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Synergistic Antibacterial Effect between Silybin and N,N'-Dicyclohexylcarbodiimide in Clinical *Pseudomonas aeruginosa* Isolates

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Silybin is a composition of the silymarin group as a hepatoprotective agent, and it exhibits various biological activities, including an antibacterial activity. In this study, the effects of a combination of silybin with N,N′-dicyclohexylcarbodiimide (DCCD) against clinical isolates of *Pseudomonas aeruginosa* were investigated. In the results of susceptibility assay, silybin showed more potent antibacterial activity in methicilin-resistant *Staphylococcus aureus* (MRSA) than in *P. aeruginosa*, but DCCD significantly increased the antibacterial activity of silybin in *P. aeruginosa*. The antibacterial activity of silybin was affected by the strong action of multidrug-resistant pumps rather than by a permeable disruption of lipopolysaccharide and silybin showed a remarkable synergistic activity in combination with some antibiotic agents against drug-resistant bacteria. Therefore, silybin has a potential as a combination therapeutic agent for treatment of infectious diseases by multidrug-resistant bacteria.

***Keywords*:** silybin, antibacterial activity, MDR pump, synergistic effect, N,N′-dicyclohexylcarbodiimide

Silybin is a main flavonolignan of the silymarin complex, with silydianin and silychristin extracted from the fruits of *Silybum marianum* (milk thistle). It is a well-known hepatoprotective agent in Europe and Asia (Perez-Victoria *et al.*, 2001; Kren and Walterova, 2005). Recently, *in vitro* and *in vivo* studies have reported that silybin possesses antioxidant, anti-inflammatory, and anti-arthritic activities (Gupta *et al.*, 2000), and it has chemopreventive efficacy on lung carcinoma (Sharma *et al.*, 2003), prostate cancer (Singh and Agarwal, 2004), breast carcinoma (Tyagi *et al.*, 2004), hepatic disorder (Wellington and Jarvis, 2001), and colon carcimoma (Agarwal *et al.*, 2003). In a previous study, silybin showed antibacterial activity against Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus epidermidis*, but not against Gram-negative bacteria *Escherichia coli* and *Proteus vulgaris*. Silybin significantly inhibited macromolecule synthesis such as RNA and proteins in Gram-positive bacteria (Lee *et al.*, 2003a). The different capacity of silybin against Gram-positive bacteria and Gram-negative bacteria remains unknown.

Several articles have provided helpful clues regarding the differences of the permeability barrier between Gram-positive and Gram-negative bacteria. The outer membrane barrier and drug-efflux pumps of Gram-negative bacteria have enabled the penetration of amphipathic molecules to be tolerable. ATP-binding cassette (ABC) transporters that couple the energy generated from ATP hydrolysis function in efflux of toxic substances across the cell membrane in mammalian cells and bacteria (Chen *et al.*, 1986). ABC transporters are located in the periplasm of Gram-negative bacteria and in the cell surface in Gram-positive bacteria (Van Der Heide and Poolman, 2002). Though it has been well-known Gram-positive bacteria with ABC transporters as drug-efflux transporters, like NorA from *S. aureus* (Yu *et al.*, 2002) and BmrA from *B. subtilis* (Steinfels *et al.*, 2004), it is not clear whether ABC transporters have contributed to antimicrobial resistance of Gram-positive bacteria from clinical specimens. Many articles have revealed that Gram-positive bacteria was more sensitive to plant antimicrobials than Gram-negative bacteria, suggesting that the results are due to the difference between the presence and absence of the outer membrane which can limit drug diffusion in harmony with multidrug transporters (Renau *et al.*, 1999; Zgurskaya and Nikaido, 1999; Tegos *et al.*, 2002; Hooper, 2005).

Here, the *in vitro* activities of silybin alone and in combination with inhibitors for disturbing drug penetration against clinical isolates were investigated and a way to overcome discrepancies in the activity of silybin was examined. Thus, this study promotes the effects of silybin as a therapeutic agent regarding its activity toward Gram-negative bacteria.

Materials and Methods

Agents, bacterial strains, and growth

Silybin and all agents used in this study, were purchased from the Sigma-Aldrich Chemical Company (USA). *S. aureus* (ATCC 25923), *Enterococcus faecium* (ATCC 29212), *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853) were obtained from the American Type Culture Collection (ATCC). The clinical isolates of drug-resistant bacterial strains were obtained from Kyungpook National University Hospital (Korea). *S. aureus* and *P. aeruginosa* was grown in Luria-Bertani (LB) medium (Difco) at 37°C, *E. coli* was grown in MacConkey medium (Difco), and *E. faecium* was grown in Brain Heart Infusion (BHI) medium (Difco). Cell growth

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was monitored by measuring the optical density at 620 nm.

