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Antifungal effect and mode of action of glochidioboside against *Candida albicans* membranes

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ABSTRACT

Glochidioboside was obtained from *Sambucus williamsii* and its biological effect has not been reported. Its antifungal activity against pathogenic fungi and the mode of action involved in its effect were examined. Glochidioboside exerted antifungal effect with almost no hemolytic effect against human erythrocytes. To understand its antifungal mechanisms, membrane studies were done. Using two dyes, 3,3′-dipropylthiacarbocyanine iodide [$DiSC_3(5)$] and propidium iodide, membrane depolarization and permeabilization by glochidioboside were confirmed. Furthermore, the membrane-active mechanism was proven by synthesizing a model membrane, calcein-encapsulating large unilamellar vesicles (LUVs), and also by observing the influx of different sized fluorescent dyes, such as calcein, FD4 and FD10, into the fungal cells. The membrane-active action was pore-forming action with radii between 1.4 and 2.3 nm. Finally, three dimensional (3D) flow cytometric analysis showed the shrinkage of the fungal cells from the membrane damage. In conclusion, this study suggests that glochidioboside exerts an antifungal activity through a membrane-disruptive mechanism.

1. Introduction

The importance of antifungal agents is continually highlighted by increasing invasive fungal diseases. However, the discovery and development of antifungal drugs is extremely slow [1,2]. To date, only a few groups of antifungal drugs such as polyenes, azoles and echinocandins have been used. Among them, azoles and polyenes, the largest group of antifungal drugs, act on ergosterol in the fungal membrane, but each has a different mechanism. Azoles inhibit ergosterol biosynthesis and polyenes disrupt the fungal membrane by binding to ergosterol. Additionally, echinocandins inhibit the synthesis of glucan in the fungal cell wall [2].

However, incurable fungi, such as azole-resistant *Candida* spp. seem to be common nowdays. Even amphotericin B-resistant *Candida* and *Cryptococcus* strains have been reported [3]. Partients infected by antibiotics-resistant organisms continually fight the infection but it is difficult to treat fungal infections due to delays in diagnosis, restrictions in the route of administration and cytotoxicity of the drugs [1,4,5]. In view of this situation, the discovery of novel antifungal agents that are safer is necessary [2,5].

Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, flavonoids, lectins [6–8] and lignans [9], which have been found *in vitro* to have antimicrobial properties [10]. These compounds called phytochemicals serve as the plant immune system against microorganisms, insects, and herbivores [11]. Phytochemicals are an important natural resource for the discovery of antifungal agents because of their long-term use without human toxicity [12]. The herb *Sambucus williamsii* is a folk plant with medicinal properties. The genus *Sambucus*, widely found in Europe, Asia and North Africa, has been used in pharmaceutical products as an analgesic, antivirus, anti-inflammatory, homoeostatic, and diuretic drugs, which act on bruises, fractures, and edema by traditional users [12,13]. In this study, glochidioboside, a neolignan glucoside, was derived from *S. williamsii*. To date, glochidioboside has been extracted from *Acer truncatum* and *Glochidion obovatum* [14,15]. However, its bioactive property has not been reported. Therefore, its antifungal effect and mode of action(s) were investigated.

Abbreviations: MeOH, methanol; CH_2Cl_2, dichloromethane; EtOAc, ethyl acetate; *n*-BuOH, *n*-butanol; ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; KRIBB, Korea Research Institute of Bioscience and Biotechnology; MIC, minimum inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PBS, phosphate buffered saline; $DiSC_3(5)$, 3,3′-dipropylthiacarbocyanine iodide; LUV, large unilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; 3D, three dimensional; FSC, forward scatter; SSC, side scatter.

