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Gram-Positive Bacteria Specific Properties of Silybin Derived from *Silybum marianum*

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Silybin has a potent antibacterial activity, more potent than silymarin II, against gram-positive bacteria without hemolytic activity, whereas it has no antimicrobial activity against gram-negative bacteria or fungi. The mode of action of silybin against the gram-positive bacterial cell was examined by investigating the change in plasma membrane dynamics of bacterial cells using 1,6-diphenyl-1,3,5-hextriene (DPH) as a membrane probe and by assessing the inhibition of macromolecular synthesis using radiolabeled incorporation assay. The results showed that silybin inhibited RNA and protein synthesis on gram-positive bacteria.

Key words: Silybin, Gram-positive bacteria, 1,6-Diphenyl-1,3,5-hextriene (DPH)

INTRODUCTION

Silybin (formerly called silymarin) is one of the components of the "silymarin group". It is extracted from the fruits of *Silybum marianum* (Compositae). A mixture of flavolignans, the silymarin group consists of three major structural isomers, silybin, silidianin, and silichristin (Letteron *et al.*, 1990). Among them, silybin has strong antihepatotoxic activity, especially against phalloidin, galactosamine (Park *et al.*, 2003), halothane (Lecomppte, 1975) and carbon tetrachloride (Pavanato *et al.*, 2003). It increases the RNA synthesis in isolated rat liver nuclei *in vitro*. As a consequence, the formation of ribosomes is accelerated and protein synthesis is increased. In addition, silybin is used medicinally to treat liver disease and cases of *Amanita* poisoning (Choppin *et al.*, 1979). Many studies focused on the elucidation of the hepatoprotective action of silybin at the cellular level. To our knowledge, no previous investigation has been done on the antimicrobial and hemolytic activity of silybin (Stermitz *et al.*, 2000).

In this study, we report the antimicrobial activity of silybin against various strains of bacteria and fungi, and its hemolytic activity against human erythrocytes. Moreover, to elucidate its mode of antimicrobial action, the change in plasma membrane dynamics and the result of radiolabel incorporation assay are discussed.

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MATERIALS AND METHODS

Materials preparation and peptide synthesis

Silybin and Silymarin II were purchased from Sigma Chemical Co. (St. Louis, MO) (Fig. 1). Melittin, used as a positive control, was synthesized by the solid phase

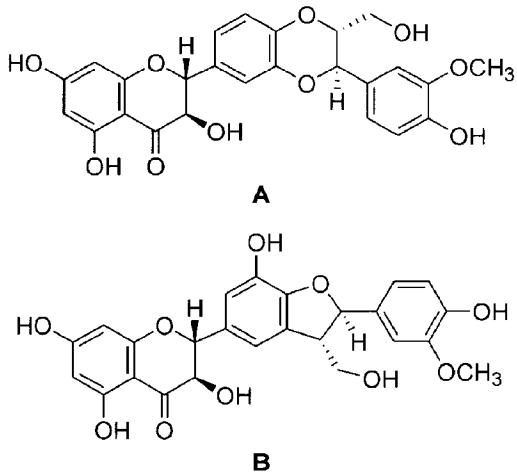

Fig. 1. Chemical structures of Silybin (A) and Silymarin II (B)

method using Fmoc (9-fluorenyl-methoxycarbonyl)-chemistry (Merrifield 1986).

Antibacterial activity assay

Escherichia coli (KCTC 1682), *Bacillus subtilis* (KCTC 1918), *Staphylococcus epidermidis* (KCTC 1917), *Proteus vulgaris* (KCTC 2433), *Candida albicans* (KCTC 7270), *Saccharomyces cerevisiae* (KCTC 7296), and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology (KRIBB) (Taejon, Korea). Silybin and silymarin II were stepwise diluted in a medium for antimicrobial activity. Three replicates for each test solution were carried out. Antibacterial activity was determined by the increase in optical density at 620 nm after incubating at 37°C for 10 h. The inhibitory concentration (IC_{50}) was defined as the lowest concentration of silybin or silymarin II at which no change in optical density was observed.

