Lupeol-induced Nitric Oxide Elicits Apoptosis-like Death within

*Escherichia coli* in a DNA Fragmentation-independent Manner

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Abstract

Lupeol is known to be plentiful in fruits or plant barks and has an antimicrobial effect, however, its mode of action(s) has yet to be determined. To elucidate lupeol generates nitric oxide (NO), which is recognized for possessing an antimicrobial activity, intracellular NO was measured in *Escherichia coli* using DAF-FM. Using the properties of NO passing through plasma membrane easily, increased malondialdehyde levels have shown that lupeol causes lipid peroxidation, and the resulting membrane depolarization was confirmed by DiBAC4(3). These data indicated that lupeol-induced NO is related to destruction of bacterial membrane. Further study was performed to examine whether NO, known as a cell proliferation inhibitor, affects bacterial cell division. As a result, DAPI staining verified that lupeol promotes cell division arrest, and followed by early apoptosis is observed in Annexin V/PI double staining. Even though these apoptotic hallmarks appeared, the endonuclease failed to perform properly with supporting data of decreased intracellular Mg2+ and Ca2+ levels without DNA fragmentation, which is confirmed using a TUNEL assay. These findings indicated that lupeol-induced NO occurs DNA fragmentation-independent bacterial apoptosis-like death (ALD). Additionally, lupeol triggers DNA filamentation and morphological changes in response to DNA repair system called SOS system. In accordance with the fact that ALD deems to SOS response, and that the RecA is considered as a caspase-like protein, increase of caspase-like protein activation occurred in *E. coli* wild-type, and no ΔRecA mutant. In conclusion, these results demonstrated that the antibacterial mode of action(s) of lupeol is an ALD while generating NO.

Keywords: lupeol; nitric oxide; *Escherichia coli*; apoptosis-like death;

Abbreviations: ALD, Apoptosis-like death; CLSI, Clinical and Laboratory Standard Institute; L-NAME, Nω-nitro-L-arginine methyl ester hydrochloride; MIC, Minimum inhibitory concentrations; NO, Nitric oxide; NOS, Nitric oxide synthase; PBS, Phosphate-buffered saline; PCD, Programmed cell death; PS, Phosphatidylserine; ROS, Reactive oxygen species; TUNEL, Transferase dUTP nick end labeling.
Introduction

Lupeol is a pentacyclic triterpene abundant in mango pulp, eggplant, cucumber, and soybean [1-3]. It is widely used as a chemo-preventive compound for prevention or treating of some diseases, as it has a broad range of pharmacological activities, including anti-inflammatory, anti-tumor, anti-cancer, and anti-angiogenic activities [3, 4]. Typically, lupeol is cytotoxic to cancer cells while also inhibiting the function of topoisomerase II, [5, 6] which prevents from binding to DNA and thereby prevents supercoiled DNA from being converted into relaxed DNA, making it impossible for DNA replication to take place [4, 5]. Lupeol can also act as an anti-cancer and anti-inflammatory agent by interfering with DNA polymerase β and the enzyme farnesyltransferase [7, 8]. In addition, lupeol induces Fas-receptor or cell cycle arrest-mediated apoptosis and decreases the inflammatory response, thereby reducing the expression of NF-κB and TNF [9, 10]. Furthermore, lupeol exhibits antimicrobial activity by inhibiting cell growth in human pathogenic fungi and bacteria. It is also known to interfere with mitochondrial dysfunction and ergosterol biosynthesis in fungi, however, the antibacterial mode of action(s) of lupeol is yet to be elucidated [11, 12].

Nitric oxide (NO) is a small, gaseous, and hydrophobic free radical with a broad range of reactivities involving various molecules. NO plays an important role in host immunity against pathogens; it can pass rapidly through lipid membranes [13-15]. In addition, NO affects cell proliferation, by inducing cell cycle arrest [16-18]. In breast cancer, promonocytic and vascular smooth muscle cells, NO-induced G1 arrest occurs when the transition from G1 to S phase is interrupted. When NO arrests the cell cycle, the downregulation of anti-apoptotic protein Bcl-2 occurs, and NO-induced apoptosis begins. NO is a biological messenger that plays important roles in vasodilatation, neurotransmission and angiogenesis; it has also been used as an antimicrobial agent for many years. In bacteria, NO and its byproducts negatively impact membrane integrity and disrupt cellular functions, thereby causing oxidative and nitrosative stress [19]. Given the properties of NO, NO-releasing devices or nanoparticles have recently been developed as antibacterial agents. Therefore, evidence suggests that compounds that generate NO can be effective therapies for bacterial infections.

Apoptosis, i.e., programmed cell death (PCD), is vital for the maintenance of life as it removes damaged or unnecessary cells; [20] it typically occurs when cells are unable to function normally due to severe DNA damage. As such, apoptosis involves the spontaneous and active action of cells, including features such as cell shrinkage, DNA fragmentation by endonuclease, and phosphatidylserine (PS) exposure [21, 22]. Unlike necrosis, the cells themselves are normally involved in death during apoptosis, but this becomes disordered
when they are exposed to extreme external factors. While apoptosis is typically an orderly process that does not harm cells, when it is dysfunctional it disrupts homeostasis, promotes chronic inflammation and cancer cell growth, and causes diseases such as Parkinson's disease [23].

