

Antifungal Activity of C-27 Steroidal Saponins

Chong-Ren Yang,¹ Ying Zhang,¹ Melissa R. Jacob,² Shabana I. Khan,² Ying-Jun Zhang,^{1*}
and Xing-Cong Li^{2*}

State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, People's Republic of China,¹ and National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi, University, Mississippi 38677²

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As part of our search for new antifungal agents from natural resources, 22 C-27 steroidal saponins and 6 steroidal sapogenins isolated from several monocotyledonous plants were tested for their antifungal activity against the opportunistic pathogens *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. The results showed that the antifungal activity of the steroidal saponins was associated with their aglycone moieties and the number and structure of monosaccharide units in their sugar chains. Within the 10 active saponins, four tigogenin saponins (compounds 1 to 4) with a sugar moiety of four or five monosaccharide units exhibited significant activity against *C. neoformans* and *A. fumigatus*, comparable to the positive control amphotericin B. The antifungal potency of these compounds was not associated with cytotoxicity to mammalian cells. This suggests that the C-27 steroidal saponins may be considered potential antifungal leads for further preclinical study.

Opportunistic fungal infections, mainly resulting from *Candida*, *Cryptococcus*, and *Aspergillus*, are life-threatening in immunocompromised patients (with AIDS, cancer, or organ transplant) (21). The current antifungal armamentarium includes five major drug classes: polyenes (interacting with fungal ergosterol, thereby disrupting the cytoplasmic membrane), azoles (inhibiting 14 α -lanosterol demethylase in ergosterol biosynthesis pathway), allylamines (inhibiting squalene epoxidase in ergosterol synthesis pathway), echinocandins (inhibiting synthesis of β -1,3-glucan, which is a required component of the cell wall of many fungi), and flucytosine (incorporated into RNA and thus inhibiting DNA synthesis) (27). Representatives from the five classes are amphotericin B (AMB), fluconazole (FLU), terbinafine, caspofungin, and flucytosine, respectively. However, each drug class suffers one or more major shortcomings (for example, significant dose-limiting toxicities for AMB, rapid development of resistance for the azole drugs, limitation of the use to dermatophytoses for terbinafine, and lack of effectiveness in cryptococcosis for caspofungin) (20, 23, 24). This has highlighted the need to discover new antifungal agents, preferably with new modes of action.

C-27 steroidal saponins are an important class of natural products which are probably best known as starting materials for the synthesis of steroidal hormones. A C-27 steroidal saponin is composed of a C-27 aglycone moiety (or referred to as a C-27 steroidal sapogenin) and a sugar chain of one or more monosaccharides. These compounds are further classified as two submajor classes: spirostanol saponins with a hexacyclic

ABCDEF-ring system, as shown for the compounds in Fig. 1, and furostanol saponins with an open F-ring skeleton, which are considered biosynthetic precursors of the former. According to conservative statistics, there are at least 90 plant families containing such compounds, particularly in monocotyledons (Liliaceae, Convallariaceae, Dioscoreaceae, Agavaceae, Smilacaceae, and Amaryllidaceae, etc.) (31). The antifungal activity of steroidal saponins, particularly against agricultural pathogens, has been known for a long time (5, 7, 34, 35), while other reported activities for this class of compounds include antitumor, hypoglycemic, immunoregulatory, and cardiovascular disease prevention and treatment (31). Although saponins are generally considered to possess detergent-like surfactant properties, the classic viewpoint that saponins make poor drug candidates has been challenged in recent years. Several steroidal saponin-based drugs have been seen on the market. For example, "Di-ao-xin-xue-kang," which has an ingredient composition of several steroidal saponins from *Dioscorea panthaica* administered orally, has dominated the market of drugs for the treatment and prevention of cardio- and cerebrovascular diseases in China for a decade (6, 18, 38). Another steroidal saponin-based drug, "Chuan-shan-long injection," made from *Dioscorea nipponica* and used for rheumatism may be administered intraperitoneally (19). In addition, numerous patents on the therapeutic potentials of steroidal saponins, such as for hepatitis (28) and fungal infections (3, 4, 22, 29) have been released. The above information indicates that some steroidal saponins do have low toxicity and may become therapeutic agents. This prompted us to explore the potential of this class of compounds as antifungal leads for the development of systemic antifungal drugs.

