Reactive Oxygen Species Depletion by Silibinin Stimulates Apoptosis-Like Death in *Escherichia coli*

Bin Lee and Dong Gun Lee*

School of Life Sciences, KNU Creative BioResearch Group (BK21 Plus Program), College of Natural Sciences, Kyungpook National University, Daegu 41566, Republic of Korea

Silibinin is the major active component of silymarin, extracted from the medicinal plant *Silybum marianum*. Silibinin has potent antibacterial activity; however, the exact mechanism underlying its activity has not been elucidated. Here, we investigated the novel mechanism of silibinin against *Escherichia coli*. Time-kill kinetic assay showed that silibinin possess a bactericidal effect at minimal inhibitory concentration (MIC) and higher concentrations (2- and 4-fold MIC). At the membrane, depolarization and increased intracellular Ca\(^{2+}\) levels were observed, considered as characteristics of bacterial apoptosis. Additionally, cells treated with MIC and higher concentrations showed apoptotic features like DNA fragmentation, phosphatidylserine exposure, and caspase-like protein expression. Generally, apoptotic death is closely related with ROS generation; however, silibinin did not induce ROS generation but acted as a scavenger of intracellular ROS. These results indicate that silibinin dose-dependently induces bacterial apoptosis-like death, which was affected by ROS depletion, suggesting that silibinin is a potential candidate for controlling bacteria.

**Keywords:** Bacterial apoptosis-like death, *Escherichia coli*, silibinin, reactive oxygen species depletion

Introduction

Resistance to preexisting antibiotics is supervening and increasing globally, leading to neutralization of conventional antibiotic therapies and resulting in higher medical costs, longer hospital stays, and increased mortality [1]. Several new antibiotic compounds currently in development seem to be promising; however, there are concerns regarding rapid development of resistance to new compounds based on existing antibiotics [2]. Therefore, development of new antibiotics with novel mode of action has become a significant problem in public health care. Among candidates, plant-derived natural products are attracting more attention owing to their potential antimicrobial efficacy and no side effects [3]. These natural products are called phytochemicals, which are necessary for survival and proper functioning in plants [4]. Owing to their antimicrobial properties and no side effects, many medicinal plants have been traditionally used for centuries to thwart microbial growth. Indeed, phytochemicals produced by medicinal plants are active against a wide range of microorganisms, including fungi, yeasts, and bacteria [5].

*Silybum marianum*, also known as milk thistle, is a famous medicinal plant that has traditionally been used for detoxification and for treating liver disease [6]. Silibinin is the major active component in silymarin, an extract of *S. marianum* that contains a mixture of flavonolignans, including that of silibinin, isosilibinin, silicristin, silidianin, and others [7]. It has been reported that silibinin has many biological activities, such as antioxidant effect and anticancer effects by inducing cell cycle arrest and apoptosis in human carcinoma cells [8]. Like other phytochemicals, silibinin was found to have broad-spectrum antimicrobial activity. A number of studies have reported the synergistic antibacterial effect of silibinin on pathogenic bacteria [9–12] and silibinin is also reported to induce apoptosis mediated by mitochondrial Ca\(^{2+}\) signaling in fungi [13].

Recently, a novel cell death mechanism analogous to
apoptotic death was suggested to exist in prokaryotic cells [14]. Apoptosis is generally known to occur in metazoan organisms; it is a process of programmed cell death that occurs commonly during development and aging, and acts to maintain normal cellular homeostasis. It also has the advantage of removing damaged or abnormal cells without releasing detrimental substances to the surrounding cells [15]. Similar to the concept of conventional apoptosis, Dwyer et al. [16] demonstrated the occurrence of apoptosis-like death in the prokaryote *Escherichia coli*, and detected specific features of apoptosis in *E. coli* cells treated with antibacterial agents. They also demonstrated that apoptosis-like death in *E. coli* was mediated by RecA protein, which is known to be involved in the SOS repair system [17, 18]. Based on the apoptotic effect of silibinin on carcinoma cells and yeast, and the lack of information regarding its exact mechanism of antibacterial activity, we investigated the antibacterial mechanism of silibinin focusing on bacterial apoptosis-like death.

