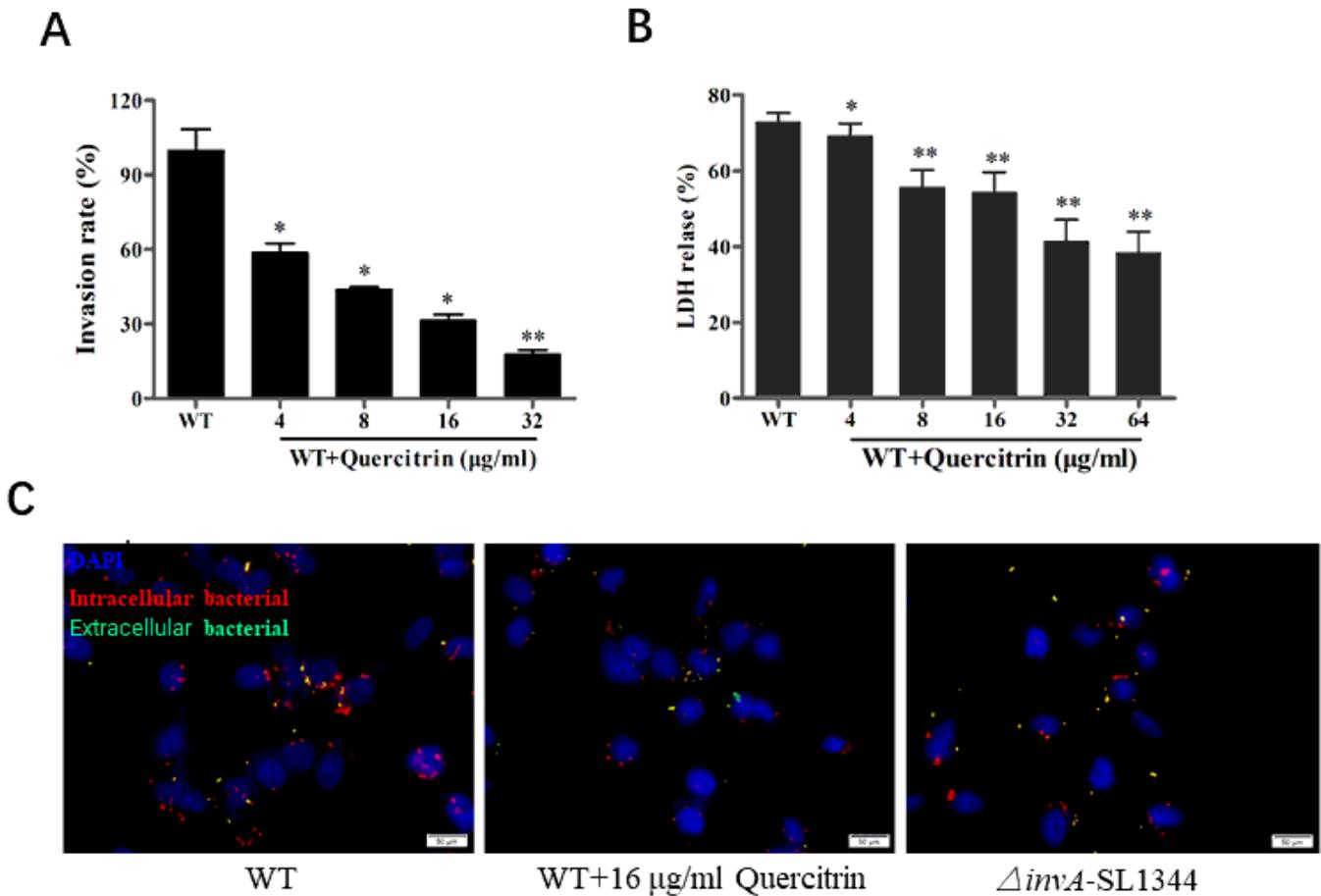


Figure 4

Quercitrin inhibits the invasion of host cells by *S. Typhimurium*. **(A)** Inhibitory effect of quercitrin on the invasion of *S. Typhimurium*. HeLa cells were infected with *S. Typhimurium* SL1344 pretreated with quercitrin. **(B)** Quercitrin protects HeLa cells from *S. Typhimurium* mediated injury. The LDH release data showed that the degree of cell damage caused by *S. Typhimurium* was significantly reduced after pretreatment with quercitrin. All data are shown as the mean \pm SEM from three independent experiments. **(C)** Quercitrin significantly inhibits the invasion of *S. Typhimurium*. Red fluorescence, intracellular bacteria; green fluorescence, extracellular bacteria; blue fluorescence, nuclei. * $p < 0.05$; ** $p < 0.01$ compared to the control group.

