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Silymarin exerts antifungal effects via membrane-targeted mode of action by increasing permeability and inducing oxidative stress

Dae Gyu Yun, Dong Gun Lee *

School of Life Sciences, BK 21 Plus KNU Creative BioResearch Group, College of Natural Sciences, Kyungpook National University, 80 Daehakro, Bukgu, Daegu, 41566, Republic of Korea

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ABSTRACT

Silymarin, which is derived from the seeds of *Silybum marianum*, has been widely used to prevent and treat liver disorders. It is also consumed as a dietary supplement to improve liver function, as it does not exhibit any toxic effects in humans. Recently, silymarin has been reported to show antimicrobial effects against various pathogenic microorganisms, but the mode of action remains unknown. Thus, we investigated the antifungal activity of silymarin and aimed to determine the underlying mechanism. Initially, a propidium iodide assay was carried out; the results indicated that silymarin induced injury to the fungal plasma membrane. Subsequently, large unilamellar vesicles encapsulating calcein and fluorescein isothiocyanate-labeled dextrans (FDs) 4, 10, and 20 were prepared to analyze whether silymarin affects an artificial membrane model. The results indicated that silymarin increased membrane permeability by disturbing the membrane structure, thereby allowing free access to molecules smaller than FD20 (approximately 3.3 nm). The accumulation of reactive oxygen species (ROS) results in deleterious effects to various cellular components. In particular, ROS easily react with the membrane lipids and induce lipid peroxidation, which increases membrane permeability and disturbs hydrophobic phospholipids. Using 2′,7′-dichlorodihydrofluorescein diacetate and thiobarbituric acid, we confirmed that silymarin induced harmful effects on the plasma membrane. Membrane depolarization and K^+ leakage, which were associated with an increase in membrane permeability, were also observed in *Candida albicans* cells. An assay using 1,6-diphenyl-1,3,5-hexatriene showed that silymarin decreased membrane fluidity. Taken together, we suggest that silymarin exerts its antifungal activity by targeting the *C. albicans* plasma membrane.

1. Introduction

Phytochemicals are chemical compounds that occur naturally in plants. They are defined as bioactive, non-nutrient plant compounds found in fruits, vegetables, grains, and other plant-derived foods. More than 5,000 different phytochemicals have been identified so far, and they have been used as potential drugs for millennia [1]. Many studies have demonstrated the therapeutic effects of phytochemicals in the prevention of cancer, inflammatory bowel disease, and infectious diseases [2]. In addition, the interest in phytochemical research is steadily increasing because of its low toxicity and safety [3].

Silybum marianum (milk thistle) has been used as both food and herbal medicine for a long time in China and Europe, especially for treating liver disorders and protecting the liver against toxic agents [4]. Silymarin, which is a multicomponent extract derived from the seeds of *S. marianum*, mainly consists of silibinin, isosilibinin, silychristin, and silydianin [4]. Silibinin, the most bioactive component of the extract, is known to exert strong antioxidative and antiviral activities against hepatitis C virus, which makes it a useful drug for the treatment of chronic liver diseases [5–7]. Isosilibinin is reported to induce G_1 arrest and cause apoptosis in human prostate cancer LNCaP and 22Rv1 cells [8]. Silychristin has been shown to increase proliferation rate as well as protein and DNA biosynthesis [9], and silydianin has been shown to induce caspase-3 activation and decrease super oxide generation [10]. Silymarin is also well known to exert anti-inflammatory, antitumor, and antidiabetic effects [11,12]. Recently, animal and human studies with silymarin are increasingly being carried out, and the hepatoprotective effects of silymarin have been demonstrated with minimal side effects in these studies [13]. Besides, silymarin has shown antimicrobial effects against various microorganisms, such as *Candida parapsilosis*, *Escherichia coli*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*, which make it even more attractive as a potential medicine [14].