**Materials and Methods**

**Preparation of Silibinin and Cell Growth Conditions**

Silibinin was purchased from Sigma–Aldrich (USA) and *E. coli* MG1655 (ATCC 700926) was obtained from the American Type Culture Collection (ATCC, USA). For all experiments, cells were grown in Luria-Bertani (LB) broth (BD Pharmingen, USA) under aerobic conditions at 37°C and 120 rpm. Cultures were grown to an optical density between 0.3 and 0.4. Optical density measurements were carried out using an ELISA Microplate Reader (BioTek, USA) at 600 nm. The bacterial cells were then centrifuged at 5,000×g using Microfuge 16 (Beckman Coulter, USA), and suspended in phosphate-buffered saline (PBS).

**Antibacterial Susceptibility Test**

The minimum inhibitory concentration (MIC) test for silibinin was performed in *E. coli* MG1655. MIC values were determined using 2-fold serial dilutions of silibinin or norfloxacin, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, with slight modifications. *E. coli* cell suspensions (5 × 10⁶ cells/ml) were inoculated into the wells of microtiter plates (0.1 ml/well), and test compounds were then added. The MIC values were determined after 24 h of incubation at 37°C. All experiments were performed three times independently.

**Time-Kill Kinetic Analysis**

*E. coli* cells (5 × 10⁷ cells/ml) were incubated with silibinin (20, 40, 80, and 160 μg/ml) or norfloxacin (0.75 μg/ml). After 0, 2, 4, 8, 16, and 24 h of incubation, cultures were collected and spread onto LB agar plates. Colony forming units were counted after incubation at 37°C for 24 h. Experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation (SD).

**Membrane Integrity Assessment**

Membrane integrity was assessed using SYTOX green (Molecular Probes, USA). Melittin, a membrane-active antimicrobial peptide, was used as the positive control for comparing the physiological change in membrane status elicited by silibinin [19]. *E. coli* cells were treated with silibinin (20, 40, 80, and 160 μg/ml) or melittin (16 μg/ml), and incubated for 4 h at 37°C and 120 rpm. After incubation, the cells were harvested and washed with PBS. The cells were then treated with 0.5 μM SYTOX green. Fluorescence intensity was measured on a FACSVerse flow cytometer (Becton–Dickinson, USA) [20].

**Membrane Potential Change Analysis**

Membrane potential disturbance was assessed using bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3)) (Molecular Probes). *E. coli* cells were treated with silibinin (20, 40, 80, and 160 μg/ml) or norfloxacin (0.75 μg/ml), and incubated for 4 h at 37°C and 120 rpm. After incubation, the cells were harvested by centrifugation at 10,000 xg using the SORVALL Biofuge fresco (Kendro Laboratory Products Inc., USA) and then washed with PBS. The cells were then treated with 5 μg/ml DiBAC₄(3) and their fluorescence intensity was measured on a FACSVerse flow cytometer [20].

**Analysis of Intracellular Ca²⁺ Levels**

To evaluate the changes in intracellular Ca²⁺ levels, we used the intracellular calcium indicator, Fura-2 AM. When Fura-2 AM enters the cell, its acetoxymethyl groups are removed by cellular esterases, and the calcium-sensitive indicator Fura-2 is generated. *E. coli* cells were incubated with silibinin (20, 40, 80, and 160 μg/ml) or norfloxacin (0.75 μg/ml), and incubated for 4 h at 37°C and 120 rpm. The cells were washed three times with Krebs buffer (4 mM KCl, 132 mM NaCl, 10 mM NaHCO₃, 6 mM glucose, 1.4 mM MgCl₂, 10 mM HEPES, and 1 mM CaCl₂, pH 7.4) and treated with 1% bovine serum albumin and 0.01% Pluronic F-127 (Molecular Probes). The suspensions were then incubated with the Ca²⁺-specific dye, Fura-2 AM (5 μM) (Molecular Probes) at 37°C for 40 min. The samples were washed three times with calcium-free Krebs buffer and analyzed using a spectrofluorophotometer (Shimadzu RF-5301PC, Shimadzu, Japan) at the wavelengths 340 nm (excitation) and 510 nm (emission) [21].