Previous studies indicate that, when spirostanol saponins show antifungal activity, their corresponding furostanol saponins usually show little to no activity (30, 37). Thus, from the perspective of structure-activity relationships (SAR), 22 steroidal saponins with a spirostanol skeleton and 6 steroidal sapogenins isolated from several monocotyledonous plants (8–16)

* Corresponding author. Mailing address for Ying-Jun Zhang: State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, CAS, Kunming 650204, People's Republic of China. Phone: 86 871 5223235. Fax: 86 871 5150124. E-mail: zhangyj@mail.kib.ac.cn. Mailing address for Xing-Cong Li: National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi, University, MS 38677. Phone: (662) 915-6742. Fax: (662) 915-7989. E-mail: xcli7@olemiss.edu.

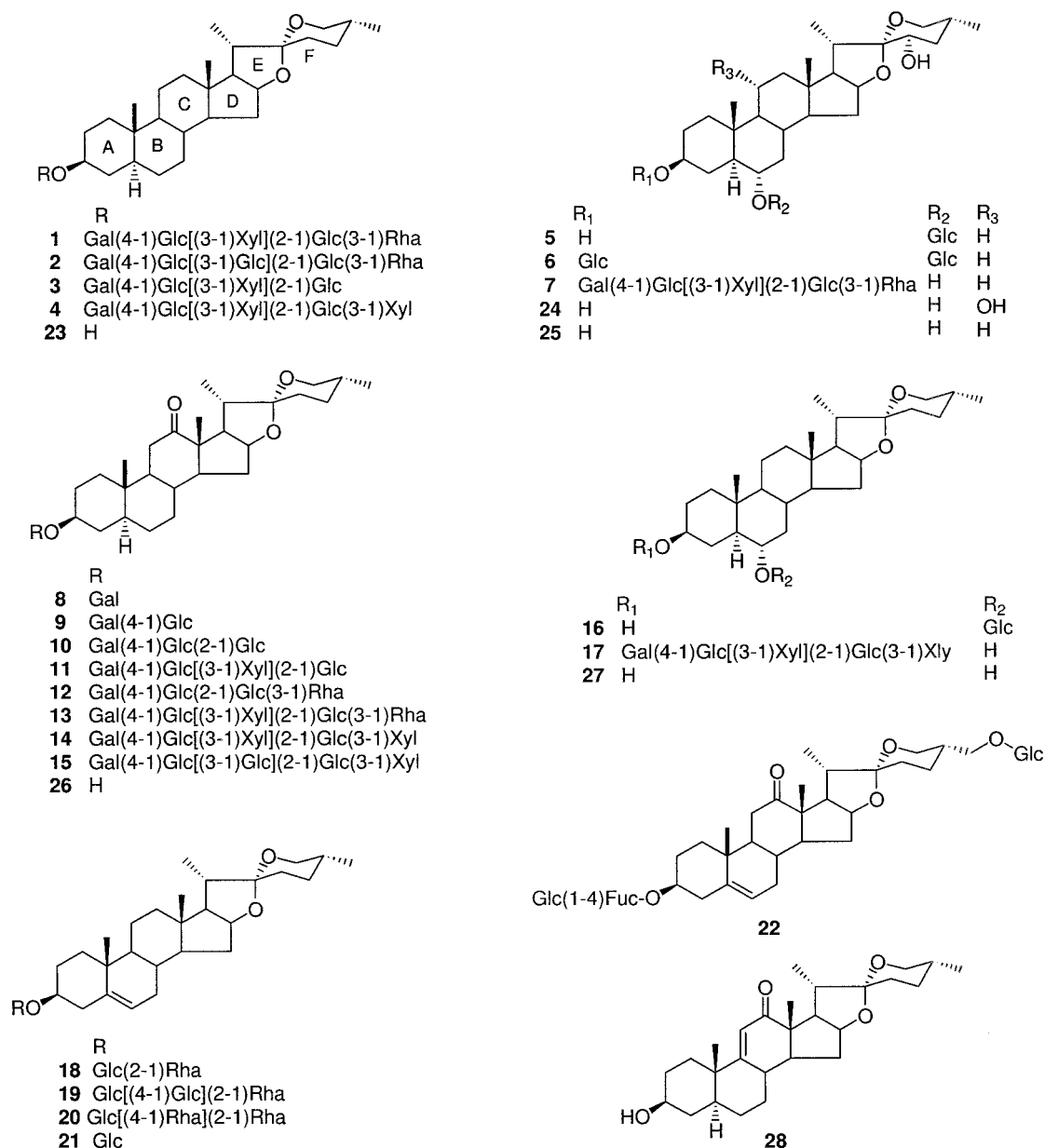


FIG. 1. Structures of compounds 1 to 28.

and belonging to different chemotypes were selected for this study. This selection was particularly based on the structures of several saponins containing hecogenin, neohecogenin, tigogenin, neotigogenin, chlorogenin, or diosgenin as an aglycone moiety that have been demonstrated to be antifungal (1, 30, 32, 37, 39).

MATERIALS AND METHODS

Chemicals and materials. Twenty-two steroidal saponins (compounds 1 to 22) and six steroidal saponinins (compounds 23 to 28) for antifungal testing were isolated from *Agave americana*, *Polianthes tuberosa*, *Polygonatum zanlanscianense*, and *Dioscorea parviflora* by the group at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences (8–16). These include (25*R*)-5 α -spirost-3 β -hydroxy-3-*O*- α -L-rhamnopyranosyl-(1-3)- β -D-glucopyranosyl-(1-2)-[β -D-xylopyranosyl-(1-3)]-