Apoptosis is a form of PCD that occurs only in eukaryotes and some fungi. Recently, however, Escherichia coli has been found to have a novel death pathway, characterized by features such as DNA fragmentation and membrane depolarization, known as apoptosis-like death (ALD) [24, 25]. ALD is recognized as a bacterial SOS response that occurs when cells are subjected to severe DNA damage; this response involves a broad range of cellular reactions in response to DNA damage, particularly related to RecA and LexA protein [26, 27]. Under DNA damage stress, the exposed single strand DNA and RecA combine; RecA induces the SOS response while weakening the activity of LexA. Thus, the SOS response in E. coli, namely bacterial ALD, could be used as a mode of action(s) for destroying bacteria in a novel, i.e., not in bacteriostatic or bactericidal, system.

In the present study, we investigated whether lupeol generates NO in E. coli and whether lupeol-induced NO has effects similar to eukaryotic apoptosis but in E. coli. Our results were also evaluated in relation to a novel mechanism(s) involving ALD.
Material and Methods

Analysis of cell viability

In accordance with the Clinical and Laboratory Standard Institute (CLSI) guidelines, the MIC values were measured. In the compound and strain preparation stages, lupeol (Sigma Chemical Co., St. Louis, MO, USA) or norfloxacin were dissolved using ethanol or acetic acid, respectively. The following bacterial strains were used in this study: Enterococcus faecium (ATCC 19434), Enterococcus faecalis (ATCC 29212), Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), and Salmonella enteritidis (ATCC 13076). These 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 allocated into 96-microwell plates (0.1 mL/well). Lupeol and norfloxacin treatments were applied via a two-fold serial dilution. After 24 h of incubation at 37 °C, cell proliferation was determined by optical density at 600 nm using a microtiter ELISA reader. (BioTek Instruments, Winooski, VT, USA).

Measurement of intracellular NO and O_2^- 

E. coli MG 1655, acquired from Coli Genetic Stock Center, was used for measuring NO generation. We used 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA, Molecular Probes, Eugene, OR, US) as an NO indicator. Bacterial cells (2×10^6 cells/mL) were treated with lupeol (5 μg/mL) or norfloxacin (2.5 μg/mL) at 37 °C for 2 h. Following incubation, the cells were centrifuged at 12,000 rpm for 5 min and the supernatants were eliminated. The cells were then resuspended in phosphate-buffered saline (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 DAF-FM at 37 °C for 30 min. After washing twice with PBS, PBS was used to adjust the total volume to 1 mL. The samples were then evaluated utilizing a FACSVerse flow cytometer (Becton Dickinson, NJ, USA). O_2^- levels were estimated using a dihydrorhodamine 123 (DHR-123) (Sigma Chemical Co.). The E. coli cells were first incubated with lupeol (5 μg/ml) or norfloxacin (2.5 μg/ml) at 37 °C for 2 h. Following incubation, the cells were collected via centrifugation at 12,000 rpm for 5 min and then stained with 5 μM DHR-123. Samples were assessed using a FACSVerse flow cytometer.
Evaluation of lipid peroxidation

The thiobarbituric acid-reactive substances (TBARS) assay was employed to detect malondialdehyde (MDA) levels. MDA, the end product of lipid peroxidation, was evaluated from standard curves (created based on the suggestions of the manufacturer, Sigma-Aldrich). No-nitro-L-arginine methyl ester hydrochloride (L-NAME), an analog of arginine that inhibits NO production, was dissolved in H₂O. Bacterial cells (2×10⁶ cells/mL) were then incubated for 2 h at 37 °C with lupeol (5 μg/mL), lupeol pretreated with L-NAME (0.5 μg/mL), or norfloxacin (2.5 μg/mL). Following incubation, the cells were centrifuged at 12,000 rpm for 5 min and the cell pellets were mixed with lysis buffer (pH 8.0, 10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 2% Triton-X 100, and 1% SDS). Cells were then sonicated on ice using an ultrasonic sonicator (10 pulses of 1 min each at amplitude 38) (Sonics, Newtown, CT, USA). Subsequently, the mixture was centrifuged, and the supernatant was mixed with thiobarbituric acid in 5% trichloroacetic acid. The mixture was heated at 95 °C for 30 min, and then cooled on ice. The absorbance of the reaction mixture was measured at 532 nm.

Assessment of membrane depolarization

Bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3)] (Molecular Probes) is a translational membrane potential dye that redistributes within the cell membrane when membrane potential changes. The fluorescence of the dye is enhanced when it enters the cell membrane as a result of membrane depolarization. To assess membrane depolarization, DiBAC₄(3) was dissolved in DMSO at a concentration of 5 μg/mL. The bacterial cells (2×10⁶ cells/mL) were treated with lupeol (5 μg/mL), lupeol pretreated with L-NAME (0.5 μg/mL), or norfloxacin (2.5 μg/mL) and then incubated for 2 h at 37 °C. Following incubation, the cells were centrifuged at 12,000 rpm for 5 min and the supernatants were removed. The cell pellets were resuspended twice in PBS and the final volume was adjusted to 1 mL with PBS. These bacterial solutions were stained with 5 μg/mL DiBAC₄(3), and the fluorescence intensity of each sample was analyzed using a FACSVerse flow cytometer.

