Biomolecules & Therapeutics, **18(4)**, 420-425 (2010)
www.biomolther.org

ISSN: 1976-9148(print)/2005-4483(online)
DOI: 10.4062/biomolther.2010.18.4.420

Styraxjaponoside A and B, Antifungal Lignan Glycosides Isolated from *Styrax japonica* S. et Z.

Cana Park[1], Jaeyong Cho[1], Bomi Hwang[1], In-sok Hwang[1], Mi Ran Kim[2], Eun-Rhan Woo[2,*], and Dong Gun Lee[1,*]

[1]*School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu 702-701,*
[2]*College of Pharmacy, Chosun University, Gwangju 501-759, Republic of Korea*

(Received July 13, 2010; Revised July 13, 2010; Accepted August 18, 2010)

Abstract − The antifungal effects and action mechanisms of styraxjaponoside A and B were investigated. Devoid of hemolytic effect, the compounds had significant effect against several human pathogenic fungal strains, with energy-independent manners. To understand the action mechanisms of the compounds, the flow cytometric analysis plotting the forward scatter and the side scatter, $DiBAC_4(3)$ staining and DPH fluorescence analysis were conducted. The results indicated that the actions of the compounds were dependent upon the membrane-active mechanisms. The present study suggests that styraxjaponoside A and B exert their antimicrobial effects *via* membrane-disruptive mechanisms.

Keywords: Antifungal activity, Lignans, Styraxjaponoside A, Styraxjaponoside B, *Styrax japonica* S. et Z.

INTRODUCTION

Antibiotics resistance limits therapeutic options and requires the use of more expensive agents. Multi-antibiotic resistance may result in a complete lack of treatment options (Projan and Shlaes, 2004; Fox, 2006). An additional desire for the identification of novel antimicrobials has come from the demand for new drugs ensuing from the emergence of many multi-drug resistant pathogens (Davies, 2007). Above all, the investigation of natural products which possibly may be templates for novel antimicrobial targets is widely accepted as one of the alternatives for the antibiotics development (Butler and Buss, 2006; Saleem *et al*., 2010). In this study, novel templates for antimicrobial compounds, along with their mechanism of action were investigated.

Styraxjaponoside A and B are lignan glycoside derivatives that are isolated from the stem bark of *Styrax japonica* S. et Z. (Kim *et al*., 2004). *Styrax* species are found to be a rich source of lignans, benzofurans, and saponins (Ayres and Loike, 1990; Saleem, 2005). Styraxjaponoside A and B, the structurally unique lignans, were tested for intrinsic antifungal activity. It is possible that this study presents the first investigation of the *in vitro* antimicrobial effect of the compounds and its mechanism of action.

*Corresponding authors
Tel: +82-53-950-5373 (Lee DG), +82-62-230-6369 (Woo ER)
Fax: +82-53-955-5522 (Lee DG), +82-62-222-5414 (Woo ER)
E-mail: dglee222@knu.ac.kr (Lee DG),
wooer@chosun.ac.kr (Woo ER)

MATERIALS AND METHODS

Extraction and isolation of compounds from *Styrax japonica*

The stem bark of the *S. japonica* was collected from Jogyesan, Suncheon, Chonnam, Korea, in September 2002. Voucher specimens were deposited in the Herbarium of the College of Pharmacy, Chosun University, South Korea (CSU-964-17). The air-dried stem bark of *S. japonica* (654 g) was then cut and extracted with MeOH at 80°C for 4 h. The MeOH extract (120.32 g) was suspended in water and then partitioned sequentially with equal volumes of dichloromethane, ethyl acetate, and *n*-butanol. Each fraction was evaporated *in vaccuo* to yield the following amounts of residues of CH_2Cl_2 (13.98 g), EtOAc (12.16 g), *n*-BuOH (75.88 g), and water (2.90 g) extract. The EtOAc extract (4 g) was subjected to column chromatography over a silica gel by eluting it with EtOAc-*i*-PrOH-H_2O (20:1:0.5). The groups were then divided into 7 separate groups based on their TLC profiles. The subgroup, E4 (430.4 mg) was then purified by column chromatography on a silica gel eluting it

