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Research Article

Ginsenoside Rg3 reduces the adhesion, invasion, and intracellular survival of *Salmonella enterica* serovar Typhimurium

Abraham F. Mechesso, Yixian Quah, Seung-Chun Park*

Laboratory of Veterinary Pharmacokinetics and Pharmacodynamics, College of Veterinary Medicine, Kyungpook National University, Daegu, Republic of Korea

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ABSTRACT

Background: Invasive infections due to foodborne pathogens, including *Salmonella enterica* serovar Typhimurium, are prevalent and life-threatening. This study aimed to evaluate the effects of ginsenoside Rg3 (Rg3) on the adhesion, invasion, and intracellular survival of *S.* Typhimurium.
Methods: The impacts of Rg3 on bacterial growth and host cell viability were determined using the time kill and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assays, respectively. Gentamicin assay and confocal microscopic examination were undertaken to determine the effects of Rg3 on the adhesive and invasive abilities of *S.* Typhimurium to Caco-2 and RAW 264.7 cells. Quantitative reverse transcription polymerase chain reaction was performed to assess the expression of genes correlated with the adhesion, invasion, and virulence of *S.* Typhimurium.
Results: Subinhibitory concentrations of Rg3 significantly reduced ($p < 0.05$) the adhesion, invasion, and intracellular survival of *S.* Typhimurium. Rg3 considerably reduced ($p < 0.05$) the bacterial motility as well as the release of nitrite from infected macrophages in a concentration-dependent manner. The expression of genes related to the adhesion, invasion, quorum sensing, and virulence of *S.* Typhimurium including *cheY*, *hilA*, *OmpD*, *PrgK*, *rsgE*, *SdiA*, and *SipB* was significantly reduced after Rg3 treatment. Besides, the compound downregulated *rac-1* and *Cdc*-42 that are essential for actin remodeling and membrane ruffling, thereby facilitating *Salmonella* entry into host cells. This report is the first to describe the effects of Rg3 on "trigger" entry mechanism and intracellular survival *S.* Typhimurium.
Conclusion: Rg3 could be considered as a supplement agent to prevent *S. Typhimurium* infection.

1. Introduction

Salmonella enterica serovar Typhimurium is the main cause of foodborne gastroenteritis in human [1]. It is also an important pathogen in food-producing animals, including cattle, pigs, and chicken. Infection is commonly acquired after ingestion of contaminated food or water. The bacteria colonize in the intestine and adhere to and invade the enterocytes. Then, it penetrates the basolateral membrane, internalized by phagocytic and non-phagocytic cells, and rapidly disseminates through the bloodstream [2].

The ability of *Salmonella* to cross the intestinal barrier and invade mammalian cells is a crucial step to initiate infection. Invasion of *Salmonella* into nonphagocytic cells is mediated by a type III secretion system (T3SS). T3SS1 is encoded by *Salmonella* pathogenicity islands (SPIs) especially SPI-1 [3]. SPI-1 genome expression activates the release of bacterial effector proteins such as *Salmonella* invasive protein (Sip)A and the *Salmonella* outer proteins, including (Sop)E/E2, SopB, and SopA, which mediates the "trigger" entry mechanism [4]. In addition, *Salmonella* uses Rck (resistance to complement killing), an outer membrane protein, to invade cells via a "zipper" process [5]. Once inside the cell, the survival of *Salmonella* and the outcome of infection are dependent on various bacterial and host factors [6]. Besides, quorum sensing (QS) enables *Salmonella* to communicate with one another and regulate its pathogenicity including biofilm formation, sporulation, virulence factor production, and motility [7].

* Corresponding author. Laboratory of Veterinary Pharmacokinetics and Pharmacodynamics, College of Veterinary Medicine, Kyungpook National University, 80 Daehakro, 41566, Bukgu, Daegu, Republic of Korea
E-mail address: parksch@knu.ac.kr (S.-C. Park).

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The problem of emergence of antibiotic-resistant *S.* Typhimurium necessitates alternative therapeutic options. Various studies have demonstrated the antimicrobial activity of plant components against a wide range of microorganisms [8,9]. Interestingly, the T3SS and QS systems become an ideal target for the development of antibacterial agents. Studies on baicalin [10] and thymol [11] have demonstrated the potentials of plant-based natural compounds in inhibiting the T3SS-1 of *S.* Typhimurium. For the past few years, the authors have been working on large-scale screening of compounds of plant origin that are capable of inhibiting or reducing the QS and invasive abilities of various pathogens including *S.* Typhimurium. Compounds that possess anti-QS and antiinvasive effects such as ginsenoside Rg3 (Rg3) (Supplementary Fig. 1), pyrogallol, and methyl gallate were identified when applied alone or in combination with various antibiotics. Our recent studies verified the anti-QS and antiinvasive activities of methyl gallate in the presence and absence of marbofloxacin in *Chromobacterium violaceum* and *Pseudomonas aeruginosa* [12] and *S.* Typhimurium [13].

This study was undertaken to evaluate the effects of Rg3 on the adhesion, invasion, and survival of *S.* Typhimurium into host cells. Ginseng is widely used in Korea for the treatment and prevention of various diseases. It is available in the market in various forms, such as syrups and tablets. The steroidal structure of ginsenosides enables them to interact with cell membranes, membrane-bound ion channels, and cellular receptors. Previous studies have confirmed the *in vitro* and *in vivo* efficacies of ginsenosides mainly Rb1, Rg1 and Rg3, in the treatment of neurological, cardiovascular, diabetic, and immune disorders [13–16]. A study on *Panax ginseng* confirmed its anti-QS activity in *P. aeruginosa* [17]. Besides, Lee et al [18] revealed the inhibitory potential of polysaccharides from *Panax ginseng* on the adhesion of various pathogens to host cells. Moreover, Chang et al [19] and Huy et al [20] reported the inhibition of *Brucella abortus* and *S.* Typhimurium invasion into RAW 264.7 cells by Rb1 and Rg3, respectively. Despite the widespread uses of ginsenosides for various purposes, including a dietary supplement, no studies have been conducted so far on its impacts on the invasive capacity of *S.* Typhimurium in intestinal epithelial cells. Thus, this study aimed to evaluate the effects of Rg3 on the cellular adhesion, invasion, and QS activities of *S.* Typhimurium.

2. Materials and methods

2.1. Chemicals and reagents

Unless specified, the chemicals and reagents used in this study were purchased from Sigma (St. Louis, MO, USA). A stock preparation of Rg3 was prepared in dimethyl sulfoxide (DMSO) and diluted in the appropriate medium to the desired concentration. To avoid the cytotoxic and antibacterial effects of DMSO, the proportion in the final diluent never exceeded 0.1% (v/v).

2.2. Salmonella strains and culture conditions

S. Typhimurium (LVPP-STI15) and *S.* Typhimurium (ATCC 14028) strains were maintained in Luria–Bertani (LB) agar (Difco, Sparks, MD, USA) at 37°C with shaking. The strains of *Salmonella* were chosen based on a previous study on the antibiotic susceptibility profiles of *Salmonella* isolates to the commonly used antibiotics in veterinary medicine especially marbofloxacin. The minimum inhibitory concentrations of marbofloxacin against *S.* Typhimurium ATCC 14028 and LVPP-STI15 were 0.03 and 2 μg/mL, respectively [21]. Before each experiment, *S.* Typhimurium was grown overnight in LB broth at 37°C.

