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Nitric oxide–inducing Genistein elicits apoptosis-like death via an intense SOS response in *Escherichia coli*

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Abstract

Increasing prevalence of multidrug-resistant untreatable infections has prompted researchers to trial alternative treatments such as a substitute for traditional antibiotics. This study endeavored to elucidate the antibacterial mechanism(s) of this isoflavone, via analysis of relationship between genistein and *Escherichia coli*. Furthermore, this investigation analyzed whether genistein generates nitric oxide (NO) in *E. coli* as NO contributes to cell death. RecA, an essential protein for the bacterial SOS response, was detected through western blot, and the activated caspases decreased without RecA. The results showed that the NO induced by genistein affected the bacterial DNA. Under conditions of acute DNA damage, an SOS response called apoptosis-like death occurred, affecting DNA repair. These results suggested that RecA was bacterial caspase-like protein. In addition, NO was toxic to the bacterial cells and induced dysfunction of the plasma membrane. Thus, membrane depolarization and phosphatidylserine exposure were observed similarly to eukaryotic apoptosis. In conclusion, the combined results demonstrated that the antibacterial mode of action(s) of genistein was a NO-induced apoptosis-like death, and the role of RecA suggested that it contributed to the SOS response of NO defense.

Key points

- *Genistein generates nitric oxide in E. coli.*
- *Genistein exhibits intense SOS response in E. coli.*
- *Genistein-induced NO causes apoptosis-like death in E. coli.*

Keywords Genistein · *Escherichia coli* · Bacterial apoptosis-like death · Nitric oxide · RecA

Introduction

The hazards of antibiotic resistance are increasing sharply due to the abuse of antibiotics and inappropriate antibiotic prescription (Aslam et al. 2018; Ventola 2015). Recently, antibiotic-resistant bacteria have emerged and are difficult to treat (Ahmed and Baptiste 2018; McGuinness et al. 2017; Ventola 2015). Furthermore, since antibiotic-resistant bacteria are dangerous enough to take a patient's life, resistant forms are regarded as one of the major threats to health of the world (Chaudhary 2016; Laxminarayan et al. 2013). Therefore, antibiotics modified by renowned drugs have been developed; however, efficacy is not always apparent (Walsh 2003). Therefore, it is significant to discover and develop more treatments with a novel mechanism(s), which are more effective and safer to replace the antibiotics currently in use (Aslam et al. 2018; Butler and Buss 2006; Chaudhary 2016; Livermore 2004; Wright 2017; Yang et al. 2018).

Genistein, soy-derived isoflavonoid, is broadly contained in leguminous plant foods, such as soybean, chickpeas, tofu, and lupin (Ganai and Farooqi 2015; Hong et al. 2006; Khan et al. 2015; Squadrito and Bitto 2012; Węgrzyn et al. 2010). It has been used as an adequate agent in remedy of chronic illness and cancer (Chatterjee et al. 2015; Ganai and Farooqi 2015; Li et al. 2013; Valles et al. 2010). This isoflavonoid exhibited various therapeutic effects in human cancer due to genistein's ability to trigger apoptosis through caspase activation, NF-κB inactivation, and downregulation of Bcl-2 and Bcl-xL known as anti-apoptotic factors (Banerjee et al.

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2008; Dhandayuthapani et al. 2013; Lee et al. 2012; Zhang et al. 2010). Apoptosis mediated by genistein also triggers disruption of the mitochondria membrane potential (de Oliveira 2016; Salvi et al. 2002). In addition, although this flavonoid is an antioxidant, it acts as a pro-oxidant by leading to DNA impairment in the presence of nitric oxide (NO) (Muzandu et al. 2005). This compound also activates nitric oxide synthase (NOS) that produces nitric oxide in mammalian cells (Ganai and Farooqi 2015; Liu et al. 2004; Si and Liu 2008; Verdrengh et al. 2004). Genistein is informed that it has antimicrobial activity and acts as poisons of bacterial topoisomerases (DNA gyrase) playing a vital role in DNA replication and repair (Hong et al. 2006; Pommier et al. 2010; Tse-Dinh 2009; Ulanowska et al. 2006). Except for this mode of action, the antibacterial mechanism of genistein remains largely indistinct.

Nitric oxide (NO) is a diatomic molecule created by NOS and the reduction of inorganic nitrate. Previously, it was regarded as a modulator of apoptosis (Kim et al. 2001; Li and Wogan 2005). This pro-apoptotic molecule promotes single-stranded DNA breaks in the bacterial and mammalian cells that inhibit ribonucleotide reductase, which blocks DNA synthesis and induces mitochondrial dysfunction and membrane depolarization (Brown and Borutaite 2001; Habib and Ali 2011; Poderoso et al. 2019; Spek et al. 2001). NO itself has a short half-life due to its reactivity with biological molecules, such as oxygen or superoxide radical to form reactive NO species (RNOS) (Sawa and Ohshima 2006; Schairer et al. 2012). NO displays antimicrobial activity and covalently binds to DNA and proteins, thereby destabilizing the target pathogens (Mihu et al. 2010; Schairer et al. 2012). Antimicrobial mechanisms mediated by NO are widely known today, and chemical change of DNA induced by RNOS is the one of the main modes of action, leading to nitrosative stresses in *E. coli* (Carpenter and Schoenfisch 2012). In addition, NO induces the modification of proteins related to the synthesis and repair of *E. coli* DNA (Ren et al. 2008).

In programmed cell death (PCD), apoptosis is the one of general mode, which is characterized by several stereotyped aspects (Wlodkowic et al. 2011). Apoptosis occurs to sustain cells homeostatically and acts as a defense mechanism when cells are devastated by various stimuli and conditions (Baar et al. 2017). During the process of apoptosis, chromatin condensation, DNA fragmentation, activated caspases, membrane potential loss, and the existence of phosphatidylserine in the outside leaflet of the cell membrane are visible (Bakshi et al. 2010; Elmore 2007; Wlodkowic et al. 2011). Although apoptosis occurs generally in eukaryotic cells, recent studies have indicated that prokaryotic cells could also go through an apoptosis-like response (Erental et al. 2014; Lee and Lee 2014). When treated with norfloxacin, a second-generation fluoroquinolone antibiotic, *E. coli*, exhibited apoptotic markers (Choi et al. 2016; Erental et al. 2014; Lee and Lee 2014; Yun and Lee 2016).

In this investigation, several experiments were conducted to confirm that genistein induces bacterial apoptosis-like death in response to genistein treatment. Furthermore, we evaluated the overexpression of RecA protein which concerned the bacterial SOS response to recover impaired DNA.