with a $CHCl_3$-MeOH-Me_2CO-H_2O (50:4:2:0.3→30:4:2:0.3) and was followed with a gel filtration column chromatography (Sephadex LH-20, MeOH-H_2O, 50:50→65:35), preparative TLC (RP-18, MeOH-H_2O, 50:50), and finally with TOYOPEARL HW-40© (MeOH-H_2O, 50:50) to give styraxjaponoside A (50 mg). The subgroup, E6 (1.1 g) was purified by column chromatography on a silica gel eluting it with a $CHCl_3$-MeOH-Me_2CO-H_2O (30:3:2:0.3) and was followed with a gel filtration column chromatography (Sephadex LH-20, MeOH-H_2O, 50:50) to give white crystals of styraxjaponoside B (150 mg) (Kim *et al*., 2004).

Antifungal susceptibility testing

Candida albicans (ATCC 90028) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Malassezia furfur* (KCTC 7744) and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC) (Daejeon, Korea). Log-phased fungal cells (2×10^4 cells/ml) were inoculated into YPD broth and aliquoted into 0.1 ml/well microtiter plates. MICs were determined by a two-fold serial dilution compound, following a micro-dilution method, and by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay. After 48 h at 28°C, the minimal compound concentration that prevented the growth of a given test organism was determined, and defined as the MIC. Growth was recorded with a microtiter ELISA Reader (Molecular Devices Emax, CA, U.S.A.) at 580 nm. MIC values were determined from three independent assays (Jain *et al*., 1995).

Hemolysis assay against human erythrocytes

Human erythrocytes were rinsed with PBS, by repeated centrifugation and suspension trials (5 min at 1,500 g). 4% suspension of the erythrocytes was incubated with the 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 was measured at 414 nm with an ELISA plate reader. The hemolysis percentage was calculated by using the following equation: Percentage hemolysis=[($Abs_{414\ nm}$ in the compound solution − $Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100 − $Abs_{414\ nm}$ in PBS)]× 100 (Lee and Lee, 2009).

The effect of compounds on the dimorphic transition in *C. albicans*

C. albicans cells were maintained by periodic sub-culturing in YPD media. To induce the formation of mycelia, the cultures were directly supplemented with 20% fetal bovine serum (FBS). The dimorphic transition in *C. albicans* was investigated by observing the presence or absence of the compound and was incubated for 48 h at 37°C (Lee *et al*., 2009). The dimorphic transition to mycelial forms was detected by phase contrast light microscopy (NIKON, ECLIPSE TE 300, Japan).

The effect of sodium azide (NaN_3) on antifungal activity

To examine the effect of NaN_3 on antifungal activity of styraxjaponoside A and B, the activity was investigated in the presence or absence of 0.1 mM NaN_3. Cell cultures were seeded on a 96-well microtiter plate at a density of 2×10^4 cells per well. Fungal cells were added 20 μg/ml of the compounds with or without 0.1 mM NaN_3, and MTT assay was performed. The percentage of cell viability was determined by comparing the absorbance of compound-treated cell culture with that of compound-untreated cell culture. The values were the average of triplicate measurements in three independent assays (Lee *et al*., 2009).

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

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 1,500 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. The protoplasts, resuspended in the YPD medium containing 1 M sorbitol, were treated with the compounds and incubated for 2 h at 28°C with constant steady shaking. After incubation, the protoplast cells were harvested by centrifugation and suspended in the PBS containing 1 M sorbitol. Flow cytometry was then performed via a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) (Zelezetsky *et al*., 2005).

Measurement of plasma membrane potential

Log-phased *C. albicans* (2×10^4 cells/ml, YPD) was treated with the compounds or 10 μM CCCP. After incubation for 3 h, the cells were washed with, and suspended in the PBS. This was finished before staining it with 10 μg of $DiBAC_4(3)$ for 30 min at 4°C in the dark (Liao *et al*., 1999). Flow cytometry was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Measurement of plasma membrane fluorescence intensity

Fluorescence from the plasma membrane of the *C. albicans* cells labeled by 1,6-diphenyl-1,3,5-hexatriene (DPH)

(Molecular probes, Eugene, Oregon, USA) were used to monitor changes in membrane dynamics. *C. albicans* cells (2×10^4 cells/ml, in 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 then incubated with 0.6 mM DPH for 45 min at 28°C and this was followed by washing with PBS. The fluorescence intensity of the DPH was measured by a Spectrofluorophotometer (Shimadzu RF-5301PC, Shimadzu, Japan) at 350 nm excitation and 425 nm emission wavelengths (Vincent *et al*., 2004).