β -D-glucopyranosyl-(1-4)- β -D-galactopyranoside (compound 1) (10), agamenoside G (compound 2) (10), degalactotigonin (compound 3) (15), (25*R*)-5 α -spirost-3 β -hydroxy-3-*O*- β -D-xylopyranosyl-(1-3)- β -D-glucopyranosyl-(1-2)-[β -D-xylopyranosyl-(1-3)]- β -D-glucopyranosyl-(1-4)- β -D-galactopyranoside (compound 4) (15), agamenoside C (compound 5) (9), cantalasaponin 1 (compound 6) (9), agamenoside A (compound 7) (12), agavesides A (compound 8) (11) and B (compound 9) (11), (25*R*)-5 α -spirost-3 β -hydroxy-12-oxo-3-*O*- β -D-glucopyranosyl-(1-2)- β -D-glucopyranosyl-(1-4)- β -D-galactopyranoside (compound 10) (11), (25*R*)-5 α -spirost-3 β -hydroxy-12-oxo-3-*O*- β -D-glucopyranosyl-(1-2)-[β -D-xylopyranosyl-(1-3)]- β -D-glucopyranosyl-(1-4)- β -D-galactopyranoside (compound 11) (11), agamenosides D (compound 12) (11) and E (compound 13) (11), (25*R*)-5 α -spirost-3 β -hydroxy-12-oxo-3-*O*- β -D-xylopyranosyl-(1-3)- β -D-glucopyranosyl-(1-2)-[β -D-xylopyranosyl-(1-3)]- β -D-glucopyranosyl-(1-4)- β -D-galactopyranoside (compound 14) (15), agamenoside F (compound 15) (11, 15), (25*R*)-5 α -spirost-3 β ,6 α -diol-6-*O*- β -D-glucopyranoside (compound 16) (14), (25*R*)-5 α -spirost-3 β ,6 α -diol-3-*O*- β -D-xylopyranosyl-(1-3)- β -D-glucopyranosyl-(1-2)-[β -D-xylopyranosyl-(1-3)]- β -D-glucopyranosyl-(1-4)- β -D-galactopyranoside (compound 17) (15), prosapogenin A of

dioscin (compound 18) (16), deltonin (compound 19), dioscin (compound 20) (13), colletiside I (compound 21), polygonatoside A (compound 22) (16), tigenin (compound 23) (10), agavegenin A (compound 24) (8), hongguanggenin (compound 25) (9), hecogenin (compound 26) (11), chlorogenin (compound 27) (14), and 9(11)-dehydrohecogenin (compound 28) (14). AMB and doxorubicin, used as control drugs for antifungal and cytotoxicity testing, respectively, were purchased from ICN Biomedicals, Ohio. FLU, used as a control drug for antifungal testing, was obtained from Pfizer, Morris Plains, N.J.