2.3. Cell culture

Human colorectal cancer (Caco-2) and murine macrophage (RAW 264.7) cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Caco-2 cells were cultivated in minimum essential medium (MEM; Gibco, Grand Island, NY, USA) containing 1% penicillin–streptomycin, 1% nonessential amino acid, and 20% fetal bovine serum, whereas RAW 264.7 macrophages were maintained in Roswell Park Memorial Institute medium containing 1% penicillin–streptomycin and 10% fetal bovine serum. Cells were grown in monolayers at 37°C in 5% CO_2, and the medium was changed every other day.

2.4. Bacterial growth curve

The effect of Rg3 on the growth curve of *S.* Typhimurium was determined according to the method described by Brunelle et al [22], with a slight modification. Briefly, overnight cultures of *S.* Typhimurium were diluted and grown up to an early log phase ($OD_{600} = 0.15$) in 96-well plates. Serial dilutions of Rg3 (15.6–500 μM) were added and incubated at 37°C for 24 h with continuous shaking. LB broth in the presence and absence of *S.* Typhimurium was included as controls. The growth of *S.* Typhimurium was evaluated based on the OD_{600} measurements taken every hour using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Cytotoxicity assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay was performed to evaluate the impacts of Rg3 on the viability of Caco-2 and RAW 264.7 cells [23]. Briefly, cells (10^5/mL) were grown onto 96-well plates in their respective antibiotic-free medium for 24–48 h. Except for the control wells, cells were treated with various concentrations of Rg3 (0.2–100 μM) and incubated at 37°C in 5% CO_2 for 24 h. Then, the medium containing Rg3 was replaced by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (0.5 mg/mL) solution. After 4 h of incubation at 37°C, 100 μL of DMSO was added to dissolve the formazan precipitate. The absorbance was determined at 570 nm using a VersaMax microplate reader. The percentage survival was determined using the following formula:

Percentage viability = (OD value of treated cells/OD value of control cells) *100

where OD is the optical density.

2.6. Motility assay

The effect of Rg3 on the bacterial motility was assessed on LB agar (0.4% w/v) containing 0.5% glucose [24]. Rg3 (25–100 μM) was mixed with the medium and allowed to dry at 24°C. Then, plates were inoculated with 5 μL of an 8-h culture of *S.* Typhimurium. The diameter of bacterial spread was determined after incubation at 37°C for 10 h.

2.7. Adhesion and invasion assay

The effects of Rg3 on the adhesive and invasive abilities of *S.* Typhimurium were evaluated using the gentamicin protection assay [25]. Caco-2 cells (10^5/mL) were cultured in the antibiotic-free medium on 24-well plates. After 24–48 h, the cells were treated with different concentrations of Rg3 (12.5–50 μM) for 45 min. Then, 4-h cultures of *S.* Typhimurium were centrifuged

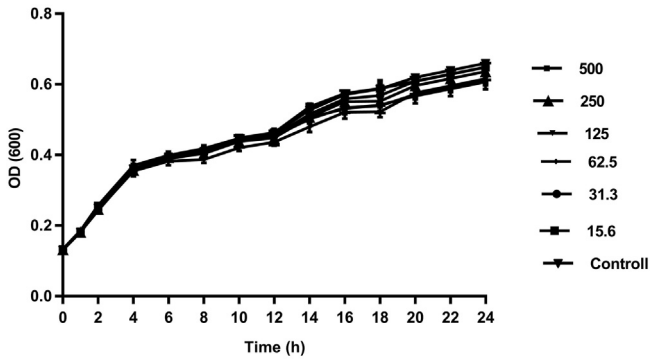

Fig. 1. Growth curve of *S.* Typhimurium in the presence of various concentrations of Rg3. The growth curves of both strains of *S.* Typhimurium were almost similar. Thus, a representative figure is displayed. Values represent mean ± SD of five independent experiments. OD, optical density; SD, standard deviation.

(10,000 *g* for 10 min), washed twice with 1 × phosphate-buffered saline (PBS), and resuspended in antibiotic-free medium. Caco-2 cells were infected at a multiplicity of infection of 1:10, centrifuged at 1000 *g* for 2 min and incubated at 37°C for 45 min. The supernatant was removed, and cells were washed three times (1 × PBS) to remove unattached bacteria. Cells were lysed with 1% Triton X-100, and the suspensions were serially diluted in agar saline. The total number of adhering bacteria (CFU/mL) was determined after incubation at 37°C for 16–18 h.

In the invasion assay, Rg3-pretreated and infected Caco-2 cells, as described previously, were incubated at 37°C for 60 min. The cells were washed three times with 1 × PBS and incubated for 60 min in the MEM medium supplemented with gentamicin (100 μg/mL). Cell lysis and the total number (CFU/mL) of intracellular bacteria were determined following a similar procedure described previously in the adhesion assay.

2.8. Intracellular survival of Salmonella Typhimurium

Survival of *S.* Typhimurium in macrophages pretreated with Rg3 (12.5–50 μM) was determined by slight modifications of a previously described method [26]. RAW 264.7 cells (10^5/mL) grown in antibiotic-free Roswell Park Memorial Institute medium was treated and infected as described previously for Caco-2 cells in the invasion assay. After incubation with gentamicin (100 μg/mL) at 37°C for 60 min, cells were washed with 1 × PBS, and 25 μg/mL of gentamicin in MEM was added for the remaining incubation time. At 6 and 12 h after incubation, the total number of surviving bacteria was determined as described previously.

2.9. Confocal microscopic examination of intracellular S. Typhimurium

Confocal microscopic examination was performed to visualize intracellular *S.* Typhimurium in Caco-2 cells pretreated with 25 and 50 μM of Rg3 [27]. Briefly, Caco-2 cells (10^4/mL) were seeded onto chambered glass slides in antibiotic-free MEM. After incubation at 37°C for 24 h, cells were treated and infected by a method similar to the invasion assay described previously. Then, the medium was aspirated and cells were washed twice with 3-(N-morpholino) propanesulfonic acid (0.1 M MOPS, pH 7.2) buffer mixed with magnesium chloride (MgC_{l2}, 1 mM) (MOPS/MgC_{l2}). The medium was replaced by 400 μL of BacLight staining solution (Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA) containing 5 μM SYTO9 and 30 μM propidium iodide mixed with saponin (0.1%) in MOPS/MgC_{l2}. After 15–30 min of incubation at room temperature, cells were washed (2 ×) in MOPS/MgC_{l2} and covered with coverslips. Finally, slides were examined using a Zeiss confocal microscope (LSM700; Carl-Zeiss, Jena, Germany).

