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Molecules and Cells

Anti-Candida Property of a Lignan Glycoside Derived from *Styrax japonica* S. et Z. *via* Membrane-Active Mechanisms

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Styraxjaponoside C was investigated with respect to its antifungal activity and mechanisms of action. Devoid of hemolytic activity, Styraxjaponoside C demonstrated an antifungal effect against the human pathogenic yeast *Candida albicans* in an energy-independent manner. To characterize the mechanisms of the antifungal activity of Styraxjaponoside C, fluorescence analysis with membrane probe 1,6-diphenyl-1,3,5-hexatriene, and flow cytometric analysis on *C. albicans* were conducted. The results showed that Styraxjaponosdie C induced cytoplasmic membrane perturbation. The current study suggested that Styraxjaponoside C was active against *C. albicans* with membrane-active mechanisms.

INTRODUCTION

Candida albicans is a dimorphous fungus colonizing the mucous membranes of the mouth and vagina in a saprophytic manner. However, *C. albicans* is also known to be involved in several diseases such as cutaneous infections and systemic infections in individuals undergoing immunosuppressive therapy for cancer or organ transplantation and in human immunodeficiency virus (HIV)-infected patients (Lupetti et al., 2002; Richardson, 2005; Romani, 2000). Infections of this nature are frequently associated with AIDS and can be considered a criterion for the progression towards AIDS (Coleman et al., 1993). Unfortunately, long-term therapies have led to the emergence of fluconazole-resistant *C. albicans* strains that are cross resistant not only to triazole but also to amphotericin B (Kelly et al., 1993). This points to a pressing need for new antifungal agents, e.g., antimicrobial proteins or natural products (Hancock, 1999; Hancock and Chapple, 1999).

Styraxjaponoside C is a glycoside derivatives of lignans derived from the stem bark of *Styrax japonica* S. et Z (Kim et al., 2007). *Styrax japonica*, widely distributed in Korea, Japan, and Central America, and was traditionally used to treat inflammatory diseases (Costa, 1968). A variety of second metabolites, such as, benzofurans (Li et al., 2007), saponins (Yoshikawa et al., 2000) and lignans (Kinoshita et al., 2005) have been isolated from the *Styrax* species and investigated to characterize their bioactive properties.

In this study, Styraxjaponoside C was investigated for its bioactive properties with regard to antifungal effect against *C. albicans*.

MATERIALS AND METHODS

Extraction and isolation of compound from *Styrax japonica*

The stem bark of *S. japonica* was collected in Korea. The MeOH extract (120.32 g) was suspended in water and then partitioned sequentially with equal volumes of dichloromethane, ethyl acetate, and *n*-butanol. The *n*-BuOH extract (10 g) was purified by column chromatography on a silica gel eluting with a $CHCl_3$-MeOH (50:1 $\rightarrow$ 0:100) in a step-wise system. The subfraction, Bu3 (3.34 g) was purified by column chromatography on a silica gel ($CHCl_3$-MeOH, 50:1 $\rightarrow$ 0:100) followed by gel filtration column chromatography (Sephadex LH-20, MeOH-H_2O, 35:65) and preparative TLC (RP-18 F_{254}s, 1.0 mm, MeOH-H_2O, 1:1) that yielded Styraxjaponoside C (28 mg) (Kim et al., 2007).

Fungal strains and culture conditions

C. albicans (ATCC 90028) was obtained from the American Type Culture Collection (USA). Fungal cells were cultured in a YPD broth (Difco, USA) containing yeast extract, peptone and dextrose (50 g/L) with aeration at 28°C.

Time-killing kinetics

Log-phase fungal cells (2×10^4/ml, YPD) were incubated with compounds. The cultures were obtained and spread on an YPD agar plate, and then the colony forming units (CFUs) were counted after 24 h incubation at 28°C (Gazit et al., 1994).

Effect of Styrajxaponoside C on the dimorphic transition in *C. albicans*

C. albicans cells were maintained by periodic subculturing in a YPD medium. To induce the formation of mycelia, the cultures were directly supplemented with 20% fetal bovine serum (FBS).