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2. Materials and methods

2.1. Extraction and isolation of glochidioboside

The air-dried stem bark of *S. williamsii* (840 g) was cut and extracted with methanol (MeOH) at 80 °C for 4 h. The MeOH extract (57.1 g) was suspended in water and then partitioned sequentially with equal volumes of dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH). The EtOAc fraction (4.2 g) was subjected to column chromatography over a HP-20 column by eluting it with a MeOH:H_2O (5:95 → 25:75 → 40:60 → 100:0) gradient system. Based on their TLC pattern, the fractions were combined to yield subfractions, which were designated as E1–E4. The subfraction E1 (1.49 g) was then purified by column chromatography over a HP-20 column with a MeOH:H_2O (0:100 → 50:50 → 15:85 → 100:0) gradient system to yield four subfractions (E1-1–E1-4). The subfraction E1-1 was subjected to repeated column chromatography (Silica gel, $CHCl_3$:MeOH:H_2O, 10:1:0.1 → 1:1:0.1) to yield glochidioboside (2.3 mg). The physico-chemical data including ^{1}H NMR, ^{13}C NMR, and HSQC of the glochidioboside were identical with those reported in the literature [14,16].

2.2. Antifungal activity assay

Candida albicans (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Trichosporon beigelii* (KCTC 7707) and *Malassezia furfur* (KCTC 7744) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIBB) (Daejeon, Korea). The fungal strains were cultured in YPD broth (Difco) with aeration at 28 °C, and *M. furfur* was cultured at 32 °C in a modified YM broth (Difco) containing 1% olive oil. Fungal cell suspensions were adjusted to obtain standardized populations by measuring the turbidity with a spectrophotometer (DU530; Beckman, Fullerton, CA, USA). Fungal cells (1×10^6 cells/ml) were incubated in 0.1 ml/wells containing YPD or YM broth. The minimum inhibitory concentrations (MICs) were determined with a twofold serial dilution of the test compounds, based on the Clinical and Laboratory Standards Institute (CLSI) method [17] and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [18]. After 48 h of incubation, the minimal concentration of the compounds required to prevent the growth of the microorganisms was determined, and defined as the MIC. The growth was measured with a microtiter ELISA Reader (Molecular Devices Emax, CA, USA) by monitoring the absorption at 580 nm. The MIC values were determined by three independent assays [19].

2.3. Hemolytic activity assay

Fresh human erythrocytes were centrifuged at 2000 rpm for 10 min and washed three times with phosphate buffered saline (PBS: 35 mM phosphate buffer/150 mM NaCl, pH 7.4). The final concentration of the erythrocytes was 4%. The erythrocyte suspension was transferred to sterilized 96-well plates and twofold serial dilutions of the compound were added to the wells of a 96-well plate. The samples were then incubated with the compounds at 37 °C for 1 h and the plate was centrifuged at 1500 rpm for 10 min. An aliquot of the supernatant was taken, and then, the hemolytic activity of the compounds was evaluated by measuring the release of hemoglobin from a 4% suspension of human erythrocytes at 414 nm with an ELISA reader. Hemolytic levels of zero and 100% were determined in PBS alone and with 0.1 Triton X-100, respectively. The hemolysis percentage was calculated with 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 [20].

2.4. Detection of changes in membrane electrical potential

C. albicans cells (1×10^6 cells/ml) were washed with Ca^{2+} and Mg^{2+} free PBS and changes in the membrane potential were measured with membrane potential sensitive probe 3,3′-dipropylthiacarbocyanine iodide [$DiSC_3(5)$]. Changes in fluorescence due to the collapse of the cytoplasmic membrane potential by 6.5 μg/ml of compounds were continuously monitored using a spectrofluorophotometer (Shimadzu, RF-5301PC, Shimadzu, Kyoto, Japan) at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. The experiment was repeated three times under each condition to ensure reproducibility [21].

2.5. Propidium iodide influx assay

To analyze fungal membrane permeabilization after treatment with the compound, *C. albicans* cells (1×10^6 cells/ml), suspended in PBS, were treated with 6.5 μg/ml of the compounds and incubated for 4 h at 28 °C. After incubation, the cells were harvested by centrifugation and resuspended in PBS. Subsequently, the cells were treated with 6 μM of propidium iodide and incubated for 5 min at room temperature. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) [22].