2.10. Measurement of nitric oxide production

The level of nitric oxide (NO) in the cell culture supernatant was measured to evaluate the effects of Rg3 (12.5–50 μM) on *S.* Typhimurium–induced oxidative stress. RAW 264.7 cells (10^5/mL) were pretreated and infected by a method similar to the adhesion assay described previously. The level of NO produced after 12 h of incubation was determined using the Griess assay [28]. The experiment was conducted in duplicate in three independent experiments, and absorbance was determined at 550 nm using a VersaMax microplate reader (Molecular Devices).

2.11. Virulence gene expression

S. Typhimurium (10^6/mL) was treated with Rg3 (12.5–50 μM) in LB broth and incubated at 37°C for 13 h. TRIzol (Ambion Life Technologies, Carlsbad, CA, USA) was used to extract total RNA. The purity of RNA was confirmed using a Nanophotometer (Implen GmbH, Munich, Germany). A random hexamer was added to the bacterial RNA, and cDNA was synthesized by quantitative reverse transcription polymerase chain reaction (qRT-PCR) premix

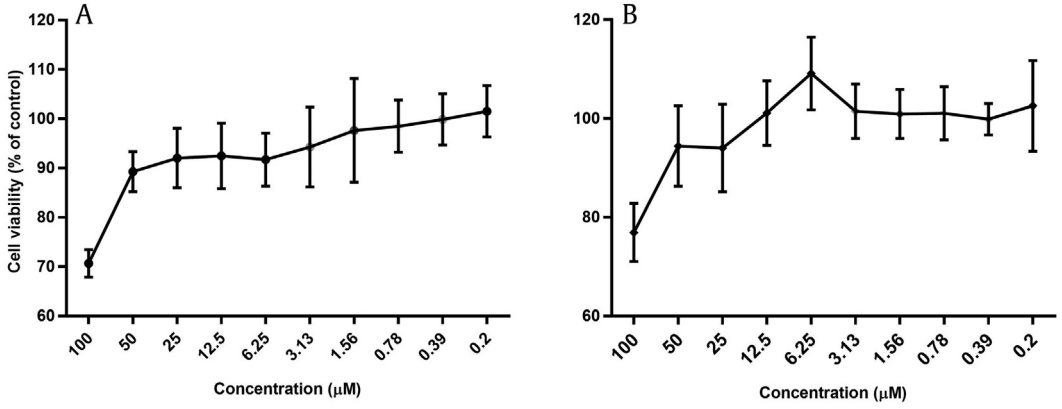

Fig. 2. Viability of RAW 264.7 macrophages (**A**) and Caco-2 intestinal cells (**B**) exposed to various concentrations of Rg3. Data are presented as the mean ± SD of five independent experiments. SD, standard deviation.

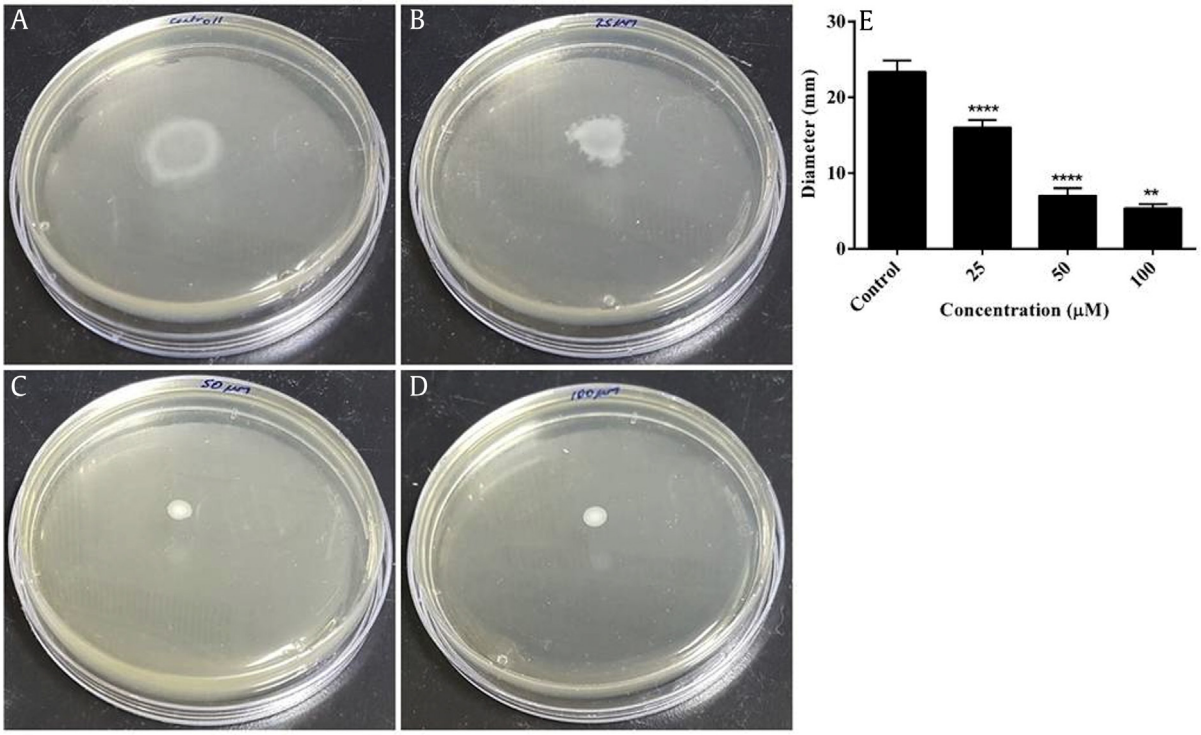

Fig. 3. Motility of *S.* Typhimurium ATCC 14028 in the presence of 0 μM (**A**), 25 μM (**B**), 50 μM (**C**), and 100 μM (**D**) of Rg3. Bar graphs depicted mean ± SD of three independent experiments (**E**). The differences in the diameter of spread were insignificant ($p > 0.05$) between strains (data not shown). Therefore, representative photographs of on LB agar plates are displayed. $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the non-treated *S.* Typhimurium. LB, Luria–Bertani; SD, standard deviation.

(Bioneer, Korea). Gene expression levels of *hilA, PrgK, sipB, OmpA, OmpD*, *cheY*, *lexA,* and *rck* were determined by using a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using IQ SYBR Green Supermix [Bio-Rad Laboratories (Singapore) Pte. Ltd.]. The reaction conditions include denaturation at 95°C for 3 min, 40 cycles of amplification at 95°C for 10 s and at 58°C for 30 s, and dissociation at 95°C for 15 s and at 60°C for 30 s. The sequences of all primers used in this experiment are listed in supplementary file 2 (Table 1). The housekeeping gene *rrsG* was used to normalize the gene expression ($2^{-\Delta\Delta CT}$).

2.12. QS gene expression

Gram-negative bacteria such as *Salmonella* uses the acetylated homoserine lactone (AHL) signaling molecule to activate the QS system [29]. The effects of Rg3 on the expression AHL-induced QS genes was assessed following a previous method [24]. Briefly, *S.* Typhimurium (10^6/mL) was supplemented with the AHL (1 μmol/mL) in the presence and absence of Rg3 (12.5-50 μM) in LB broth. Then, it was incubated at 37°C for 13 h. Total RNA extraction, cDNA synthesis, and PCR analysis were undertaken following similar procedures explained previously in the virulence gene expression assay. The gene expression levels of *sdiA, rsgE,* and *rck* were determined by using IQ SYBR Green Supermix (Bio-Rad). The sequences of all primers used in this experiment are listed in the supplementary file 2 (Table 1).