Detection of DNA filamentation and morphological change due to cell division arrest

To identify whether cell division arrest occurred, chromosomal condensation was detected using 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma Chemical Co.). Cells (2×10⁶ cells/mL) were first treated with lupeol (5 μg/mL), lupeol pretreated with L-NAME (0.5 μg/mL), or norfloxacin (2.5 μg/mL) for 2 h at 37 °C. For nuclear staining, the cells were washed with PBS and incubated with 1 μg/mL DAPI in a darkroom.
for 20 min. The intensity of fluorescence was measured with a spectrofluorophotometer (Shimadzu RF-5301PC; Shimadzu, Japan) at wavelengths of 340 nm (excitation) and 488 nm (emission). To ascertain DNA filamentation and morphological changes caused by cell cycle arrest in *E. coli*, bacterial cells were incubated with lupeol (5 μg/mL), lupeol pretreated with L-NAME (0.5 μg/mL), or norfloxacin (2.5 μg/mL) for 2 h at 37 °C. The cells were then harvested by centrifugation and resuspended in PBS. DNA filamentation was observed using a microscope (Nikon Eclipse Ti-s; Tokyo, Japan) and morphological change was analyzed using a FACSVerse flow cytometer.

**Estimation of PS exposure and bacterial caspase-like protein**

PS exposure was detected using the Annexin V–FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). Cells (2×10^6 cells/mL) were first treated with lupeol (5 μg/mL), L-NAME (12.5 ng/mL) pretreated lupeol, or norfloxacin (60 ng/mL) and then incubated for 2 h at 37 °C. After incubation, the cells were gathered and resuspended in 100 μL of 1× Annexin V binding buffer; 50 μL/ml of Annexin V–FITC was then added to the cell suspensions. The mixtures were incubated for 15 min in the dark before being assessed utilizing a FACSVerse flow cytometer. To investigate homologous of eukaryotic caspase, the CaspACE FITC-VAD-FMK In Situ Marker (Promega, Fitchburg, WI, USA) was employed and the ΔRecA mutant, obtained from *E. coli* K-12 collection, was used. *E. coli* wild-type and ΔRecA cells were incubated with lupeol (5 μg/mL), L-NAME (0.5 μg/mL) pretreated lupeol, or norfloxacin (2.5 μg/mL) for 2 h at 37 °C. The cells were then washed twice with PBS and stained with 5 μM FITC-VAD-FMK for 30 min. The total volume was adjusted to 1mL with PBS, and fluorescence was estimated utilizing a FACSVerse flow cytometer.

**Determination of DNA fragmentation**

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to detect DNA cleavage. This assay was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Basel, Switzerland). The 3’-OH termini of the nucleotide were enzymatically labeled and mediated by terminal deoxynucleotidyl transferase; the fragmented DNA was then identified. Cells (2×10^6 cells/mL) were incubated for 2 h at 37 °C with lupeol (5 μg/mL), L-NAME (0.5 μg/mL) pre-treated lupeol, 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. Subsequently, the fixed cells were incubated with permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) on ice for 2 min. The cells were then incubated with a TUNEL
reaction mixture for 1 h at 37 °C. Finally, fluorescence intensity was estimated using a spectrofluorophotometer (Shimadzu RF-5301PC; Shimadzu, Japan) at wavelengths of 495 nm (excitation) and 519 nm (emission).

**Measurement of intracellular Mg**$^{2+}$**and Ca**$^{2+}$**levels**

Intracellular Mg$^{2+}$ levels were measured using Magnesium Green (Molecular Probes). *E. coli* cells were incubated with lupeol (10, 20, 40, and 80 μg/mL) or norfloxacin (2.5 μg/mL) at 37 °C for 2 h. Using Krebs buffer (132 mM NaCl, 10 mM HEPES, 4 mM KCl, 10 mM NaHCO$_3$, 6 mM glucose, 1.4 mM MgCl$_2$ and 1 mM CaCl$_2$; pH 7.2), to which 1 % bovine serum albumin and 0.01 % pluronic F-127 (Molecular Probes) were added, the cells were washed three times. The suspensions were stained with 5 μM Magnesium Green and then washed in triplicate with Ca$^{2+}$- and Mg$^{2+}$-free Krebs buffer. Ca$^{2+}$ levels were measured using Fura-2AM. *E. coli* cells were treated with lupeol (10, 20, 40, and 80 μg/mL) or norfloxacin (2.5 μg/mL) and incubated for 2 h at 37 °C. The cells were then washed three times with Krebs buffer containing 1 % bovine serum albumin and 0.01 % pluronic F-127. Fura-2AM (10 μM) (Molecular Probes) was added in a subsequent suspension incubation and then cells were washed in three times using Ca$^{2+}$-free Krebs buffer. The Magnesium Green (excitation = 506 nm; emission = 531 nm) and Fura-2AM (excitation = 552 nm; emission = 581 nm) intensities were both measured using a spectrofluorophotometer.