RESULTS AND DISCUSSION

Antimicrobial and hemolytic activities of styraxjaponoside A and B

Styraxjaponoside A and B, derivatives of the lignan glycosides, were obtained from the stem bark of *S. japonica* S. et Z (Fig. 1). NMR showed that each of the structures were determined to be 7,7'-dihydroxybursehernin 4-β-D-glucoside and 4-methoxymatairesinol 4'-β-D-glucoside, respectively (Kim *et al*., 2004). In this study, the antimicrobial activity and action mechanisms of these two lignan glycosides were investigated.

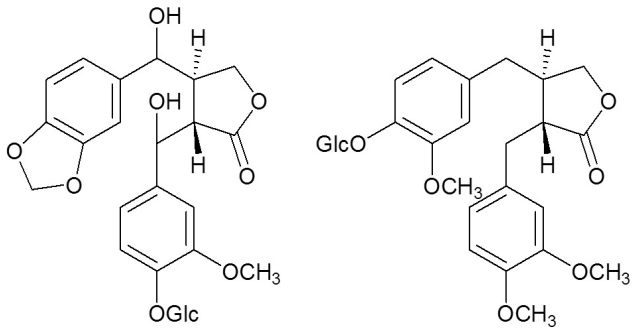

Fig. 1. Chemical structures of styraxjaponoside A and B.

The antifungal activities of the compounds against the human fungal pathogens were determined by the MTT assay (Jain *et al*., 1995). Amphotericin B was used as a positive control to compare the extent of the antimicrobial effects of the compounds (Kleinberg, 2006). The result showed that all fungal strains tested were susceptible to the compounds with the MIC value ranging 5-40 μg/ml (Table I).

The *in vitro* cell cytotoxicity of the compounds against the human erythrocytes was evaluated (Lee and Lee, 2009). Even though it exhibits an antimicrobial effect, the efficacy of the antibiotics such as amphotericin B for clinical usage has been limited due to its significant cytotoxicity. As indicated in Table II, styraxjaponoside A and B showed no hemolysis, whereas amphotericin B induced potent hemolysis, which reached up to a complete hemolysis at a concentration of 40 μg. These results suggested that it was unlikely that the compounds showed signs of cytotoxicity detrimental to mammalian cells and that its activity was directed against *C. albicans*.

Significantly, it was also observed that the compounds were active against *C. albicans*, a well-known yeast model system which is the most prevalent systemic fungal pathogen (Fröhlich and Madeo, 2000). *C. albicans* was then selected to be used as the test organism for subsequent experiments. With respect to *C. albicans*, dimorphism

Table I. Antifungal activity of styraxjaponoside A and B

Fungal strains	MIC (μg/ml)		
	Styraxjaponoside A	Styraxjaponoside B	Amphotericin B
C. albicans	20 ± 2.72	20 ± 2.72	5 ± 1.36
T. beigelii	20 ± 7.2	40 ± 5.44	5 ± 3.60
M. furfur	20 ± 7.2	20 ± 2.72	10 ± 1.36

MIC values are mean ± SEM from three independent assays.

Table II. Hemolytic activity of styraxjaponoside A and B against human erythrocytes

Compounds	% Hemolysis (μg/ml)					
	40	20	10	5	2.5	1.25
Styraxjaponoside A	0	0	0	0	0	0
Styraxjaponoside B	0	0	0	0	0	0
Amphotericin B	100	92.3 ± 1.2	86.6 ± 2.0	71.2 ± 2.6	62.2 ± 1.8	28.6 ± 1.6

Values were determined from three independent assays. Value are mean ± SEM.