2.13. Invasion-related host cell gene expression

The impacts of Rg3 (12.5–50 μM) on *S.* Typhimurium–induced expression of genes related to invasion was assessed in Caco-2 cells. Pretreated cells were infected by a method similar to the invasion assay. Extraction of total RNA and synthesis of cDNA was performed as described previously. Oligo (dT) primer was used to synthesize cDNA. Gene expression levels of *RhoA, RhoG, Cdc-42,* and *rac-1* were assessed using a real-time PCR detection system as described previously. The reaction conditions include denaturation at 95°C for 7 min, followed by 35 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 30 s. The housekeeping gene β-actin was used to normalize the gene expression ($2^{-\Delta\Delta CT}$). The sequences of all primers used in this experiment are listed in the supplementary file 2 (Table 1).

2.14. Data analysis

Data were analyzed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). One-way analyses of variance (ANOVAs) followed by the Tukey Honest significant difference (HSD) test were conducted to compare the mean values among treatment groups. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of Rg3 on bacterial growth

The growth of *S.* Typhimurium strains was not affected by the presence of Rg3 up to a maximum tested concentration of 500 μM (Fig. 1). The growth curves of both strains exposed to varying concentrations of Rg3 were almost similar. The figure shows a representative growth curve of *S.* Typhimurium in the presence of various concentrations of Rg3.

3.2. Cytotoxicity of Rg3

Exposure to 100 μM of Rg3 killed 29.3% of RAW 264.7 cells and 24.1% of Caco-2 epithelial cells, suggesting the toxicity of the

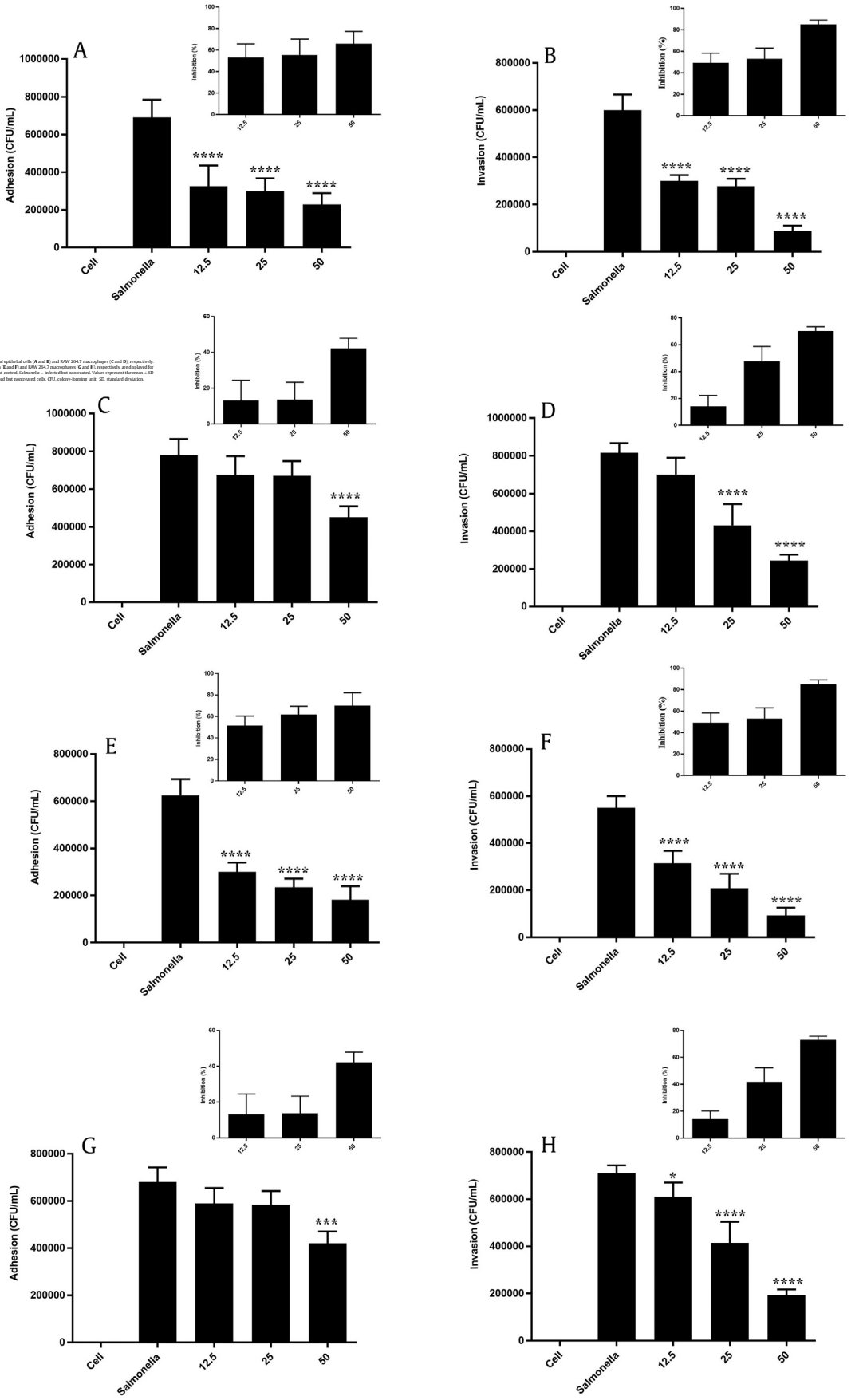

Fig. 4. Effects of Rg3 on adhesion and invasion of *S.* Typhimurium (ATCC 14028) to Caco-2 intestinal epithelial cells (**A** and **B**) and RAW 264.7 macrophages (**C** and **D**), respectively. The counts of adhered and invaded *S.* Typhimurium (SVPP-SIT15) to Caco-2 intestinal epithelial cells (**E** and **F**) and RAW 264.7 macrophages (**G** and **H**), respectively, are displayed for comparison. The upper graphs represent the percentage inhibition. Cell –: noninfected and untreated control; Salmonella +: infected but nontreated. Values represent the mean ± SD of five independent experiments. $^{*}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ compared to the infected but nontreated cells. CFU, colony-forming unit; SD, standard deviation.