**PS Externalization Analysis**

Phosphatidylserine (PS) exposure was detected using the Annexin V–FITC apoptosis detection kit (BD Pharmingen). *E. coli* cells were treated with silibinin (20, 40, 80, and 160 μg/ml) or norfloxacin (0.75 μg/ml), and incubated for 4 h at 37°C and 120 rpm. After incubation, the cells were collected and resuspended in 100 μl of 1× Annexin V binding buffer, followed by 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. Next, the total volume was raised to 1 ml with PBS and the cells were analyzed using the FACSVerse flow cytometer [20].

**DNA Fragmentation Analysis**

To evaluate DNA fragmentation, a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed, using an In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Germany). The TUNEL assay measures apoptotic DNA fragmentation, which is a late apoptosis feature. Terminal deoxynucleotidyl transferase recognizes the blunt ends of double-stranded DNA breaks, and catalyzes the addition of labeled dUTPs to the 3’-hydroxyl termini of the DNA ends, which can be detected by flow cytometry. *E. coli* cells were treated with silibinin (20, 40, 80, and 160 μg/ml) or norfloxacin (0.75 μg/ml), and incubated for 4 h at 37°C and 120 rpm. The cells were then fixed with 2% paraformaldehyde for 1 h. The fixed cells were collected, suspended in PBS, treated with 100 μl of permeabilization solution (0.1% sodium citrate and 0.1% Triton X-100), and incubated on ice for 2 min. The suspensions were collected and resuspended twice in PBS, and the label solution and enzyme solution were then added. After incubating for 1 h at 37°C, the samples were collected and suspended, and the percentages of fluorescent cells were measured with the FACSVerse flow cytometer [20].

**Detecting the Expression of Bacterial Caspase-Like Protein**

Caspases have a specific cysteine protease activity, which cleaves a target protein only after an aspartic acid residue. Owing to their ability for selective cleavage, caspases can be considered a hallmark for apoptosis detection [22]. The CaspACE FITC-VAD-FMK In Situ Marker (Promega, USA) was used to detect a bacterial caspase-like protein homolog of the eukaryotic caspase. FITC-VAD-FMK is cell-permeable, and can be delivered into the bacterial caspase-like protein homolog of the eukaryotic caspase. FMK In Situ Marker (Promega, USA) was used to detect a hallmark for apoptosis detection [22]. The CaspACE FITC-VAD-FMK assay was performed alone or in the presence of 100 ng of extracted DNA. Fluorescence spectra of silibinin (100 ng in sterile distilled water) was stained with 50 μM, and 1 mM EDTA at pH 8.0). After electrophoresis, the agarose gel in 1× Tris-acetate-EDTA buffer (40 mM Tris-acetate at pH 8.0). DNA electrophoresis was performed on 0.8% agarose gel in 1× Tris-acetate-EDTA buffer (40 mM Tris-acetate and 1 mM EDTA at pH 8.0). After electrophoresis, the agarose gel was stained with 50 μg/ml ethidium bromide in 1× Tris-acetate-EDTA buffer. For the size marker, the 1 kb Plus DNA Ladder Marker (Doctor Protein, Korea) was used.

The fluorescence spectrum change effect of silibinin on the addition of extracted *E. coli* DNA was assessed using a spectro-fluorophotometer (Shimadzu RF-5301PC). The emission spectra were assessed from 220 to 400 nm with 288 nm excitation [25]. The fluorescence spectra of silibinin (100 ng in sterile distilled water) was measured alone in the presence of 100 ng of extracted DNA.