Antifungal activity assay

The fungal cells were seeded on 96-well microtiter plates at a density of 2×10^3 cells per well in 100 μL of YPD (Yeast extract: Peptone: Dextrose, 10 g: 20 g: 20 g per liter) media. Ten microliters of the serially diluted silybin or silymarin II were added to each well and incubated at 28°C for 24 h. After incubation, 5 μL of a solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) [5 mg/mL MTT in PBS, pH 7.4] was added to each well, and the plates were incubated at 37°C for a further 4 h. The absorbance of each well was measured at 570 nm using a microtiter ELISA reader (Merck, Germany).

Hemolytic activity

The hemolytic activity of silybin and silymarin II were evaluated by determining hemoglobin release from 8% suspensions of fresh human erythrocytes. Hemolysis was measured at 414 nm with an ELISA plate reader. The hemolysis percentage was calculated using the following equation: hemolysis % = [(Abs_{414nm} in the peptide solution Abs_{414nm} in PBS)/(Abs_{414nm} in 0.1% Triton X-100 Abs_{414nm} in PBS)]×100 (Shin *et al.*, 1998).

Steady-state anisotropy measurements of living cells

The anisotropy of fluorescence from *B. subtilis* and *S. epidermidis* cells labeled by 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular Probes, Eugene, Oregon, USA) was used to monitor changes in membrane dynamics. The cells (2×10^6 cells in 1% bactopeptone) containing silybin or silymarin II were incubated at a physiological temperature of 37°C on a rotary shaker at 150 rpm for 2 h. The labeling and fluorescence measurement were performed by the previously described method (Binenbaum *et al.* 1999).

[^{3}H]-Thymidine, [^{3}H]-uridine and [^{14}C]-leucine incorporation assays

Bacillus subtilis and *Staphylococcus epidermidis* were grown in LB broth. Three hundred microliters of silybin and silymarin II (100 μg/mL) were added to 5.7 mL culture, then [^{3}H]-thymidine (60 μL, 84 Ci/mmol; NEN Amersham Pharmacia Biotech, UK), [^{3}H]-uridine (30 μL, 40 Ci/mmol; NEN) or [^{14}C]-leucine (10 μL, 295 mCi/mmol; NEN) was added to the culture, which was mixed and incubated at 37°C for 6 h. Three 1.5 mL aliquots were then removed. After 30 min the trichloroacetic acid (TCA) precipitated materials were collected by centrifugation (10000×g, 10 min) and washed three times with 10% (w/v) TCA. The radioactivities with precipitates were counted using a liquid scintillation counter (Loyola-Rodriguez *et al.*, 1992).

RESULTS AND DISCUSSION

Medically, silybin and silymarin II have been known for their anti-carcinogenic effects and specifically as hepatoprotective standards (Saliou *et al.*, 1998). Silybins antimicrobial activity and mechanism of action have not yet been elucidated. In this study, the antimicrobial activity of silybin was determined and the IC_{50} values are shown in Tables I and II. The IC_{50} values of silybin for *B. subtilis* and *S. epidermidis* were 11.8 and 15.7 μg/mL, respectively, and of silymarin II they were 320 and 200 μg/mL, respectively. This indicates that silybin, although about 30-fold more potent than silymarin II, remains less potent than melittin, which was used as a positive control (Lee *et al.*, 1997). The antifungal activities showed that neither silybin nor silymarin II had antifungal activity against yeast (Table II).

Table I. Antibacterial activities of silybin and silymarin II

Cell types		Silybin	Silymarin II	Melittin
Gram-positive bacteria (IC_{50} : μg/mL)	*B. subtilis*	11.8	360	0.03
	S. epidermidis	15.7	320	1.85
Gram-negative bacteria (IC_{50} : μg/mL)	*E. coli*	–*	–	0.23
	P. vulgaris	–	–	1.85

*– : not detected.

Table II. Antifungal activities of silybin and silymarin II

Cell types		Silybin	Silymarin II	Melittin
Fungal strains (IC_{50} : μg/mL)	*C. albicans*	–	–	0.37
	T. beigelii	–	–	0.37
	S. cerevisae	–	–	0.37

*– : not detected.