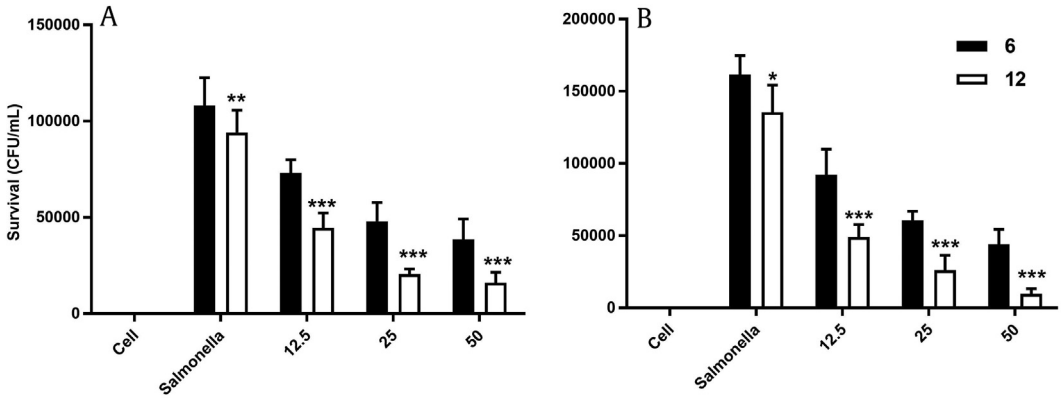

Fig. 5. Intracellular survival of *S.* Typhimurium ATCC (14028) (**A**) and (LVPP-STI15) (**B**) in RAW 264.7 macrophages, respectively, after 6 and 12 h of incubation with various concentrations of Rg3. Cell = noninfected and nontreated control, *Salmonella* = infected but nontreated. The data are given as mean ± SD of five independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the corresponding intracellular *S.* Typhimurium at 6 h. CFU, colony-forming unit; SD, standard deviation.

compound at this concentration (Fig. 2). However, more than 89% and 94% of RAW 264.7 and Caco-2 cells, respectively, survived after treatment with 50 μM Rg3. Therefore, only subinhibitory concentrations of Rg3 (≤50 μM) that were not lethal to the bacteria and host cells were selected for subsequent experiments.

3.3. Inhibitory effect of Rg3 on the S. Typhimurium motility

Rg3 significantly reduced ($p < 0.01$) the motility of both *S.* Typhimurium isolates in a concentration-dependent manner (Fig. 3A). Exposure of *S.* Typhimurium to 100 and 50 μM of Rg3 reduced the bacterial spread by more than threefold than in the nontreated control (Fig. 3B).

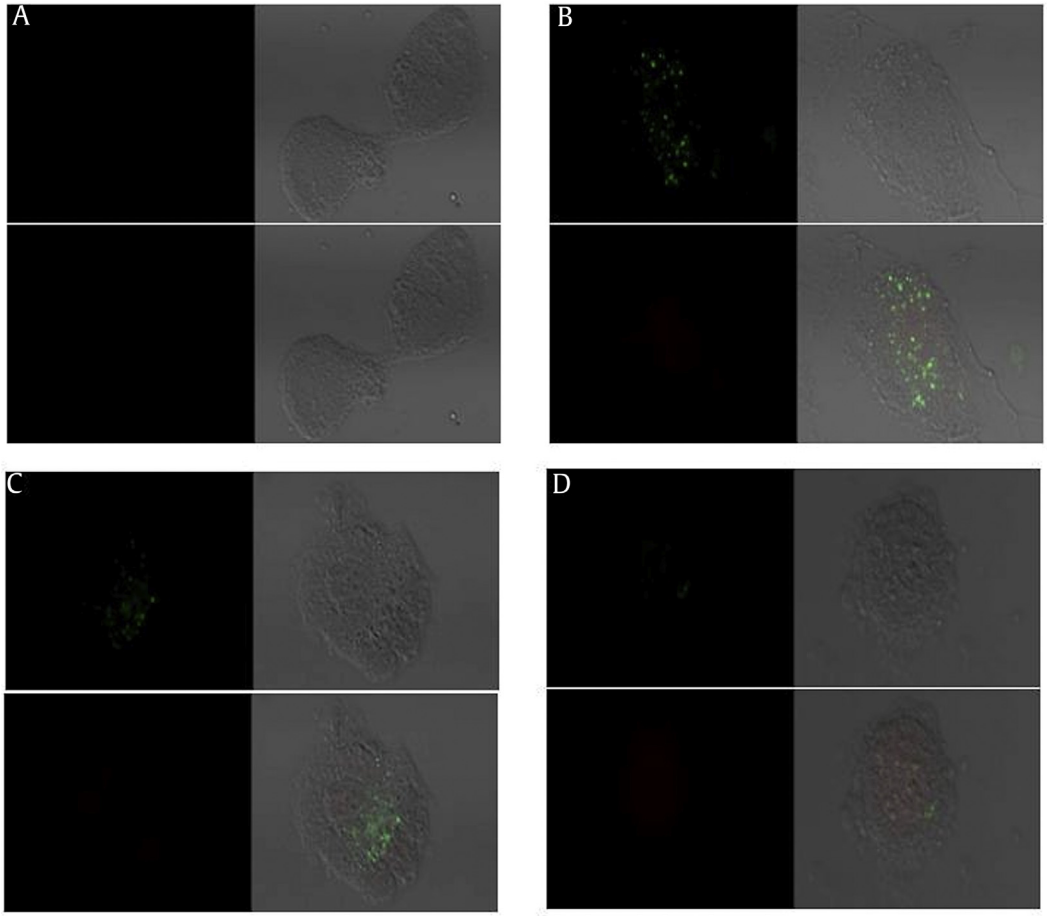

Fig. 6. Confocal images of Caco-2 cells pretreated with various concentrations of Rg3 and infected with *S.* Typhimurium ATCC 14028. Uninfected cell (**A**), Infected but nontreated (**B**), infected and treated with 25 μM of Rg3 (**C**), and infected and treated with 50 μM of Rg3 (**D**). Parts of each image represent SYTO-9, upper left; cell structure, upper right; PI, lower left; and SYTO-9/PI merged image, lower right. (Green, live bacteria).

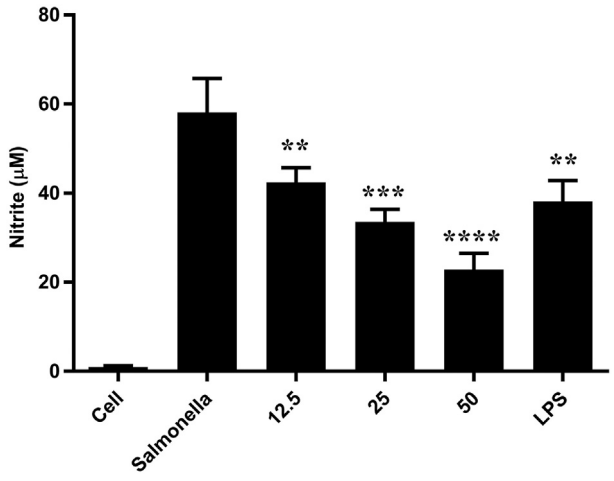

Fig. 7. Effect of Rg3 *S.* Typhimurium–induced nitric oxide production in RAW 264.7 cells. Results are depicted as the mean ± SD of three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the infected and nontreated cells. SD, standard deviation.