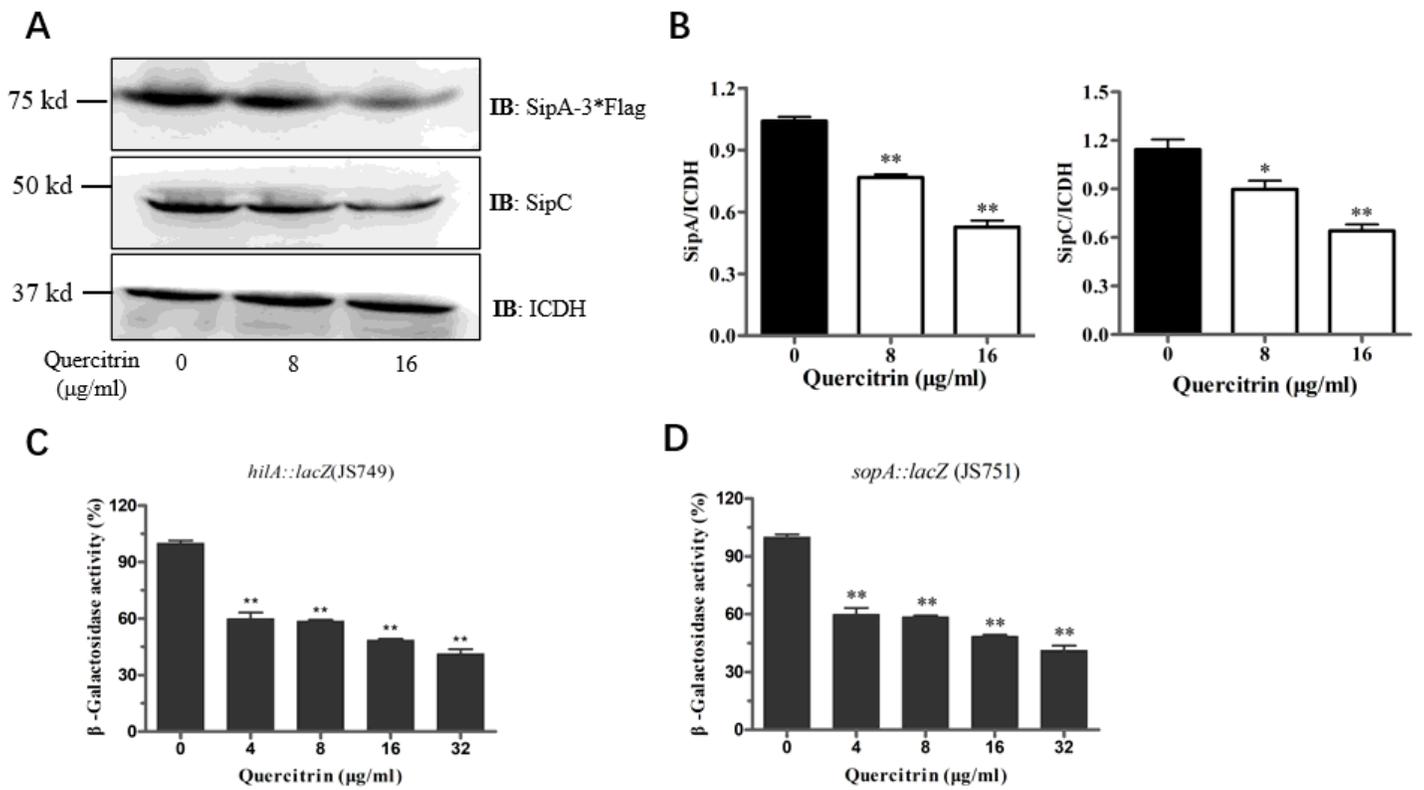


Figure 5

Quercitrin inhibits the expression of T3SS effectors and key SPI-1 gene. **(A)** Quercitrin pretreatment reduced the expression of SipA-3×Flag and SipC encoded by *S. Typhimurium*. **(B)** Quantitative analysis of the expression levels of effectors with ImageJ software. **(C~D)** The activity of β-galactosidase was measured in cultures of *S. Typhimurium* expressing *hilA::lacZ* (JS749) and *sopA::lacZ* (JS751). * $p < 0.05$; ** $p < 0.01$ compared to the control group.

