Quercetin attenuates the production of pro-inflammatory cytokines in H292 human lung epithelial cells infected with *Pseudomonas aeruginosa*, by modulating ExoS production

Hye In Ahn
Korea Research Institute of Bioscience and Biotechnology https://orcid.org/0000-0002-6477-5396
Ji-Won Park
Korea Research Institute of Bioscience and Biotechnology
Hyun-Jae Jang
Korea Research Institute of Bioscience and Biotechnology
Ok-kyoung Kwon
Korea Research Institute of Bioscience and Biotechnology
Jung Hee Kim
Korea Research Institute of Bioscience and Biotechnology
Sei-Ryang Oh
Korea Research Institute of Bioscience and Biotechnology
Sang-Bae Han
Chungbuk National University College of Medicine
Kyung-Seop Ahn (ksahn@kribb.re.kr)

Research article

Keywords: Pseudomonas aeruginosa (P. aeruginosa), type three secretion system (T3SS), exoenzyme S (ExoS), ExsA, PopD, quercetin

Posted Date: July 24th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-33039/v2

Abstract

Background: The type three secretion system (T3SS) is a major virulence system of *Pseudomonas aeruginosa* (*P. aeruginosa*). The effector protein Exotoxin S (ExoS) produced by *P. aeruginosa* is secreted into the host cells via the T3SS. For the purpose of screening the inhibitors with regard to ExoS secretion, we developed the sandwich-type enzyme-linked immunosorbent assay (ELISA) system. From the initial screening, quercetin was selected because it has the prominent effect of ExoS inhibition and also is known to have anti-inflammatory and antioxidant effects on mammalian cells.

Results: In this study, we investigated the effects of quercetin on the expression and secretion of ExoS using the ELISA and Western blot analysis methods. The results showed that the secretion of ExoS was significantly decreased by 10, 20uM quercetin. Also, pscF and popD, which are composed of the T3SS needle, are reduced by quercetin at the mRNA level, and we confirmed the inhibitory effect of quercetin on cytokines in *P. aeruginosa*-infected H292 cells by real-time polymerase chain reaction (PCR).

Conclusion: Collectively, quercetin inhibits the secretion of ExoS by reducing both ExoS production and the expression of the needle protein of T3SS. Furthermore, these results suggest that quercetin has the potential to be used as an anti-toxic treatment for the disease caused by *P. aeruginosa* infection.

Background

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the most common gram-negative bacteria and is an opportunistic pathogen that causes secondary infections in immuno-compromised patients, including pneumonia and cystic fibrosis (CF). In particular, *P. aeruginosa* accounts for more than 60% of the causes of CF [1-3]

P. aeruginosa has a variety of systems by which it can cause infection. The type three secretion system (T3SS) is a major cause in various human infections of *P. aeruginosa*. T3SS forms a needle complex that consists of proteins, including pscF, pcrV, and popB/D on the bacterial membrane and is regulated by ExsA, the transcription factor of T3SS. In addition, T3SS is regulated by various environmental factors, such as contact with the host cells and a low concentration of calcium. T3SS needle complex acts as a translocator of four T3SS toxins, exoenzymes S, T, U, and Y, and these toxins play key roles in the virulence of *P. aeruginosa* [4-6]. Exoenzyme S (ExoS) shows ADP-ribosyltransferase (ADPRT) activity, and it is a major characteristic of patients with acute lung injuries who are also infected with *P. aeruginosa* among these secretory toxins [7, 8]. Injected ExoS is known to convert pro-interleukin (IL-1β) into IL-1β via NLRC4 or NLRP3 and caspase-1 in infected mammalian cells by *P. aeruginosa* [9, 10]. In addition, it produces other pro-inflammatory cytokines IL-18, IL-6, and tumor necrosis factor α (TNF-α) through the nuclear factor κB (NF-κB) pathway [11].

Quercetin is a flavonoid found in many fruits, leaves, grains, and vegetables; kale and red onions are common foods containing appreciable content of quercetin [12, 13]. Quercetin has a bitter flavor and is used as an ingredient in foods, beverages, and dietary supplements [14]. This compound has been

reported to have pharmacological effects, such as anti-oxidant [15], anti-inflammatory [16, 17], anti-virus [18], anti-diabetic effect [19]and anti-tumor activities [15, 20, 21]. However, the ExoS inhibitory effect of quercetin has not been reported.

Therefore, in this experiment, we studied effect of quercetin on the inhibitory activity of ExoS secretion and the expression of T3SS. This study confirms the effect of quercetin on ExoS and T3SS and demonstrates its potential for use as an anti-virulence substance for *P. aeruginosa*.

Results

Effect of quercetin on bacterial growth

To confirm the effect of quercetin on bacterial growth, the growth of the *P. aeruginosa* PAK strain with quercetin was determined at optical density [22] 600 for 48 h by a spectrophotometer. As a result, there was no effect on the growth rate compared to untreated PAK (Fig. 1B). Therefore, this data indicates that the data presented later is not related to the growth of *P. aeruginosa*.

Quercetin reduced the secretion of ExoS-FLAG from the *P. aeruginosa*

To measure the amount of secreted ExoS, an ExoS-FLAG ELISA was conducted. quercetin has a definite inhibitory effect of more than 40% in 2.5 and 5uM and 50% in 10 and 20uM (Fig. 2A). We also conducted a western analysis to detect any remaining ExoS in the bacteria. As a result, the expression of ExoS in bacteria was found to be inhibited (Fig. 2B and Fig. S2). Quercetin has the effect of inhibition of ExoS as identified in the ExoS-FLAG ELISA test. Thus, these results confirmed that quercetin inhibits both the secretion and expression of ExoS. Through this result, we thought that ExoS was related to the transcription of ExsCEBA controlling the expression of ExoS, or the expression of popB/D controlling the secretion of ExoS.

Quercetin reduced the expression of ExoS, pscF, and popD in *P. aeruginosa*.

To evaluate the expression of the effector protein and regulators of the T3SS at the mRNA level, quantitative real-time PCR of factors related to T3SS was conducted. As a result, the expression of ExoS was inhibited, and the expression of needle proteins pscF and popD was also suppressed (Fig. 3A-C). However, the transcription factor ExsA had only a slight effect of inhibition (Fig. 3D). These results show that quercetin ultimately reduces the secretion of ExoS by influencing both the expression of ExoS and the secretion system. Through these results, it can be expected that quercetin can effectively suppress inflammatory reactions to host cells by reducing the exoS secreted by *P.aeruginosa*.

Cytotoxic effect of quercetin in H292 cells

MTT assays were used to identify the toxicity of quercetin on H292 cells. When quercetin (2.5, 5, 10, and 20 μM) alone was used, all concentrations of quercetin showed no toxicity on H292 cells (Fig. 4A). When

quercetin was exposed to infected H292 cells, it reduced the toxicity of the *P. aeruginosa* PAK ExoS strain (Fig. 4B). Therefore, these results suggest that quercetin is non-toxic and has an anti-toxic effect.

Quercetin reduced the pro-inflammatory cytokines in *P. aeruginosa*-infected H292 cells.

We aimed to identify how the reduced expression and secretion of ExoS by quercetin affected infected H292 cells. Therefore, the secretion and expression of cytokines induced by ExoS were verified with ELISA and real-time PCR, respectively (Fig. 5A-F) [23]. As a result, the treatment of quercetin was found to decrease the secretion and expression levels of cytokines (IL-1β, IL-6, IL-18), which also suggests that the decreased levels of cytokines were due to the decreased ExoS secretion levels.

Effect of quercetin on NF-κB pathway in *P. aeruginosa*-infected H292 cells.