Materials and methods

Minimum inhibitory concentration

Depending on the Clinical and Laboratory Standard Institute (CLSI) guidelines, the minimum inhibitory concentration (MIC) values were assessed. In the first phase, genistein (Sigma Chemical Co., St. Louis, MO, USA) or norfloxacin were dissolved using the universal solvent, dimethyl sulfoxide (DMSO) or acetic acid (Merck KGaA, Darmstadt, Germany), respectively. The following bacterial strains were used for this experiment: *Enterococcus faecium* (ATCC 19434), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Salmonella enteritidis* (ATCC 13076) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). *Staphylococcus epidermidis* (KCTC 1917), *Streptococcus mutans* (KCTC 3065), and *Salmonella typhimurium* (KCTC 1926) were obtained from the Korean Collection for Type Cultures (KCTC, Jeongeup-si, Jeollabuk-do, Korea). Growing bacterial cells (2×10^6 cells/mL) were allotted into microwell plates (0.1 mL/well). Genistein and norfloxacin were treated via two-fold serial dilution. After incubation at 37 °C for 24 h, cell proliferation was determined by optical density at 600 nm using a microtiter ELISA Reader (BioTek Instruments, Winooski, VT, USA).

Estimation of intracellular NO and superoxide (O_2^-) generation

To estimate NO generation, *E. coli* MG 1655 were used, which is acquired from Coli Genetic Stock Center. Bacterial cells (2×10^6 cells/mL) were treated with genistein (5 μg/mL) or norfloxacin (2.5 μg/mL) at 37 °C for 2 h. Then the cells were resuspended in PBS (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4) and incubated with 10 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Molecular Probes) at 37 °C for 30 min. Ensuing centrifugation and resuspension in PBS, the samples were assessed using a FACSVerse flow cytometer (Becton Dickinson, NJ, USA). Evaluation of the O_2^- levels was measured using a Dihydrorhodamine 123 (DHR-123) (Sigma Chemical Co.,

St. Louis, MO, USA) dissolved in DMSO. The *E. coli* cells were incubated with genistein (5 μg/ml) or norfloxacin (2.5 μg/ml) at 37 °C for 2 h. Following incubation, the cells were collected via centrifugation at 12000 rpm for 5 min and then stained with 5 μM DHR-123. Samples were assessed using a FACSVerse flow cytometer.

Detection of peroxynitrite ($ONOO^-$) formation

$ONOO^-$ formation was detected using a 3′-(p-hydroxyphenyl) fluorescein (HPF, Molecular Probes) (Invitrogen, Carlsbad, CA, USA) which is a cell-permeable fluorescent reporter dye. HPF itself was not very fluorescent; however, when reacted with $ONOO^-$, this compound exhibited strong dose-dependent fluorescence. Bacterial cells (2×10^6 cells/mL) were incubated with genistein (5 μg/mL), L-NAME (Nω-nitro-L-arginine methyl ester hydrochloride, 0.5 μg/mL)-pre-treated genistein, or norfloxacin (2.5 μg/mL) treatment for 2 h at 37 °C. Following incubation, the cells were centrifuged 12000 rpm for 5 min. Then, the cells were washed with PBS and dyed with 5 μM HPF, which was dissolved in Dimethylformamide (DMF) (JUNSEI Chemical Co., Tokyo, Japan). Following treatment with HPF, the intensity of fluorescence was determined by utilizing a FACSVerse flow cytometer.

Measurement of DNA fragmentation and chromosomal condensation

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining is the method for detecting the DNA cleavage. This assay was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Basel, Switzerland). 3′-OH termini of the nucleotide were enzymatically labeled and mediated by the terminal deoxynucleotidyl transferase and, then, the fragmented DNA was identified. Cells (2×10^6 cells/mL) were incubated for 2 h at 37 °C with genistein (5 μg/mL), L-NAME (0.5 μg/mL)-pre-treated genistein, or norfloxacin (2.5 μg/mL). Following incubation, the cells were washed with PBS and then fixed with 2% paraformaldehyde for 1 h on ice. Succeeding this washing step, the fixed cells were incubated with permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) on ice for 2 min. Then, the cells were incubated with a TUNEL reaction mixture for 1 h at 37 °C. The fluorescence intensity was estimated using a spectrofluorophotometer (Shimadzu RF-5301PC; Shimadzu, Japan) at wavelengths of 495 nm (excitation) and 519 nm (emission). Chromosomal condensation was measured using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Chemical Co., St. Louis, MO, USA). The *E. coli* (2×10^6 cells/mL) were treated with genistein (5 μg/mL), L-NAME (0.5 μg/mL)-pre-treated genistein, or norfloxacin (2.5 μg/mL) for 2 h. Cells were resuspended twice with PBS and incubated with 1 μg/mL DAPI. The intensity of fluorescence was assessed by utilizing a FACSVerse flow cytometer.

Protein extraction and western blotting

The harvested *E. coli* cells were treated with genistein (5 μg/mL), L-NAME (0.5 μg/mL)-pre-treated genistein, or norfloxacin (2.5 μg/mL). Cells were incubated at 37 °C on an incubator shaker (120 rpm) for 2 h and resuspended in PBS. The suspensions underwent lysis using an ultrasonic sonicator (10 pulses of 2 min each at amplitude 38) (Sonics, Newtown, CT, USA) and then centrifuged at 12000 rpm for 20 min to eliminate undamaged cells. The supernatants were gathered, and the proteins were precipitated with 5% trichloroacetic acid (TCA) at 4 °C for 10 min. The precipitated proteins were washed with cold acetone and dissolved in H_2O. Quantitation of the protein was estimated with a Bradford assay (Bio-Rad, Hercules, CA, USA). Each 10 μg protein sample was transferred to a nitrocellulose membrane. The membranes were blocked in 3% skim milk at room temperature for 1 h and incubated with a rabbit polyclonal anti-RecA antibody (Abcam, Cambridge, UK), then diluted to 1:2000, for 16 h at 4 °C. Then the samples were incubated for 1 h at room temperature with a secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Biovision, Milpitas, CA, USA), which is diluted 1:2000. Pierce ECL Plus Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA) was added, and the membranes were exposed to an X-ray film. The relative amount of RecA was numerically quantified with the ImageJ program (http://rsb.info.nih.gov/ij).

Determination of bacterial caspase-like protein

With the aim of detecting a homologous of eukaryotic caspase (cysteine-dependent aspartate-directed proteases), the CaspACE FITC-VAD-FMK In Situ Marker (Promega, Fitchburg, WI, USA) was employed and *ΔRecA* mutant was obtained from *E. coli* K-12 collection. FITC-VAD-FMK is cell-permeable and irreversibly binds to activated caspases. VAD-FMK, a FITC-conjugated peptide pan-caspase inhibitor, is shifted into cells and combines to the active site of caspase to identify expression of the bacterial caspase-like protein. A stock of FITC-VAD-FMK was dissolved in DMSO at a concentration of 50 μM. *E. coli* wild-type and *ΔRecA* cells were incubated with genistein (5 μg/mL), (0.5 μg/mL)-pre-treated genistein, or norfloxacin (2.5 μg/mL) for 2 h at 37 °C. The cells were centrifuged 12000 rpm for 5 min and resuspended in 1 mL of PBS. Cells were centrifuged again to make cells cleaner and stained with 5 μM FITC-VAD-FMK for 30 min at 37 °C.

After setting the total volume to 1 mL with PBS, intensity of fluorescence was assessed utilizing a FACSVerse flow cytometer.