Assay for antifungal activity. Reference strains were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and included *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906. Susceptibility testing was performed using a modified version of the CLSI (formerly NCCLS) methods (25, 26). Samples (dissolved in dimethyl sulfoxide) were serially diluted using 20% dimethyl sulfoxide in 0.9% saline and transferred in duplicate to 96-well flat-bottom microplates. *Candida* spp. and *C. neoformans* inocula were prepared by picking 1 to 3 colonies from agar plates and resuspending in ~5 ml 0.9% sterile saline. The optical density at 630 nm of the saline suspensions was compared to the 0.5 McFarland standard. The microorganisms were diluted in broth (RPMI 1640-2% dextrose-0.165 M morpholinepropane-sulfonic acid [MOPS] at pH 4.5 [Cellgro] for *Candida* spp. and Sabouraud dextrose [Difco] for *C. neoformans*) to afford final target inocula of 5.0×10^3 for *Candida* spp. and 5.0×10^5 for *C. neoformans*. The *A. fumigatus* inoculum was prepared by gently removing spores from an agar slant, transferring to ~5 ml 0.9% saline, and filtering through Miracloth (Calbiochem, La Jolla, CA). The filtrate was diluted appropriately (via comparison to a standard curve) in 5% Alamar blue (BioSource International, Camarillo, CA)-RPMI 1640 broth (2% dextrose buffered with 0.165 M MOPS at pH 7.3) to afford a final target inoculum of 4.0×10^4 CFU/ml. The fungal inocula were added to the samples to achieve a final volume of 200 μ l and final sample concentrations starting with 20 μ g/ml. Growth (saline only), solvent, and blank (media only) controls were included on each test plate. Fungicidal AMB and fungistatic FLU were included as positive controls. All organisms were read at 630 nm using the EL-340 Biokinetics reader (Bio-Tek Instruments, Vermont) prior to and after incubation (*Candida* spp. at 37°C for 18 to 24 h; *C. neoformans* and *A. fumigatus* at 30°C for 72 h), except *A. fumigatus*, which was read at 544-nm excitation/590-nm emission using the Polarstar Galaxy plate reader (BMG LabTechnologies, Germany). Percent growth was calculated and plotted versus test concentration to afford the IC_{50} (sample concentration that affords 50% growth of the organism). The MIC was defined as the lowest test concentration that allowed no detectable growth (or no more than 20% growth for FLU; for *A. fumigatus*, no color change from blue to pink). Minimum fungicidal concentrations (MFCs) were determined by removing 5 μ l from each clear (or blue) well, transferring to agar, and incubating as previously mentioned. The MFC was defined as the lowest test concentration that allows no growth of the organism on agar.

Assay for in vitro cytotoxicity. A panel of mammalian cells obtained from ATCC (Manassas, VA) included five human cancer cell lines (SK-MEL [melanoma], KB [epidermal carcinoma, oral], BT-549 [ductal carcinoma, breast], SK-OV-3 [ovary carcinoma], and HepG2 [human hepatic carcinoma]) and one noncancerous Vero cell line (African green monkey kidney fibroblast). The cells were cultured in 75-cm² culture flasks in RPMI 1640 medium (Gibco, Invitrogen Corp., CA) supplemented with bovine calf serum (10%) and amikacin (60 mg/liter), at 37°C in an atmosphere of 95% humidity, 5% CO₂. The assay was performed in 96-well microplates. Cells were seeded to the wells of the plate at a density of 25,000 cells/well and grown for 24 h at 37°C. Samples, diluted appropriately in RPMI 1640 medium, were added to the cells and again incubated for 48 h. The number of viable cells was determined by neutral red procedure (2). Briefly, after incubation with the samples, the cells were washed with saline and incubated for 90 min with the medium containing neutral red (166 μ g/ml). After washing the plate to remove extracellular dye, a solution of acidified isopropanol (0.33% HCl) was then added to lyse the viable cells. The absorbance was read at 540 nm. The IC_{50} (the concentration of the test compound that caused a growth inhibition of 50% after 48 h exposure of the cells) was calculated from the dose curves generated by plotting percent growth versus the test concentration on a logarithmic scale using Microsoft Excel. Doxorubicin was used as a positive control in the cytotoxicity assay.