2.6. Calcein leakage measurement

Large unilamellar vesicles (LUVs) encapsulating calcein, composed of phosphatidylcholine (PC)/phosphatidylethanolamine (PE)/phosphatidylinositol (PI)/ergosterol (5:4:1:2, w/w/w/w), were prepared by vortexing dried lipids in a dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA [pH 7.4]). The suspension was freeze-thawed in liquid nitrogen 13 times and extruded through polycarbonate filters (two stacked 200 nm pore size filters) with a LiposoFast extruder (Avestin Inc., Ottawa, Canada). Untrapped calcein was removed using a gel filtration process on a Sephadex G-50 column. For the assay, a suspension of liposomes including calcein was treated with the compounds. The mixture (1 ml, final volume) was stirred for 10 min in the dark and centrifuged at 13,000 rpm for 10 min. The supernatant was transferred, and the release of calcein from the LUVs was monitored at 25 °C by measuring the fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm with a spectrofluorophotometer (Shimadzu, RF-5301PC, Shimadzu, Kyoto, Japan). Twenty microliters of 10% Triton X-100 was added to the vesicles to determine 100% calcein leakage. The percentage of calcein leakage caused by the compounds was calculated as follows: calcein leakage (%) = $100 \times (F - F_0)/(F_t - F_0)$, where F represents the fluorescence intensity achieved after addition of the compounds and F_0 and F_t represent the fluorescence intensities without the compounds and with Triton X-100, respectively [23].

2.7. Estimation of the pore size in C. albicans membrane

C. albicans cells (1×10^6 cells/ml) were suspended in PBS, treated with 6.5 μg/ml of the compound and incubated for 4 h at 28 °C. After incubation, the cells were harvested by centrifugation and resuspended in PBS. Subsequently, the soluble fluorescent molecules calcein, FD4 and FD10, were added to the *C. albicans* cells to a final concentration of 2 μM, 0.1, and 0.1 mg/ml, respectively [24]. All FDs were purchased from Sigma Chemical Co. (USA). The

influx of the fluorescent molecules was observed with a fluorescent microscope (Nikon eclipse Ti-S, Japan).

2.8. Three dimensional (3D) flow cytometric contour plot analysis

C. albicans cells (1×10^6 cells/ml), suspended in PBS, were treated with 6.5 μg/ml of the compounds and incubated for 4 h at 28 °C. After incubation, the cells were harvested by centrifugation and resuspended in PBS. The morphological changes were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Non-stained living cells were evaluated for each sample by excitation with 488 nm light from an argon ion laser by determining their position on a forward scatter (FSC) versus side scatter (SSC) contour plot [22].

3. Results and discussion

3.1. Antifungal and hemolytic effects of glochidioboside

Antimicrobial and anti-inflammatory effects of phytochemicals have been taken advantage of humans due to low human toxicity [25–27]. In this sense, the antifungal properties of glochidioboside, which is a phytochemical was derived from *S. williamsii*, were investigated. First, Glochidioboside possessed an inhibitory effect against various pathogenic fungi including *C. albicans*, *C. parapsilosis*, *T. beigelii*, and *M. furfur*. They are representative fungal strains which cause diverse infections in humans. Particularly, *C. albicans*, one of the fungal strains against which the susceptibility to glochidioboside was tested, is not only the most widespread fungal pathogen but also the primary cause of candidiasis [28,29]. Therefore, *C. albicans* was selected as a model organism for the experiments in this study. Amphotericin B used as a positive control is widely known to form pores, by binding the sterol in the cell membrane, and then, the immediate action induces cell death [30]. In antifungal activity assay, the fungal strains are highly susceptible to glochidioboside with a MIC range of 3.3–6.5 μg/ml (Table 1). Amphotericin B shows a more potent activity with a range of 1.4–2.9 μg/ml.

Furthermore, to observe hemolytic effect of glochidioboside against human erythrocytes, the release of hemoglobin was measured. As shown in Table 1, glochidioboside has no hemolytic activity until concentration of 50 μM whereas amphotericin B showed 100% of hemolysis at 50 μM. These results suggest that glochidioboside has more activity against fungal cells compared to human erythrocytes (Table 1).

