Antifungal Jujubogenin Saponins from *Colubrina retusa*

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Antifungal assay-guided isolation of the 95% ethanol extract of the stems of *Colubrina retusa* yielded jujubogenin 3-*O*-α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl (1→3)]-α-L-arabinopyranoside (**1**), which showed modest growth-inhibitory effects against *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (MICs, 50 μg/mL). In addition, two new minor saponins, jujubogenin 3-*O*-α-L-arabinofuranosyl(1→2)-[2-*O*-(*trans*,*cis*)*p*-coumaroyl-β-D-glucopyranosyl(1→3)]-α-L-arabinopyranoside (**2**), and jujubogenin 3-*O*-(5-*O*-malonyl)-α-L-arabinofuranosyl (1→2)-[β-D-glucopyranosyl(1→3)]-α-L-arabinopyranoside (**3**), were obtained. Saponin **2** was marginally active against only *C. neoformans*, with a MIC of 50 μg/mL, while **3** was inactive. NMR spectroscopy was used extensively for the structure determination of these compounds. The previously reported ambiguity of the NMR assignments of jujubogenin saponins for carbons -26 to -29 was clarified by a comprehensive analysis of the NMR spectra of **1**.

During our screening program searching for antifungal agents from higher plants, *Colubrina retusa*, a rhamnaceous plant growing in Venezuela, was selected for bioassay-guided fractionation on the basis of its activity against *Cryptococcus neoformans*. It has been reported that some species of *Colubrina* contain saponins,[1−4] alkaloids,[5−9] triterpenes, [10,11] phenolics,[12] and essential oils;[13] however, no work has been reported on the chemical constituents of *C. retusa*. In this paper, we present the isolation, structure determination, and antifungal activity of three jujubogenin saponins (**1**−**3**) from the stems of this plant.

Results and Discussion

The crude 95% EtOH extract was partitioned between $CHCl_3$ and H_2O. The water phase was further partitioned between 1-butanol and H_2O. The 1-butanol-soluble portion was fractionated by column chromatography on Si gel. The fraction showing activity against *Candida albicans* and *C. neoformans* was further chromatographed on Si gel to yield compound **1**. Chromatography of the other two less active fractions afforded compounds **2** and **3**. All three compounds were predicted to be triterpenoid saponins by a brief analysis of their ^1H and ^{13}C NMR spectra.

Compound **1**, the major saponin in this plant, was identified as jujubogenin 3-*O*-α-L-arabinofuranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-α-L-arabinopyranoside by comparison of its acid hydrolysis products, FABMS, and ^{13}C NMR spectra with those of a saponin isolated by Higuchi et al. from *Zizyphus joazeiro* (Rhamnaceae).[14] In the present study, complete assignments of the ^1H and ^{13}C NMR signals of **1** were accomplished by 2D NMR, including COSY, HMQC, HMBC, and NOESY (Table 1). Key HMBC and NOE correlations were summarized in Figure 1. The assignments of the ^{13}C NMR signals for the aglycon moiety were in agreement with those reported[14] except for C-18 and C-27. In addition, we have found that some ^{13}C NMR signals due to the sugar moiety should be revised. For example, the assignments for the anomeric carbon of arabinopyranose and that of glucose should be interchanged. Also, the signal of C-3 of arabinopyranose should be reversed with that of C-2 of arabinofuranose. The signals for C-8 and C-10 were reported by Tinto et al.[15] to be at δ 37.3 and 37.5, respectively. However, our data for these signals indicated that these values should be reversed, based on HMBC correlations with H-18 and H-19. Some ambiguities related to the NMR assignments of jujubogenin saponins for carbon-26 to carbon-29 were found in the literature.[15−18] For instance, Kawai et al.[16] numbered the four carbons of jujubogenin (**4**) as depicted in parentheses, inconsistent with the general dammarane triterpene sequence. On the other hand, Inoue et al.[17] and Kimura et al.[18] assigned the ^{13}C NMR signals for the above-mentioned carbons according to the general dammarane triterpene numbering sequence (i.e., δ 25.8, 18.8, 28.6, and 16.3, respectively) without indicating clearly the numbering positions of these four carbons. In addition, two jujubogenin saponins, isolated by Wagner et al.[2] had these four carbons numbered according to Kawai et al.,[16] but their ^{13}C NMR assignments followed the dammarane triterpene numbering sequence. Only Higuchi et al.[14] clearly denoted the numbering positions for carbons 26−29 (dammarane triterpene sequence) with correct assignments of their ^{13}C NMR signals. However, these assignments were based only on 1D ^{13}C NMR. The current study provided substantial information for the NOE correlations of H-29 (δ 1.10) and H-26 (δ 1.68) with other protons (Figure 1). Thus, we have clarified the NMR ambiguities reported above for these four