3.4. Impacts of Rg3 on S. Typhimurium adhesion and invasion

Preincubation of Caco-2 cells with 12.5–50 μM Rg3 significantly reduced ($p < 0.0001$) the adhesion and invasion of *S.* Typhimurium by at least twofold compared with those without Rg3 (Fig. 4). Fifty micromolar of Rg3 strongly decreased the adhesion (34 and 29.9%) and invasion (30.8 and 26.9%) of *S.* Typhimurium (ATCC 14028 and LVPP-STI15), respectively, to Caco-2 cells. Similarly, the invasion of *S.* Typhimurium (ATCC 14028 and LVPP-STI15) to RAW 264.7 cells was lowered significantly ($p < 0.001$) in the presence of 25 μM (52.3 and 59.1%) and 50 μM (29.7 and 27%) of Rg3. However, only 50 μM of Rg3 produced a significant reduction (61.2% to 57.8%, $p < 0.001$) in the adhesion of *S.* Typhimurium strains to RAW 264.7 cells.

3.5. Effect of Rg3 on intracellular survival of S. Typhimurium

RAW 264.7 cells preincubated with Rg3 demonstrated concentration- and time-dependent killing of intracellular *S.* Typhimurium (Fig. 5). After 6 and 12 h of incubation with Rg3 (50 μM), the intracellular load of *S.* Typhimurium strains was drastically reduced ($p < 0.001$) by 65.5% and 86.5% (ATCC 14028) and 72.4% and 93% (LVPP-STI15). After 12 h of exposure, the bacterial count in nontreated cells was also reduced significantly ($p < 0.01$) but to a lesser extent than that in the Rg3-treated cells.

3.6. Confocal microscopic examination of Rg3-pretreated and infected cells

The invasion-preventive ability of Rg3 was confirmed by confocal microscopic examination of SYTO-9/PI–Propidium iodide

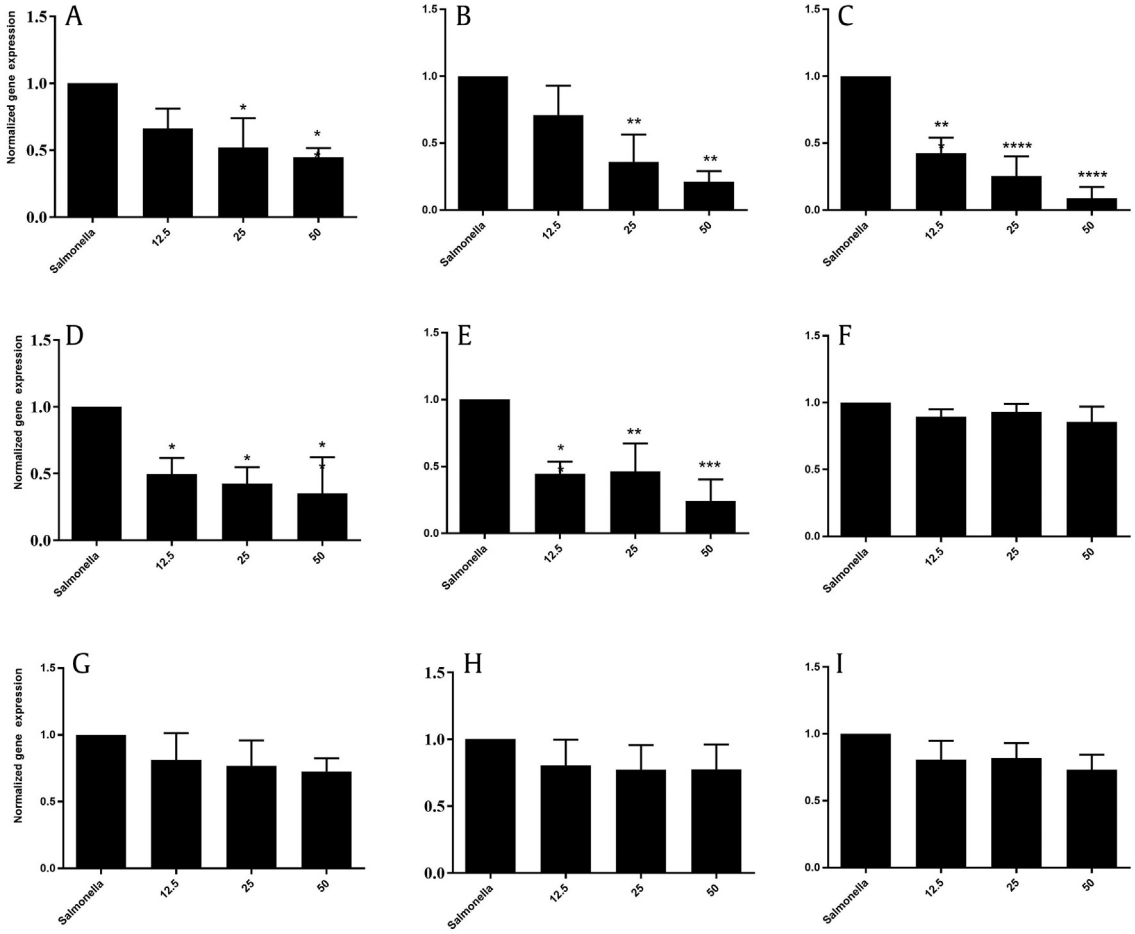

Fig. 8. Effects of Rg3 on virulence gene expressions of *S.* Typhimurium ATCC 14028. qRT-PCR was performed to evaluate the gene expressions of *OmpD* (**A**), *SipB* (**B**), *PrgK* (**C**), *cheY* (**D**), *hilA* (**E**), *OmpA* (**F**), *invA* (**G**), *LexA* (**H**), and *rck* (**I**). *Salmonella* = nontreated *S.* Typhimurium. Results are the mean ± SD of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the nontreated *S.* Typhimurium. SD, standard deviation.

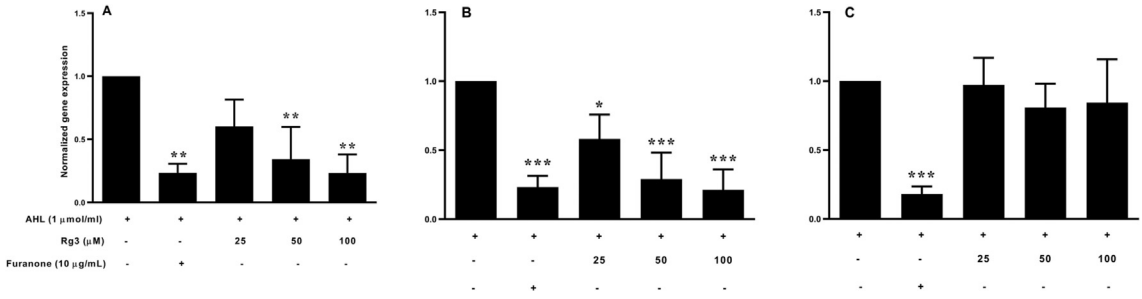

Fig. 9. Quorum sensing gene expressions of *S.* Typhimurium ATCC 14028 treated with various concentrations Rg3. The bar graph represents gene expressions of *sdiA* (**A**), *srgE* (**B**), and *rck* (**C**) genes. Furanone (10 μg/mL) was used as a positive control. Results are the mean ± SD of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with the expressions in AHL-stimulated but nontreated *S.* Typhimurium. AHL, acetylated homoserine lactone; SD, standard deviation.

stained Caco-2 cells. Accordingly, a relatively small number of viable bacteria (fluoresced green) were observed in infected cells preincubated with 25 and 50 μM of Rg3 than the infected but nontreated cells (Fig. 6).