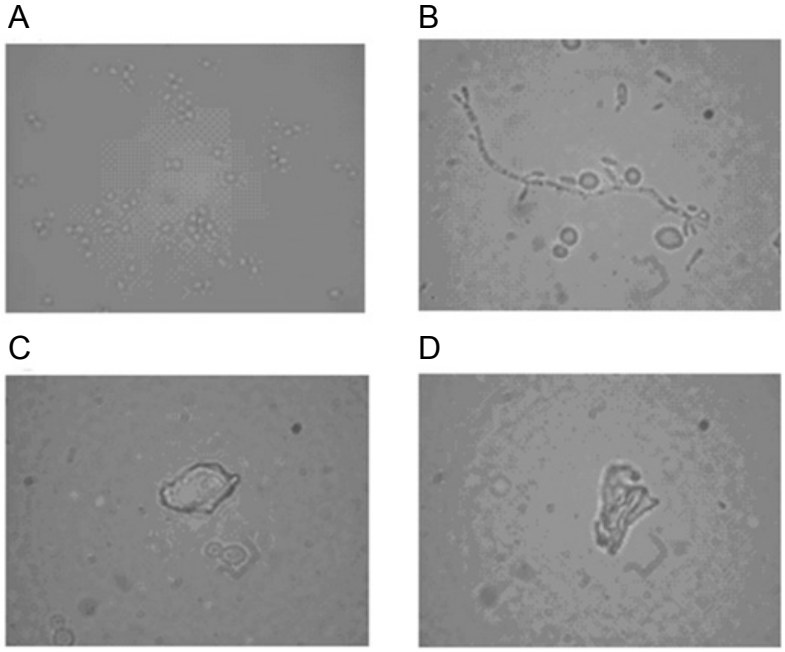

Fig. 2. The effect of the compounds on the dimorphic transition in *C. albicans*. *C. albicans* (2×10^4 cells/ml, in YPD) was incubated with 20% fetal bovine serum (FBS). (A) Yeast control without FBS and compounds. (B) Cells treated with only 20% FBS. (C) Cells treated with 20% FBS and 40 μg styraxjaponoside A. (D) Cells treated with 20% FBS and 40 μg styraxjaponoside B.

plays a crucial role in the pathogenesis, with mycelial shapes being mainly found during a host tissue invasion (Fig. 2A, B) (Lee *et al*., 2009; Okamoto-Shibayama *et al*., 2010). The compounds exerted an inhibitory effect against the mycelial form of the *C. albicans* cells. The serum-induced mycelia *C. albicans* cells which were treated with the compounds were destroyed (Fig. 2C, D). These results indicated that the compounds have an antimicrobial property against human pathogenic strains as its intrinsic biological activity.

The effect of energy metabolism on antifungal activity of styraxjaponoside A and B

The metabolic inhibitor regarding the ATP depletion can inhibit an imported system from extracellular environments into the cell interior and also disrupt the proton gradient, resulting in membrane depolarization (Veerman *et al*., 2007). Therefore, to examine the relationship between the cellular energy consumption and the transport pathway of styraxjaponoside A and B, an energy-dependence test was conducted. *C. albicans* cells were incubated with styraxjaponoside A and B in the absence or presence of 0.1 mM NaN_3. Sodium azide (NaN_3) is known as one of the metabolic inhibitors blocking the intracellular ATP synthesis and the ability of ATPase by inhibiting the activity of cytochrome oxidase, which further prevents membrane-active transport (Wilson and Chance, 1967). The result showed that the viability of the cells was almost not affected by NaN_3 (Fig. 3). However, it was shown to be affected by styraxjaponoside A and B, regardless of the presence of NaN_3. This result indicated that the activity of styraxjaponoside A and B was independent to the metabolic inhibitor like NaN_3, suggesting that their activities were mediated by a cellular function, which does not need energy consumption (Lee *et al*., 2009).

Action mechanisms of styraxjaponoside A and B

The well-known antifungal agent, amphotericin B is thought to form membrane-spanning channels which ultimately result in fungal cell death (Kleinberg, 2006). To understand the action mechanisms of the two lignan glycosides, a series of experiments measuring the membrane-active activity of the compounds were conducted. The morphological effects of the compounds on *C. albicans* were investigated by flow cytometric analysis plotting the forward scatter and side scatter of the treated and untreated cells. As shown in Fig. 4, in the absence of the compounds, a control population of undamaged cells was observed. In the cultures treated with the compounds, the population of damaged and permeabilized cells, showing decreased FS, became dominant. This indicates that the damage by the compounds in cellular membrane resulted in decrease in cell size (Zelezetsky *et al*., 2005).