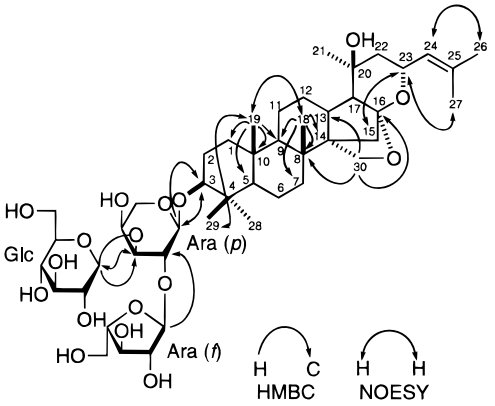

Figure 1. Key HMBC and NOESY correlations of **1**.

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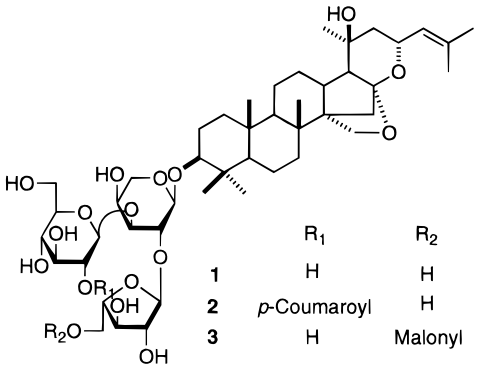
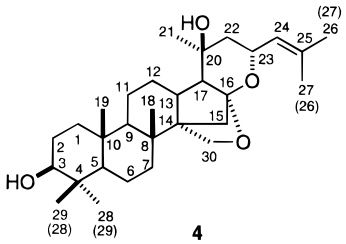
carbons by a comprehensive analysis of the 2D NMR spectra of **1**.





Compound **2** showed a molecular ion peak at *m*/*z* 1044 $[M]^-$ in the negative FABMS. In conjunction with the analysis of the ^{13}C NMR spectrum, its molecular formula was deduced to be $C_{55}H_{80}O_{19}$. The ^1H and ^{13}C NMR spectra of **2** showed great similarities with compound **1**, indicating it was related to the 3-*O*-glycoside of jujubogenin. The ^{13}C NMR spectrum of **2** displayed three anomeric carbon signals at δ 101.7, 105.1, and 109.2 as well as a typical carboxylic ester carbon signal at δ 167.6, along with some aromatic and olefinic carbon signals. Aromatic and olefinic proton signals were also observed in the downfield region of the ^1H NMR spectrum. On alkaline hydrolysis, **2** afforded **1** and *p*-coumaric acid. The *p*-coumaroyl group was indicated by the carbon signals at δ 115.8 (d), 116.7 (d), 126.4 (d), 130.9 (d), 145.7 (d), 161.3 (s), and 167.6 (s).[19] Meanwhile, it was observed that these carbon signals were accompanied by a set of less intense signals with close chemical shifts. The intensity ratio of the two sets of signals, for instance, δ 167.6 and 166.6 was about 3:1. In the ^1H NMR spectrum of **2**, the aromatic proton signals appeared as two *ortho*-coupled doublets at δ 7.61 (2H, J = 8.5 Hz) and 7.10 (2H, J = 8.5 Hz), together with another two doublets at δ 6.94 (1H, J = 15.9 Hz) and 7.98 (1H, J = 15.9 Hz) integrating for only one proton each, which were assigned to the olefinic protons. This confirmed the presence of a *trans*-*p*-coumaroyl group. Similarly, less intense aromatic proton signals at δ 8.04 (d, J = 8.3 Hz) and 7.10 (d, J = 8.5 Hz) and olefinic proton signals at δ 6.89 (d, J = 12.8 Hz) and 6.45 (d, J = 12.8 Hz) also appeared in the ^1H NMR spectrum of **2**, indicating the presence of a *cis*-*p*-coumaroyl moiety. The integration ratio for trans- and cis-signals was approximately 3:1. Thus, **2** was a mixture of *trans*- and *cis*-*p*-coumaroyl derivatives of **1**. Further separation of compound **2** was not carried out, because it has been reported that the *cis*- and *trans*-*p*-coumaroyl derivatives can isomerize in solution.[19]