3.7. Inhibitory effect of Rg3 on S. Typhimurium–induced nitrite release

Rg3 significantly ($p < 0.01$) reduced the release of nitrite from infected macrophages in a concentration-dependent manner (Fig. 7). The amount of nitrite released by *S.* Typhimurium (ATCC 14028)–infected cells preincubated with 12.5, 25, and 50 μM of Rg3 was 42.4, 33.6, and 22.8 μM, respectively. The differences in Rg3-induced reductions in nitrite levels were not significant between the two strains ($p > 0.05$) (data not shown).

3.8. Effects of Rg3 on S. Typhimurium virulence gene expression

Rg3 significantly ($p < 0.05$) downregulated several genes that are associated with virulence of *S.* Typhimurium ATCC 14028 (Fig. 8). The expression of *OmpD*, *SipB*, and *PrgK* was reduced ($p < 0.01$) by 55.3, 78.8, and 91.1%, respectively, after exposed to 50 μM of Rg3. Similarly, Rg3 downregulated the expression of *cheY* and *hilA* by at least 50%. However, treatment did not produce a significant change ($p > 0.05$) on the expression of *OmpA*, *invA*, *lexA,* and *rck*.

3.9. Inhibitory effect of Rg3 on S. Typhimurium QS gene expression

Salmonella cannot synthesize AHLs which are crucial for the activation of QS genes, but it can detect AHLs produced by other bacteria [30]. Therefore, *S.* Typhimurium ATCC 14028 was incubated with AHL to induce the expression of QS genes. Rg3 downregulated the expression of *S.* Typhimurium QS genes in a concentration-dependent manner ($p < 0.01$) (Fig. 9). qRT-PCR analysis depicted at least a twofold reduction in the AHL-induced expression of *SdiA* and *rsgE* in *S.* Typhimurium exposed to 25 and 50 μM of Rg3. The downregulations of *sdiA and rsgE* caused by 50 μM of Rg3 were comparable with that of furanone (positive control). In contrast, Rg3 did not affect the *rck* gene expression.

3.10. Impacts of Rg3 on invasion-related host cell protein

To elucidate the contribution of host cells in *S.* Typhimurium ATCC 14028 invasion, the gene expression of invasion-related host cell proteins and virulence factors was evaluated in Caco-2 cells (Fig. 10). Pretreatment of cells with 50 μM of Rg3 downregulated ($p < 0.01$) the expressions of *rac-1* and *Cdc*-42 by 58.6 and 51.3%, respectively. However, treatment did not produce a significant ($p > 0.05$) effect on the expression of *RhoA* and *RhoG*.

4. Discussion

S. Typhimurium is a global public health threat affecting both human and animals. The emergence of resistant strains necessitates alternative treatment options targeting various virulence mechanisms of the bacteria [31]. Despite the enormous potential of natural compounds in providing novel antimicrobial leads, only a small proportion (less than 10%) of the world's biodiversity has been studied for potential antibacterial activity [32]. In this study, we have attempted to evaluate the impacts of Rg3 on the adhesion, invasion, and survival of *S.* Typhimurium in various cell lines. In addition, the effects of Rg3 on *S.* Typhimurium motility, QS ability, and the expression of genes related to host cell invasion and virulence were determined.

Various authors reported the antibacterial activities of crude extracts of ginseng against major foodborne pathogens including *S.* Typhimurium. A study by Lee et al [33] demonstrated that steamed extract leaves of ginseng inhibited the growth of *S. enteritidis* by disrupting bacterial membrane. Chung et al [34] showed the inhibitory effects of Korean Red Ginseng marc with aluminum sulfate on the fecal load of *S. enterica.* In this study, Rg3 did not affect the growth of *S.* Typhimurium up to 500 μM concentrations. In addition, Lee and Jeong [35] and Yang et al [36] reported that Rg3 did not affect the viability of RAW 264.7 cells at a maximum tested concentration of 100 μg/mL. In the present study; however, 100 μM of Rg3 reduced the viability of Caco-2 and RAW 264.7 cells. Thus, considering the direct effects of the compound on the bacteria and mammalian cells, concentrations ≤ 50 μM were used for subsequent experiments.

For successful initiation of infection, *Salmonella* has to adhere, invade, and multiply in various cells and tissues. Coordinated expression of various virulence factors is vital for adhesion, invasion, and multiplication of *S.* Typhimurium in host cells. The absence of one or more of these factors reduces the adhesive and invasive abilities of *S.* Typhimurium, which subsequently reduces bacteria-induced inflammatory host response [24,37]. Rg3 significantly reduced the gene expressions of *ompD*, *PrgK*, and *Sip-B*. *ompD* is correlated with the adhesion of *Salmonella* to macrophages and intestinal epithelial cells [38,39]. The inability of bacterial adherence facilitates its physical elimination and impedes the initiation of infection [40]. *PrgK* is one of those genes that encode for SPI-1 secretion apparatus proteins and assembly of the needle complex. Consequently, it facilitates the entry of *Salmonella* into the host cell [41,42], whereas *Sip-B*–encoded proteins are mainly important for translocation of effector proteins through this needle into the host cell and facilitate invasion [42]. Therefore, the reduction in the