Assessment of membrane depolarization and PS exposure

The bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] (Molecular Probes, Eugene, OR, US) was used to evaluate membrane depolarization. Cells (2×10^6 cells/mL) were treated with genistein (5 μg/mL), L-NAME (0.5 μg/mL)-pre-treated genistein, or norfloxacin (2.5 μg/mL) and incubated for 2 h at 37 °C. Following this incubation, the cells were washed with PBS and stained with 5 μg/mL $DiBAC_4(3)$. Intensity of fluorescence was analyzed utilizing a FACSVerse flow cytometer. Phosphatidylserine (PS) exposure was detected using the Annexin V–FITC apoptosis detection kit (BD Pharmingen, San Diego, CA, USA). Cells (2×10^6 cells/mL) treated with genistein (5 μg/mL), L-NAME (12.5 ng/mL)-pre-treated genistein, or norfloxacin (60 ng/mL) were incubated for 2 h at 37 °C. Ensuing incubation, the cells were gathered and resuspended in 100 μl of 1 × Annexin V binding buffer, followed by the addition of 50 μl/ml of Annexin V–FITC to the cell suspensions. The mixtures were then incubated at room temperature for 15 min in the dark. Thereafter, the total volume was raised to 1 ml with PBS and the cells were assessed utilizing a FACSVerse flow cytometer.

Statistical analysis

All the experiments were performed in triplicates and the values were expressed as the means ± standard deviation. After confirming the normality of distribution using the Shapiro-Wilk test, statistical comparisons between various groups were carried out by analysis of variance (ANOVA) followed by Tukey's post hoc test for three-group comparisons using SPSS software (SPSS, version 25, SPSS/IBM. Chicago, IL, USA). Intergroup differences were considered statistically significant at p values < 0.05.

Results

Genistein exhibits antibacterial effect

Genistein is known to exhibit an antimicrobial effect with lesser toxicity (Ganai and Farooqi 2015). Therefore, to estimate the antibacterial effect of genistein, the MIC values were performed based on CLSI method. MIC values between 2.5 and 5 μg/mL confirmed that genistein possessed potent antibacterial activity (Table 1). Hence, to better understand the antibacterial mechanism of genistein, *E. coli* was utilized as a bacterial model organism. Many papers have long proved that NO exhibits broad-spectrum antimicrobial activity by exacting oxidative and nitrosative damage on pathogens (Jones et al. 2010; Regev-Shoshani et al. 2010; Schairer et al. 2012). Several researches have shown that genistein induced NOS activation, increasing NO formation (Liu et al. 2004; Si and Liu 2008). This prompted us to determine whether the antibacterial mechanism of genistein is associated with NO production in *E. coli*. The cells treated with genistein (5 μg/mL) or norfloxacin (2.5 μg/mL) accounted for 55.70% and 87.69%, respectively, compared to 21.93% for untreated cells (Fig. 1). This result indicates that genistein induced the creation of NO.

Table 1 The antimicrobial activity of genistein and norfloxacin

Microbial strains	MIC (μg/ml)	
	Genistein	Norfloxacin
Gram-positive bacteria		
Enterococcus faecium ATCC 19434	2.5	2.5
Enterococcus faecalis ATCC 29212	5	2.5
Staphylococcus epidermidis KCTC 1917	5	2.5
Streptococcus mutans KCTC 3065	5	2.5
Gram-negative bacteria		
Escherichia coli ATCC 25922	5	2.5
Pseudomonas aeruginosa ATCC 27853	2.5	1.25
Salmonella typhimurium KCTC 1926	5	2.5
Salmonella enteritidis ATCC 13076	5	2.5–5

Genistein produces O_2^- by inducing $ONOO^-$ formation

The free radical NO is considered a signaling molecule in a biological reaction. NO is toxic if it combines with O_2^- to form $ONOO^-$ which dissolves rapidly to a highly reactive oxidant species. A previous study reported that O_2^- reacted readily with NO, facilitating $ONOO^-$ formation. $ONOO^-$ is associated with killing bacteria as it produced oxidative and nitrosative stresses in *E. coli* (McLean et al. 2010). Initially, the level of intracellular O_2^-, which was necessary to form $ONOO^-$, was estimated using the DHR-123 reactive oxygen species sensor. DHR-123 is used an indicator as it can passively diffuse across the membrane. Compared with untreated cell (12.37%), cells treated with genistein or norfloxacin exhibited 83.85% and 96.78%, respectively (Fig. 2). Moreover, HPF was used to measure the formation of $ONOO^-$. The *E. coli* treated with genistein (5 μg/mL) or norfloxacin (2.5 μg/mL) exhibited increases in $ONOO^-$ formation, and the cells

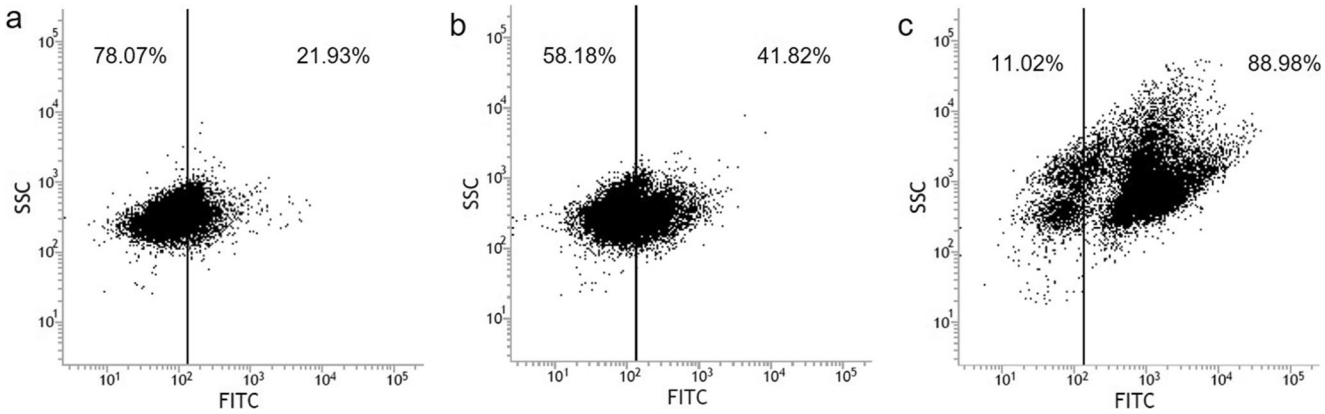

Fig. 1 Detection of nitric oxide in genistein-treated *E. coli* cells. After treatment with 5 μg/ml genistein and 2.5 μg/ml norfloxacin, nitric oxide was measured by DAF-FM

pre-treated with L-NAME in genistein showed a similar trend in the untreated cells (Fig. 3). These results demonstrated that genistein produced O_2^- and this O_2^- reacted with the NO induced by genistein to form $ONOO^-$.