Previously, the effects of caspase-1 pathways were checked, and also, the results of NF-κB pathways in cells infected with *P. aeruginosa* were tested. IκB and phosphorylation of p65 were checked through the western blot and found that both targets' phosphorylation decreased compared to normal form. Therefore, it was confirmed that the additional NF-κB pathways due to infection of *P. aeruginosa* were inhibited through quercetin. (Fig. 6 and Fig. S3)

Quercetin inhibits the induction of the NLRC4-caspase-1 pathway by *P. aeruginosa* infection.

Pro-inflammatory cytokines induced by ExoS were confirmed to be reduced by quercetin. Therefore, western blots were conducted to determine the expression of NLRC4, caspase-1, which induced pro-inflammatory cytokines. As a result, increased caspase-1 in infected cells was reduced when quercetin was treated, and NLRC4 had the same result (Fig. 7 and Fig. S4). These results suggest that the quercetin reduces ExoS and further affects NLRC4 and caspase-1 pathways.

Discussion

Multi-drug resistant strains of *P. aeruginosa* continue to present a persistent and critical danger to immunocompromised patients, particularly in intensive care settings [24]. These problems can be resolved with the development of a therapeutic agent such as *P. aeruginosa* infection or virulence blockers. *P. aeruginosa* has a variety of systems necessary for infection, and T3SS can act as a major cause during various *P. aeruginosa* infections. The T3SS activation requires specific environments, such as contact with host cells and low concentration of calcium [25]. We have already demonstrated that ExoS can cause an inflammatory response and improve clearance in the lungs in vitro and vivo [26]. It is also well known that quercetin is a useful substance for various diseases [27-29]. However, it is not known whether quercetin prevents disease by inhibiting the expression of ExoS due to infection of the host via T3SS of *P. aeruginosa*. Therefore, we aimed to elucidate the mechanism of the anti-inflammatory activity of quercetin.

Among the toxic substances secreted by T3SS, ExoS has a similar structure to the ExoT and has similar functions, ADPRT and GTPase [1]. However, ExoS has higher toxicity than the ExoT and is expressed in

some *P. aeruginosa* clinical isolates [30, 31]. Conversely, because the ExoT is expressed in most *P. aeruginosa* clinical isolates, this experiment used a strain inserted with plasmid so that only ExoS can be expressed while knocking out the ExoT.

T3SS is transcribed through the ExsCEBA site, which can be called the T3SS regulator. The secretion activity is carried out by ExsC, ExsD, ExsE, and ExsA. In the T3SS inactivation conditions, ExsD (anti-activator) and ExsC (anti-anti-activator) binds to ExsA and ExsE, respectively. In the T3SS activation conditions, ExsC binds to ExsD. So, free-ExsA activates the T3SS operons, and free-ExsE is secreted into the host cell. Thus, ExsA enables the transcriptional activation of T3SS [32]. We investigated the inhibitory effect of quercetin on the expression of ExsA, the transcriptional activator of T3SS operons, among others, in mRNA level. As a result, quercetin decreased the expression of ExsA, and ExoS production of T3SS followed by ExsA also decreased. The results, we know that quercetin has the effect of suppressing the overall expression of T3SS.

The needle protein, which acts as a translocator so that the secretory toxins of T3SS can be secreted, consists of pscF, pcrV, and popB/D. pscF serves as a major component for needle proteins among them, while popD is responsible for direct insertion into host cells, and popB is responsible for assisting in insertion of popD [33, 34]. Therefore, we investigated whether quercetin affects the expression of pscF and popD, which account for the most important region of needle protein. The results show that quercetin has reduced the expression of T3SS needle protein, which can be directly toxic to host cells by injecting the generated secretory toxins. Through this result, we demonstrate that quercetin has an inhibitory effect on virulence factors of *P. aeruginosa*. Host cells infected with *P. aeruginosa* produce cytokines such as IL-1β and IL-18 resulting from the activation of caspase-1, and additionally activates inflammatory signals, such as NF-κB pathways, by injecting ExoS [26]. Caspase-1 has a proteolytic activity for the precursor of IL-1β and IL-18 and converts them into IL-1β and IL-18. Also, after caspase-1 is activated, it causes cell death by proptosis [35, 36]. Therefore, the secretion and expression levels of cytokines (IL-1β, IL-6, and IL-18) induced by ExoS were verified with ELISA and real-time PCR assessments, respectively. As a result, treatment of quercetin decreased the secretion and expression levels of the cytokines, which suggests that reduced cytokines in H292 cells are due to the decreased ExoS secretion by quercetin. According to the previous study, caspase-1 is activated by NLRC4 or NLRP3 forming an inflammasome [37]. Quercetin inhibits the NLRC4 inflammasome and caspase-1 induced by *P. aeruginosa* on western blot. Additionally, further research is needed on whether ExoS reduced by quercetin also affects NLRP3. All these findings suggest that quercetin alleviates cytotoxicity caused by infection by reducing the generation of ExoS, and it is expected that its effect on the ExoS of T3SS will prevent *P. aeruginosa* from developing lung-related diseases caused by the infection of host cells. It is thought that in vivo experiments will be needed to support this finding.

In summary, quercetin has been shown to decrease ExoS production. It also inhibits the expression of needle proteins and reduces the secretion of ExoS. In addition, it was confirmed that there is an effect of reducing the pro-inflammatory cytokines (IL-6, IL-1β, and IL-18) by reducing the activation of the NLRC4-

caspase-1 pathway in the H292 cells (Fig. 8). However, suppression of ExoS is not due to ExsA. Thus, further study of another factor in which quercetin affects the suppression of ExoS is likely to be needed.

Conclusions

In conclusion, quercetin not only reduces the generation of ExoS but also inhibits the expression of needle protein, reducing the secretion of ExoS. Furthermore, the activation of caspase-1 pathways was decreased within eukaryotic cells. Therefore, it is expected that quercetin can be used as a treatment for anti-toxic, anti-inflammatory activity even in lung diseases resulting from infection with *P. aeruginosa*.

Methods

Quercetin

Quercetin was supplied as a purified compound (code No. NPFL10051.1) from the *Styphnolobiumjaponicum* (L.) Schott in The Korea Plant Extract Bank (https://extract.kribb.re.kr) at The Korea Research Institute of Bioscience & Biotechnology (Daejeon, Korea). The structural information of authentic compound was listed as a supplementary data. (Fig. 1A and Fig. S1)

Bacterial strains

All strains and plasmids used in this study are listed in Table S1. Chromosomal mutants were all derived from the same parental PAK (*P. aeruginosa* strain K; wild-type strain) strain [26, 38, 39] and were generated by allelic exchange.

Bacterial culture and growth curve

All bacterial strains were grown overnight in Luria-Bertani (LB) media at 37°C on a rotary shaker if not otherwise specified. Bacterial cells were harvested at 12,000 g at 4°C for 10 min after overnight LB broth culture. Antibiotics were used at concentrations of spectinomycin (Sp; 200 μg/ml, Cat. No. S9007-5G), streptomycin (Sm; 200 μg/ml, Cat. No. S6501-5G), gentamycin (Gm; 150 μg/ml, Cat. No. G1264, Sigma-Aldrich, St. Louis, MO, USA) and carbenicillin disodium (Cb; 150 μg/ml, Cat. No. 2485-1G, BioVision, CA, USA) [39, 40]. Overnight cultures of untreated *P. aeruginosa* PAK strain and *P. aeruginosa* treated with a 20 μM concentration of quercetin were re-inoculated and diluted 1:1,000 in fresh LB broth. Growth of the bacteria was determined at OD 600 using an ultraviolet–visible (UV/VIS) spectrophotometer (Cat. No. Optizen 2120UV, Mecasys Co., Ltd., Daejeon, Korea) [41].