RESULTS AND DISCUSSION

Twenty-two naturally occurring C-27 steroidal saponins for antifungal evaluation comprised four tigenin saponins (compounds 1 to 4), three hongguanggenin saponins (compounds 5

to 7), eight hecogenin saponins (compounds 8 to 15), two chlorogenin saponins (compounds 16 and 17), four diosgenin saponins (compounds 18 to 21), and one polygonatogenin saponin (compound 22). In addition, six steroidal sapogenins: tigenin (compound 23), agavegenin A (compound 24), hongguanggenin (compound 25), hecogenin (compound 26), chlorogenin (compound 27), and 9(11)-dehydrohecogenin (compound 28) were included (structures are shown in Fig. 1). Among these compounds, hecogenin saponins 8 to 11 and diosgenin saponins 18 to 21 have been reported to show some antifungal activity against agricultural pathogens such as *Piricularia oryzae* and the human pathogenic yeast *Candida* species (3, 5, 7, 30, 32, 33). However, inclusion of these compounds in this study will provide comparable evidence regarding relative antifungal potency and meaningful SAR information for this class of compounds by, for example, comparing the effect of the number and structure of monosaccharide units in the hecogenin saponin series (compounds 8 to 15).

The antifungal testing results indicated that steroidal saponins 1 to 4, 6, 11, 14, 17, and 19 to 20 were active against *Candida albicans*, *C. glabrata*, *C. krusei*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, while the remaining steroidal saponins (compounds 5, 7 to 10, 12, 13, 15, 16, 18, and 21 to 22) and all the steroidal sapogenins (compounds 23 to 28) were inactive at the highest test concentration of 20 μ g/ml. The antifungal activities of the active saponins are shown in Table 1. The lack of antifungal activity of the steroidal sapogenins is consistent with the previous report that similar compounds show little activity against agricultural pathogens *P. oryzae* and *Hansenula anomala* (7). Among the 10 active saponins that exhibited various degrees of activity against the five human pathogenic fungi, tigenin saponins 1 to 4 with a sugar moiety of four or five monosaccharide units displayed remarkable activity against *C. neoformans* with MFCs comparable to the positive control AMB (MFC, 1.25 μ g/ml). Compounds 1, 3, and 4 also exhibited potent activity against *A. fumigatus* with MICs of 2.5 to 5 μ g/ml. Two diosgenin saponins, compounds 19 and 20, showed activity against *C. albicans* and *C. glabrata* similar to the four tigenin saponins but were not active in *C. neoformans* and *A. fumigatus* at the highest test concentration of 20 μ g/ml. To our knowledge, the in vitro antifungal activity of compounds 1, 3, and 4 against *C. neoformans* is the most potent within the steroidal saponin class that has so far been reported, while their antifungal potency against *A. fumigatus* is comparable to that of diuranthoside A, a neotigenin saponin with the same sugar moiety as in compound 3 (37). In view of limited antifungal drugs for cryptococcosis and aspergillosis (17, 36), it is of particular significance for this discovery.