3.2. Membrane damage induced by glochidioboside

The targets of antifungal agents are various such as membranes, cell walls, nuclei and microtubules. Considering most antifungal agents possess a membrane-active action [31], the effect of glochidioboside was investigated as to whether it can affect the functions of the fungal plasma membrane. $DiSC_3(5)$ is a membrane potential sensitive fluorescent probe and detects changes in the membrane potential. Its fluorescence intensity is increased due to membrane depolarization by the compound [32,33]. After quenching the fluorescence of the $DiSC_3(5)$, treatment with glochidioboside (at 180 s) resulted in a gradually increase in the fluorescence intensity, whereas amphotericin B resulted in a rapid increase in the fluorescence intensity, indicating membrane depolarization. Additionally, NaCl, as a negative control, was added, and an increase in the fluorescence intensity was not observed (Fig. 1A). The results support that glochidioboside has the ability to dissipate the cytoplasmic membrane potential of *C. albicans*.

Generally, the disruption of intracellular electrochemical gradients is associated with an increase in plasma membrane permeability [34]. Hence, change in *C. albicans* membrane permeability induced by the addition of glochidioboside was determined using flow cytometry analysis with propidium iodide. Propidium iodide penetrates cells with membrane damage and then fluoresces [35–37]. In the flow cytometric analysis, 14.9% and 75.0% of the cells treated with glochidioboside and amphotericin B showed an influx of propidium iodide, respectively, compared to the untreated cells (Fig. 1B). In other words, $DiSC_3(5)$ assay and propidium iodide influx assay certainly suggest that glochidioboside causes membrane damage such as membrane depolarization and permeabilization.

Table 1
The antifungal and hemolytic effect of glochidioboside and amphotericin B.

Cells	MIC (μg/ml)	
	Glochidioboside	Amphotericin B
C. albicans ATCC90028	6.5	2.9
C. parapsilosis ATCC22019	3.3	1.4
T. beigelii KCTC7707	6.5	1.4
M. furfur KCTC7744	6.5	2.9
Human erythrocyte cells	0%[a]	100%[a]

[a] Percentage of hemolytic effect induced by the compound at 50 μM concentration.

3.3. Pore-forming action of glochidioboside

Propidium iodide influx is considered pore formation in the cell membrane [38]. Therefore, the membrane-active mechanism of glochidioboside was examined by forming a model membrane. LUV mimics the outer leaflets of the plasma membrane of *C. albicans* composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w). The interaction between liposomes and various substances is tested by measuring the leakage of internal contents such as fluorescent probes from a LUV suspension [39]. Calcein leakage caused by glochidioboside and amphotericin B at 6.5 μg/ml was 33.8% and 58.3%, respectively, and occurred in a dose-dependent manner. The level of calcein leakage by glochidioboside was lower than that of amphotericin B, which is in agreement with the results from a previous experiment (Fig. 2A). The leakage of water soluble fluorescent probes, such as calcein, from the LUVs induced by a substance means the formation of pores in the lipid membrane [40,41]. This calcein leakage from the LUVs clearly suggests that the membrane-destabilizing effect of glochidioboside is through pore formation in the fungal membrane.

To determine the size of the pores caused by glochidioboside in the membrane, we observed the fluorescent influx into viable *C. albicans* cells using the water soluble fluorescent molecules, such as calcein, FD4 and FD10 which have different radii. After the addition of glochidioboside to the *C. albicans* cells, the influx of calcein (average molecular weight = 623 Da, Stokes–Einstein radius = 0.74 nm) [42] and FD4 (average molecular weight = 4 kDa, Stokes–Einstein radius = 1.4 nm) were observed. However, FD10 (average molecular weight = 10 kDa, Stokes–Einstein radius = 2.3 nm) [43] did not enter the cytoplasm of the cells (Fig. 2B). The radii of the pores formed by glochidioboside could be between 1.4 and 2.3 nm. Amphotericin B caused the leakage of all fluorescent dyes and the result indicates that the pore size by amphotericin B is bigger than 2.3 nm (Fig. 2B). The size of the pore induced by glochidioboside is smaller than a ribosome, the smallest cell organelle; thus, it has no alternative but to allow only the migration of just ions and small molecules. Taken together, when *C. albicans* was exposed to glochidioboside, pores were formed in its membrane, resulting in membrane permeabilization and depolarization. With membrane damage like pore formation, it is hard to maintain important functions of the cell membrane to selectively