Cell culture

The H292 cell line (ATCC; American Type Culture Collection, Manassas, VA, USA) is human epithelial cells derived from human lung carcinoma. H292 cells were cultured in the RPMI 1640 medium (Cat. No. LM 011-01, Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand

Island, New York) and antibiotics (1% penicillin-streptomycin; Invitrogen, Grand Island, New York) and incubated at 37℃ in a 5% CO_2 incubator.

MTT assay

Cell viability was measured via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. H292 cells were seeded into 96-well plates (SPL, Gyeonggi-do, Korea) at a concentration of 1×10^4 cells/well. After incubation for 5 h, the cells incubated with various concentrations of quercetin for 20 h. In the infection cases, H292 cells were infected by MOI 100 of the PAK ExoS strain after a quercetin treatment for 1 h. Subsequently, 5 μl of 5 mg/ml MTT solution (Amresco, LLC, solon, OH, USA) was added to each well for 4 h. The supernatant was removed, and 100 μl of dimethyl sulfoxide (DMSO) was added to each well for dissolution of the formazan crystals.

ExoS-FLAG enzyme-linked immunosorbent assay (ELISA) assay

To detect ExoS secretion, the secreted ExoS-FLAG amounts in bacterial overnight culture supernatants supplemented with quercetin and 1 mM of EGTA were determined using an ExoS-FLAG sandwich ELISA system designed according to a previous study [25]. An anti-ExoS antibody as a capture antibody commissioned by Koma Biotech (Seoul, Korea), Inc. and produced by Prosci, Inc. (CA, USA). The ExoS amino acid sequence was diluted to 1:5,000 in a carbonate-bicarbonate buffer (0.05 M, pH 9.6), coated into 96-well microplates at 4℃, and kept overnight. The plates were incubated with a blocking buffer (2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)) for 1 h, after which they were washed, and a bacterial supernatant was added to each well. After incubation for 2 h, the plates were washed and mouse anti-FLAG antibody (Anti-DDDDK antibody; Cat. No. ARG62342, Arigo Biolaboratories, Taiwan, ROC), a detection antibody diluted to 1:5,000 in 1% BSA (Bovine Serum Albumin; Cat. No. BSAS0.1, Bovogen Biologicals Pty. Ltd., AUSTRALIA) in PBS (assay diluent), was added to each well. After incubation for 1 h, the plates were washed, and horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) diluted to 1:2,000 in an assay diluent was then added to each well for 1 h. After incubation, the plates were washed, and a substrate solution was added to each well. Lastly, the color reaction of the plates was stopped with H_2SO_4 and the absorbance was determined at 450-570 nm using a plate reader instrument. All steps except for the incubation of the capture antibody were performed at room temperature.

Cytokine ELISA assay

H292 cells were seeded into 12-well plates at a concentration of 4×10^6 cells/well and cultured overnight at 37℃. After incubation, the media in the plates were changed to RPMI 1640 without supplementation, and the cells were pretreated with various concentrations of quercetin for 1 h. The H292 cells were then infected by moment of infection (MOI) 100 of the PAK ExoS strain at 37℃ for 6 h. Cytokines of the culture supernatant of the infected cells were measured using ELISA kits (IL-18 set, Cat. No. DY318-05, R&D system, Minneapolis, USA ; IL-1β set, Cat. No. 557953; IL-6 set, Cat. No. 555220; BD biosciences, CA, USA). Each experiment was conducted according to the manufacturer's protocol.

Real-time polymerase chain reaction (PCR) analysis

Briefly, H292 cells were seeded into 12-well plates at a concentration of 4×10^6 cells/well and cultured at 37℃ overnights. The cells were treated with quercetin and PAK ExoS for 6 h. In contrast, bacterial cells were grown overnight at 37℃ in a rotary shaker and re-inoculated in fresh LB broth supplemented with quercetin and 1 mM of EGTA for 4 h. After all of the steps, the H292 cells and bacterial cells were harvested. The total RNA was isolated using the TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc., MA, USA). The synthesis of the complementary DNA was performed using a ReverTra Ace® qPCR RT Master Mix kit (Cat. No. FSQ-301, TOYOBO, Japan). cDNA was used for real-time PCR using primers (Table. S2, Bioneer, Daejeon). This experiment was conducted using SYBR Green PCR Master Mix (KAPA Bio-systems, Woburn, Massachusetts).

Western blot analysis

For the ExoS-FLAG detection, the p30 and p137 strains (Table. S1) were grown overnight in LB broth supplemented with quercetin and 1 mM of EGTA at 37℃ in a rotary shaker. Bacterial cells were then harvested into 1.5-ml tubes and lysed using a diluted 5× bacterial protein extraction reagent (Cat. No. AKR-180, Cell Biolabs, Inc., CA, USA) for protein extraction. The bacterial protein quantity was measured using a Pierce™ BCA Protein assay kit (Cat. No. 23225, Thermo Fisher Scientific Inc., MA, USA).

Equal amounts of protein were loaded and separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Loaded proteins were transferred to a hydrophilic polyvinylidene fluoride membrane (PVDF; EMD Millipore, Billerica, MA, USA). Each membrane was blocked with 5% skim milk in tris-buffered saline with Tween (TBS-T) for 1 h at room temperature. Primary antibodies used ExoS-FLAG (Anti-DDDDK antibody; Cat. No. ARG62342, Arigo Biolaboratories, Taiwan, ROC), β-actin (MA5-15739), IκBα (MA5-15132), p-IκBα (MA5-15087, Thermo Fisher Scientific Inc., MA, USA), p65 (8242S), p-p65 (3033S), caspase-1 (3866S), IL-1β (83186S), NLRC4 (124215S, Cell Signaling Technology, MA, USA) was diluted to 1:1,000 in 5% skim milk in TBS-T, added to the membrane rack, and then incubated overnight at 4℃. After incubation, the membranes were washed with TBS-T and incubated with secondary antibody HRP-conjugated anti-mouse IgG (Cat. No. sc-2005, Santa Cruz Biotechnology Inc., Texas, USA), anti-rabbit IgG (Cat. No. 111-035-003, Jackson Immune Research Laboratories, Inc, PA, USA) diluted to 1:5,000 in 5% skim milk in TBS-T for 1 h at room temperature. The protein bands were visualized using a chemiluminescence (ECL; Cat. No. 32106, Thermo Fisher Scientific Inc., MA, USA) kit and a luminescent image analyzer (LAS-4000, Fujifilm, Tokyo, Japan).

Statistical analysis

The data are expressed as mean ± SEM. Statistical significance was determined using two-tailed Student's t-test. A value of $p<0.05$ was considered to indicate a statistically significant difference.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw data from this study are available upon request. Please contact the corresponding author.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the Bio and Medical Technology Development Program of the National Research Foundation (NRF) and funded by the Korean government (grant number: NRF-2017R1A2B2011555) and awarded to the Korea Research Institute of Bioscience and Biotechnology Research Initiative program (KGM 5521911) of the Republic of Korea. The funders had no role in data analysis, study design or preparation of the manuscript.

Authors' contributions

HIA carried out all experiments and prepared the manuscript. JWP contributed to design of the study and prepared the manuscript. OKK contributed to prepare the manuscript. HJJ and JHK contributed to perform UPLC-QTOF-MS and NMR spectrum analysis. SRO, SBH and KSA contributed to prepare and review the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Not applicable.