Genistein causes DNA fragmentation and chromosomal condensation

DNA cleavage is a general apoptosis feature that could be assessed by TUNEL assay, which binds to the 3′-ends of fragmented DNA. The TUNEL assay was performed to evaluate whether genistein induced DNA fragmentation. Compared to the intensity of the untreated cells, genistein (5 μg/mL) and norfloxacin (2.5 μg/mL) showed an increase in fluorescence intensity. Meanwhile, the intensity of the cells pre-treated with L-NAME in genistein tended to reduce the fragmented DNA levels which indicated that NO was caused by genistein and led to DNA fragmentation (Fig. 4). DAPI, which binds to the minor groove of A-T-rich regions in DNA sequences, was used to monitor the chromosomal condensation. DAPI staining involved the following; cells with genistein (5 μg/mL) or norfloxacin (2.5 μg/mL) displayed a mightier fluorescent intensity (Fig. 5). Overall, these data demonstrated that the production of genistein-induced NO influences the DNA and the chromosome.

Genistein exerts expression of RecA as a caspase-like protein

RecA, which is known as a caspase-like protein concerned with the bacterial SOS response, was identified by western blotting (Erental et al. 2014). In the genistein (5 μg/mL)- or norfloxacin (2.5 μg/mL)-treated cells, the band equivalent to the RecA protein was more intense than that from the untreated cells or the pretreatment with L-NAME cells (Fig. 6). This study indicated that genistein induces over-occurrence of the RecA protein, inducing the SOS response. To examine whether genistein-induced RecA acted as a caspase-like protein, FITC-VAD-FMK was applied in the *E. coli* wild-type and

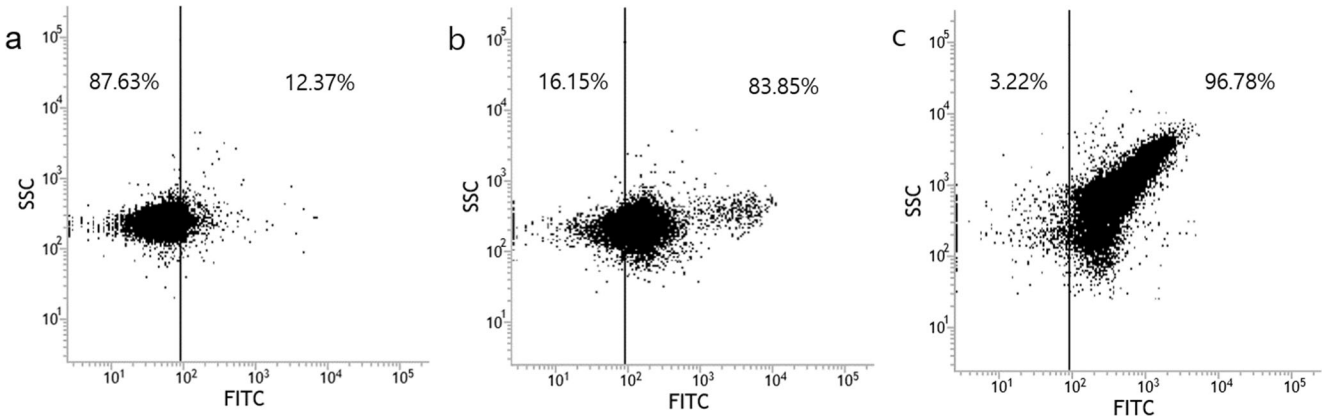

Fig. 2 Superoxide generation was measured using Dihydrorhodamine 123 in *E. coli*. **a** Untreated cells, **b** cells were treated with 5 μg/mL genistein, and **c** cells were treated with 2.5 μg/mL norfloxacin

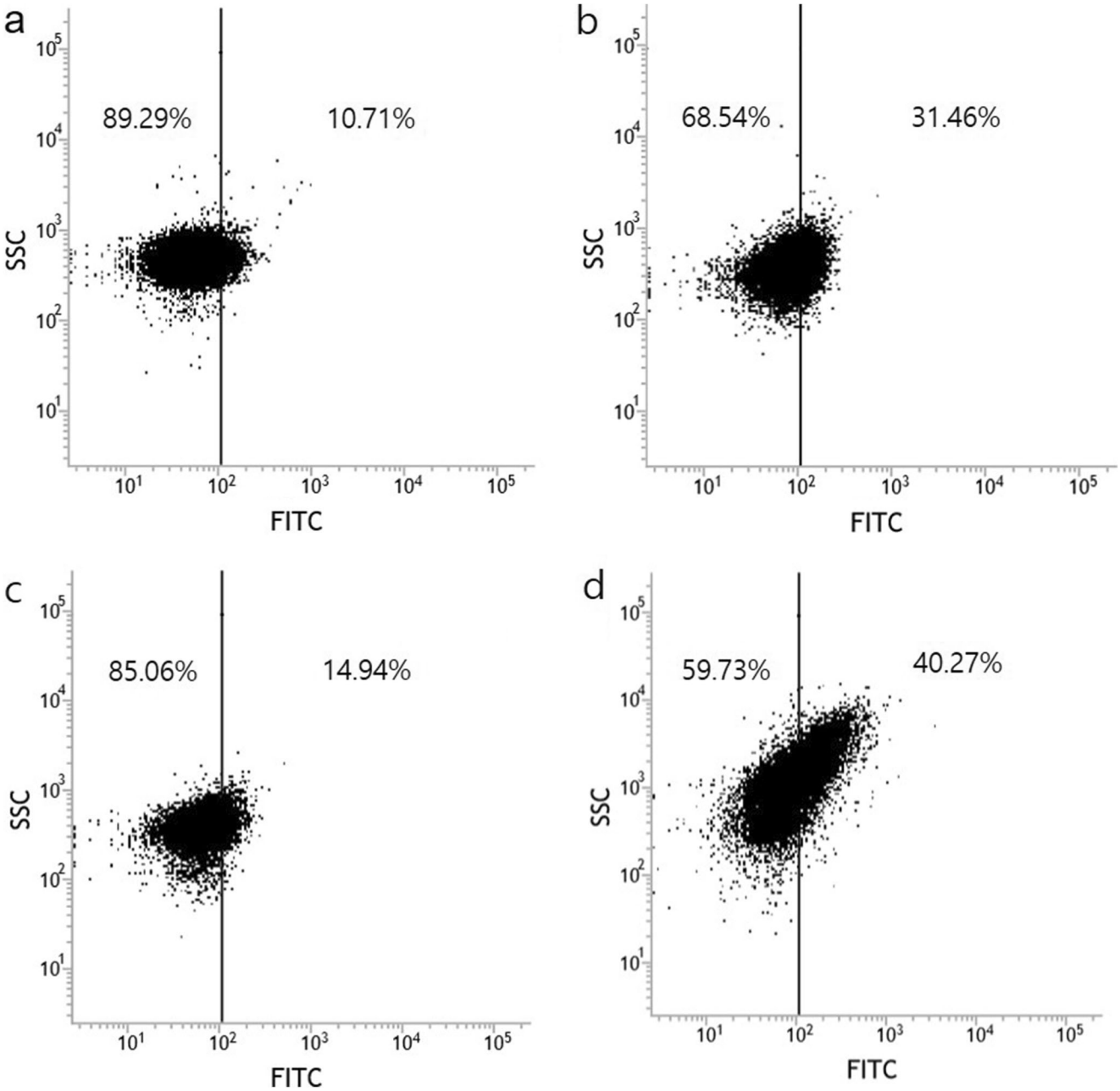

Fig. 3 Peroxynitrite formation was measured using HPF in *E. coli*. **a** Untreated cells, **b** cells were treated with 5 μg/mL genistein, **c** cells were treated with 5 μg/mL genistein and 0.5 μg/mL L-NAME, and **d** cells were treated with 2.5 μg/mL norfloxacin