The effect of the compounds against biomembranes was confirmed by staining it with $DiBAC_4(3)$. $DiBAC_4(3)$, which is also used as a motality dye to evaluate antimicrobial susceptibility has a high voltage sensitivity and enters depolarized cells, where localized in the membrane

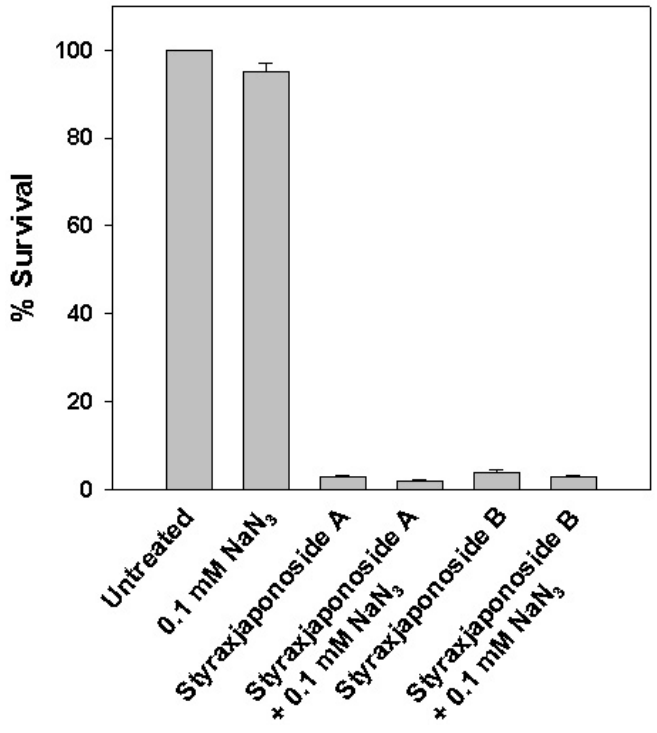

Fig. 3. The effect of sodium azide (NaN_3) on antifungal activity of styraxjaponoside A and B against *C. albicans*. The error bars represent the standard deviation (SD) values for three independent experiments.

(Liao *et al*., 1999). As shown in Fig. 5, the styraxjaponoside A and B-treated cells showed very weak fluorescence compared to CCCP (Lou *et al*., 2007), an inhibitor of the cell membrane potential. It was speculated that the membrane defects caused by the compounds resulted in the disruption of the structure of the membrane, and the dye might then be released from the membrane.

To characterize the extent of membrane-disruption in cells by the compounds, changes in membrane dynamics were examined with a fluorescent membrane probe, DPH. The DPH is a hydrophobic molecule, and this characteristic allows it to associate with the hydrocarbon tail region of phospholipids, within the cytoplasmic membrane, without disturbing the structure of the lipid bilayer (Vincent *et al*., 2004). 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 (Fig. 6). This reduction of DPH fluorescence intensity corresponded to the antimicrobial effect of the compounds against *C. albicans* cells by perturbing the plasma membrane. The results of the membrane-active activity suggested that the compounds were likely to have membrane-disruption mechanisms.

Recently, it has been reported that styraxjaponoside C isolated from the stem bark of *S. japonica* had antifungal activity *via* membrane-active mechanism (Park *et al*., 2010). Being based on this fact, we investigated the antimicrobial

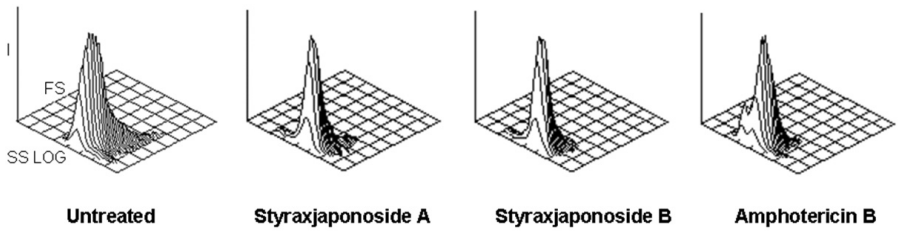

Fig. 4. Three-dimensional flow cytometric contour-plot analysis of *C. albicans* protoplasts treated with styraxjaponoside A, styraxjaponoside B or amphotericin B. FS (y-axis) is an indicator of size and SS (90° scattering, SS LOG, x-axis) is an indicator of granularity. The z-axis represents the cellular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