The linkage position of the *p*-coumaroyl to the sugar moiety was determined by 2D NMR spectra as follows. $^1H-^1H$ COSY and HMQC spectra of **2** established each sugar's spin-coupling network. Their ^1H and ^{13}C NMR signals were assigned as shown in Table 1. It could be observed that the signal of H-2 of glucose was significantly shifted to a downfield value of δ 5.75 (dd, J = 8.0, 9.3 Hz) when compared with compound **1** in which it resonated at δ 3.97 (t, 8.0). This indicated that the *p*-coumaroyl group was linked at the C-2 position of glucose. In addition, two less intense proton signals with close chemical shifts and the same coupling patterns (Table 1) as those of H-1 and H-2 of glucose were observed, indicating that the *cis*-*p*-coumaroyl group was substituted at the same position. Finally, the direct connectivity information was obtained from the HMBC spectrum, which showed a correlation between the proton signal of H-2 of glucose and the carboxylic carbon signal of the *p*-coumaroyl residue (Figure 2). It can be seen from the above data that esterification of the hydroxyl group of C-2 of the glucose moiety by a *p*-coumaroyl residue resulted in upfield shifts of the carbons α to that bearing the ester function ($\Delta\delta$ −3.4 ppm for C-1 and $\Delta\delta$ −1.6 ppm for C-3), although the carbon carrying the substituent (C-2) remained almost unaffected (0.1 ppm as compared to the expected 1.5−4 ppm).[20] There are some cases in the literature that support our findings.[21,22] In addition, it was observed that the introduction of the *p*-coumaroyl residue also led to upfield shifts, to various degrees, of all the carbons of the arabinopyranose and C-1 of the arabinofuranose (Table 1). This could be explained by the steric interaction of the *p*-coumaroyl residue and the 2-*O*-linked arabinofuranose, which resulted in a slight change in the conformation of the arabinopyranose, as indicated by the coupling constant of the anomeric proton (J = 5.6 Hz) when compared to that of compound **1** (J = 7.1 Hz). Based on the above evidence, the structure of **2** was established to be jujubogenin 3-*O*-α-L-arabinofuranosyl-(1→2)-[2-*O*-(*trans*,*cis*)*p*-coumaroyl-*β*-D-glucopyranosyl(1→3)]-α-L-arabinopyranoside.

The third compound, **3**, displayed a ^1H NMR spectrum similar to **1** except for the presence of a signal at δ 3.73 (2H, s). The ^{13}C NMR spectrum of **3** was also similar to that of **1**, with the exception of additional signals at δ170.3 (s), 168.6 (s), and 43.4 (t). In the negative FABMS, **3** showed a molecular ion peak at *m*/*z* 984 $[M(C_{49}H_{76}O_{20})]^-$, 86 mass units (corresponding to a molecular composition $C_3H_2O_3$) more than **1**. The above data indicated the presence of a malonyl residue in **3**.[23] Alkaline hydrolysis of **3** yielded **1** and malonic acid, suggesting that **3** was a malonyl glycoside of **1**. The linkage position of the malonyl group to **1** was deduced to be at the hydroxy group of C-5 of arabinofuranose, because this carbon signal was shifted downfield to δ 65.4, whereas the carbon signal of C-4 of arabinofuranose was displaced upfield to δ 81.4. Confirmation was made with the analyses of the $^1H-^1H$ COSY, HMQC, and HMBC spectra of **3** (Figure 3). Therefore, **3** was shown to be jujubogenin 3-*O*-(5-*O*-malonyl)-α-L-arabinofuranosyl(1→2)-[*β*-D-glucopyranosyl(1→3)]-α-L-arabinopyranoside.