List Of Abbreviations

PAK: Pseudomonas aeruginosa K

T3SS: Type three secretion system

ExoS: Exoenzyme S

pscF: pseudomonas secretion protein F

ADPRT: ADP-ribosyltransferase

NLRC4: NLR family CARD domain-containing protein 4

DMSO: Dimethyl sulfoxide

EGTA: Ethylene glycol tetraacetic acid

References

1. Engel J, Balachandran P. Role of Pseudomonas aeruginosa type III effectors in disease. Curr Opin Microbiol. 2009;12(1):61-6; doi: 10.1016/j.mib.2008.12.007.
2. Sousa AM, Pereira MO. Pseudomonas aeruginosa Diversification during Infection Development in Cystic Fibrosis Lungs-A Review. Pathogens. 2014;3(3):680-703; doi: 10.3390/pathogens3030680.
3. Sawa T. The molecular mechanism of acute lung injury caused by Pseudomonas aeruginosa: from bacterial pathogenesis to host response. J Intensive Care. 2014;2(1):10; doi: 10.1186/2052-0492-2-10.
4. Hauser AR. The type III secretion system of Pseudomonas aeruginosa: infection by injection. Nat Rev Microbiol. 2009;7(9):654-65; doi: 10.1038/nrmicro2199.
5. Romano FB, Tang Y, Rossi KC, Monopoli KR, Ross JL, Heuck AP. Type 3 Secretion Translocators Spontaneously Assemble a Hexadecameric Transmembrane Complex. J Biol Chem. 2016;291(12):6304-15; doi: 10.1074/jbc.M115.681031.
6. Galle M, Carpentier I, Beyaert R. Structure and function of the Type III secretion system of Pseudomonas aeruginosa. Curr Protein Pept Sci. 2012;13(8):831-42.
7. Kaufman MR, Jia J, Zeng L, Ha U, Chow M, Jin S. Pseudomonas aeruginosa mediated apoptosis requires the ADP-ribosylating activity of exoS. Microbiology. 2000;146 (Pt 10):2531-41; doi: 10.1099/00221287-146-10-2531.
8. Iglewski BH, Sadoff J, Bjorn MJ, Maxwell ES. Pseudomonas aeruginosa exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. Proc Natl Acad Sci U S A. 1978;75(7):3211-5.
9. Grandjean T, Boucher A, Thepaut M, Monlezun L, Guery B, Faudry E, et al. The human NAIP-NLRC4-inflammasome senses the Pseudomonas aeruginosa T3SS inner-rod protein. Int Immunol. 2017;29(8):377-84; doi: 10.1093/intimm/dxx047.
10. Sutterwala FS, Mijares LA, Li L, Ogura Y, Kazmierczak BI, Flavell RA. Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome. J Exp Med. 2007;204(13):3235-45; doi: 10.1084/jem.20071239.
11. Anantharajah A, Mingeot-Leclercq MP, Van Bambeke F. Targeting the Type Three Secretion System in Pseudomonas aeruginosa. Trends Pharmacol Sci. 2016;37(9):734-49; doi: 10.1016/j.tips.2016.05.011.
12. Formica JV, Regelson W. Review of the biology of Quercetin and related bioflavonoids. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 1995;33(12):1061-80; doi: 10.1016/0278-6915(95)00077-1.

13. Kawabata K, Mukai R, Ishisaka A. Quercetin and related polyphenols: new insights and implications for their bioactivity and bioavailability. Food & function. 2015;6(5):1399-417; doi: 10.1039/c4fo01178c.
14. Marchetti F, De Santi C, Vietri M, Pietrabissa A, Spisni R, Mosca F, et al. Differential inhibition of human liver and duodenum sulphotransferase activities by quercetin, a flavonoid present in vegetables, fruit and wine. Xenobiotica; the fate of foreign compounds in biological systems. 2001;31(12):841-7; doi: 10.1080/00498250110069159.
15. Luo M, Tian R, Yang Z, Peng YY, Lu N. Quercetin suppressed NADPH oxidase-derived oxidative stress via heme oxygenase-1 induction in macrophages. Arch Biochem Biophys. 2019;671:69-76; doi: 10.1016/j.abb.2019.06.007.
16. Cheng SC, Huang WC, JH SP, Wu YH, Cheng CY. Quercetin Inhibits the Production of IL-1beta-Induced Inflammatory Cytokines and Chemokines in ARPE-19 Cells via the MAPK and NF-kappaB Signaling Pathways. International journal of molecular sciences. 2019;20(12); doi: 10.3390/ijms20122957.
17. Cheng SC, Wu YH, Huang WC, Pang JS, Huang TH, Cheng CY. Anti-inflammatory property of quercetin through downregulation of ICAM-1 and MMP-9 in TNF-alpha-activated retinal pigment epithelial cells. Cytokine. 2019;116:48-60; doi: 10.1016/j.cyto.2019.01.001.
18. Lei D, Chengcheng L, Xuan Q, Yibing C, Lei W, Hao Y, et al. Quercetin inhibited mesangial cell proliferation of early diabetic nephropathy through the Hippo pathway. Pharmacological research. 2019;146:104320; doi: 10.1016/j.phrs.2019.104320.
19. Bule M, Abdurahman A, Nikfar S, Abdollahi M, Amini M. Antidiabetic effect of quercetin: A systematic review and meta-analysis of animal studies. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2019;125:494-502; doi: 10.1016/j.fct.2019.01.037.
20. Ward AB, Mir H, Kapur N, Gales DN, Carriere PP, Singh S. Quercetin inhibits prostate cancer by attenuating cell survival and inhibiting anti-apoptotic pathways. World J Surg Oncol. 2018;16(1):108; doi: 10.1186/s12957-018-1400-z.
21. Chen CY, Kao CL, Liu CM. The Cancer Prevention, Anti-Inflammatory and Anti-Oxidation of Bioactive Phytochemicals Targeting the TLR4 Signaling Pathway. Int J Mol Sci. 2018;19(9); doi: 10.3390/ijms19092729.
22. Anantharajah A, Buyck JM, Faure E, Glupczynski Y, Rodriguez-Villalobos H, De Vos D, et al. Correlation between cytotoxicity induced by Pseudomonas aeruginosa clinical isolates from acute infections and IL-1beta secretion in a model of human THP-1 monocytes. Pathog Dis. 2015;73(7); doi: 10.1093/femspd/ftv049.
23. Dosunmu EF, Emeh RO, Dixit S, Bakeer MK, Coats MT, Owen DR, et al. The anti-microbial peptide TP359 attenuates inflammation in human lung cells infected with Pseudomonas aeruginosa via TLR5 and MAPK pathways. PLoS One. 2017;12(5):e0176640; doi: 10.1371/journal.pone.0176640.
24. Lin CK, Kazmierczak BI. Inflammation: A Double-Edged Sword in the Response to Pseudomonas aeruginosa Infection. J Innate Immun. 2017;9(3):250-61; doi: 10.1159/000455857.