**Western Blotting for RecA Protein**

Harvested *E. coli* cells were incubated with silibinin (20, 40, 80, and 160 μg/ml, respectively) or norfloxacin (0.75 μg/ml), and incubated for 4 h at 37°C and 120 rpm. The cells were collected and resuspended in PBS. The suspensions were then lysed using a sonicator (10 pulses of 2 min each, at amplitude 38) (Sonics, USA) and centrifuged on a SORVALL Biofuge fresco at 10,000 × g for 20 min to remove the intact cells and cell debris. The supernatants were collected and proteins were precipitated with 5% trichloroacetic acid (TCA) at 4°C for 10 min. The TCA-precipitated proteins were washed twice with cold acetone, harvested, and dissolved in H2O. Quantitation of the solubilized proteins was performed using a Bradford assay (Bio-Rad, USA) [23]. Each 50 μg sample of proteins was resolved by 7.5% SDS-PAGE, followed by transfer to a nitrocellulose membrane, and Ponceau S staining was done to confirm equal protein loading. After decolorizing, the membrane was blocked in 3% skim milk for 1 h at room temperature. The membrane was incubated with 1:3,000 diluted rabbit polyclonal anti-RecA antibody (Abcam, UK) for 12 h at 4°C. Subsequently, the membrane was incubated with 1:2,000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Biovision, USA) as the secondary antibody for 6 h at 4°C. Pierce ECL Plus Western Blotting Substrate (Thermo Scientific, USA) was then added, and the membranes were exposed to an X-ray film [24].

**Silibinin-DNA Interaction Analysis**

To extract DNA, *E. coli* cells were harvested and lysed in 200 μl of cell lysis buffer containing 100 mM NaCl, 1% SDS, 2% Triton X-100, 1 mM EDTA (pH 8.0), and 10 mM Tris-HCl (pH 8.0). The lysate was incubated with 0.2 mg/ml proteinase K for 1 h at 37°C, and then with 0.5 mg/ml RNase A for 1 h at 37°C. Extracted DNA (100 ng) was mixed with silibinin (100, 200, 300, and 400 ng respectively) in 12 μl of TE buffer (1 mM EDTA at pH 8.0, 10 mM Tris at pH 8.0). DNA electrophoresis was performed on 0.8% agarose gel in 1× Tris-acetate-EDTA buffer (40 mM Tris-acetate and 1 mM EDTA at pH 8.0). After electrophoresis, the agarose gel was stained with 50 μg/ml ethidium bromide in 1× Tris-acetate-EDTA buffer. For the size marker, the 1 kb Plus DNA Ladder Marker (Doctor Protein, Korea) was used.

**Statistical Analysis**

Measurements were performed in triplicate, and values are reported as the means ± SD. Group statistical comparisons were
performed by analysis of variance, after confirming the normality of distribution (Shapiro-Wilk test), followed by a post-hoc analysis (Tukey's test) for 3-group comparisons in SPSS (ver. 23; SPSS/IBM, USA). Differences between samples were considered significant at $p < 0.05$.

**Results**

**Silibinin Exhibited Antibacterial Activity**

To determine the antibacterial activity of silibinin against *E. coli*, antibacterial susceptibility was determined before performing a time-kill kinetic assay according to CLSI guidelines (Table 1). The effect of antimicrobial agents can vary depending on their concentration. Considering the MIC (40 µg/ml), the antibacterial activity of silibinin was then evaluated at various concentrations (20, 40, 80, and 160 µg/ml) in a time-kill kinetic assay. As shown in Fig. 1, S1 (20 µg/ml) inhibited cell growth, whereas the remaining concentrations had a bactericidal effect.

**Silibinin Caused Membrane Disintegration**

To investigate whether silibinin causes changes in membrane integrity, the fluorescent dye SYTOX green was employed. SYTOX green only penetrates cells with compromised plasma membranes. As shown in Fig. 2A, S1 and S2 (20 and 40 µg/ml, respectively)-treated cells showed fluorescence intensity analogous to that of control cells (~1.21% and 0.61% respectively), whereas S3 and S4 (80 and 160 µg/ml, respectively)-treated cells showed increased fluorescence compared with that of control cells; the fluorescence intensity was higher with S4 treatment (8.85% and 16.28%). These results indicate that silibinin may cause membrane disruption only at relatively high concentrations (80 and 160 µg/ml).

**The Plasma Membrane Potential Was Changed by Silibinin**

Upon severe membrane damage, intracellular ions can migrate through the ruptured membrane, leading to a breakdown of ion homeostasis, which causes membrane depolarization. DiBAC$_4$(3) has been used for monitoring changes in plasma membrane potential, as it can enter depolarized cells where it binds to intracellular proteins or membranes and exhibits enhanced fluorescence [26]. Compared with untreated cells (16.20%), S1 (20 µg/ml)-treated cells did not show a significant increase in fluorescence (17.65%). However, S2, S3, and S4 (40, 80, and 160 µg/ml, respectively)-treated cells showed a dose-dependent increase in fluorescence intensity (38.04%, 44.30%, and 54.51% respectively) (Fig. 2B). These results demonstrate that silibinin causes membrane potential collapse at all concentrations except 1/2 MIC.