ΔRecA cells. In the genistein-treated *E. coli* wild-type cells (5 μg/mL), fluorescence was increased by 50.62% compared to the untreated cells (10.39%) and the cells pre-treated with L-NAME in genistein (11.28%), and the norfloxacin-treated (2.5 μg/mL) cells increased by 64.39%. In the *ΔRecA* cells, the fluorescence remained unaltered ensuing treatment with genistein (5 μg/mL) or norfloxacin (2.5 μg/mL) as well as the untreated cells and the cells pre-treated with L-NAME in genistein (Fig. 7). These observations indicated that a caspase-like protein, which shared the same substrate with the eukaryotic caspase, was increased by genistein, and suggested the potentiality that RecA induced by genistein could act as a caspase-like protein.

Genistein induces apoptosis-like death

Cells treated with genistein exhibited DNA fragmentation and caspase-like proteins. Furthermore, increased levels of fragmented DNA and caspase-like proteins were considered an apoptotic marker. Moreover, we confirmed the induction of apoptosis-like death by observing the membrane

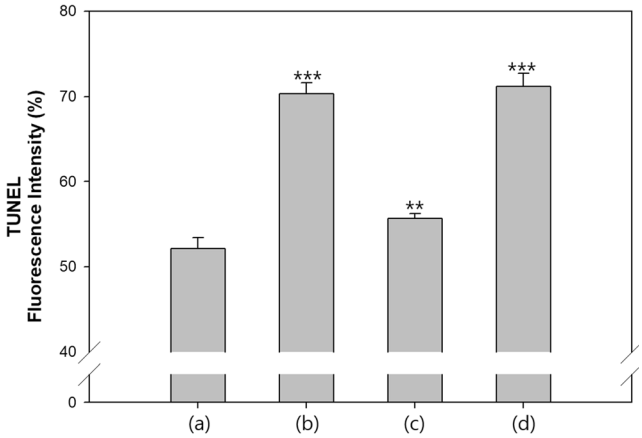

Fig. 4 Spectrofluorophotometric analysis of DNA strand break was measured by TUNEL assay in *E. coli*. (a) Untreated cell, (b) genistein was treated with 5 μg/mL, (c) genistein was treated with 5 μg/mL and L-NAME was treated with 0.5 μg/mL, and (d) norfloxacin was treated with 2.5 μg/mL. Experiments were held triplicate independently and the results represent the average, standard deviation, and *p* values from three different experiments (**$p < 0.05$; ***$p < 0.01$)

depolarization, one of the characteristics of eukaryotic apoptosis. In eukaryotic cells, the membrane potential was constantly maintained, but when cells suffer apoptosis, this potential was interrupted, and membrane depolarization was triggered. Furthermore, NO mediated the mitochondrial membrane depolarization, which induced eukaryotic apoptosis (Brown 2010). Similarly, recent studies have shown that the membrane potential loss was associated with apoptosis-like death. Thus, we postulated that genistein induces apoptosis-like death in bacteria cells. To investigate this objective, we assessed membrane

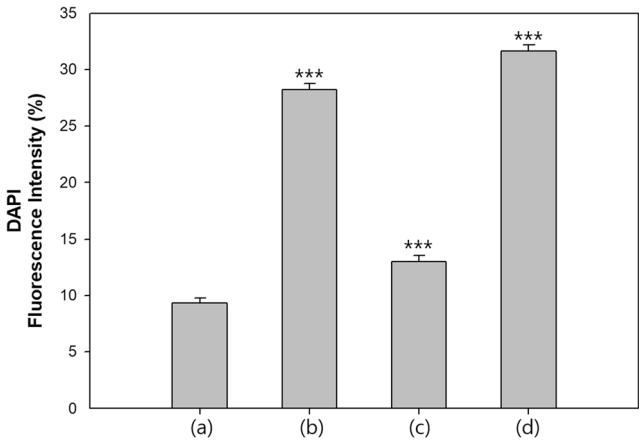

Fig. 5 Flow cytometric analysis of chromosomal condensation was measured using DAPI in *E. coli*. (a) Untreated cell, (b) genistein was treated with 5 μg/mL, (c) genistein was treated with 5 μg/mL and L-NAME was treated with 0.5 μg/mL, and (d) norfloxacin was treated with 2.5 μg/mL. Experiments were held triplicate independently and the results represent the average, standard deviation, and *p* values from three different experiments (**$p < 0.05$; ***$p < 0.01$)

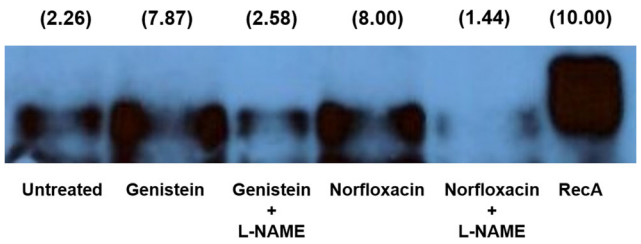

Fig. 6 Analysis of RecA expression levels by western blotting. *E. coli* cells were treated genistein (5 μg/mL) or norfloxacin (2.5 μg/mL) and L-NAME was treated with 0.5 μg/mL. Each band was compared with the band of RecA, which is the 5 μg/mL concentration on the far right. The relative amount of RecA, indicated with number above western blot, was quantified in comparison with RecA using ImageJ (http://rsb.info.nih.gov/ij)

depolarization by using the potential-sensitive $DiBAC_4(3)$ dye. Compared to the untreated cells (17.05%), the cells depolarized by genistein (5 μg/mL) or norfloxacin (2.5 μg/mL) exhibited 59.41% and 97.36%, respectively (Fig. 8). However, L-NAME attenuated the genistein-induced apoptotic signals by inhibiting membrane depolarization. These observations (DNA fragmentation, caspase-like protein, and membrane depolarization) demonstrated persuasive evidence that the mechanism triggered by the genistein-induced NO is a certainly apoptosis-like death.

Genistein mediates PS exposure

PS is a phospholipid that composes cell membrane and primarily resided in the inside leaflet of the cell membrane. In apoptosis, PS is no longer restricted to the inner side by flippase and becomes revealed on the outside leaflet. Detection of the displaced PS was accomplished using annexin V–FITC that binds to PS. Annexin V/PI double staining (annexin V positive and PI negative; early apoptosis, annexin V positive, and PI-positive; necrosis) was succeeded by flow cytometric analysis. PS exposure of 35.75% and 37.00% was derived in the *E. coli* cells treated with genistein (5 μg/mL) or norfloxacin (60 ng/mL), whereas the L-NAME diminished genistein-induced PS exposure (Fig. 9). These results suggested that genistein could lead to PS exposure on the outside leaflet without necrosis (upper right quadrant) by generating NO.