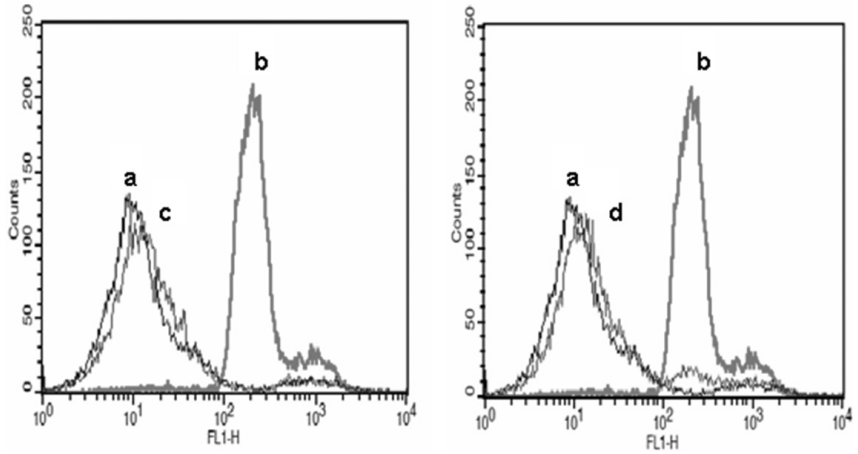

Fig. 5. Flow cytometric analysis of $DiBAC_4(3)$ staining in *C. albicans*. *C. albicans* (2×10^4 cells/ml, in YPD) was incubated for 3 h with the compounds; (a) control, (b) CCCP, (c) styraxjaponoside A, (d) styraxjaponoside B.

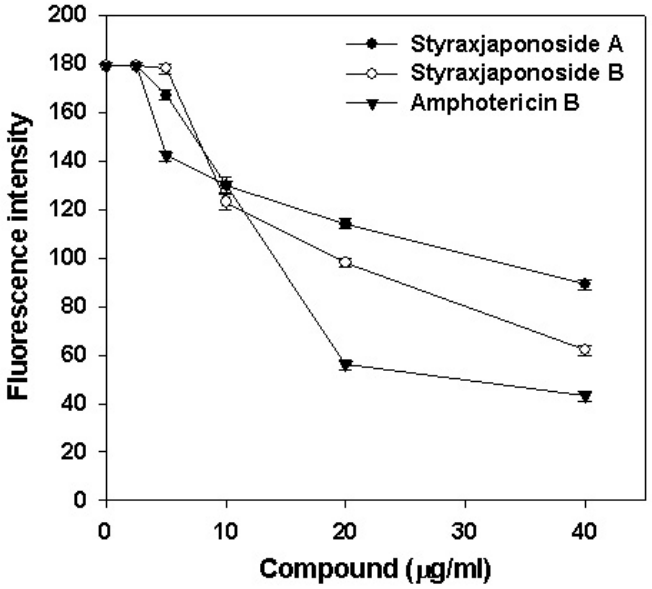

Fig. 6. DPH fluorescence analysis of *C. albicans* treated with the compounds. The error bars represent the standard deviation (SD) values for three independent experiments.

effect and action mechanisms of styraxjaponoside A and B. Without any hemolytic activity, the compounds exhibited an antimicrobial effect against human pathogens. Although the exact mechanisms of the compounds at the molecular level are still elusive, the effectivity of the antimicrobial activity of the compound could be correlated with its capacity to disrupt the microbial membrane.

ACKNOWLEDGMENTS

This work was supported by the Marine and Extreme Genome Research Center Program of the Ministry of Land, Transportation and Maritime Affairs, Republic of Korea.

REFERENCES

Ayres, D. and Loike, J. D. (1990). *Chemistry and pharmacology of natural products. Lignans: chemical, biological and clinical properties.* Cambridge University Press., Cambridge.

Butler, M. S. and Buss, A. D. (2006). Natural products-the future scaffolds for novel antibiotics. *Biochem. Pharmacol.* **71**, 919-929.

Davies, J. (2007). Microbes have the last word. A drastic re-evaluation of antimicrobial treatment is needed to overcome the threat of antibiotic-resistant bacteria. *EMBO Rep.* **8**, 616-621.