**Statistical analysis**

All the experiments were performed in triplicate 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 followed by Tukey's post-hoc test for three-group comparisons using SPSS software (version 25, SPSS/IBM, Chicago, IL, USA). Intergroup differences were considered statistically significant at $p < 0.05$. 
Results

Antibacterial effects of lupeol in the NO-generating manner

Lupeol is a dietary triterpene and has several potential biological activity and medical properties. It has previously been reported that lupeol has antimicrobial activities while inhibiting the proliferation of microorganisms. To assess whether lupeol has antibacterial effects on pathogenic bacteria, the MIC values were performed based on the CLSI method. It was confirmed that lupeol showed potential antibacterial effects at MIC values from 2.5 to 5 μg/mL in eight hospital bacterial strains (Table 1). Thus, this data indicated that lupeol has antibacterial effects similar to norfloxacin which is antibiotics for gram-negative. Among others, E. coli was also used as a bacterial model to examine the antibacterial mode of action of lupeol. NO also has been known to have antimicrobial properties and regarded as antimicrobial agent. Therefore, we assessed whether lupeol produced NO in E. coli by estimating the fluorescence intensity of DAF-FM. This reagent reacts with NO to form fluorescence benzotriazole and helps detect NO quantitative. Cells treated with lupeol (5 μg/mL) and norfloxacin (2.5 μg/mL) accounted for 33.80 % and 90.89 %, respectively, compared to 14.52 % for untreated cells (Fig. 1A). Given that both lupeol and norfloxacin produce NO at its MIC values, this investigation implied that lupeol has antibacterial effect, apparently generating intracellular NO in E. coli cells. As a result, NO-producing method could explain the mode of antibacterial action of lupeol.

Induction peroxidation of bacterial membrane lipids

NO is a known free radical molecule that interferes with membrane lipid and plays a role in lipid biochemistry. When it is present with superoxide (O$_2^-$), it strongly promotes the peroxidation of phospholipids by forming prooxidant species such as peroxynitrite (ONOO$^-$) [28]. Their by-product, ONOO$^-$, as well as NO and O$_2^-$, rapidly permeates phospholipid membrane and initiates lipid oxidation. For this reason, the following examinations were accompanied to detect whether NO and its byproduct affect the oxidation of bacterial plasma membrane. In the initial stage, an experiment was conducted using DHR-123 to determine whether lupeol and norfloxacin produce O$_2^-$ in bacterial cells. Lupeol (5 μg/mL) or norfloxacin (2.5 μg/mL) treated cells showed 43.28 % and 98.04 % fluorescence intensity, respectively, while untreated cells showed 13.13 % (Fig. 1B).
Accordingly, peroxidation of phospholipids changes the permeability and fluidity of cell membranes, affecting the interaction between lipids. High levels of malondialdehyde (MDA), a lipid oxidation indicator generated by peroxidation of membrane polyunsaturated fatty acids, were observed after treatment with lupeol (5 μg/mL) or norfloxacin (2.5 μg/mL), relative to the untreated cells and the cells treated with L-NAME pretreated lupeol (Fig. 2). Therefore, these results indicated that lupeol triggers lipid peroxidation of bacterial plasma membrane, thereby producing intracellular O₂⁻ and NO within bacterial cells.

Appearance of plasma membrane depolarization

Lipid peroxidation changes the physiological properties of the cell membrane, causing deformation of proteins or nucleic acid. In particular, oxidation of the cell membrane changes mitochondrial membrane potential leading to membrane depolarization in eukaryotes [29, 30]. It is also the basis that the oxidation of bacterial plasma membranes can also change the membrane potential of bacteria, in the view of the evolution of mitochondria in eukaryotic organisms from prokaryotes. DiBAC₄(3), a sensitive slow-response probe for measuring cellular membrane potential that and can easily enter depolarized cells, was used to assess whether lupeol-induced NO causes bacterial membrane depolarization similar to eukaryotes. The untreated cells exhibited a fluorescence intensity of 11.61 %, while those treated with lupeol (5 μg/mL) or norfloxacin (2.5 μg/mL) exhibited fluorescence intensities of 36.11 % and 99.60 %, respectively (Fig. 3). On the other hand, cells treated with L-NAME-pretreated lupeol exhibited 12.17 % fluorescence intensity, i.e., similar to untreated cells. Decreased fluorescence intensity following pretreated with L-NAME suggested that lupeol-induced NO mediates bacterial membrane depolarization. Collectively, this observation suggests that lupeol-induced NO changes the bacterial membrane potential and induced a resulting membrane depolarization in E. coli.