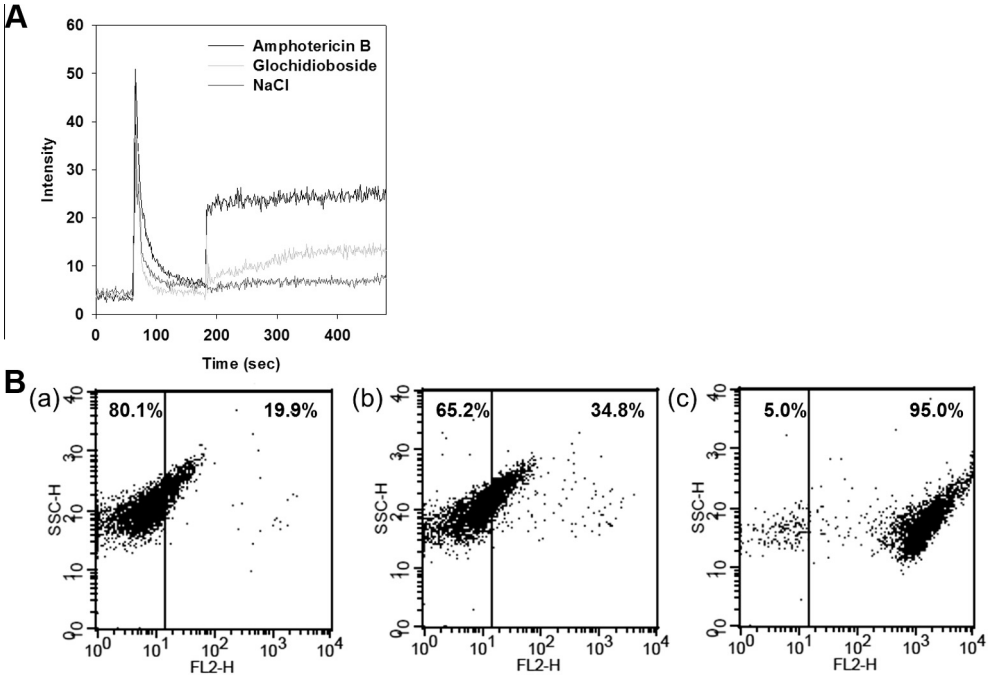

Fig. 1. (A) Depolarization of the membrane potential was detected by $DiSC_3(5)$. $DiSC_3(5)$ was added at $t = 60$ s. After internalization of the probe, at $t = 180$ s, 6.5 μg/ml of the compounds were added to monitor the changes in fluorescence (Ex. 622 nm and Em. 670 nm). (B) Flow cytometric analysis of membrane permeabilization detected by the propidium iodide influx assay. *C. albicans* cells (1×10^6 cells/ml) were treated with 6.5 μg/ml of the compounds for 4 h at 28 °C. (a) Untreated cells, (b) cells treated with glochidioboside and (c) cells treated with amphotericin B.