Antibacterial susceptibility assay

Susceptibility tests with silybin, kanamycin, and chloramphenicol were carried out in 96-well microtiter plates by a two-fold standard broth-microdilution of antibacterial agents in Mueller-Hinton (MH) Broth (Difco) by the Clinical and Laboratory Standards Institute (CLSI) guideline. Briefly, Bacterial cells were grown to the mid-log phase in an MH medium, and seeded in the wells of a 96-well microtiter plate in LB medium at a density of 1×10^6 cells (100 μl/well). The bacterial cells were added to 10 μl each of the serially-diluted solutions of the compounds, and incubated for 18 h at 37°C. The MIC was defined as the lowest concentration of drug-inhibiting visible growth after overnight incubation at 37°C.

Antibacterial assay with detergents or ATPase inhibitors

To elucidate whether antibacterial activity of silybin was associated with the altered membrane permeability or the action of multidrug-resistant pumps, antibacterial susceptibility of silybin was examined in the presence of detergents or ATPase-inhibiting agents. To increase the permeability of the outer membrane, the concentration of silybin, as a fractional inhibitory concentration (FIC) determined in a combination assay with other therapeutic agents, was added to bacterial cells in the presence of 0.1 mM EDTA, 0.001% Triton X-100, and 30 mM Tris, respectively. NaN_3 and N,N′-dicyclohexylcarbodiimide (DCCD) were used as a inhibitor of ATPase (Linnett and Beechey, 1979). The antibacterial susceptibility of silybin, in the presence of 0.001% NaN_3 and 25 μM DCCD, was also carried out at the same condition. Experiments were performed in triplicate of a plate by 3-independent assay, and the results are expressed as Mean±SD.

Combination assay

Combinations of silybin with antibiotics were investigated as previously described (Cha *et al.*, 2007). Susceptibility tests were carried out in 96-well microtiter plates by a two-fold standard broth microdilution, with the FIC levels of two antibacterial agents in MH Broth. The inoculum concentration levels in the combination assay were 10^6 cells/ml. The FIC was calculated as follows: (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). These assays were performed in triplicate, and the results are expressed as Mean ±SD.

Statistical analysis

Data were presented as the Mean±SE for the indicated number of separate experiments. Statistical analysis of data was performed using sigmaplot (SPSS), and significance was set at P values less than 0.05.

Results

Antibacterial activity of silybin against clinical isolates

The antibacterial activity of silybin and conventional antibiotics were tested against several bacterial isolates from patients at Kyungpook National University Hospital by the CLSI method, and was described as a MIC (Table 1). Gram-

Table 1. Antibacterial activity of silybin and antibiotics against clinical isolates

Bacterial strains		MIC (μg/ml)		
		Silybin	Chloramphenicol	Kanamycin
S. aureus	ATCC 25923	10	<0.625	<0.625
	1	1.25	<0.625	160
	2	5	<0.625	>320
	3	2.5	<0.625	160
	4	5	<0.625	160
MRSA	5	5	<0.625	160
	6	2.5	<0.625	320
	7	2.5	<0.625	>320
	8	5	<0.625	320
	9	5	<0.625	320
	ATCC 29212	20	1.25	20
E. faecium	1	20	<0.625	>320
	2	20	<0.625	>320
	ATCC 25922	20	<0.625	<0.625
E. coli	1	20	10	<0.625
	2	20	40	10
	ATCC 27853	10	1.25	10
P. aeruginosa	1	20	40	80
	2	20	40	40