Table III. Hemolytic Activity of silybin and silymarin II. The hemolytic activity of silybin and silymarin II was evaluated by determining the hemoglobin release of 8% suspensions of fresh human erythrocytes at 414 nm.

	Silybin	Silymarin II	Melittin
% Hemolysis	0	0	100

Tested concentrations: silybin and silymarin II, both 200 mg/mL; melittin, 1.85 μg/mL.

These results indicate that silybin, among the isomers of silymarin II, has an essential role in the antibacterial activity against *B. subtilis* and *S. epidermidis* without hemolytic activity (Table III).

The antibacterial effects of silybin or silymarin II were further confirmed by using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a plasma membrane probe. If the bactericidal activities exerted by the silybin or silymarin II, on *B. subtilis* and *S. epidermidis* are at the level of the plasma membrane, and DPH, which interacts with an acyl group of plasma membrane lipid bilayers, could not be inserted into the membrane. As shown in Fig. 2, silybin or silymarin II did not affect the plasma membrane of the bacterial cells. The uptake of silybin and silymarin II into bacterial cells may occur in a similar way to the internalization characteristics with receptor mediated endocytosis or fluid-phase pinocytosis.

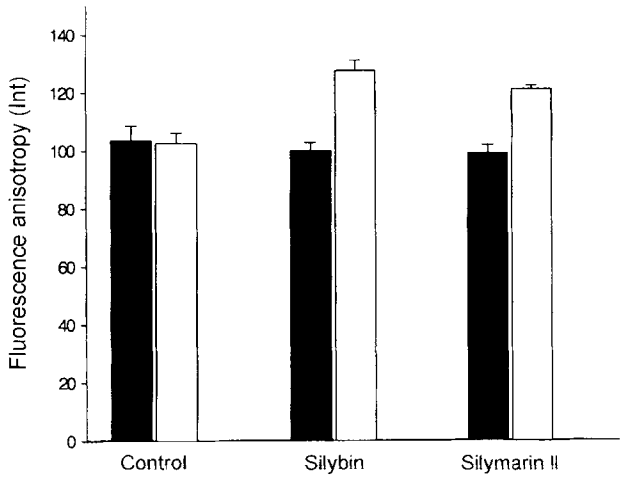

Fig. 2. DPH fluorescence anisotropy after the addition of silybin or silymarin II on *B. subtilis* (■) and *S. epidermidis* (□). Silybin and silymarin II, both at 200 μg/mL concentration, were added to the *B. subtilis* and *S. epidermidis* suspensions and incubated for 2 h. Anisotropy data are shown as a percentage of a corresponding untreated control.

To investigate the cellular matrix, macromolecular synthesis inhibition by silybin and silymarin II was performed using a radiolabeled incorporation assay (Shin *et al.* 1998). As shown in Table IV, silybin and silymarin II inhibited the RNA and protein synthesis.

In summary, the gram-positive bacteria specific properties of silybin may be caused by the inhibition of RNA and protein synthesis, rather than by attacking the bacterial membrane.

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Table IV. Effect of silybin and silymarin II on DNA, RNA, and protein biosynthesis of *B. subtilis*, *S. epidermidis* and *E. coli*

Cell types	Macromolecule (cpm10^3/mg)	Control	Silybin	Silymarin II
B. subtilis	DNA	3.94±0.25[a]	3.85±0.06*	3.92±0.15
	RNA	3.46±0.23	0.37±0.06	0.61±0.02
	Protein	18.21±0.22	7.61±0.05*	9.70±0.10
S. epidermidis	DNA	8.22±0.08	7.41±0.01*	8.22±0.06
	RNA	0.52±0.01	0.19±0.01	0.20±0.05
	Protein	47.73±0.02	23.94±0.05*	28.31±0.06
E. coli	DNA	4.49±0.36	5.94±0.56	5.27±0.46
	RNA	1,017 ±85.8	1,103 ±66.4	1,152 ±57.9
	Protein	20.91±0.61	19.31±0.75	20.23±1.95

[a]Each value is expressed as mean±S.E. of three independent determinations. Asterisks designate significant differences (*P<0.05) compared with the control group.

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