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The dimorphic transition in *C. albicans* was investigated from the cultures in the presence or absence of the compound and incubated for 48 h at 37°C (Alvarez-Peral and Arguelles, 2000). The dimorphic transition to mycelial forms was detected by phase contrast light microscopy (NIKON, ECLIPSE TE300, Japan).

Effect of energy metabolism on the antifungal activity of Styraxjaponoside C

C. albicans cells (2×10^6/ml, YPD), pre-incubated for 1 h with 0.1 mM NaN_3, were then treated with compound and incubated for 24 h at 28°C. The cell population was illustrated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sung et al., 2008). The absorbance of each well was measured at 580 nm by using a Microtiter ELISA Reader (Molecular Devices Emax). The equation of the cell viability percentage was illustrated as follows: [(the absorbance of the compound-treated cells)/(the absorbance of non-treated cells)] × 100. The error bars represent the standard deviation (S.D.) values for three independent experiments, performed in triplicate.

Hemolytic activity against human erythrocytes

Human erythrocytes were rinsed with PBS, by repeated centrifugation and suspension (5 min at 1500 g). 4% suspension of erythrocytes was incubated with compound at 37°C for 1 h. After centrifugation at 1,500 g for 10 min, the supernatant was separated from the pellet and the absorbance measured at 414 nm with an ELISA plate reader (Veerman et al., 2007). The hemolysis percentage was calculated by using the following equation: Percentage hemolysis = [($Abs_{414\,nm}$ in the compound solution - $Abs_{414\,nm}$ in a PBS (phosphate buffered saline))/($Abs_{414\,nm}$ in 0.1% Triton X-100 - $Abs_{414\,nm}$ in a PBS)] × 100.

Flow cytometric analysis for morphological changes of *C. albicans* protoplasts

For the preparation of *C. albicans* protoplasts, cells were digested with a 0.1 M phosphate buffer (pH 6.0) containing 1 M sorbitol, and lysing enzyme (Sigma) (20 mg/ml) for 1 h at 30°C by gentle agitation. The filtrated protoplasts were gathered by centrifugation at 1500 rpm for 10 min. The protoplasts were resuspended in a washing buffer (1 M sorbitol, 0.8 M NaCl, 10 mM $CaCl_2$, and 50 mM Tris–HCl, pH 7.5) and centrifuged (Park and Lee, 2009). The protoplasts, resuspended in YPD medium containing 1 M sorbitol, were treated with the compounds and incubated for 2 h at 28°C with constant shaking. After incubation, the protoplast cells were harvested by centrifugation and suspended in PBS containing 1 M sorbitol. Flow cytometry was performed via a FACSCalibur flow cytometer (Becton Dickinson, USA) (Zelezetsky et al., 2005).

Measurement of plasma membrane fluorescence intensity

Fluorescence from the plasma membrane of *C. albicans* cells labeled by 1,6-diphenyl-1,3,5-hexatriene (DPH) (Molecular probes, USA.) was used to monitor changes in membrane dynamics. *C. albicans* cells (2×10^6/ml, YPD) were treated with the compounds and incubated for 2 h at 28°C. Samples of the fungal culture were fixed by 0.37% formaldehyde, collected and washed with PBS and the cells were frozen by liquid nitrogen. For labeling, cells were thawed with PBS and resuspended in PBS. The suspension was incubated with 0.6 mM DPH for 45 min at 28°C and this was followed by washing with PBS. The fluorescence intensity of DPH was measured by a Spectrofluorometer (Shimadzu RF-5301PC, Shimadzu, Japan) at 350 nm excitation and 425 nm emission wavelengths (Lee et al., 2009a; Sung and Lee, 2008).