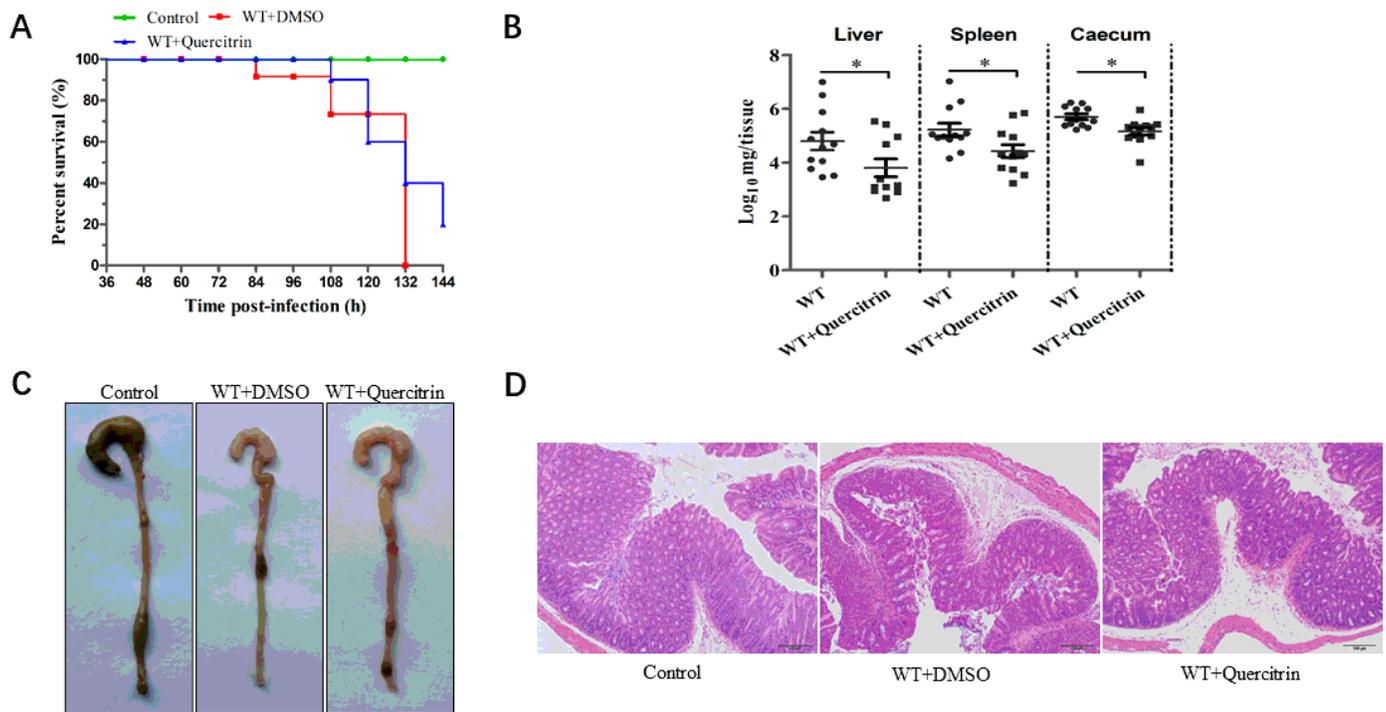


Figure 6

Quercitrin provides protection against *S. Typhimurium* infection in mice. **(A)** Quercitrin improves the survival rate of mice infected with *S. Typhimurium*. **(B)** The bacterial load in several organs of infected mice at 96 h post infection. **(C)** Gross lesion observations of cecal tissue sections from each group. **(D)** Histopathology observations (200×, hematoxylin and eosin staining) of cecal tissue. * $p < 0.05$; ** $p < 0.01$ compared to the control group.

Supplementary Files

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