Discussion

The crisis of antibiotic resistance has been ascribed to the misuse of medicines, as well as the shortage of new drug development. Thus, resistance of bacteria to renowned antibiotics is a growing problem globally now and in the future, and it is one of the major challenges facing health care providers in the 21st century (Aslam et al. 2018; Ventola 2015). Although various strategies have been proposed to confront this problem, it

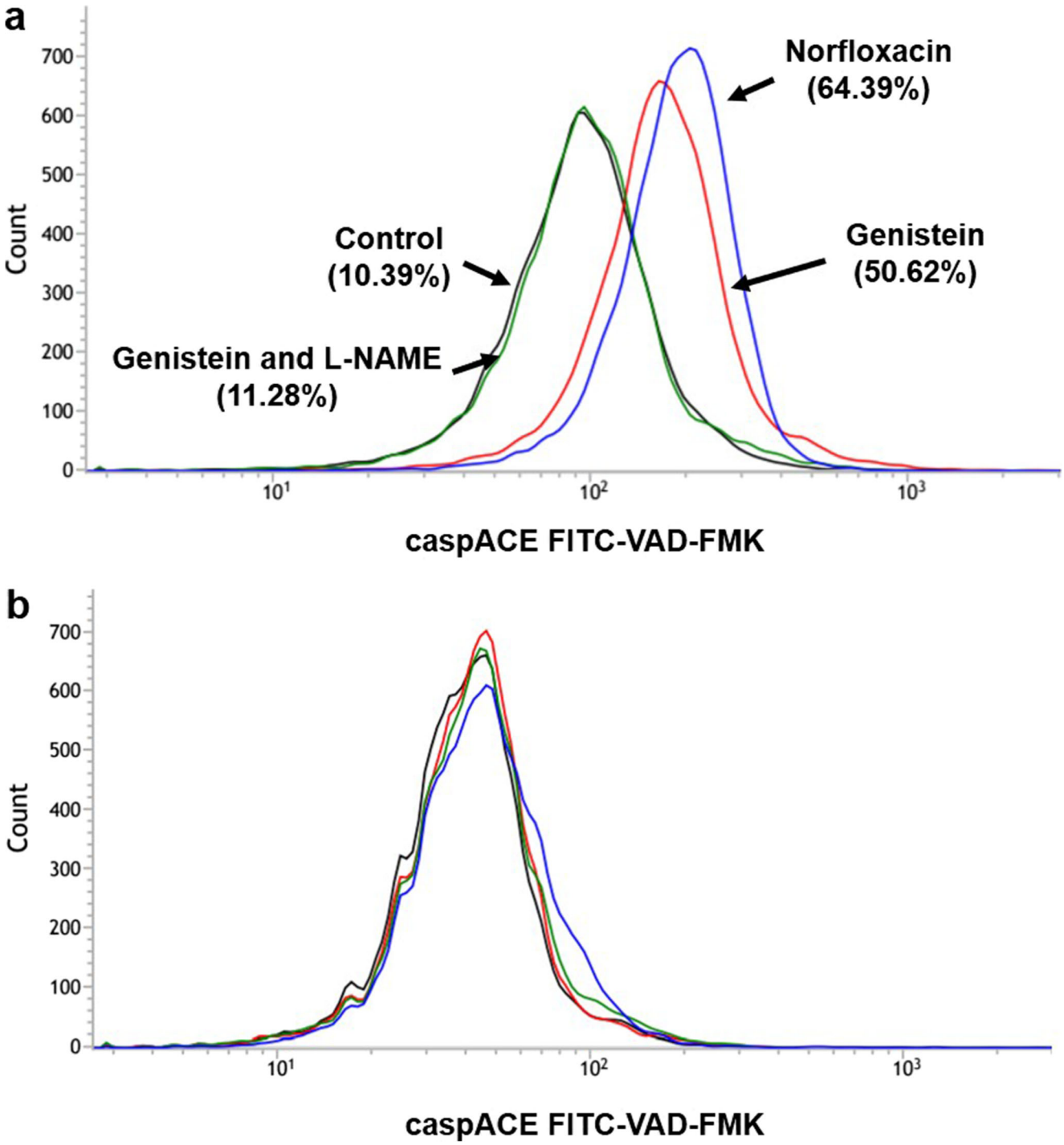

Fig. 7 Flow cytometric analysis of caspase-like protein expression by caspACE FITC-VAD-FMK in *E. coli*. **a** *E. coli* wild-type cells were treated with genistein (5 μg/mL) or norfloxacin (2.5 μg/mL), and **b** *ΔRecA* cells were treated with genistein (5 μg/mL) or norfloxacin (2.5 μg/mL). L-NAME was pre-treated both *E. coli* wild-type and *ΔRecA* cells

seems difficult to expect a distinct effect. Consequently, novel antimicrobial agents are required urgently due to the appearance of antibiotic-resistant bacteria like MRSA and VRE (Ahmed and Baptiste 2018; McGuinness et al. 2017). Genistein, a soy-derived flavonoid, was known to exhibit antimicrobial activity against various microorganisms (Ganai and Farooqi 2015; Hong et al. 2006; Ozcelik et al. 2011; Rahman et al. 2008; Sauter et al. 2014; Węgrzyn et al. 2010). In current trend, natural phenolic compounds such as genistein were associated with bacterial topoisomerase IV by disturbing DNA synthesis similar to norfloxacin, a bacterial topoisomerase IV inhibitor (Alt et al. 2011; Deibler et al. 2001; Fournier et al.

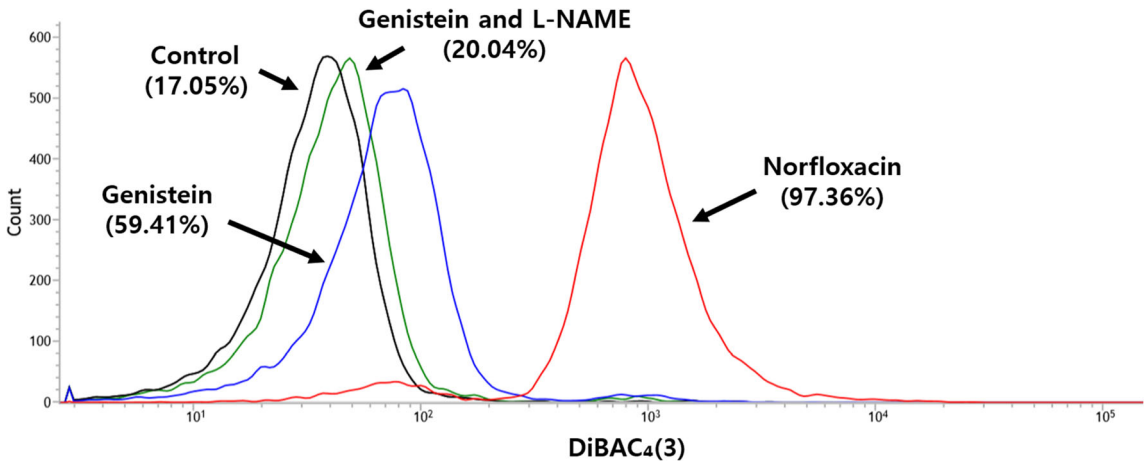

Fig. 8 Flow cytometric analysis of membrane depolarization by $DiBAC_4(3)$ in *E. coli*. Genistein was treated with 5 μg/mL, genistein, and L-NAME was treated with 5 μg/mL genistein and 0.5 μg/mL L-NAME. Norfloxacin was treated with 2.5 μg/mL