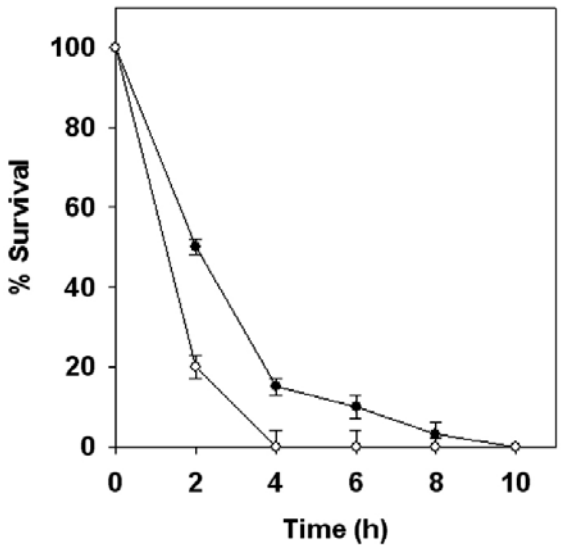

Fig. 1. Time-killing kinetics of Styraxjaponoside C against *C. albicans*. *C. albicans* (2×10^4 cells/ml YPD) was incubated with 80 μg Styraxjaponoside C (●) or 10 μg Amphotericin B (○). Viability was determined every 2 h by counting colony forming units (CFUs) and expressed as percentage of survivals.

RESULTS AND DISCUSSION

Antifungal and hemolytic activity

Styraxjaponoside C, a novel glycoside derivative of lignans, was isolated from the stem bark of *Styrax japonica* S. et Z. and its structure was determined to be (-)-fargesin-4-O-β-D-glucopyranoside by NMR (Kim et al., 2007). In this study, Styraxjaponoside C was investigated for antifungal effect against *C. albicans* and its mechanism of action.

C. albicans is the most prevalent systemic fungal pathogen of man and is associated with a range of clinical conditions ranging from irritating superficial infections of the oral and vaginal mucosa to life-threatening systemic disease in immunocompromised patients (Hancock, 1999; Hancock and Chapple, 1999). Considering its medical importance, *C. albicans* was selected as a model organism for examination.

Initially, the *in vitro* antifungal activity of Styraxjaponoside C against *C. albicans* was investigated. The antifungal activity test of Styraxjaponoside C against *C. albicans* was determined by the micro-dilution method and MTT assay. MIC value of the Styraxjaponoside C and a positive control, amphotericin B (a polyene antibiotics) was 40 μg/ml and 5 μg/ml, respectively (data not shown). In time-killing kinetics, Styraxjaponoside C showed significant antifungal effect against *C. albicans*. As shown in Fig. 1, the CFUs of the *C. albicans* cells exposed to Styraxjaponoside C decreased, which was less potent than that of amphotericin B. Also, Styraxjaponoside C exerted an inhibitory effect even in the dimorphic transition state of *C. albicans* cells. With respect to *C. albicans*, dimorphism plays a crucial role in the pathogenesis, with mycelial shapes being predominantly found during a host tissue invasion (Alvarez-Peral and Arguelles, 2000). The serum-induced mycelia form of *C. albicans* cells were inhibited from being fluourishing and were destroyed in the presence of Styraxjaponside C (Fig. 2D), while the mycelia in the absence of the compound formed normally (Fig. 2B). These results indicated that *C. albicans* was highly susceptible to Styraxjaponoside C.