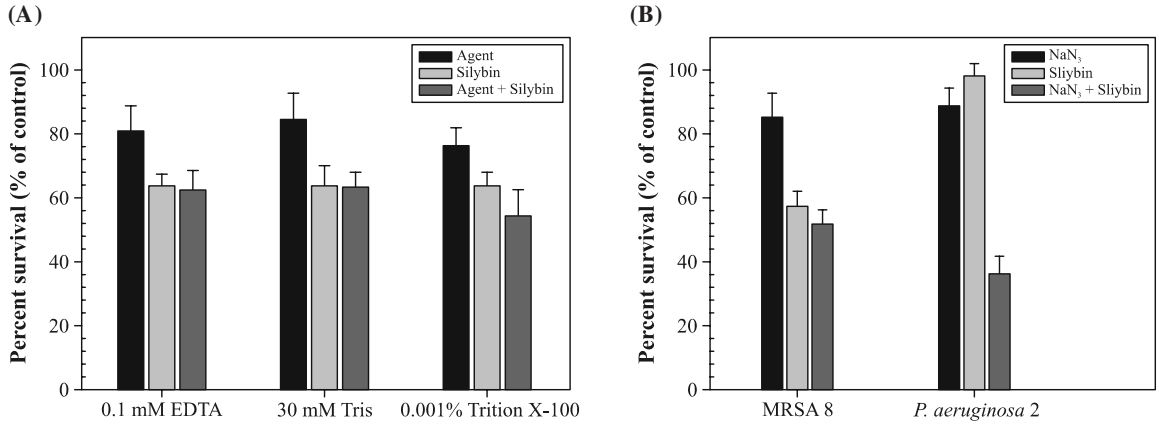

Fig. 1. Effects of membrane-permeabilizing agents (A) and NaN_3 (B) on the susceptibility of clinical isolates to silybin. The viability of bacteria was determined by counting CFU on MH agar plates after incubation for 18 h with 2.5 μg/ml silybin and the indicated concentration of each permeabilizing agent in *P. aseruginosa* 2 (A) and with the level of silybin (0.625 μg/ml to MRSA 8; 2.5 μg/ml to *P. aeruginosa* 2) in the presence or absence of 0.001% NaN_3 (B). The data are Mean±SD for triple-independent experiments.

positive bacteria *S. aureus* and *E. faecium* strains were significantly resistant to kanamycin (KAN), but extremely sensitive to chloramphenicol (CHL). Gram-negative bacteria *E. coli* and *P. aeruginosa* strains were less sensitive to KAN and CHL than Gram-positive bacteria strains. As shown in Table 1, Gram-negative bacteria were resistant to more antibiotics than Gram-positive bacteria. Silybin showed an equal level of MICs in each strain and it exhibited more potent activity toward methicilin-resistant *S. aureus* (MRSA) than to other strains including normal *S. aureus*.

Increasing the permeability of the outer membrane is not required for the silybin activity

To investigate the effects of enhanced membrane permeability on the activity of silybin using detergents, the antibacterial activity of silybin under increased membrane permeability was examined using 0.1 mM EDTA, 30 mM Tris, and 0.001% Triton X-100. EDTA, Tris, and Triton X-100 all of which are membrane-permeabilizing agents which can increase the permeability of the outer membrane in Gram-negative bacteria by binding lipopolysaccharide (LPS) (Leive, 1965; Irvin *et al.*, 1981). These agents did not reduce the viability of *P. aeruginosa* 2 treated with silybin in less than about 10% (Fig. 1A).

Another component of the permeability barrier in Gram-negative bacteria is the use of MDR pumps within the periplasmic membrane. In several drug-resistant bacteria, MDR pumps, which can extrude toxic substances, including antibiotics, to extracellular environment, have been reported as being a major cause of antimicrobial resistance (Levy, 1992). The bacterial viability in the presence of silybin with 0.001% NaN_3 as a metabolic inhibitor which can decrease ATP levels by disrupting electrochemical proton gradients in a bacterial environment was investigated (Swallow *et al.*, 1990; Jernaes and Steen, 1994; Goncalves *et al.*, 1999). MRSA, which exhibited a high susceptibility toward silybin, maintained its viability in the presence of silybin with 0.001% NaN_3 (Fig. 1B). Contrary to one of the MRSA, silybin in combination with NaN_3 significantly decreased the viability of *P. aeruginosa* over 50%.

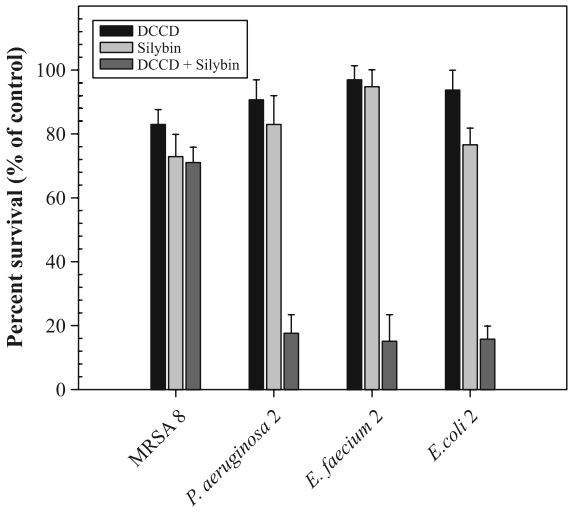

Fig. 2. Effects of DCCD on the susceptibility of bacterial isolates. The viability of bacteria was determined by counting CFU on MH agar plates after incubation with the level of silybin FIC (0.625 μg/ml to MRSA 8; 2.5 μg/ml to other strains) in the presence or absence of 25 μM DCCD.