Confirmation of cell division arrest and PS exposure

Cell cycle arrest is representative mechanism by which lupeol induces apoptosis in human cancer cells. NO has been reported to block the cell cycle by acting as a cell proliferation inhibitor. Based on the characteristics of lupeol and NO associated with cell cycle inhibition, DAPI was used to monitor the cell division arrest in E. coli. Compared with untreated cells that showed slight fluorescence, lupeol (5 μg/mL)- or norfloxacin (2.5 μg/mL)- treated cells displayed strong fluorescence, indicating the presence of cell division arrest (Fig. 4). In contrast, L-NAME pretreated lupeol cells had lower fluorescence than those treated with only lupeol. This observation proved that lupeol-induced NO inhibits cell division. PS is a phospholipid that usually
exists in the inner leaflet of the plasma membrane, acts as a unique marker of apoptosis since it is exposed on the outer leaflet while the cell undergoes apoptosis. Thus, to assess whether ALD appears in *E. coli*, Annexin V/PI double staining (Annexin V+/PI−; = early apoptosis, Annexin V+/PI+; = necrosis) was performed. PS exposures of 26.49 % and 44.45 % was derived in *E. coli* cells treated with lupeol (5 μg/mL) or norfloxacin (60 ng/mL), respectively, whereas L-NAME pretreated diminished lupeol-induced PS exposure (Fig. 5). Therefore, this data identified that lupeol could lead to PS exposure on the outside leaflet, without necrosis, by generating NO. To sum up, it is demonstrated that lupeol-induced NO triggers ALD of *E. coli* by inhibiting cell division.

**Occurrence of ALD in absence of DNA fragmentation**

The biochemical hallmark of apoptosis is DNA degradation by DNA endonucleases, which create double-stranded oligonucleosomal DNA breaks. TUNEL assay, which is designed to detect apoptotic cells that undergo considerable DNA degradation during the late stages of apoptosis, was employed to estimate the percentage of apoptosis. Therefore, the experiment was conducted to assess lupeol-induced NO elicits DNA fragmentation in *E. coli* cells. As shown in Fig. 6A, cells treated with norfloxacin (53.13 %) exhibited DNA fragmentation but lupeol-treated cells (15.32 %) did not, similar to untreated cells (10.37 %) and the L-NAME pretreated lupeol cells (10.93 %). As shown in Fig. 6B, lupeol-induced DNA did not appear only in the MIC values, but also when the MIC values of lupeol were increased 2-, 4-, 8- or 16-fold (result of 15.32 %, 15.75 %, 15.89 %, and 16.21 %, respectively). Similar results were observed with L-NAME pretreated lupeol (2-, 4-, 8-, and 16-fold MICs gave 12.17 %, 14.22 %, 14.61 %, and 14.71 %, respectively). These results suggest that lupeol induces early apoptosis without fragmentation of DNA into oligonucleosomal fragments and lupeol-induced NO does not affect DNA fragmentation. Thus, it is indicated that there are other factors than NO as a reason for the absence of DNA fragmentation.

**Inhibition of endonuclease activation involved in DNA cleavage**

Apoptotic endonuclease is a key enzyme that mediates regulated DNA fragmentation and chromatin condensation in response to apoptotic signals. The endonuclease involved in DNA fragmentation is an enzyme dependent on Mg$^{2+}$ and Ca$^{2+}$. *E. coli* has endonuclease III, proteins that regulated DNA cleavage. These Mg$^{2+}$- and Ca$^{2+}$-dependent endonucleases that break oligonucleosomal DNA are inhibited by NO [31, 32]. Therefore, we investigated whether lupeol (unlike norfloxacin) could distribute the function of endonuclease III while inhibiting the increase in intracellular Mg$^{2+}$ and Ca$^{2+}$. Magnesium Green (a cell-impermeant dye and
intracellular Mg\(^{2+}\) indicator) and Fura-2AM (a cell-permeant dye and intracellular Ca\(^{2+}\) indicator) were applied to detect intracellular Mg\(^{2+}\) and Ca\(^{2+}\) concentration changes in the *E. coli* cells treated with lupeol or norfloxacin. In this observation, the cells treated with lupeol showed a greater reduction in the concentrations of Mg\(^{2+}\) and Ca\(^{2+}\) than did untreated cells as MIC values increased. On the other hand, norfloxacin increased intracellular Mg\(^{2+}\) and Ca\(^{2+}\) at MIC values (Fig. 7 and 8). With the absence of DNA fragmentation (described earlier), and these results demonstrate that lupeol-induced Mg\(^{2+}\) and Ca\(^{2+}\) deficiency in *E. coli* cells prevents endonuclease III form working properly, inhibiting DNA fragmentation.