The above results indicate that the antifungal activity of these steroidal saponins is associated with their individual aglycone moieties (or referred as steroidal sapogenins) as well as the number and structure of monosaccharide units in their sugar chains. Tigenin (compound 23) is considered the basic steroidal skeleton in the following SAR discussion. For hongguanggenin saponins (compounds 5 to 7) which possess multi-hydroxy groups on the tigenin skeleton, only compound 6 shows marginal activity against *C. albicans* and *C. neoformans*. Saponin 7 lacks antifungal activity, although it has the same sugar chain as that in the potent tigenin saponin 1. For chlorogenin saponins (compounds 16 and 17) and hecogenin

TABLE 1. Antifungal activity of steroidal saponins 1 to 4, 6, 11, 14, 17, and 19 to 20 (IC₅₀/MIC/MFC,^a µg/ml)

Sample no. or control drug	<i>C. albicans</i>			<i>C. glabrata</i>			<i>C. krusei</i>			<i>C. neoformans</i>			<i>A. fumigatus</i>		
	IC ₅₀	MIC	MFC	IC ₅₀	MIC	MFC	IC ₅₀	MIC	MFC	IC ₅₀	MIC	MFC	IC ₅₀	MIC	MFC
1	3.0	5.0	5.0	3.5	5.0	5.0	5.0	10	10	0.40	1.25	1.25	1.5	2.5	NA ^b
2	3.5	10	10	3.5	5.0	10	7.0	20	20	0.70	1.25	1.25	10	20	NA ^b
3	2.0	5.0	5.0	2.0	5.0	5.0	5.0	NA ^b	NA ^b	0.40	0.63	0.63	1.5	2.5	10
4	5.0	20	NA ^b	15	20	NA ^b	15	NA ^b	NA ^b	0.40	0.63	0.63	2.0	5.0	10
6	10	20	20	15	NA ^b	NA ^b	NA ^b	NA ^b	NA ^b	8.5	20	20	NA ^b	NA ^b	NA ^b
11	15	20	20	NA ^b	NA ^b	NA ^b	NA ^b	NA ^b	NA ^b	10	20	20	NA ^b	NA ^b	NA ^b
14	9.0	20	NA ^b	10	20	NA ^b	15	NA ^b	NA ^b	6.0	10	10	8.5	20	20
17	7.0	10	10	7.5	10	20	10	20	NA	4.5	10	10	10	20	20
19	3.0	10	NA ^b	2.0	5.0	NA ^b	NA ^b	NA ^b	NA ^b	15	NA ^b	NA ^b	NA ^b	NA ^b	NA ^b
20	1.5	2.5	2.5	2.0	5.0	NA ^b	NA ^b	NA ^b	NA ^b	NA ^b	NA ^b	NA ^b	NA ^b	NA ^b	NA ^b
AMB	0.06	0.16	0.63	0.10	0.31	0.63	0.50	1.25	1.25	0.50	1.25	1.25	0.45	0.63	2.50
FLU	0.35	0.78	NA ^c	35	50	NA ^c	45	100	100	6.5	25	NA ^c	NA ^c	NA ^c	NA ^c

^a Results are given as micrograms/milliter.
^b Not active (NA) at the highest test concentration of 20 µg/ml.
^c Not active at the highest test concentration of 100 µg/ml.

saponins (compounds 8 to 15), which have an α-hydroxy at C-6 and a ketone functionality at C-12 of the tigogenin skeleton, respectively, only compounds 11, 14, and 17 show moderate/marginal antifungal activity, although they have the same sugar chains as in the potent tigogenin saponin 3 or 4. Also, two antifungal diosgenin saponins (compounds 19 and 20) that have an aglycone with a double bond between C-5 and C-6 show a different antifungal profile than the four antifungal tigogenin saponins (compounds 1 to 4).

The sugar chain in each saponin is necessary and plays a key role for its antifungal activity. For example, the antifungal activity of the hecogenin saponins (compounds 8 to 15) is largely dependent on the composition of sugar moiety. No activity is detected in the saponin when its sugar moiety is less than four monosaccharide units. Pentaglycoside 14 is more active than tetraglycoside 11 and shows extended antifungal spectrum against *A. fumigatus*. In the diosgenin saponin series (compounds 18 to 21), only triglycosides 19 and 20 are active against *C. albicans* and *C. glabrata*, while the monoglycoside 21 and diglycoside 18 do not show any activity. Similarly, within the group of the four most potent tigogenin saponins (compounds 1 to 4), their antifungal capacity is slightly influenced by the composition of the sugar moiety. The replacement of a glucosyl unit with a xylosyl unit in compound 1 from compound 2 shows enhanced activity against *A. fumigatus*, while an extended xylosyl unit in compound 4 from compound 3 reduces antifungal activity in *Candida* species.