**Intracellular Ca$^{2+}$ Levels Were Increased by Silibinin**

The intracellular level of Ca$^{2+}$ is maintained homeostatically, and Ca$^{2+}$ is important for retaining membrane potential. In normal conditions, the intracellular calcium concentration is kept low and stable. However, upon cell damage, Ca$^{2+}$ homeostasis is destroyed and intracellular Ca$^{2+}$ levels are changed, and these alterations can induce membrane depolarization [27, 28]. To evaluate whether intracellular Ca$^{2+}$ concentrations are changed after silibinin treatment, the membrane-permeable and sensitive intracellular calcium indicator, Fura-2 AM, was employed. In comparison with control cells, S1 and S2 (20 and 40 µg/ml, respectively) and norfloxacin (0.75 µg/ml)-treated cells showed increased fluorescence, whereas S3 and S4 (80 and 160 µg/ml, respectively) showed decreased fluorescence intensity (Fig. 3).

**Silibinin Caused PS Externalization and DNA Fragmentation**

PS externalization is an exposure of PS to the outer leaflet
of the plasma membrane, which is a hallmark of early apoptotic cell death. Fluorescence-labeled Annexin V, which binds PS with high specificity, provides a highly specific method for detecting extracellular exposure of PS at a
single-cell level in prokaryotic models [29]. Compared with control cells, S2, S3, and S4 (40, 80, and 160 µg/ml) effectively induced PS exposure in a dose-dependent manner, whereas S1 (20 µg/ml)-treated cells did not show increased fluorescence (Fig. 4A). Next, DNA fragmentation was examined as another apoptosis hallmark [30]. To evaluate DNA fragmentation, the TUNEL assay was performed. *E. coli* cells treated with silibinin (20, 40, 80, and 160 µg/ml) showed increased TUNEL fluorescence (50.22%, 55.58%, 63.01%, and 63.02% respectively), compared with that in control cells (Fig. 4B). These results indicate that silibinin induced DNA fragmentation at all concentrations.

Caspase-Like Protein Expression

Caspases are a family of proteases that play critical roles in apoptosis. To assess whether silibinin induced caspase-like protein expression, FITC-VAD-FMK was employed.
As shown in Fig. 5A, S1 (20 µg/ml)-treated cells did not show substantial augmentation (16.01%) of fluorescence compared with that of control cells (13.45%). On the other hand, S2, S3, and S4 (40, 80, and 160 µg/ml, respectively)-treated cells showed relatively high fluorescence intensities (31.43%, 37.02%, and 44.55%, respectively). These results demonstrate that silibinin could augment the expression of caspase-like proteins at bactericidal concentrations.

RecA, which is known to be involved in the bacterial SOS response, is considered as a caspase-like protein and is involved in bacterial apoptosis-like death [16]. Expression of RecA protein was therefore evaluated by western blotting. As shown in Fig. 5B, E. coli cells treated with S2, S3, and S4 (40, 80, and 160 µg/ml, respectively) showed more intense RecA bands compared with those in S1 (20 µg/ml)-treated cells and in untreated cells. These results indicate that silibinin causes overexpression of RecA protein only at bactericidal concentrations, corresponding to the increased expression of caspase-like protein.

Silibinin Did Not Bind to E. coli DNA
To verify that silibinin-induced DNA fragmentation does result from direct silibinin-DNA interaction, a gel retardation assay was performed. Extracted E. coli DNA (100 ng) was mixed with various quantities of silibinin (100, 200, 300, and 400 ng). As shown in Fig. 6A, silibinin did not cause migration of DNA, indicating that silibinin does not interact with the DNA strand of E. coli. In addition, the fluorescence spectrum of silibinin was determined to further elucidate the interaction between DNA and silibinin. The fluorescence intensity and emission maximum depend on the specific structure of agents; hence, the fluorescence spectrum of silibinin can change when DNA is bound to silibinin [31]. DNA (100 ng) was mixed with an equal quantity of silibinin. In correspondence with the gel retardation assay result, both fluorescence intensity and
emission maximum were not changed, which indicates silibinin did not interact with DNA (Fig. 6B).