25. Kim J, Ahn K, Min S, Jia J, Ha U, Wu D, et al. Factors triggering type III secretion in Pseudomonas aeruginosa. Microbiology. 2005;151(Pt 11):3575-87; doi: 10.1099/mic.0.28277-0.
26. Park JW, Kim YJ, Shin IS, Kwon OK, Hong JM, Shin NR, et al. Type III Secretion System of Pseudomonas aeruginosa Affects Matrix Metalloproteinase 12 (MMP-12) and MMP-13 Expression via Nuclear Factor kappaB Signaling in Human Carcinoma Epithelial Cells and a Pneumonia Mouse Model. J Infect Dis. 2016;214(6):962-9; doi: 10.1093/infdis/jiw278.
27. Comalada M, Camuesco D, Sierra S, Ballester I, Xaus J, Galvez J, et al. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kappaB pathway. European journal of immunology. 2005;35(2):584-92; doi: 10.1002/eji.200425778.
28. Gerin F, Sener U, Erman H, Yilmaz A, Aydin B, Armutcu F, et al. The Effects of Quercetin on Acute Lung Injury and Biomarkers of Inflammation and Oxidative Stress in the Rat Model of Sepsis. Inflammation. 2016;39(2):700-5; doi: 10.1007/s10753-015-0296-9.
29. Li G, Shen X, Wei Y, Si X, Deng X, Wang J. Quercetin reduces Streptococcus suis virulence by inhibiting suilysin activity and inflammation. International immunopharmacology. 2019;69:71-8; doi: 10.1016/j.intimp.2019.01.017.
30. Park MH, Kim SY, Roh EY, Lee HS. Difference of Type 3 secretion system (T3SS) effector gene genotypes (exoU and exoS) and its implication to antibiotics resistances in isolates of Pseudomonas aeruginosa from chronic otitis media. Auris Nasus Larynx. 2017;44(3):258-65; doi: 10.1016/j.anl.2016.07.005.
31. Rangel SM, Diaz MH, Knoten CA, Zhang A, Hauser AR. The Role of ExoS in Dissemination of Pseudomonas aeruginosa during Pneumonia. PLoS Pathog. 2015;11(6):e1004945; doi: 10.1371/journal.ppat.1004945.
32. Yahr TL, Wolfgang MC. Transcriptional regulation of the Pseudomonas aeruginosa type III secretion system. Mol Microbiol. 2006;62(3):631-40; doi: 10.1111/j.1365-2958.2006.05412.x.
33. Lombardi C, Tolchard J, Bouillot S, Signor L, Gebus C, Liebl D, et al. Structural and Functional Characterization of the Type Three Secretion System (T3SS) Needle of Pseudomonas aeruginosa. Front Microbiol. 2019;10:573; doi: 10.3389/fmicb.2019.00573.
34. Tang Y, Romano FB, Brena M, Heuck AP. The Pseudomonas aeruginosa type III secretion translocator PopB assists the insertion of the PopD translocator into host cell membranes. J Biol Chem. 2018;293(23):8982-93; doi: 10.1074/jbc.RA118.002766.
35. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, et al. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature. 1992;356(6372):768-74; doi: 10.1038/356768a0.
36. Ulland TK, Ferguson PJ, Sutterwala FS. Evasion of inflammasome activation by microbial pathogens. J Clin Invest. 2015;125(2):469-77; doi: 10.1172/JCI75254.
37. Zhao Y, Shao F. The NAIP-NLRC4 inflammasome in innate immune detection of bacterial flagellin and type III secretion apparatus. Immunol Rev. 2015;265(1):85-102; doi: 10.1111/imr.12293.

38. Ha UH, Kim J, Badrane H, Jia J, Baker HV, Wu D, et al. An in vivo inducible gene of Pseudomonas aeruginosa encodes an anti-ExsA to suppress the type III secretion system. Molecular microbiology. 2004;54(2):307-20; doi: 10.1111/j.1365-2958.2004.04282.x.
39. Ha U, Jin S. Growth phase-dependent invasion of Pseudomonas aeruginosa and its survival within HeLa cells. Infection and immunity. 2001;69(7):4398-406; doi: 10.1128/IAI.69.7.4398-4406.2001.
40. Shin HS, Lee JH, Paek SH, Jung YW, Ha UH. Pseudomonas aeruginosa-dependent upregulation of TLR2 influences host responses to a secondary Staphylococcus aureus infection. Pathogens and disease. 2013;69(2):149-56; doi: 10.1111/2049-632X.12074.
41. El-Mowafy SA, Abd El Galil KH, El-Messery SM, Shaaban MI. Aspirin is an efficient inhibitor of quorum sensing, virulence and toxins in Pseudomonas aeruginosa. Microb Pathog. 2014;74:25-32; doi: 10.1016/j.micpath.2014.07.008.

Figures

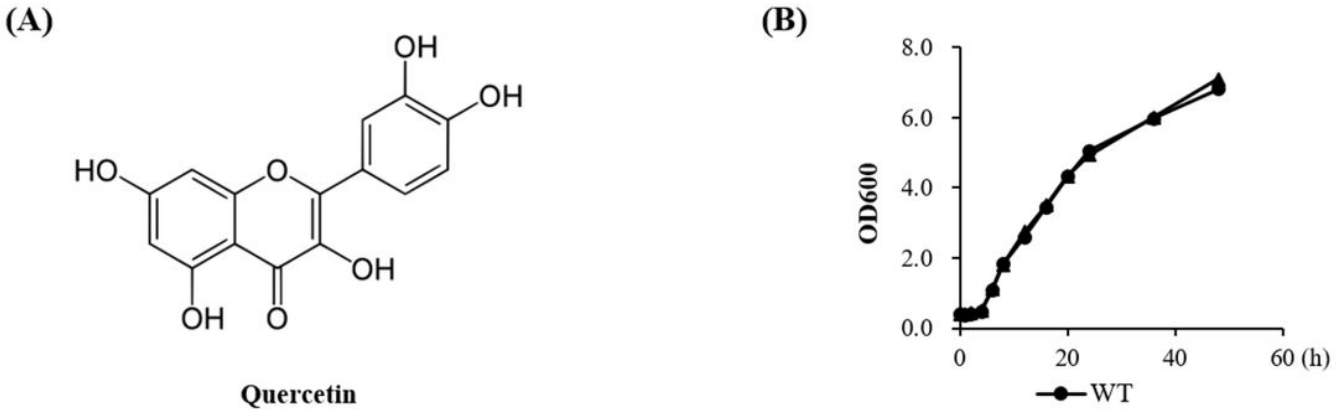

Figure 1

A compound structure and effect of quercetin on bacterial growth. (A) A compound structure of quercetin. (B) The effect of quercetin on the growth of P. aeruginosa was determined by the bacterial growth curve. PAK, P. aeruginosa wild-type strain.