The in vitro cytotoxicity of the 10 antifungal saponins 1 to 4, 6, 11, 14, 17, and 19 to 20 on the human cancer cell lines SK-MEL, KB, BT-549, SK-OV-3, and HepG2 as well as the noncancerous Vero cells was evaluated (Table 2) and compared with the positive control doxorubicin. Diosgenin saponins 19 and 20 were more cytotoxic than other saponins. Their IC₅₀s for cytotoxicity to the mammalian cells (1.9 to 6.8 µg/ml) were comparable to the IC₅₀s in the fungal cells (1.5 to 15 µg/ml), indicating a general cytotoxic effect of these compounds. However, selectivity was found in the four most potent antifungal tigogenin saponins (compounds 1 to 4). Saponins 1, 2, and 4 did not show any cytotoxicity to cancer cells up to the highest test concentration of 20 µg/ml (except compound 2 showed an IC₅₀ of 6.9 µg/ml in HepG2 cells), although mild to

moderate cytotoxicity was seen in the Vero cells (IC₅₀s, 15, 3.7, and 7.5 µg/ml, respectively). Based on this comparison, saponin 1 appears to have the most favorable activity profile with selectivity indices (IC₅₀ of Vero cells/IC₅₀ of fungal cell) of 4.6, 5.7, 2.7, 37.5, and 10 for *C. albicans*, *C. glabrata*, *C. krusei*, *C. neoformans*, and *A. fumigatus*, respectively. Although higher selectivity indices are observed in *C. neoformans* and *A. fumigatus* for saponin 1, other antifungal saponins in this study remain less selective due to their cytotoxicity to mammalian cells. It is interesting that saponin 1 was much less toxic to Vero cells than compound 2 (IC₅₀ of 15 µg/ml versus 3.7 µg/ml), whereas its antifungal activity, particularly for *A. fumigatus*, is more potent than that of compound 2 (MIC of 2.5 µg/ml versus 20 µg/ml). These observations indicate that the antifungal activity of these saponins is not necessarily correlated with their cytotoxicity to mammalian cells. The minor structural difference, for example, between compounds 1 and 2 or between compounds 1 and 4 (with the difference of one monosaccharide residue), imposes a significant difference on

TABLE 2. Cytotoxicity of antifungal saponins 1 to 4, 6, 11, 14, 17, and 19 to 20

Sample no. or control drug	IC ₅₀ (µg/ml) for:					
	SK-MEL ^a	KB ^b	BT-549 ^c	SK-OV-3 ^d	HepG2 ^e	VERO ^f
1	NA ^g	NA	NA	NA	NA	15.0
2	NA	NA	NA	NA	6.9	3.7
3	9.2	8.5	4.8	6.8	NA	6.5
4	NA	NA	NA	NA	NA	7.5
6	NA	NA	10.0	NA	NA	NA
11	NA	NA	NA	NA	NA	NA
14	10.0	4.8	4.7	10.0	NA	6.0
17	5.2	6.0	5.8	6.2	7.2	5.6
19	2.2	3.2	2.7	4.9	NA	4.7
20	2.8	1.9	3.0	4.8	6.8	3.7
Doxorubicin	0.90	0.16	0.25	1.7	0.60	8

^a Human malignant melanoma.
^b Human epidermal carcinoma, oral.
^c Ductal carcinoma, breast.
^d Human ovary carcinoma.
^e Human hepatic carcinoma.
^f Monkey kidney fibroblasts.
^g NA, not active at 20 µg/ml.

their cytotoxicity. The selectivity and specificity of a particular saponin is apparently associated with the drug target at the cellular or molecular level, which is still unknown at this point. Therefore, it is worthwhile to further study these antifungal compounds and, in a broad sense, the SAR of more steroidal saponins to explore the therapeutic potential of this important class of natural products as antifungal leads for drug discovery.

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