Abbreviations: MIC, minimal inhibitory concentration; PBS, phosphate-buffered saline; LUV, large unilamellar vesicles; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; FD, fluorescein isothiocyanate-labeled dextran; ROS, reactive oxygen species; H_2DCFDA, 2′, 7′-dichlorodihydrofluorescein diacetate; MDA, malondialdehyde; TBA, thiobarbituric acid; DiBAC$_4$(3), bis-(1,3-dibutylbarbituric acid) trimethineoxonol; DPH, 1, 6-diphenyl-1,3,5-hexatriene.

* Corresponding author at: School of Life Sciences, BK 21 Plus KNU Creative BioResearch Group, College of Natural Sciences, Kyungpook National University, 80 Daehakro, Bukgu, Daegu, 41566, Republic of Korea.
E-mail address: dglee222@knu.ac.kr (D.G. Lee).

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Conventional antibiotics are becoming ineffective owing to microbial resistance; therefore, infections caused by these microorganisms are continually increasing. Infectious diseases are emerging as a major public health problem. In particular, *Candida albicans* infection, which can affect the skin, oropharynx, lower respiratory tract, gastrointestinal tract, and genitourinary system, is one of the most frequent fungal infections. The morbidity and mortality associated with candidiasis have steadily increased [15]. To manage these infectious diseases, phytochemicals have been highlighted as alternative antimicrobial agents, and they have shown effective antimicrobial activity [16,17]. In this study, we investigated the antifungal effects of silymarin against various fungal strains. In addition, we proposed the mechanism of action of silymarin, which includes targeting the fungal plasma membrane.

2. Materials and methods

2.1. Compounds and fungal strains

Silymarin and amphotericin B were purchased from Sigma Aldrich (St. Louis, MO, USA; Lot number 107K0762 and 122K4013, respectively) and dissolved in dimethyl sulfoxide. Silymarin is mainly composed of silibinin (about 56%), isosilibinin (about 12%), silychristin (about 23%), silydianin (about 9.1%), as determined by high-pressure liquid chromatography [18,19] (Fig. 1). The fungal strains were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and Korean Collection of Type Cultures (KCTC; Daejeon, Korea). *C. albicans* (ATCC 90028), *C. parapsilosis* (ATCC 22019), *Aspergillus fumigatus* (KCTC 6145), and *Trichosporon beigelii* (KCTC 7707) were cultured at 28 °C in YPD broth (Difco, Franklin Lakes, NJ, USA), and *Malassezia furfur* (KCTC 7744) was cultured at 28 °C in YM broth (Difco) containing 1% olive oil.

2.2. Antifungal susceptibility test

The minimal inhibitory concentration (MIC) test was performed using two-fold serial dilutions of test compounds, as recommended by the Clinical and Laboratory Standards Institute (CLSI), with slight modifications [20]. The fungal cell suspensions (1×10^3 cells/mL) were dispensed into the wells of microtiter plates (0.1 mL/well), and test compounds were added. After 48 h of incubation at 28 °C, the MIC values were read and determined in three independent experiments.

2.3. Time-kill kinetics

The *C. albicans* cells (1×10^5 cells/mL) were incubated with either 10.0 μg/mL silymarin or amphotericin (Fig. 2). Aliquots were taken from the cultures after 2, 4, 6, 8, and 10 h of incubation, and spread onto YPD agar plates. The colony-forming units (CFUs) were counted after 48 h of incubation at 28 °C, and the percent survival was determined relative to those of untreated cells [21].

2.4. Propidium iodide influx

To investigate the damage to the fungal membrane, *C. albicans* cells (1×10^5 cells/mL) were treated with 5.0, 10.0, and 20.0 μg/mL silymarin (Sigma Aldrich) and 10.0 μg/mL amphotericin B (Sigma Aldrich), and incubated at 28 °C for 4 h. Then, the cells were harvested and resuspended in phosphate-buffered saline (PBS). The cells were stained with 9 μM propidium iodide (Sigma Aldrich), which can only enter cells with a compromised membrane, and analyzed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA) [22].