**Filamentation and morphological changes by SOS response**

Filamentation is a trademark of cell cycle arrest in bacteria, in which cells continue to elongate but do not divide. The SOS response is a general response to DNA damage, such as cell cycle arrest. RecA is essential to repair and maintenance of DNA in the bacterial SOS response [33]. The *E. coli* wild-type and ∆RecA mutant obtained from *E. coli* K-12 collection were applied to indicate whether DNA filamentation occurred due to cell cycle arrest and whether these features were related to the SOS response. As shown in Fig. 9A, *E. coli* wild-type cells treated with lupeol or norfloxacin displayed DNA filamentation (unlike untreated cells), but treatment with L-NAME pretreated lupeol did not have this effect. However, in ∆RecA mutant cells, lupeol, L-NAME pretreated lupeol, and norfloxacin did not induce filamentation, similar to untreated cells (Fig. 9B). Morphological changes with or without RecA. As shown in Fig. 10, the *E. coli* wild-type cells treated with lupeol or norfloxacin tended to increase FSC and SSC, which indicates the cell filamentation and granularity of cells, respectively, whereas L-NAME pretreated lupeol cells remained unchanged relative to untreated cells. In ∆RecA mutant cells, however, lupeol, L-NAME pretreated lupeol, and norfloxacin treatments did not exhibit any increased FSC and SSC in comparison to untreated cells. Summarizing the above investigations, these results suggest that lupeol-induced NO triggers a DNA damage, e.g., via cell cycle arrest, which result in DNA filamentation and increased SSC. Furthermore, the lack of DNA filamentation and increased SSC in ∆RecA mutant cells verifies that lupeol-induced NO mediates DNA filamentation and bacterial morphological changes via the SOS response.

**Expression of RecA and activation of bacterial caspase-like protein**

Caspase is an essential protein for apoptosis in eukaryotes. Activation of caspases is a very early event which occurs in all forms of apoptosis and prevention of caspase activation blocks all the morphological
features associated with apoptosis. In previous research, the RecA protein involved in the *E. coli* SOS response is considered to be a caspase-like protein that plays a similar role to that of caspase in eukaryotic organisms. To determine whether RecA in bacteria functions like eukaryotic caspase, FITC-VAD-FMK was employed in the *E. coli* wild-type and ΔRecA mutant cells. In the lupeol-treated (5 μg/mL) *E. coli* wild-type cells, fluorescence was increased by 49.73% compared to untreated cells (10.61%) and cells treated with L-NAME-pretreated lupeol (10.70%), whereas fluorescence in norfloxacin-treated (2.5 μg/mL) cells increased by 73.04%. In the ΔRecA mutant cells, fluorescence remained unchanged following treatment with lupeol (5 μg/mL) or norfloxacin (2.5 μg/mL), as well as in untreated cells and cells pretreated with L-NAME in lupeol (Fig. 11). These investigations demonstrated that a caspase-like protein (which shared the same substrate with the eukaryotic caspase) was increased by lupeol treatment, suggesting that RecA could act not only as a regulator of the SOS response but also as a bacterial caspase-like protein.
Discussion

*Escherichia coli* normally exist in the intestines of humans or animals where it is harmless bacteria that play an important role in maintaining intestinal health. However, outside of the intestinal tract, *E. coli* can cause diarrhea, pneumonia, urinary tract infection and respiratory diseases. Antibiotics have been developed to treat the disease-causing *E. coli* in a bactericidal or bacteriostatic manner; however, bacterial resistance to conventional antibiotics is increasing, and the development of treatments with new mechanism is emerging as an important challenge [34]. Lupeol, triterpene compound with pharmacological activity, is a fat-soluble and not harmful to humans; indeed, it has long been used as an effective acne treatment [35, 36]. It is also recognized as an antifungal, antiviral, and antibacterial agent. Despite its uses, it is not yet known how lupeol interferes with the growth of bacteria. Therefore, this investigation aims to reveal the novel antibacterial mode of action(s) of lupeol within *E. coli*.

Similar to lupeol, NO is also pharmacologically active in skin therapy, so NO-releasing nanoparticles are used to treat skin infections caused by MRSA (Methicillin-resistant *Staphylococcus aureus*). Intracellular NO is produced by nitric oxide synthases (NOSs) that are a family of enzymes catalyzing the production of NO from L-arginine. Arginine-derived NO synthesis has been identified in mammals and bacteria. Gram-negative bacteria do not have as much NOS as mammalian cells, but they do have bacterial NOS (bNOS) that functions similarly to mammalian NOS (mNOS) [37, 38]. The fact that *E. coli* can generate intracellular NO using its bNOS suggests that lupeol and norfloxacin exhibit antibacterial activity while producing NO known as antimicrobial agents. Therefore, in this investigation, both lupeol and norfloxacin produced NO with *E. coli* and that the way of intracellular NO generation affects *E. coli* cell proliferation.

NO itself affects the growth of bacteria, but if ONOO⁻ is created under the presence of O₂⁻, NO causes more oxidative stress on bacteria. Oxidizing agents, especially NO and ONOO⁻, facilitate the oxidation of proteins, DNA, and lipids while easily passing through the cell membrane of bacteria. In general, lipid oxidation negatively affects cell membrane fluidity or permeability, reducing the stability of the lipid bilayer [39, 40]. Similarly, lipid peroxidation destroys the structure or function of cell membranes, resulting in apoptosis or cell death. In this study, lupeol treatment increased intracellular O₂⁻ and induced peroxidation of lipids. In addition,
when NO synthesis was suppressed, the degree of lipid oxidation was reduced, indicating that NO was a factor that damaged membrane lipids. In summary, NO and its by-products devastated the bacterial cell membrane in the presence of O₂, causing peroxidation of membrane lipids.