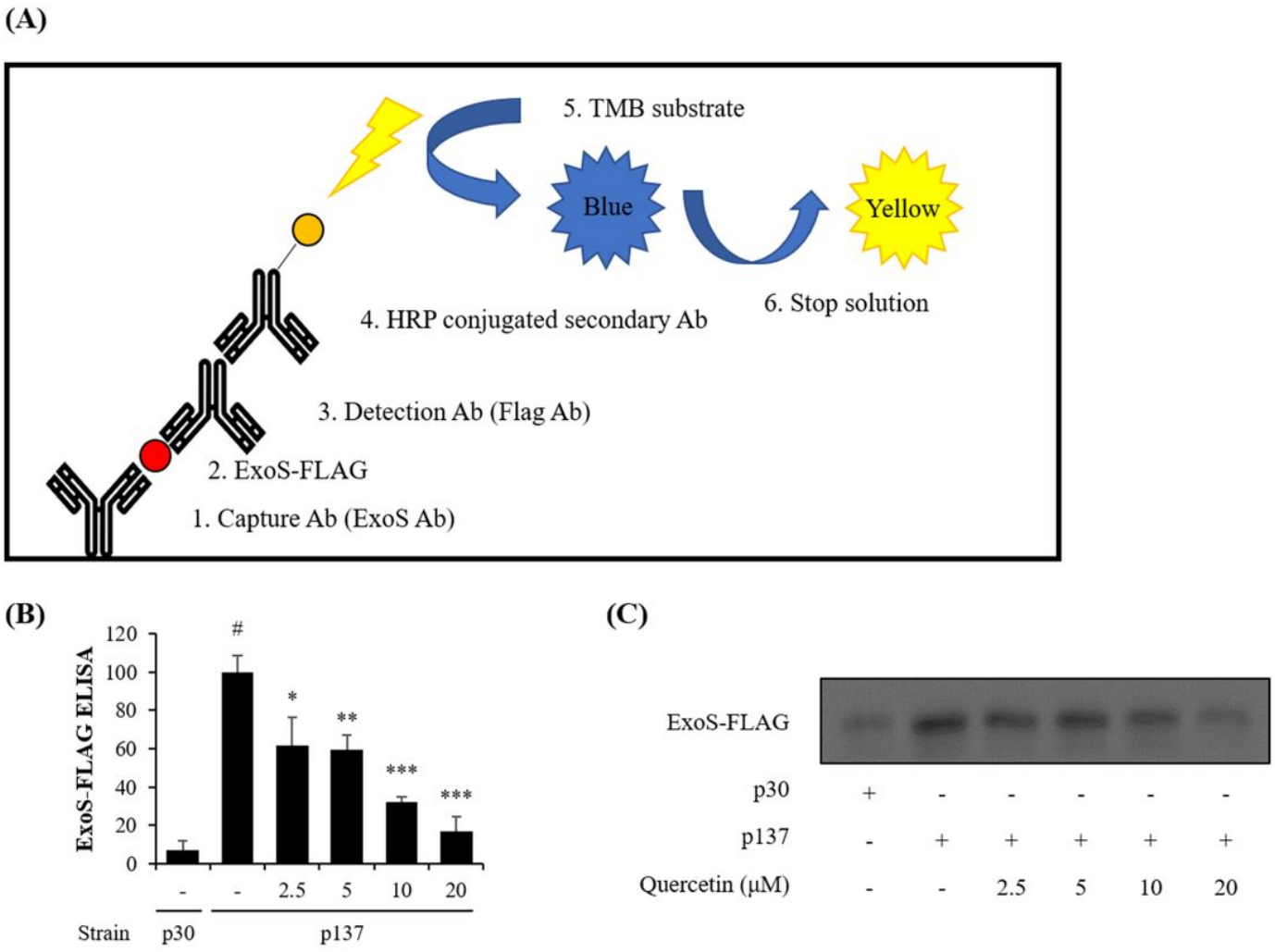

Figure 2

The effect of quercetin on produced and secreted ExoS-FLAG. (A) Overnight culture supernatant of P. aeruginosa p30 and p137 stains treated with quercetin (2.5, 5, 10, and 20 μM) used in ExoS-FLAG ELISA. (B) Lysed proteins of p30 and p137 strains are loaded by using a 10% SDS-PAGE. p30, PAK exoST::Ω; p137, PAK exoST::Ω/pHW0225; ExoS-FLAG, ExoS attached with FLAG; #significantly different from the normal control group, $P < 0.01$. *, **, ***, significantly different from the p137 group, $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

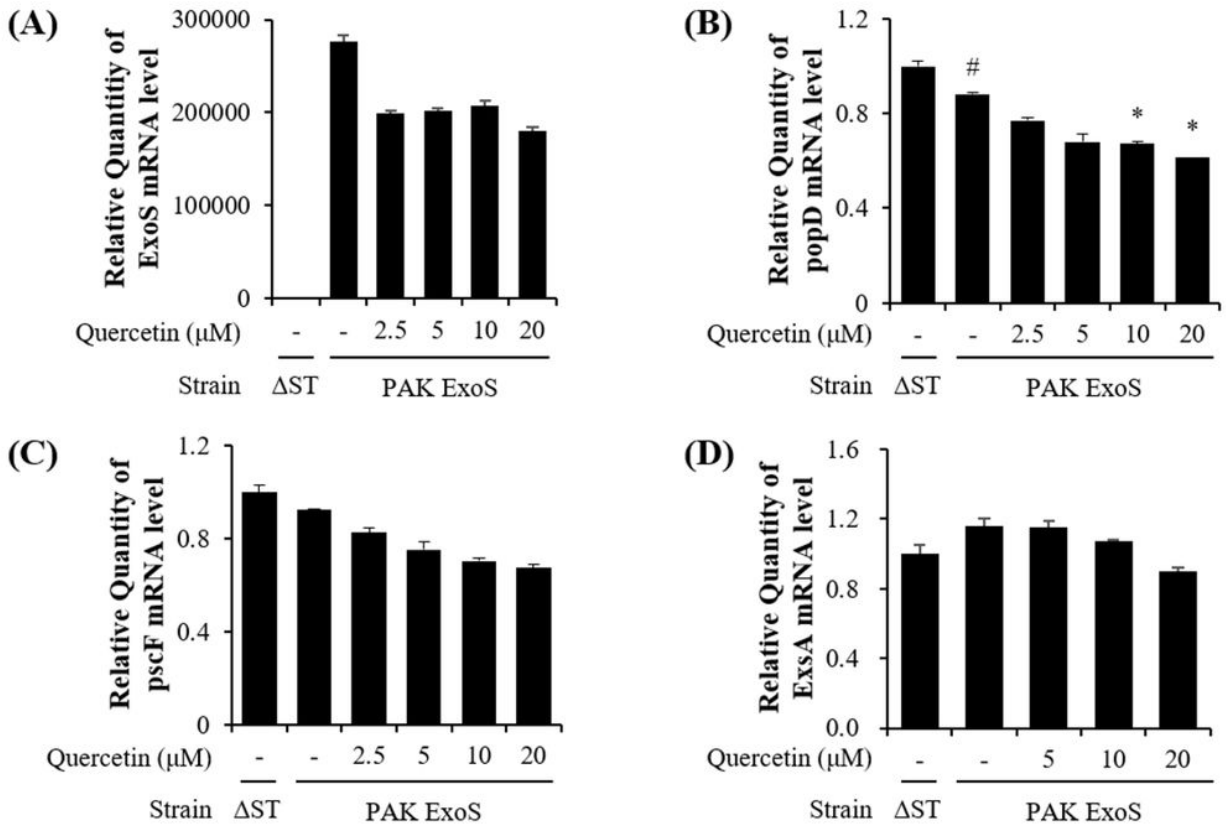

Figure 3

The effect of quercetin on type secretion system regulators and effector protein in P. aeruginosa. ΔST and PAK ExoS strains were grown overnight at 37°C and re-inoculated in fresh LB broth supplemented with quercetin (2.5, 5, 10, and 20 μM) for 4 h. (A-C) Expression of ExoS of exoenzyme, popD and pscF, and the needle protein of T3SS, was quantified by quantitative real-time PCR. (D) The expression of ExsA of transcription factor of T3SS was quantified in the same manner. ΔST, PAK ΔST; PAK ExoS, PAK ΔST/pUCP18 PAKexoS. #Significantly different from the normal control group, $P < 0.05$; *significantly different from the PAK ExoS group, $P < 0.05$.

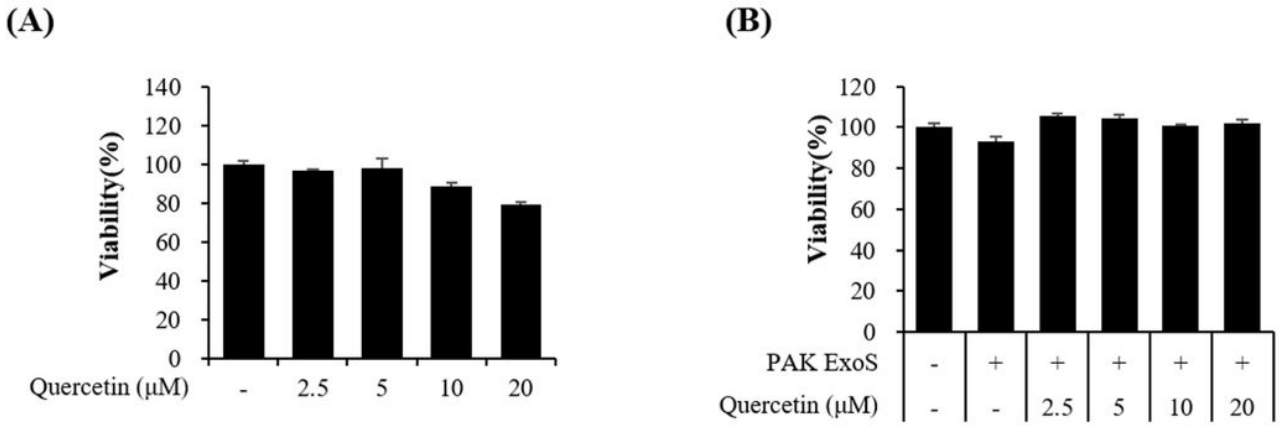

Figure 4

The cytotoxicity of quercetin in uninfected or infected H292 cells. H292 cells were incubated with quercetin (2.5, 5, 10, and 20 μM). In the case of infection, H292 cells infected by MOI 100 of PAK exoS strain after the treatment of quercetin for 1 h. The cytotoxicity of quercetin was evaluated to MTT assay. PAK ExoS, PAK ΔST/pUCP18 PAKexoS.