2000; Gradisar et al. 2007; Hooper and Jacoby 2016; Mukne et al. 2011; Phetnoo et al. 2013; Verdrengh et al. 2004). NO is also known as a substance that acts as a bactericidal and bacteriostatic (Regev-Shoshani et al. 2010). However, it is not yet clear how NO affects the cell death of bacteria. Hence, this investigation targeted to reveal the effect of NO on the *E. coli* and to disclose the novel antibacterial mechanism of genistein in the *E. coli*.

To confirm the antibacterial effects of genistein, the MIC values of genistein or norfloxacin were determined. Norfloxacin, selected for positive control, is widely used to treat bacterial infections and acts as a DNA gyrase (topoisomerase IV) inhibitor like genistein (Adjei et al. 2006; Deibler et al. 2001; Fournier et al. 2000; Moreau et al. 2018; Yim et al. 2018). This investigation demonstrated that genistein exhibited antibacterial activity similar to that of norfloxacin, which was widely used for bacterial infections. NO is known to function as an antimicrobial agent for a long time (Ghaffari et al. 2006; Schairer et al. 2012). Today, nitric oxide–releasing devices and particles that exhibit antibacterial effects are widely used as an antibacterial agent (Han et al. 2009; Mihu et al. 2010; Schairer et al. 2012). Furthermore, it is known that genistein activated mammalian NOS. Although bacterial NOS (bNOS) lacks the reductase domain, bNOS and eukaryotic NOS were mechanistically and structurally related to produce NO (Jones et al. 2010). The experiment was conducted using DAF-FM to clarify whether genistein and norfloxacin, except for role of topoisomerase IV inhibitor, produce NO that functions as antimicrobial agent in the *E. coli* cells. Genistein generated intracellular NO at MIC values and norfloxacin also showed NO generation at MIC values. Therefore, this result indicated that genistein possessed an antibacterial effect via inducing NO.

Many harmful effects of NO were not directly attributable to NO itself and were instead mediated via the production of $ONOO^-$, a byproduct of the reaction between NO and O_2^-. One of the most common RNOS, $ONOO^-$, represented an important mechanism that contributed to the DNA damage, inactivation of the metabolic enzyme, and disruption of the cell membranes and apoptosis (Ascenzi et al. 2010). Moreover, $ONOO^-$ possesses antimicrobial activity, which induces membrane damage (Genest et al. 2002; McLean et al. 2010). Therefore, we first estimated O_2^- generation using the DHR-123 dye, which was essential for RNOS creation. Cells treated with genistein exhibited an increase in intracellular O_2^-. Through use of the HPF assay, we verified that the $ONOO^-$ formation in *E. coli* was detected in compliance with genistein treatment. In this experiment, the production of peroxynitrite was similar to the untreated cells when pre-treated with substances that inhibited NO generation. Summing up the previous observation, O_2^- was identified in *E. coli* treated with genistein and this O_2^- reacted with the NO induced by genistein to create RNOS.

The observation from this study showed DNA damage in the *E. coli* cells under genistein-induced NO production. Indeed, NO has been reported to react immediately with DNA while inducing oxidation and nitration of base. Thus, it induced double-stranded DNA breaks, promoting oxidative damage of DNA (Jaiswal et al. 2001). To explain the relationship between NO and DNA, a TUNEL assay was performed. Through this assay, we confirmed that DNA fragmentation was caused by genistein-induced NO. Damage to the DNA affected the cell cycle and replication and blocked DNA synthesis; thus, degradation of chromosomal DNA indicated that DNA replication arrest has occurred, which was conducted with DAPI staining (Nagata et al. 2003). DAPI staining confirmed chromosomal condensation, and these features were diminished by NO inhibition. These results supported that genistein-induced NO plays a vital role in *E. coli* DNA damage.