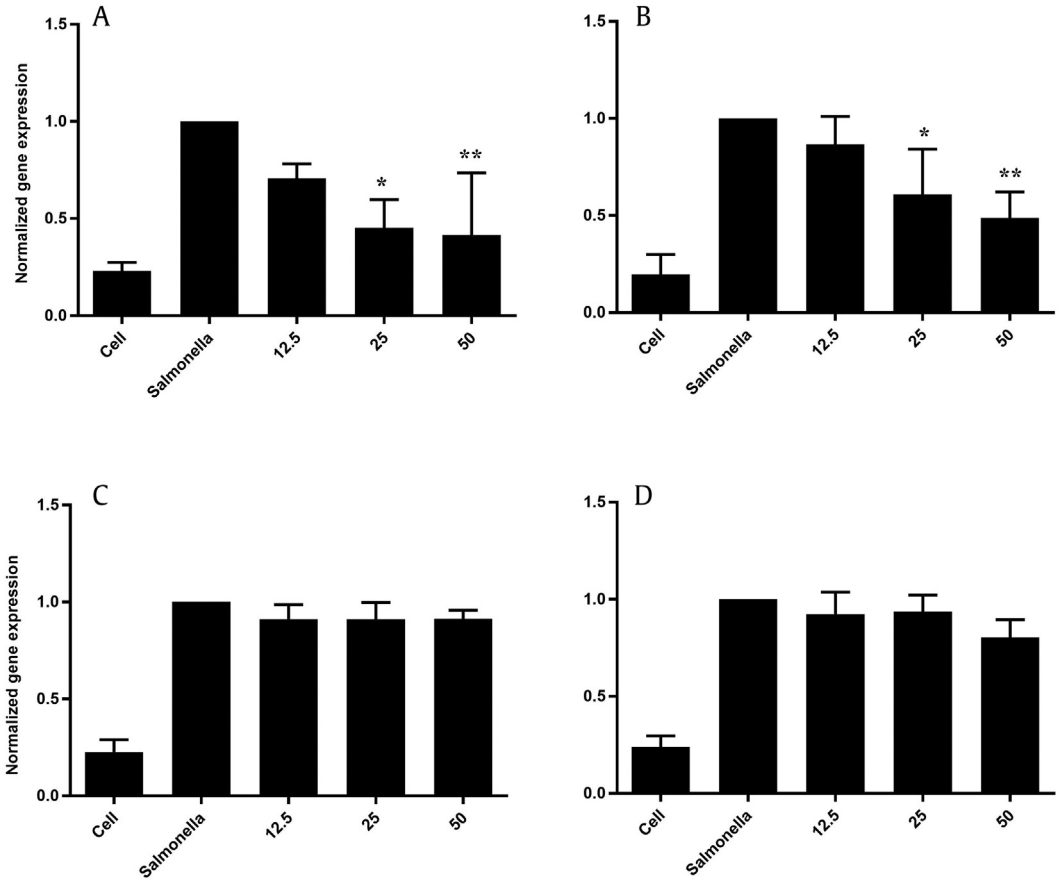

Fig. 10. Effects of Rg3 on the gene expressions of invasion-related host cell proteins and virulence factors. The gene expressions of *rac-1* (**A**), *Cdc-42* (**B**) *RhoA* (**C**), and *RhoG* (**D**) were determined in Caco-2 cells pretreated with various concentrations of Rg3 (μM). Results are the mean ± SD of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ compared with the expressions in *S.* Typhimurium ATCC 14028 infected but nontreated Caco-2 cells. SD, standard deviation.

adhesion and invasion of *S.* Typhimurium after Rg3 administration could be related to the reduced expression of *ompD*, *PrgK*, and *Sip-B* genes.

SPI-1 and SPI-2 are directly associated with host cell invasion and intracellular proliferation of *S.* Typhimurium, respectively [3]. SPIs are important for the bacterial pathogenicity because it encodes for a complex set of proteins, T3SS, that mediates the transfer of virulence factors (regulatory and effector genes) directly into the host cells [24,37]. The *hilA* gene is among those regulatory genes that was significantly downregulated by Rg3. Expression of *hilA* is essential for SPI-1 transcriptional regulation and cellular invasion of *Salmonella* through activation of invasion- and virulence-related genes (*prgH* and *invF* genes). Besides, *hilA* induces the transcription of *invF* that encodes for a transcriptional activator and targets *Salmonella* outer proteins (*sopE* and *sopB* genes). Moreover, it is involved in the regulation of SPI-1 that initiates systemic infection and shelters *Salmonella* by promoting biofilm formation [43,44]. Therefore, the downregulation of *hilA* gene and other virulence factors that correlate with the activation of *hilA* by Rg3 could contribute to the reduction in the invasion of *S. Typhimurium* into host cells.

The study confirmed that Rg3 decreased the motility of *S.* Typhimurium, which is one of the most significant events during cellular invasion [45]. The direction of flagellar rotation is the determining factors for the motility of bacteria [46]. *CheY* regulates bacterial chemotaxis, a property of motile bacteria to sense the surroundings and swim in a specific direction. *CheY* binds with motor switching complex of the flagella (FliG, FLiM, and FLiN) and causes a motor response that ensures directed flagellar rotation and bacterial motility. Therefore, Rg3-mediated suppression of *CheY* could interfere with the chemotactic and invasive ability of *S.* Typhimurium [47].

Virulence factors play an essential role in the pathogenicity of *S.* Typhimurium [24]. Under proper environmental conditions and at a sufficient population number, the QS system is critical for *Salmonella* invasion and virulence. Several plant extracts have proven to inhibit or decrease the QS activity of bacteria, including *S.* Typhimurium [12,13]. The present study demonstrated that Rg3 downregulated the AHL-induced expressions of QS genes (*sdiA* and *srgE*). Choi et al [48] have verified that activated *lsrR* reduces the expression of SPI-1 (*invF*, *sicA*, *sopB*, and *sopE)* and flagellar genes which ultimately diminish the invasive ability of *S.* Typhimurium. Therefore, *S.* Typhimurium requires a mechanism such as the QS system that reduces the overexpression of *lsrR* and enhances cellular invasion. Suppression of AHL-induced expressions of QS genes (*sdiA* and *srgE*) by Rg3 could contribute to the inhibition of *lsrR*-mediated *S. Typhimurium* invasion.

The T3SS-1—mediated injection of *Salmonella* effector proteins into the host cell is essential for invasion. The effector proteins activate the host cell Rho guanosine triphosphate (GTP)ases such as *rac-1*, *Cdc-42*, *RhoA*, and *RhoG*, which are related to various cellular functions [49]. Activation of these RhoGTPases ultimately triggers a signaling cascade that results in profuse rearrangement of the actin cytoskeleton, membrane ruffling, and internalization of bacteria [50,51]. Rg3 significantly downregulated the expression of *ac-1*r and *Cdc-42*. Activation of *rac-1* and *Cdc-42* correlates with the formation of lamellipodia and filopodia, respectively [4,52].

Consequently, Rg3 could reduce *S. Typhimurium* invasion by affecting the cytoskeletal remodeling mechanisms of the host cells, which are essential for the "trigger" entry mechanism. In addition, the insignificant difference ($p > 0.05$) in the *rck* gene expression indicates the ineffectiveness of the compound on the "zipper" entry mechanism of *S. Typhimurium*.

NO is a signaling molecule that regulates diverse physiological processes. However, excessive NO production has been implicated in a number of disease situations and chronic inflammation [53]. In this study, the level of NO in *S. Typhimurium*–infected cells was significantly higher than the nontreated cells. The excess NO might interact with superoxide anions and produce peroxynitrites. The peroxynitrites enhance the production of proinflammatory cytokines that ultimately lead to tissue injury [54]. In contrast, the *S. Typhimurium*–induced NO production was markedly reduced in cells preincubated with Rg3. The antiinflammatory and antioxidant effects of Rg3 [55] might contribute to the reduced level of NO.

In conclusion, subinhibitory concentrations of Rg3 reduced the adhesion, invasion, and intracellular survival of *S. Typhimurium*. Rg3 reduced the invasiveness of *S. Typhimurium* mainly through suppression of the "trigger" entry mechanism. Besides, it reduced the expression of virulence factors and QS-related genes. Therefore, Rg3 could be considered as a supplement agent to prevent *S. Typhimurium* infection. However, further studies are needed on the *in vivo* efficacy of the compound before clinical application.

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Author's contribution

A.F.M. designed the concept and conducted the study, analyzed and interpreted the data, and prepared the manuscript. Y.Q. reviewed the manuscript. S-.C.P. designed the concept and approved the final version of the manuscript.

Conflict of interests

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2019.09.002.

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