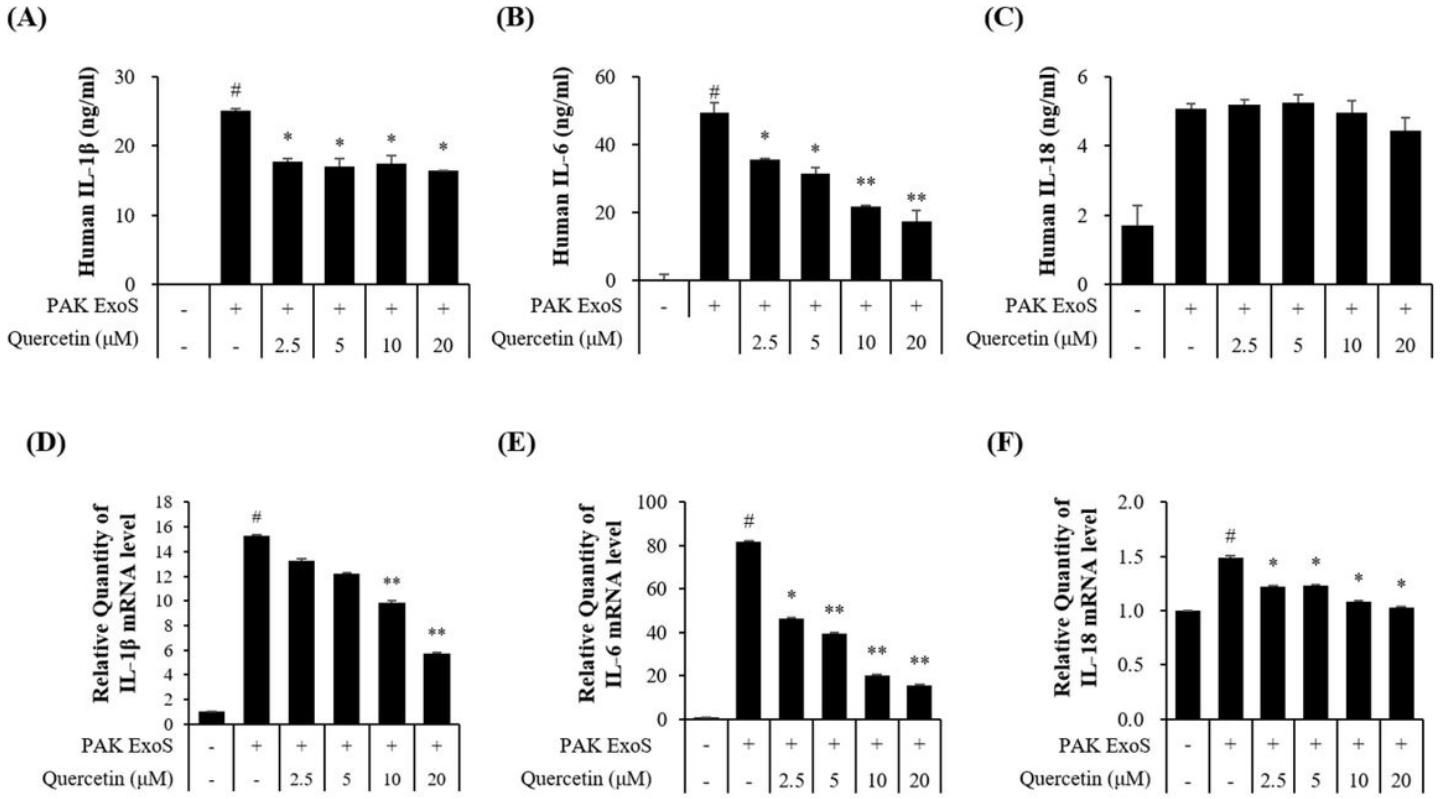

Figure 5

The effect of quercetin on cytokines in H292 cells by ELISA and real-time PCR. H292 cells were treated with quercetin and PAK ExoS for 6 h. Protein level of pro-inflammatory cytokines were evaluated to ELISA and mRNA level were evaluated to real-time PCR. PAK ExoS, PAK ΔST/pUCP18 PAKexoS. #Significantly different from the normal control group, $P < 0.01$. *, **, significantly different from the PAK ExoS group, $P < 0.05$, and $P < 0.01$gy, respectively.

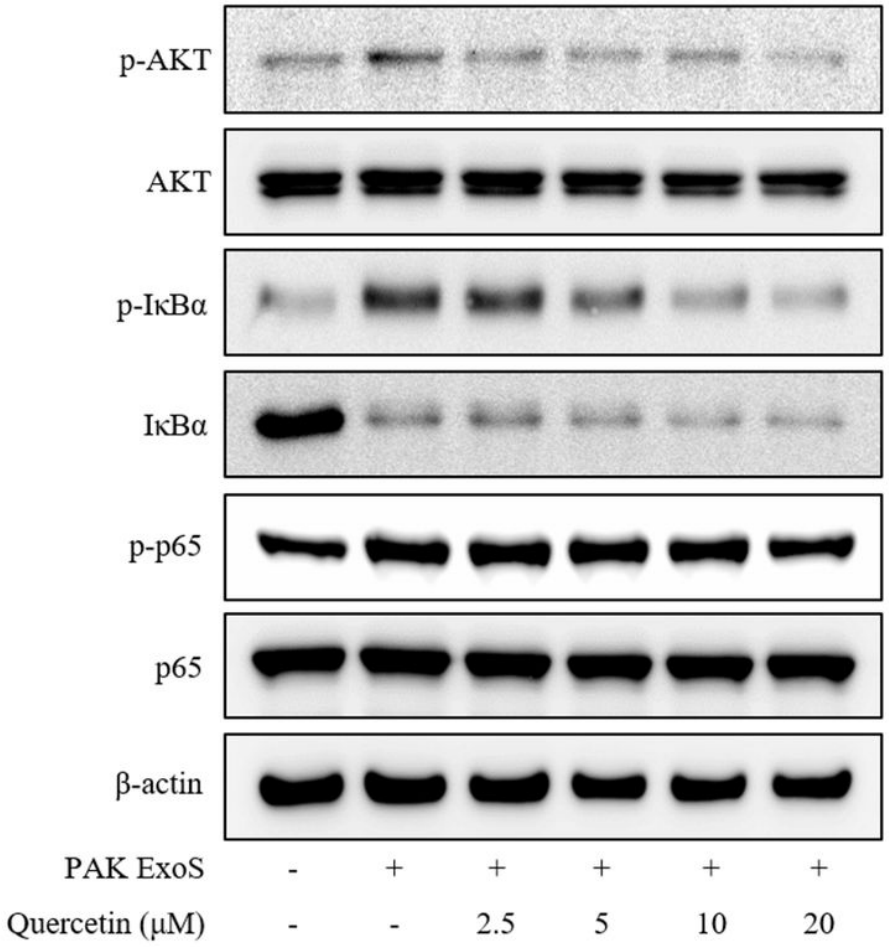

Figure 6

The effect of quercetin on P. aeruginosa-induced NF-κB activation in H292 cells. H292 cells were treated with quercetin and PAK ExoS for 4 h. The expression of NF-κB pathway was evaluated to western blotting. PAK ExoS, PAK ΔST/pUCP18 PAKexoS.