**Silibinin Decreased Intracellular ROS Levels**

Several studies have proposed that bactericidal antibiotics induce the generation of deleterious ROS, and these ROS contribute to inducing DNA damage and bacterial apoptosis-like death [16, 32]. Therefore, intracellular ROS levels were measured using H$_2$DCFDA to verify whether ROS are generated and involved in silibinin-induced bacterial apoptosis-like death. As shown in Fig. 7, all silibinin concentrations induced depletion of ROS in *E. coli*, compared with the control cells. These results demonstrate that silibinin does not generate ROS, but acts as a scavenger, and that ROS generation is not involved in silibinin-induced bacterial apoptosis-like death.

Fig. 6. DNA binding assay of silibinin.

(A) Gel retardation analysis of binding of silibinin to DNA. Various quantities of silibinin were incubated with 100 ng of *E. coli* DNA. Lanes: 1, 1 kb Plus DNA Ladder (markers); 2, Untreated; 3, 100 ng of silibinin; 4, 200 ng of silibinin; 5, 300 ng of silibinin; 6, 400 ng of silibinin. (B) Fluorescence spectrum change of silibinin in the absence (solid line) and presence (gray area) of *E. coli* DNA.

Fig. 7. Flow cytometric analysis of reactive oxygen species accumulation by H$_2$DCFDA in *E. coli*.

Cells were treated with silibinin at various concentrations, and with norfloxacin at 0.75 µg/ml. (a, untreated; b, 20 µg/ml silibinin; c, 40 µg/ml silibinin; d, 80 µg/ml silibinin; e, 160 µg/ml silibinin; f, norfloxacin). The bar graph represents the percentage of H$_2$DCFDA fluorescence intensity (N.S: not significant; **p < 0.01).
Discussion

The use of synthetic chemical preservatives or antibiotics in food products is a concern for consumers and therefore developing acceptable natural antimicrobials has become valuable [33]. Silibinin has been used as a safe dietary supplement for several decades and it is reported to demonstrate antimicrobial activity against various pathogenic microorganisms without hemolytic effect [9, 10, 34]. However, the exact mechanism behind its antibacterial activity is still unknown. In addition, resistance to existing antibiotics is increasing, posing a serious threat to public health [1]; thus, research for developing novel antimicrobial agents is crucial. Therefore, this study aimed to determine the antibacterial mechanism of silibinin using E. coli, a well-established model organism and common foodborne bacterium. Among the antibacterial mechanisms of phytochemicals and antibiotics, cytoplasmic membrane targeting is the most common [35]. Membrane disintegration was found to occur only at concentrations higher than the MIC, whereas membrane depolarization was induced dose-dependently, except at the 1/2 MIC of silibinin. Because silibinin treatment at MIC induced membrane depolarization and not disintegration, and because these concentrations exert a bactericidal effect, we considered that the antibacterial mechanism between the MIC and higher concentrations might be different.

The intracellular Ca²⁺ level is important for maintaining membrane potential. Thus, changes in the Ca²⁺ gradient can result in membrane depolarization [27, 36]. Cells treated with 2× and 4× MIC of silibinin showed that the intracellular Ca²⁺ level was similar to that of untreated cells. It was considered that Ca²⁺ was leaked across the membrane to the extracellular matrix because of membrane disintegration. However, the Ca²⁺ level was increased at the MIC of silibinin, with which an integral membrane was still present. An increase in intracellular Ca²⁺ levels with maintained membrane structure implies the occurrence of a different effect from membrane structure alteration in the cell. Ca²⁺ ions are versatile intracellular messengers that regulate many cellular processes, including cell death [27]. Both eukaryotic and prokaryotic cells maintain their intracellular Ca²⁺ homeostasis tightly through interactions among transporters, pumps, channels, and binding proteins [28]. It has been reported that when cells undergo apoptosis, Ca²⁺ homeostasis is destroyed and intracellular Ca²⁺ levels are increased [37]. Hence, we speculated the presence of another death mechanism related to apoptosis, with silibinin treated at MIC.