The antibacterial activity of silybin is potentiated by DCCD

Although *P. aeruginosa* and *E. coli* were less sensitive to silybin than MRSA, the viability of these strains dramatically was decreased in the presence of DCCD, an inhibitor of $F\text{-}F_0$ ATPase (Fig. 2). Moreover, *E. faecium* 2 also shows an increasing susceptibility toward silybin by DCCD. All strains that showed low susceptibility toward silybin were sensitive in the presence of 25 μM DCCD as well as the susceptibility of MRSA.

The synergistic effects of silybin was also assayed with some conventional antibiotics against *P. aeruginosa* 2 in the

presence of DCCD, and synergy was defined by a FIC index of less than 0.5 (Cha *et al.*, 2007). A FIC index of around 0.5 or less was found through a synergy assay between silybin and other antibiotics. Silybin showed synergy in *P. aeroginosa* 2, in combination with CHL (FIC index: 0.38) and KAN (FIC index: 0.38), and these combination activity in the presence of DCCD is also effective in reducing the viability of *P. aeruginosa*.

Discussion

In order for plant antimicrobials to protect against microbial infection, they need to be present in extremely high concentration levels (Tegos *et al.*, 2002), and these compounds display different activity against Gram-positive and Gram-negative bacteria (Lewis, 2001). In our previous study, the antibacterial activity of silybin exhibited significant specificity toward Gram-positive bacteria, by the inhibition of macromolecule synthesis within an intracellular environment (Lee *et al.*, 2003a). Several articles have reported that the low susceptibility of Gram-negative bacteria was due to the outer membrane as a permeability barrier of which the Gram-negative bacterial barriers was composed. Amphipathic molecules are contracted to permeate a cytoplasmic environment by an asymmetric bilayer and LPS (Lomovskaya and Lewis, 1992; Nikaido, 2003), and they are extruded to extracellular conditions by multidrug-resistant (MDR) pumps in the outer membrane (Lewis, 1994). Silybin has a broad spectrum of antibacterial activity against antibiotic-resistant strains but MRSA is more susceptible to silybin than other bacterial isolates in the MIC assay (Table 1). Interestingly, Gram-positive bacteria *E. faecium* as well as Gram-negative bacteria also showed low susceptibility toward silybin. Thus, this result indicates that the difference of MIC of silybin on the tested bacterial strains did not result in the differences of cellular structure properties between Gram-positive and Gram-negative bacteria.

The difference in susceptibility of antimicrobial agents against Gram-positive and Gram-negative bacteria has been suggested that was caused by a permeability barrier, such as an outer membrane or MDR pumps (Renau *et al.*, 1999). The effects of the LPS-binding agents and ABC transporter-inhibiting agents on antibacterial activity of silybin were examined. Both Tris and EDTA are useful agents which increase the membrane permeability of amphipathic molecules by reducing interaction between LPS molecules (Hancock and Wong, 1984; Jernaes and Steen, 1994). However, these agents did not show any effect on antibacterial activity of silybin (Fig. 1A). The permeability of the outer membrane was not remarkably affected by the activity of silybin, and additionally the result exhibited the low susceptibility of *E. faecium* strains, which has a different cellular structure from Gram-negative bacteria.

There are several mechanisms that can resist antibiotic and toxic molecules in bacteria (Levy, 1992), and drug efflux transporters as MDR pumps are capable of such antibiotic-resistance, because they selectively pump toxic molecules out to the extracellular condition by keeping toxic molecules to a minimum in an intracellular condition. Although there is no obvious evidence to demonstrate that these transporters play a critical role in drug-resistance (Davidson and Chen, 2004), recent studies have suggested that most bacteria produce ABC transporters and some of these transporters cause the antibiotic resistance of bacteria (Yu *et al.*, 2002; Steinfels *et al.*, 2004). This ensures survival by maintaining low amounts of toxic molecules in an intracellular environment (Hooper, 2005). The four strains that were tested in this study have known antibiotic resistance due to ABC transporters (Mcmurry *et al.*, 1980; Gibbons and Udo, 2000; De Kievit *et al.*, 2001; Leavis *et al.*, 2003; Lee *et al.*, 2003b; Hooper, 2005). Regarding the significantly decreasing viability of *P. aeruginosa* cells in the presence of NaN_3,