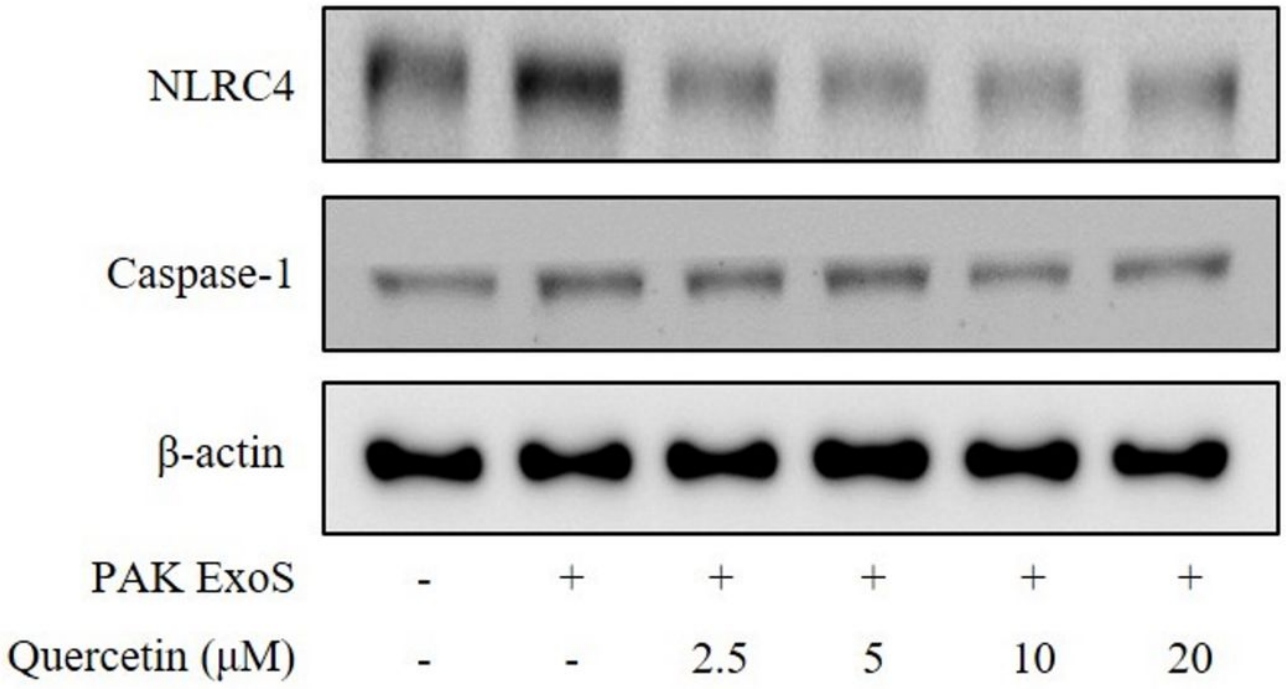

Figure 7

The effect of quercetin on P. aeruginosa-induced NLRC4-Caspase-1 pathway in H292 cells. H292 cells were treated with quercetin and PAK ExoS for 2 h. The expression of NLRC4 and Capase-1 was evaluated to western blotting. PAK ExoS, PAK ΔST/pUCP18 PAKexoS.

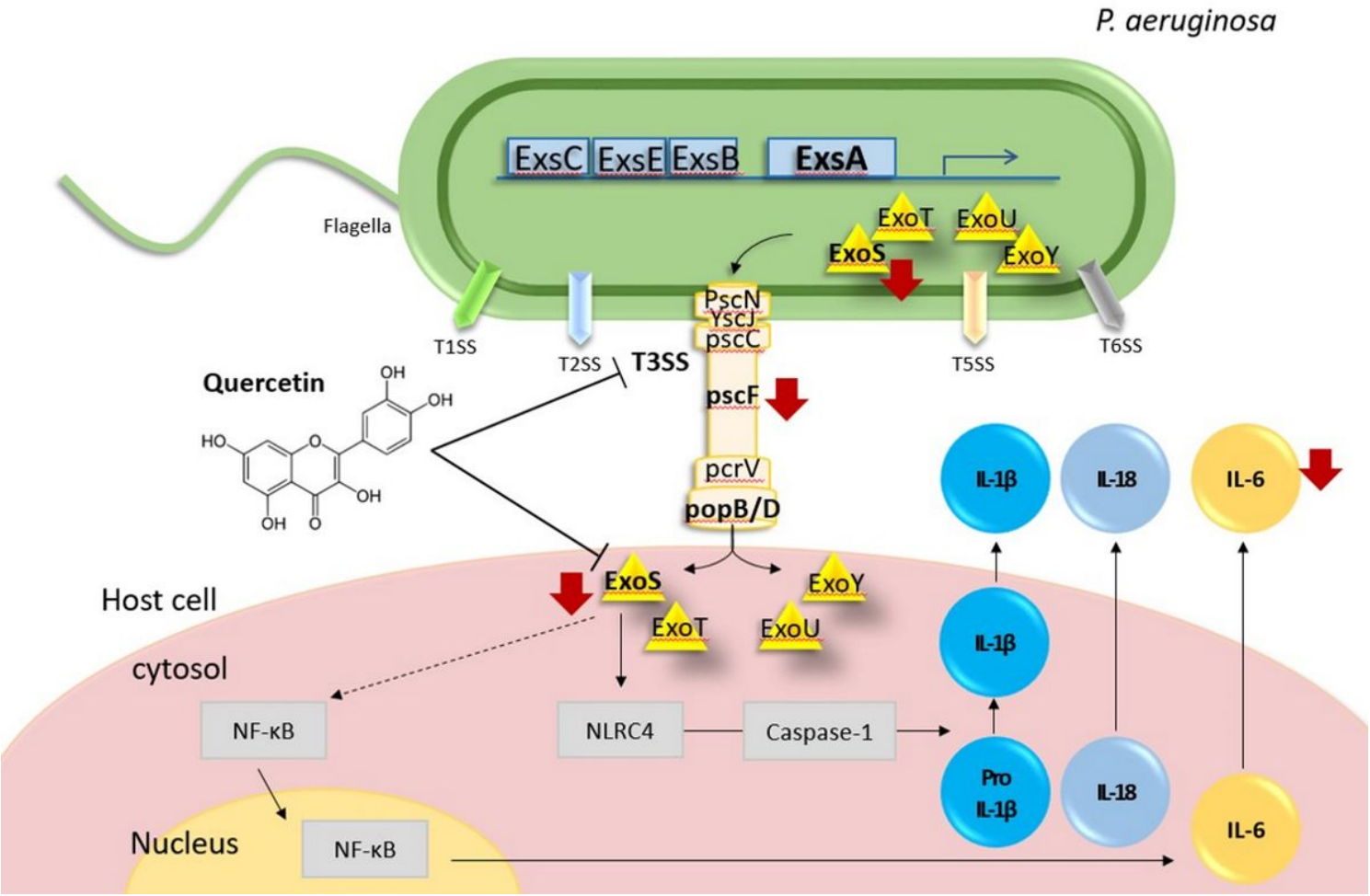

Figure 8

The mechanism of quercetin on ExoS in P. aeruginosa and infected H292 cells. ExoS production of T3SS followed by ExsA decreased and the expression of needle of T3SS (popD, pscF) are also decreased. Then, the injection of ExoS from P. aerugnosa to H292 cells is suppressed by quercetin. The expression of NLRC4-Caspase-1 pathway and the production of pro-inflammatory cytokines (IL-1β, IL-18, IL-6) induced by ExoS are decreased.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS2.xlsx
- TableS1.xlsx
- Fig.S1.docx
- Fig.S2S4.pptx