In contrast to normal cell death, apoptotic cells exhibit specific features such as DNA fragmentation, caspase activation, and PS exposure [15]. Recently, a novel cell death mechanism was suggested in prokaryotic cells, which is similar to eukaryotic apoptotic death and presents the hallmarks of apoptosis [16]. To confirm whether silibinin induced apoptosis-like cell death, externalization of PS was first investigated. PS is a phospholipid component of the cell membrane that is actively held facing the inner leaflet of the plasma membrane by the activation of flippase and floppase. However, when cells undergo apoptosis, the scramblase enzyme catalyzes a rapid exchange of PS between the inner and outer leaflets, and PS is no longer restricted to the cytosolic side [38]. PS externalization was found to occur only at the MIC and higher concentrations, and the degree of externalization was increased dose-dependently. Considering the membrane disintegration results and that Annexin V can also stain dead cells by accessing the entire plasma membrane [39], it appears that the Annexin V positivity resulting from the high concentration of silibinin treatment is caused by membrane disruption whereas that at MIC is caused by apoptosis-like death.

Another specific feature of apoptosis, DNA fragmentation, was assessed by the TUNEL assay. Silibinin was found to induce a great degree of DNA fragmentation in a dose-dependent manner. In contrast to the preceding experimental results, the lowest concentration of silibinin was also found to induce DNA damage. It is considered that the degree of DNA damage at 1/2 MIC in not quite severe, because 1/2 MIC did not exert a bactericidal activity and did not show any apoptotic feature or membrane damage. The bacteriostatic effect of the 1/2 MIC could be interpreted by cells to stop growth and repair the DNA damage caused by silibinin treatment. Similar to the characteristic of Annexin V staining, the TUNEL assay can also detect dead cells that lysed [40]. Taken together, it is suggested that apoptosis-like death is induced at MIC, whereas membrane-disrupting cell death occurs at higher concentrations.

The key factors that regulate the process of apoptosis are caspase proteins. Caspases act as molecular scissors with specific cysteine protease activity and are synthesized as inactive zymogens that are only activated after the appropriate stimulus. Through the protease activity, caspses activate target proteins and other caspases, resulting in progression of apoptosis [41]. Recent studies have shown that caspases are present in metazoans, as well as in fungi and bacteria [16, 42]. In E. coli, the protein RecA is
ROS levels were measured using DCFDA assay, intracellular ROS were scavenged by silibinin, and this result demonstrates that silibinin-induced bacterial apoptosis-like death is unrelated with ROS. Conventionally, oxidative stress occurs when cells are not capable of an antioxidant response due to excessive generation ROS. Oxidative stress brings about ROS-mediated damage to macromolecules. Paradoxically, ROS serve a significant role in maintaining homeostasis in normal healthy cells and act as intracellular signaling molecules in different organisms, from bacteria to mammalian cells. Moreover, ROS activate or deactivate various receptors, proteins, ions, and other signaling molecules. To maintain the normal physiological reactions in cells, redox equilibrium also plays a crucial role. When the redox equilibrium is interrupted because of depletion of ROS, many signaling pathways are affected, leading to cellular dysfunction. Considering that proper ROS levels are indispensable for intracellular signaling, ROS depletion by silibinin might affect cell death. This result differs from those of previous studies on bacterial apoptosis-like death. Although there are several studies about ROS-independent apoptosis in eukaryotic organisms, this result is the first finding that apoptosis occurred without generation of ROS in the bacterial system. It also alludes to a new perspective of cellular damage by intracellular ROS depletion.

In summary, silibinin exerts its antibacterial activity through apoptosis-like death only at bactericidal concentrations in a dose-dependent manner. Many hallmarks of bacterial apoptosis-like death emerged after treatment with silibinin at bactericidal concentrations, including membrane depolarization, intracellular Ca$^{2+}$ accumulation, PS externalization, DNA fragmentation, and caspase-like protein expression. In contrast to conventional bacterial apoptosis-like death, silibinin-induced apoptosis-like death is a result of ROS depletion, which is the first report of its kind in the bacterial system. These results propose that silibinin has potential for development as an antibacterial agent and food additive with novel cell death mechanism.

**Acknowledgments**

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2017R1A2B4005811)

**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

References


2140 Lee and Lee