Lipid peroxidation causes loss of polyunsaturated fatty acids and disrupts the functions of membrane-related ion channels or enzymes. Thus, changes in lipid fluidity and plasma membrane permeability cause loss of plasma membrane potential. When the plasma membrane potential changes, intracellular ionic homeostasis cannot be maintained and this results in membrane depolarization. Since altered intracellular ionic homeostasis stimulates the activity of apoptotic cascades, membrane depolarization is considered to be a marker of apoptosis; therefore, the occurrence of apoptosis in mammalian cancer cells is observed by detecting membrane depolarization. In this study, a membrane potential imbalance, such as membrane depolarization, was observed in lupeol-treated E. coli cells, indicating that lupeol-induced NO disrupts bacterial membrane potential. Therefore, it is manifested that NO is sufficient to damage bacterial membranes.

In multicellular organisms, cell cycle regulators balance cell proliferation and death through an interplay with apoptotic factors. Uncontrolled cell proliferation or cell cycle arrest can cause a homeostasis imbalance in organisms that leads to PCD due to physiological changes. Previously, NO has been reported to promote cell cycle arrest and primarily interrupt G1 to S transition. Moreover, NO-mediated proliferation arrest can induce PCD acting as pro-apoptotic factor. Unlike eukaryotic organisms, bacteria do not undergo apoptosis, but recently it is reported that they do exhibit apoptosis-like PCD called ALD. Our research identified that lupeol-treated cells have interrupted completion of cell division and that inhibiting NO synthesis tends to diminish this occurrence. Thus, these data demonstrate that lupeol-induced NO not only suppresses cell division but also leads to bacterial ALD.

In the early stages of apoptosis, DNA degradation and fragmentation are induced by endonuclease; therefore, DNA fragmentation is applied as an indicator of early apoptosis. However, apoptosis did not necessarily involve DNA fragmentation. Similar to eukaryotic cells, E. coli has endonuclease III that acts as glycosylase. In addition, [4Fe-4S] cluster of the E. coli endonuclease III can be modified by NO. Modification of the [4Fe-4S] cluster completely inhibits the DNA glycosylase activity of the endonuclease III. In our investigation, unlike the previously known ALD, DNA fragmentation caused by lupeol-induced NO did not appear. Given that the DNA fragmentation did not appear even when the MIC value of lupeol is increased by 16 times, it is demonstrated that lupeol-induced NO does not directly affect DNA cleavage. Therefore, we tried to figure out the relationship between lupeol-induced NO and endonuclease III participating
in the DNA fragmentation. The cells treated with lupeol reduced the intracellular Mg\(^{2+}\) and Ca\(^{2+}\) levels enough to undermine endonuclease III activity. As a result, these examinations indicated that lupeol exerts ALD in absence of DNA fragmentation unlike common bacterial apoptosis-like PCD.

Some bacteria have filamentous proteins that are involved in cell division and cell shape. *E. coli* controls cell division under environmental conditions that cause metabolic changes or DNA damage. To cope with these stresses, *E. coli* uses survival strategies such as cell filamentation and morphological change. In this paper, our findings demonstrate that when lupeol-induced NO initiates cell division arrest and interferes with DNA replication, filamentation and morphological change occurs in *E. coli* as a response to DNA damage. The overall reaction to DNA damage in bacteria, the SOS response, halts cell division and repairs DNA. Both filamentation and morphological changes are trademarks of SOS response in *E. coli* [46]. For example, DNA damaging and DNA synthesis-inhibiting drugs are reported to induce bacterial filamentation via the SOS response [47]. Thus, our investigations suggest that when lupeol-induced NO causes DNA damage, the SOS response occurs in *E. coli* to repair DNA.

Caspases are a family of proteases essential to apoptosis. They usually exist in an inactive pro caspase, but when an apoptotic signal, such as that from DNA damage or ROS, is received, caspase is activated. RecA is an ATP-dependent protein that formed nucleoprotein filaments by binding to the single-stranded DNA, and these filaments triggers the SOS response by inducing autocleavage of the LexA repressor [48, 49]. Under severe DNA damage, RecA combines with single-stranded DNA to maintain weakening of LexA and SOS genes are expressed by strong SOS operation. Although caspase has not been found in prokaryotes, RecA involved in the SOS response is considered to be a bacterial caspase-like protein. Therefore, our study indicated that lupeol-induced NO promotes the activation of RecA in *E. coli*, much in like eukaryotes, the SOS protein, RecA serves as a bacterial caspase-like protein.

Evidence for apoptotic death in bacteria has been found in recent years. Bacterial apoptotic death, known as ALD, is an extreme SOS response under DNA damage [26]. Similar to eukaryotic apoptosis, the ALD in *E. coli* also features membrane depolarization, PS exposure, DNA fragmentation and chromosomal condensation. In ALD, RecA also acts as a substrate for eukaryotic caspase. Taken together, our results provide evidence that ALD occurs in *E. coli* treated with lupeol. Membrane depolarization and cell division arrest are also shown because lupeol-induced NO is harmful to the bacterial membrane and DNA. In addition, caspase-like protein activation and SOS response induction were observed. Furthermore, feature of early apoptosis (PS exposure) was appeared in lupeol-treated *E. coli* cells. Unlike regular ALD, however, lupeol did not trigger
DNA fragmentation by reducing intracellular Mg\(^{2+}\) and Ca\(^{2+}\) levels. Thus, lupeol-induced ALD occurs in *E. coli* without DNA fragmentation.