Fox, J. L. (2006). The business of developing antibacterials. *Nat. Biotechnol.* **24**, 1521-1528.

Fröhlich, K. U. and Madeo, F. (2000). Apoptosis in yeast--a monocellular organism exhibits altruistic behaviour. *FEBS Lett.* **473**, 6-9.

Jain, B., Martin, E., Stueben, A. and Bhakdi, S. (1995). Susceptibility testing of *Candida albicans* and *Aspergillus species* by a simple microtiter menadione-augmented 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. *J. Clin. Microbiol.* **33**, 661-667.

Kim, M. R., Moon, H. I., Chung, J. H., Moon, Y. H., Hahm, K. S. and Woo, E. R. (2004). Matrix metalloproteinase-1 inhibitor from the stem bark of *Styrax japonica* S. et Z. *Chem. Pharm. Bull.* **52**, 1466-1469.

Kleinberg, M. (2006). What is the current and future status of conventional amphotericin B? *Int. J. Antimicrob. Agents.* **27**, 12-16.

Lee, J., Choi, Y., Woo, E. R. and Lee, D. G. (2009). Isocryptomerin, a novel membrane-active antifungal compound from Selaginella tamariscina. *Biochem. Biophys. Res. Commun.* **379**, 676-680.

Lee, J. and Lee, D. G. (2009). Antifungal properties of a peptide derived from the signal peptide of the HIV-1 regulatory protein, Rev. *FEBS Lett.* **583**, 1544-1547.

Liao, R. S., Rennie, R. P. and Talbot, J. A. (1999). Assessment of the effect of amphotericin B on the vitality of *Candida albicans. Antimicrob. Agents Chemother.* **43**, 1034-1041.

Lou, P. H., Hansen, B. S., Olsen, P. H., Tullin, S., Murphy, M. P. and Brand, M. D. (2007). Mitochondrial uncouplers with an extraordinary dynamic range. *Biochem. J.* **407**, 129-140.

Okamoto-Shibayama, K., Yutaka, S. and Toshifumi A. (2010). Resveratrol Impaired the Morphological Transition of Candida albicans Under Various Hyphae-Inducing Conditions. *J. Microbiol. Biotechnol.* **20**, 942-945.

Park, C., Woo E. R. and Lee D. G. (2010). Anti-Candida property of a lignan glycoside derived from *Styrax japonica* S. et Z. via membrane-active mechanisms. *Mol. Cells* **29**, 581-584.

Projan, S. J. and Shlaes, D. M. (2004). Antibacterial drug discovery: is it all downhill from here? *Clin. Microbiol. Infect.* **10**, 18-22.

Saleem, M., Kim, H. J., Ali, M. S. and Lee, Y. S. (2005). An update on bioactive plant lignans. *Nat. Prod. Rep.* **22**, 696-716.

Saleem, M., Nazir, M., Ali, M. S., Hussain, H., Lee, Y. S., Riaz, N. and Jabbar, A. (2010). Antimicrobial natural products: an update on future antibiotic drug candidates. *Nat. Prod. Rep.* **27**, 238-254.

Veerman, E. C., Valentijn-Benz, M., Nazmi, K., Ruissen, A. L., Walgreen-Weterings, E., Van Marle, J., Doust, A. B., Van't Hof, W., Bolscher, J. G. and Amerongen, A. V. (2007). Energy depletion protects Candida albicans against antimicrobial peptides by rigidifying its cell membrane. *J. Biol. Chem.* **282**, 18831-18841.

Vincent, M., England, L. S. and Trevors, J. T. (2004). Cytoplasmic membrane polarization in Gram-positive and Gram-negative bacteria grown in the absence and presence of tetracycline. *Biochim. Biophys. Acta.* **1672**, 131-134.

Wilson, D. F. and Chance, B. (1967). Azide inhibition of mitochondrial electron transport. I. The aerobic steady state of succinate oxidation. *Biochim. Biophys. Acta.* **131**, 421-430.

Zelezetsky, I., Pacor, S., Pag, U., Papo, N., Shai, Y., Sahl, H.-G. and Tossi, A. (2005). Controlled alteration of the shape and conformational stability of alpha-helical cell-lytic peptides: effect on mode of action and cell specificity. *Biochem.* J. **390**, 177-188.