2.5. Dye leakage from artificial large unilamellar vesicles

The major components of the fungal lipid membrane, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and ergosterol (5:4:1:2, w/w/w/w), were dissolved in chloroform. Chloroform was evaporated in a rotary vacuum evaporator for 30 min. To encapsulate calcein (Sigma Aldrich) and fluorescein isothiocyanate-labeled dextrans (FDs; Sigma Aldrich) in the liposomes, 70 mM calcein or 2 mg/mL FD in a dye-buffer solution (10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA, pH 7.4) was added to the dried membrane components. Then, the mixture was freeze-thawed for 13 cycles and extruded

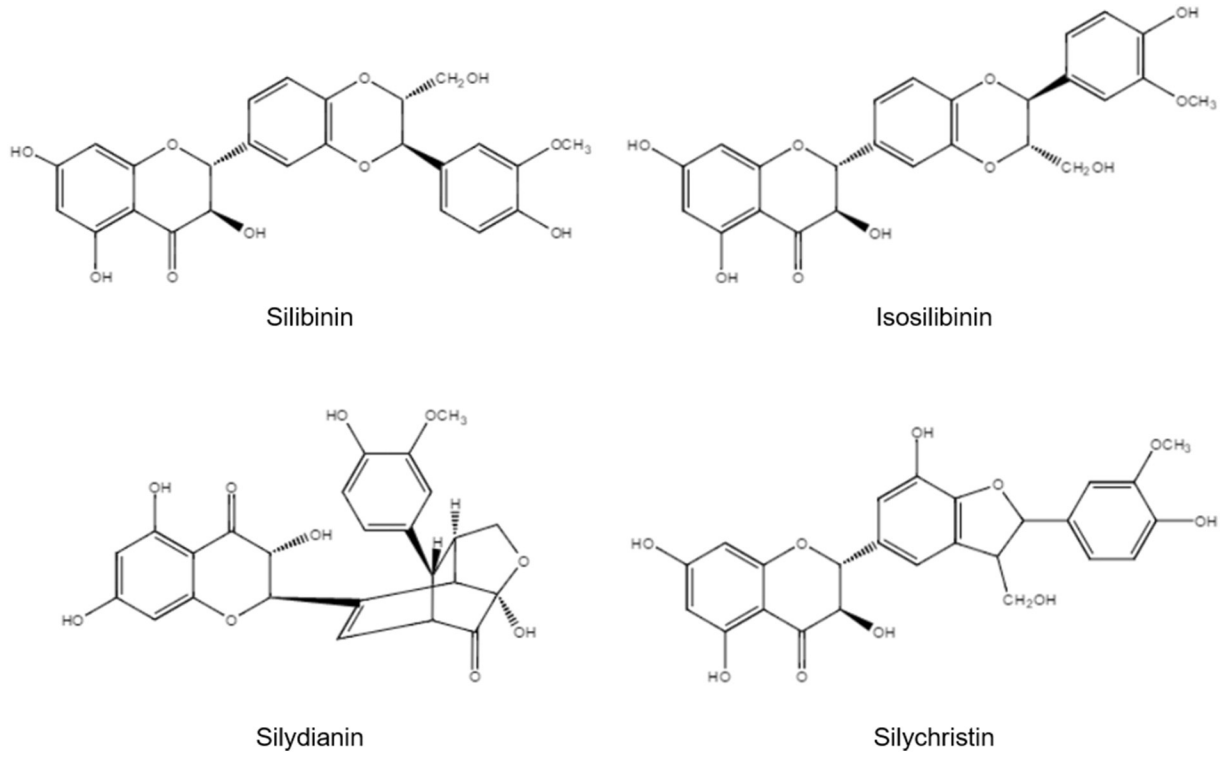

Fig. 1. Structure of silymarin components.