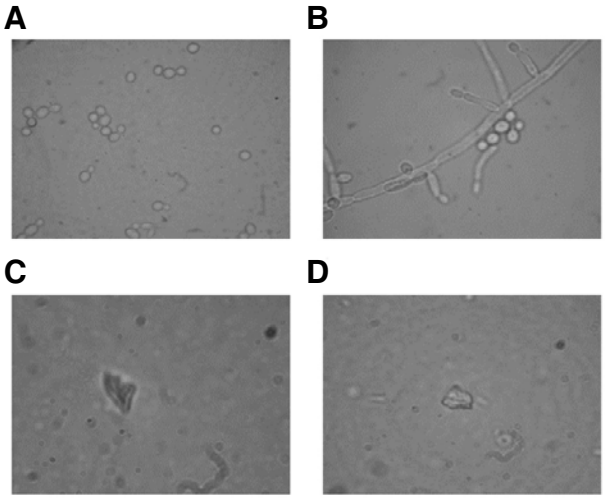

Fig. 2. Effect of Styraxjaponoside C on the dimorphic transition in *C. albicans*. *C. albicans* (2×10^6 cells/ml, YPD) was incubated with 20% fetal bovine serum (FBS). (A) Yeast control without FBS and compounds, (B) Cells treated with only 20% FBS, (C) Cells treated with 20% FBS and 80 μg Styraxjaponoside C, (D) Cells treated with 20% FBS and 10 μg Amphotericin B.

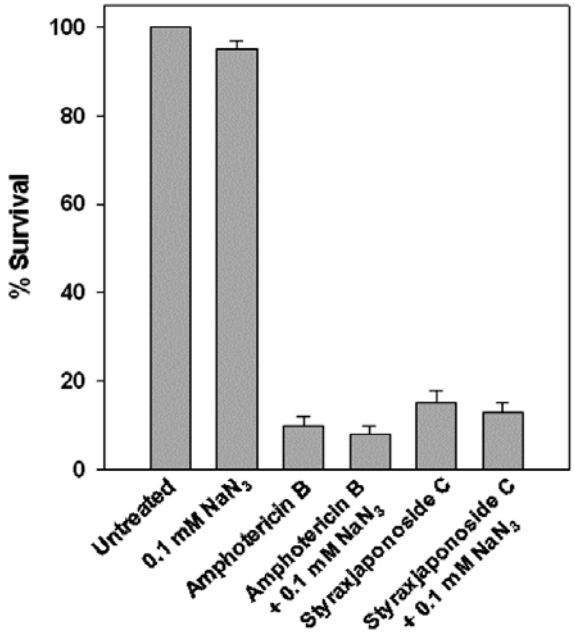

Fig. 3. Effect of energy metabolism on antifungal activity of Styraxjaponoside C. *C. albicans* (2×10^6 cells/ml, YPD) was incubated with 0.1 mM NaN_3 for 1 h. After incubation, the cells treated with 80 μg Styraxjaponoside C or 10 μg Amphotericin B.

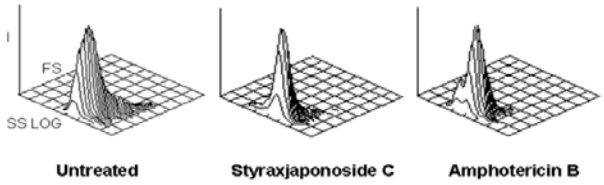

Fig. 4. Three-dimensional flow cytometric contour-plot analysis of *C. albicans* protoplasts treated with Styraxjaponoside C, or Amphotericin B. FS (y-axis) is an indicator of size and SS (90° scattering, SS LOG, x-axis) is an indicator of granularity. The z-axis represents the cellular population intensity.

To investigate whether the energy metabolism of *C. albicans* cells influenced their susceptibility to Styraxjaponoside C, the cells were treated with Styraxjaponoside C in the presence of 0.1 mM sodium azide, which is a metabolic inhibitor which blocks both the conventional as well as the alternative respiratory pathways of *C. albicans* (Sung et al., 2008). As illustrated in Fig. 3, even in the presence of the energy poison, the antifungal activity of the compound was still retained. The results suggested that Styraxjaponoside C exhibits an antifungal effect against *C. albicans* in an energy-independent manner.