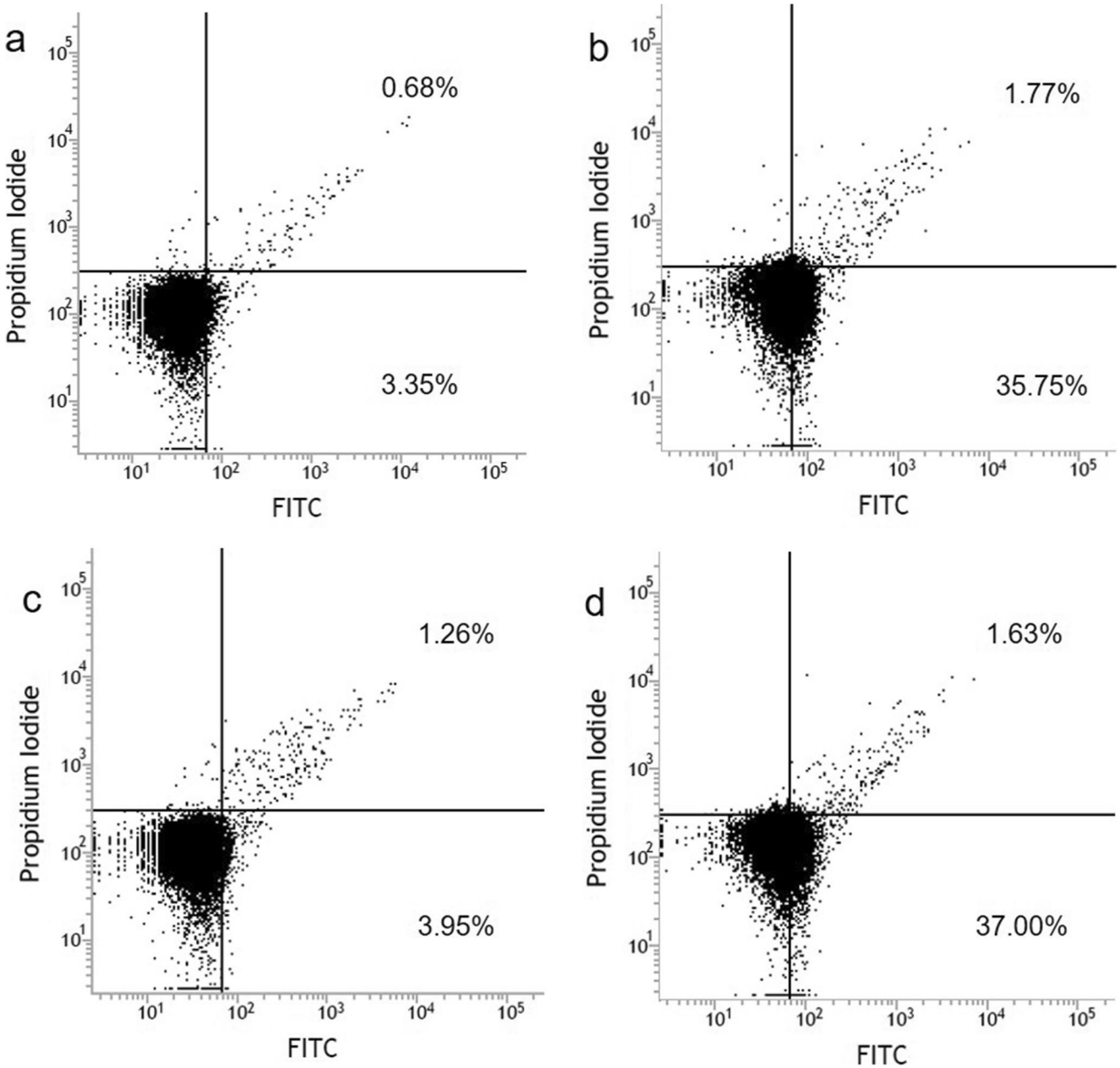

Fig. 9 Flow cytometric analysis of phosphatidylserine exposure was measured using Annexin V/propidium iodide double staining in *E. coli*. **a** Untreated cell, **b** genistein was treated with 5 μg/mL, **c** genistein was treated with 5 μg/mL and L-NAME was treated with 12.5 ng/mL, and **d** norfloxacin was treated with 60 ng/mL

During eukaryotic apoptosis, caspase amplified the apoptotic signal by activating different caspase and diverse apoptotic factors. Caspase, therefore, had a significant part in the apoptotic process. RecA is an ATP-dependent protein that formed nucleoprotein filaments by binding to the single-stranded DNA, and these filaments promoted the SOS response by inducing autocleavage of the LexA repressor. The SOS response generally occurred when DNA was damaged by various stress conditions, and if the damage was weak, the cells were repaired by this response. However, if the cells are severely damaged, they could not be repaired, and RecA contributed to the apoptosis-like response rather than an SOS response (Lee and Lee 2017). In some papers, RecA, which is required for a bacterial apoptosis-like response, is known as caspase-like protein in *E. coli* (Erental et al. 2014; Lee and Lee 2014, 2017). In *E. coli*, the SOS response is induced by promoting expression of the DNA repair proteins such as RecA (Lee and Lee 2019). Thus, we proposed that a genistein-induced NO caused DNA damage that promoted the SOS response, leading to a bacterial apoptosis-like response. Following treatment with genistein, overexpression of RecA was confirmed by a western

blot assay, and its role as a caspase-like protein was also confirmed in *E. coli*.

In western blot assay, the cells treated with genistein exhibited bands that appeared dark, while the cells pre-treated with NO inhibitor exhibited light bands. To find out whether RecA acted as a caspase-like protein in *E. coli*, FITC-VAD-FMK was employed. The *E. coli* wild-type cells treated with genistein increased the caspase-like protein activation, however, not in the cells pre-treated with the NO inhibitor. Experimenting with *ΔRecA* cells, the caspase-like protein activation in those treated with genistein and those with the pretreatment of the NO inhibitor was similar. Based on these results, we assumed that genistein-induced NO triggered overexpression of RecA, which contributed to the bacterial SOS response, and RecA acted qua a caspase-like protein in *E. coli*. Nevertheless, functional resemblances between caspase and RecA have not yet been confirmed, and further investigation was needed.

In eukaryotic PCD, the mitochondrial membrane potential was disturbed, and membrane depolarization is exhibited (Kim et al. 2011; Ly et al. 2003). Mitochondrial membrane potential decreased, activating mitochondrial apoptotic factor, such as cytochrome *c* (Garedew et al. 2010; Okada et al. 2012). In addition, NO and RNOS entered the membranes of bacteria, causing damage to the cell membranes. In *E. coli*, which is considered a unicellular organism, $DiBAC_4(3)$ was conducted to identify the characteristics of apoptosis, such as membrane depolarization. Compared to the untreated cells, the intensity of fluorescence increased in the cells treated with genistein, and in the cells pre-treated with the NO inhibitors, fluorescence intensity did not increase significantly. These results suggested that genistein caused damage to the bacterial membranes, producing NO and induced membrane depolarization, one of the characteristics of the apoptosis of eukaryotic cells.

Apoptosis is a crucial process in cell growth and homeostasis (Baar et al. 2017; Negroni et al. 2015). In the apoptotic cells, they display a signal to eat me, probably due to PS exposure (Segawa and Nagata 2015). PS was normally confined to the interior leaflet of the plasma membrane by a "flippase"; apoptosis activated a "scramblase" that rapidly exposed PS on the cell exterior (Marino and Kroemer 2013). Annexin V is a Ca^{2+}-reliant phospholipid-binding protein with high affinity for PS. Thus, this protein could be used as a sensitive probe for PS exposure upon the cell membrane. Annexin V/PI double staining was employed to confirm whether genistein-treated *E. coli* cells exhibit PS exposure, a feature of early apoptosis. Genistein-treated cells exhibit PS exposure without membrane integrity; however, this result was decreased by the NO inhibition, which indicated that NO took part in mediating genistein-induced bacterial apoptosis-like death.

The SOS response is a universal response to DNA damage in bacteria, mediated by the LexA-RecA genes that result in DNA repair and cell cycle arrest (Bellio et al. 2017; McKenzie et al. 2000). Previously, a number of studies had detailed that *E. coli* responded to DNA damage via another RecA-LexA-mediated pathway resulting in PCD (Choi et al. 2016; Lee et al. 2019). It is called an apoptosis-like death because it is characterized by DNA cleavage and membrane depolarization, which are hallmarks of eukaryotic apoptosis. In addition, under apoptosis-like death, activation of RecA led to degradation of LexA (Erental et al. 2014). The NO and RNOS generated by genistein were enough to cause membrane depolarization and severe DNA damage. These features activated the apoptosis-like death, leading to bacterial cell death.

In summary, a number of apoptotic hallmarks, such as DNA fragmentation, caspase-like protein activation, membrane depolarization, and PS exposure, were caused by genistein. Our findings suggested that genistein possesses a novel mechanism of antibacterial action and genistein-induced apoptosis-like death, which was effectuated by inordinate NO generation. Importantly, RecA was essential for the process of apoptosis-like death, acting as caspase. Consequently, genistein exerted antibacterial activity via a novel mechanism, apoptosis-like death, and genistein could be an antibiotic derived from natural substances that is easy to obtain around and could work as an antimicrobial agent while generating NO with antimicrobial activity.

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Author contributions H. Kim and D.G. Lee conceived the study and designed the experiment. H. Kim performed the experiments and collected the data. H. Kim and D.G. Lee analyzed the data. H. Kim wrote the manuscript

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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