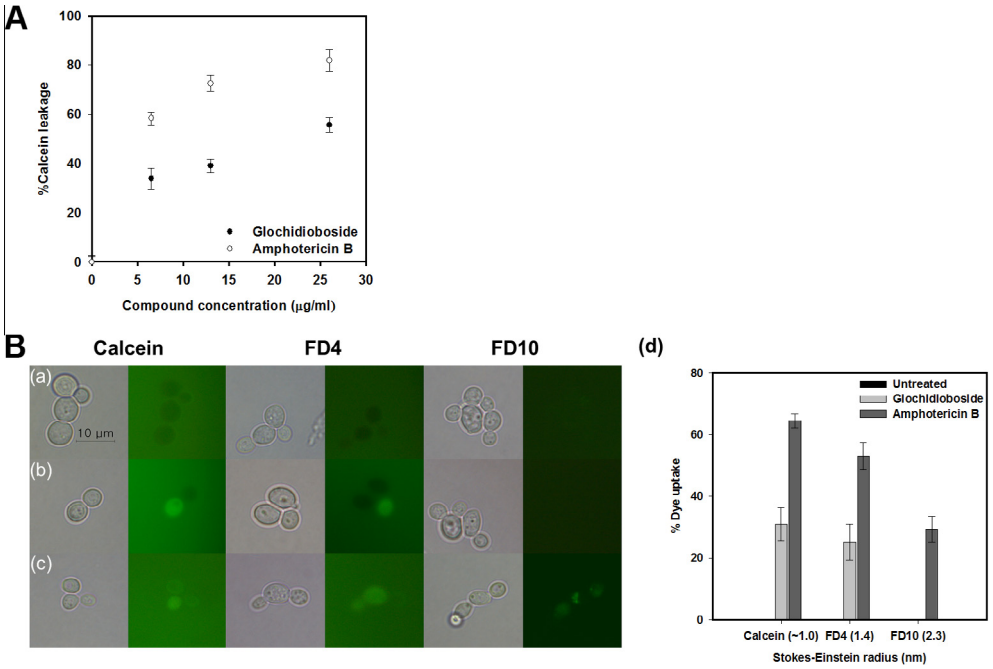

Fig. 2. (A) Calcein leakage from LUVs composed of PC/PE/PI/ergosterol (5:4:1:2, w/w/w/w), by glochidioboside (6.5, 13.0, 26.0 μg/ml). The data represent the mean ± SD for three independent experiments. (B) Determination of the size of the pores formed by 6.5 μg/ml of glochidioboside in the membranes of *C. albicans*. (a) Untreated cells, (b) cells treated with glochidioboside, (c) cells treated with amphotericin B and (d) influx percentage of fluorescent dye into *C. albicans* cells. The data represent the mean ± SD for three independent experiments.

exclude detrimental compounds and retain valuable metabolites [44].

3.4. Shrinkage of *C. albicans* cells by glochidioboside

Morphological changes in the cells were investigated by flow cytometric analysis plotting the FSC (cell size, *x*-axis) and SSC (granularity, *y*-axis) of compound-treated and -untreated cells. The result shows that in cells treated with glochidioboside and amphotericin B, 18.0% and 37.9% of the cell population showed decrease in FSC values, respectively, compared to the untreated cells, indicating cell shrinkage (Fig. 3). Generally, cell shrinkage is caused by various reasons such as the consequence of apoptosis, inhibition of biomass production and damage to the cell membrane

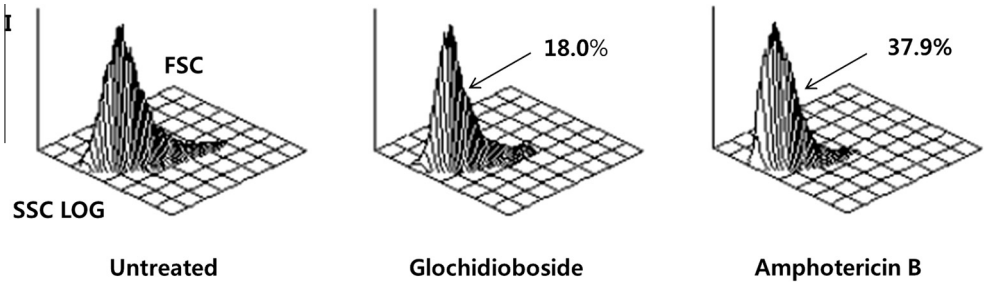

Fig. 3. Three dimensional (3D)-flow cytometric contour-plot analysis of *C. albicans* treated with 6.5 μg/ml of glochidioboside for 4 h at 28 °C. FSC is an indicator of cell size and SSC is an indicator of cell granularity. I represents the cellular population intensity.

[21,45,46]. From the above-mentioned experiments, the results strongly support that glochidioboside decreases the size of the cells due to the collapse of the electrochemical gradient balance in membrane.

In conclusion, glochidioboside was derived from *S. williamsii* and it exerted a significant antifungal activity against pathogenic fungi with low hemolysis. Further membrane studies suggested that glochidioboside exhibited a membrane-disruptive mechanism.

Acknowledgments

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