By means of a cytotoxicity test, the hemolytic activity assay was conducted against human erythrocytes (Veerman et al., 2007). Even though it exhibits a remarkable antifungal effect, the efficacy of amphotericin B for clinical usage has been limited due to its significant cytotoxicity. As indicated in Table 1, Styraxjaponoside C showed no hemolysis, whereas amphotericin B induced potent hemolysis, reaching up to 100% complete hemolysis at a concentration 80 μg. These results suggested that it was unlikely that Styraxjaponoside C showed signs of cytotoxicity detrimental to mammalian cells and that its activity was directed against *C. albicans.*

Membrane-active antifungal activity

To characterize the mechanisms of the antifungal effect of Styraxjaponoside C, a series of experiments with regards to membrane-active mechanisms were conducted. Amphotericin B is thought to form membrane-spanning channels that allow the leakage of essential components, which ultimately results in fungal cell death (Matsumori et al., 2002). The morphological effects of Styraxjaponoside C on *C. albicans* were investigated by flow cytometric analysis plotting the forward scatter and side scatter of treated and untreated cells. The protoplasts of *C. albicans* were utilized to monitor the effect of the compound at the level of the fungal cytoplasmic membrane. As shown in Fig. 4, in the absence of the compounds, a control population of undamaged cells was observed. In the presence of Styraxjaponoside C, the population of damaged and permeabilized cells, showing decreased FS, became dominant. This indicates that the damages by the compound in cellular membrane resulted in decrease in cell size (Lee et al., 2009b; Park and Lee, 2009). To confirm the membrane-active activity of Styraxjaponoside C, changes in membrane dynamics were examined with a fluorescent membrane probe, DPH. DPH is a hydrophobic molecule, and this characteristic enables it possible to associate with the hydrocarbon tail region of phospholipids, within the cytoplasmic membrane, without disturbing the structure of the lipid bilayer (Sánchez et al., 2009). The level of the fluorescence from the DPH-labeled-membrane of *C. albicans* was analyzed in the absence or in the presence of the compounds. The results showed that as compound concentrations increased, the plasma membrane DPH fluorescence intensity decreased (Fig. 5). This reduction of DPH fluorescence intensity corresponded to the antifungal effect of Styraxjaponoside C against *C. albicans* cells by perturbing the plasma membrane. The results of membrane-active activity suggested that Styraxjaponoside C was likely to have membrane-active mechanisms similar to amphotericin B, although further investigations are needed.

Table 1. Hemolytic activity of Styraxjaponoside C against human erythrocytes

Compounds	% Hemolysis (μg/ml)					
	80	40	20	10	5	2.5
Styraxjaponoside C	0	0	0	0	0	0
Amphotericin B	100	100	92.3	86.6	71.2	62.2

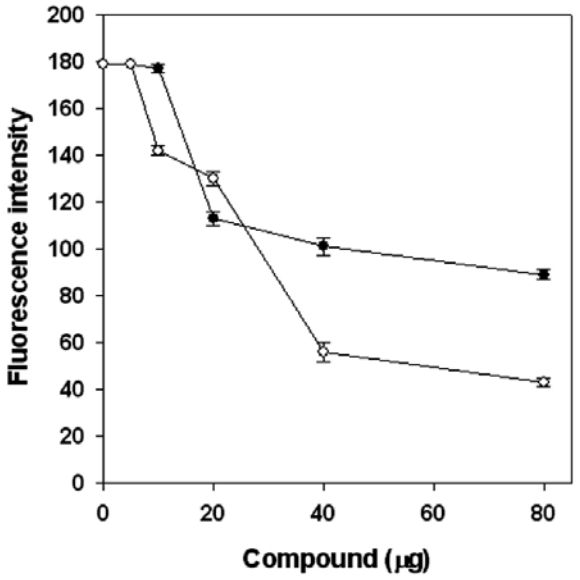

Fig. 5. DPH fluorescence analysis of *C. albicans* treated with Styraxjaponoside C (●), or Amphotericin B (○).

In summary, we investigated the anticandida effect of Styraxjaponoside C and its possible mode of action. Devoid of any hemolytic activity, Styraxjaponoside C exhibited an antifungal effect towards *C. albicans*. Although the exact mechanisms of Styraxjaponoside C at the molecular level are still elusive, the effectivity of the anticandida activity of the compound could be correlated with its capacity to perturb the cytoplasmic membrane.

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