In conclusion, a number of ALD hallmarks, including membrane depolarization, PS exposure, caspase-like protein (RecA) activation, and induction of SOS response, were mediated by lupeol-induced NO. Additional research on ALD is required, but our findings suggest that lupeol possesses a novel antibacterial mechanism that results in ALD while generating NO. Consequently, amid increasing of bacterial resistance to widely used bacteriostatic and bactericidal antibiotics, lupeol, may be suitable as a potential novel antibacterial agent.
Conflict of interest

The authors declare that they have no conflict of interests.

Data Availability Statement

No datasets were generated or analyzed during the current study.

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Figure legends

Fig. 1. *E. coli* cells were treated with 5 µg/mL lupeol and 2.5 µg/mL norfloxacin. (A) Detection of nitric oxide was measured by DAF-FM. (B) Superoxide (O$_2^-$) generation was measured using DHR-123.

Fig. 2. Lipid peroxidation in *E. coli* was measured using the TBARS assay and increased MDA levels indicate peroxidation of lipids. (a) Untreated cells, (b) lupeol treated cells (5 µg/mL), (c) lupeol treated cells (5 µg/mL) with L-NAME pretreatment (0.5 µg/mL), and (d) norfloxacin treated cells (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. 3. Membrane depolarization was measured using DiBAC$_4$(3) in *E. coli*. (a) Untreated cells, (b) cells treated with 5 µg/mL lupeol, (c) cells treated with 5 µg/mL lupeol with pretreatment of 0.5 µg/mL L-NAME, and (d) cells treated with 2.5 µg/mL norfloxacin.

Fig. 4. Spectrofluorophotometric analysis of cell division arrest measured using DAPI in *E. coli*. (a) Untreated cell, (b) cells treated with 5 µg/mL lupeol, (c) cells treated with 5 µg/mL lupeol with a pretreatment of 0.5 µg/mL L-NAME, and (d) cells treated with 2.5 µg/mL norfloxacin. Experiments were held triplicate independently and the results represent the average, standard deviation, and p values from three different experiments (**p < 0.01).

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

Fig. 6. (A) Flow cytometric analysis of DNA fragmentation in *E. coli* was measured using a TUNEL assay. (a) Untreated cell, (b) lupeol treated cells (5 µg/mL), (c) lupeol treated cells (5 µg/mL) with a pretreatment of L-NAME (0.5 µg/mL), and (d) norfloxacin treated cells (2.5 µg/mL). (B) Cells were treated with lupeol concentrations from 2-fold MIC to 16-fold MIC; the figures on the left side were treated with lupeol only, whereas those on right side were treated with lupeol pretreated with 0.5 µg/mL L-NAME.
Fig. 7. Increase in intracellular Mg\(^{2+}\) concentration was measured using Magnesium Green. *E. coli* cells were treated with 2-, 4-, 8-, and 16-fold MIC lupeol (10, 20, 40, and 80 µg/mL, respectively) or 2.5 µg/mL norfloxacin. 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. 8. Increase in intracellular Ca\(^{2+}\) concentration measured by Fura-2AM. *E. coli* cells were treated with 2-, 4-, 8-, and 16-fold MIC lupeol (10, 20, 40, and 80 µg/mL, respectively) or 2.5 µg/mL norfloxacin. 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. 9. DNA filamentation in *E. coli* treated with 5 µg/mL lupeol, 0.5 µg/mL L-NAME-pretreated lupeol, and 2.5 µg/mL norfloxacin. (A) DNA filamentation in wild-type cells. (B) DNA filamentation in ΔRecA mutant cells.  

Fig. 10. Morphological changes in *E. coli* treated with 5 µg/mL lupeol, 0.5 µg/mL L-NAME-pretreated lupeol, and 2.5 µg/mL norfloxacin. An increase in FSC and SSC indicated cell filamentation and granularity of cells, respectively. (A) Morphological changes in wild-type cells. (B) Morphological changes in ΔRecA mutant cells.  

Fig. 11. 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 lupeol (5 µg/mL) or norfloxacin (2.5 µg/mL), (b) ΔRecA mutant cells were treated with lupeol (5 µg/mL) or norfloxacin (2.5 µg/mL). L-NAME (0.5 µg/mL) pretreatment was used on both *E. coli* wild-type and ΔRecA mutant cells.
Propidium Iodide

Amean V-FITC

Propidium Iodide

Amean V-FITC

Propidium Iodide

Amean V-FITC

4.16%

1.10%

26.49%

3.19%

6.87%

1.65%

4.45%

4.45%
Table 1. The antimicrobial activity of Lupeol and Norfloxacin.

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