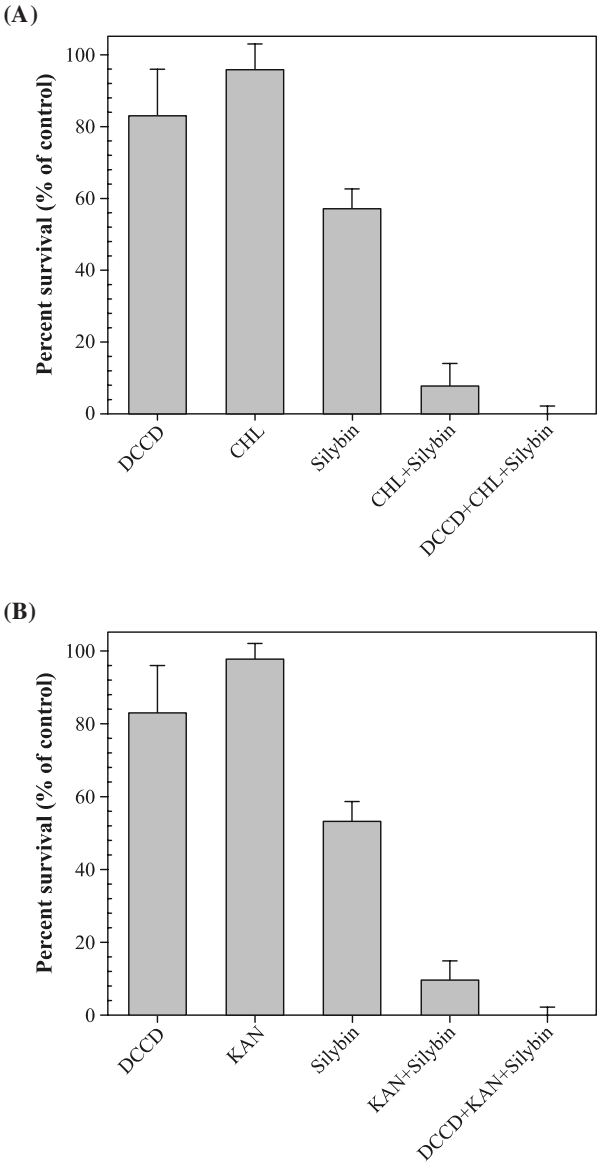

Fig. 3. Synergistic activity of silybin and conventional antibiotics against *P. aeruginosa* 2 in the presence of DCCD. The viability of bacteria was determined by counting CFU on MH agar plates after incubation for 18 h with the level of 2.5 μg/ml silybin and 5 μg/ml CHL or 5 μg/ml KAN in the presence or absence of 25 μM DCCD, and was represented as percent survival of control bacteria.

which disorders proton motive force around cellular membrane (Fig. 1B), it was theorized that the low susceptibility of the tested strains to silybin was caused by the extrusion of silybin by ABC transporters, which exist in bacterial membranes. Though *S. aureus* has efflux transporter NorA (Yu *et al.*, 2002), why is MRSA more sensitive to silybin than other tested beateria? Many researchers have reported that some plant materials act as specific efflux transporter inhibitors against some pathogenic bacteria. Interestingly, one article suggested that silybin from the Milk thistle directly inhibited NorA of *S. aureus* (Stermitz *et al.*, 2000). Considering these results, the antibacterial activity of silybin was reduced by ABC transporters of bacterial isolates, and silybin displayed more potent activity in combination with DCCD for inhibition of ABC transporters. ABC transporters have ATP- dependent transporting activity, and DCCD inhibits the H^+ translocation activity of the F_0 domain of F_0F_1-ATPase that generates proton motive force as in the presence of NaN_3 (Linnett and Beechey, 1979; Jung *et al.*, 2007) (Fig. 2). Actually, it has been reported that DCCD completely abolished the ATP-dependent transport activity of LmrA among ABC transporters derived from *Lactococcus lactis* in membrane vesicle system (Van Den Berg Van Saparoea *et al.*, 2005).

Combination antibiotic therapy has been studied to promote the effective use of antibiotics in increasing *in vivo* activity of antibiotics, in preventing the spread of drug-resistant strains, and in minimizing toxicity (Gradelski *et al.*, 2001). In *P. aeruginosa* 2, CHL and KAN showed synergistic activity in combination with silybin as indicated FIC values of 0.38 both respectively (data not shown), and the combination in the presence of DCCD was increased (Fig. 3).

Silybin as a plant antimicrobial possesses antibacterial activity against clinical isolates, and it could be strengthened by a combination treatment with DCCD for inhibition of ABC transporter activity. Regarding to another effect of silybin that inhibits efflux transporters in *S. aureus* strains, MRSA is significantly susceptible to silybin alone, and it may be considered as a therapeutic agent in combination with other conventional agents. These combinations suggest that silybin has potential as an adjuvant for antimicrobial therapy for the treatment of infectious diseases by drug-resistant bacteria.

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