Saponin **1** showed modest minimum inhibitory concentrations (MIC) of 50 μg/mL against *C. albicans*, *C. neoformans*, and *Aspergillus fumigatus*, while saponin **2** was marginally active against only *C. neoformans* (MIC 50 μg/mL). No inhibition was noted for **3** against the above fungi. Amphotericin B was used as a positive control and inhibited all growth at 2 μg/mL against *C. albicans*, *C. neoformans*, and *A. fumigatus*.

Experimental Section

General Experimental Procedure. NMR were recorded in pyridine-d_5 with TMS as an internal standard, using Bruker Avance DPX-300 (300 MHz) for the ^1H and ^{13}C NMR, DEPT, $^1H-^1H$ COSY, HMQC, HMBC (optimized for J coupling at 10

Table 1. ^{13}C and ^{1}H NMR Data for Compounds **1**−**3** in Pyridine-d_5 (ppm)[a]

	1[b]	**1**		**2**[c]		**3**	
	δ_C	δ_C	δ_H (J, Hz)	δ_C	δ_H (J, Hz)	δ_C	δ_H (J, Hz)
Aglycon-1	38.8	38.8	1.58, 0.80	38.8	1.56, 0.79	39.0	1.58, 0.79
2	26.8	26.7	2.16, 1.74	26.7	2.05, 1.83	27.0	2.12, 1.78
3	88.7	88.8	3.25 (dd, 11.7, 4.3)	88.4	3.23 (dd, 11.7, 4.3)	88.9	3.24 (dd, 11.7, 4.1)
4	39.8	39.9		39.8		40.0	
5	56.2	56.3	0.69	56.3	0.67	56.5	0.69
6	18.4	18.4	1.50, 1.37	18.4	1.48, 1.38	18.5	1.48, 1.37
7	36.0	36.2	1.51, 1.40	36.1	1.52, 1.42	36.3	1.50, 1.43
8	37.5	37.6		37.6		37.7	
9	53.0	53.1	0.92	53.1	0.89	53.2	0.89 (br d, 12.3)
10	37.2	37.3		37.3		37.4	
11	21.8	21.8	1.57, 1.34	21.8	1.56, 1.36	21.9	1.54, 1.35
12	28.5	28.6	1.93, 1.80	28.6	1.95, 1.81	28.7	1.94, 1.84
13	37.2	37.2	2.84	37.2	2.85	37.3	2.83
14	53.7	53.8		53.8		53.9	
15	36.9	37.0	2.48/1.56 (ABq, 8)	36.9	2.50/1.55(ABq, 8)	37.0	2.48/1.53 (ABq, 8)
16	110.6	110.7		110.7		110.8	
17	53.9	54.1	1.46	54.1	1.41	54.2	1.46
18	18.4	19.0	1.08 (*s*)	19.0	1.06 (*s*)	19.1	1.07 (*s*)
19	16.4	16.4	0.74 (*s*)	16.5	0.71 (*s*)	16.6	0.74 (*s*)
20	68.5	68.6		68.6		68.7	
21	30.0	30.2	1.40 (*s*)	30.2	1.39 (*s*)	30.3	1.39 (*s*)
22	45.4	45.6	1.77, 1.67	45.6	1.80, 1.70	45.7	1.78, 1.66
23	68.5	68.6	5.22 (br dd, 10, 9)	68.6	5.19 (t-like, 9.6)	68.7	5.21 (t-like, 9.8)
24	127.1	127.2	5.54 (dd, 8.1, 1.2)	127.2	5.54 (br d, 8)	127.3	5.54 (br d, 8.0)
25	134.1	134.2		134.2		134.3	
26	25.6	25.6	1.68 (*s*)	25.7	1.68 (*s*)	25.8	1.68 (*s*)
27	18.9	18.4	1.70 (*s*)	18.4	1.70 (*s*)	18.5	1.71 (*s*)
28	27.8	27.9	1.29 (*s*)	28.1	1.20 (*s*)	28.0	1.30 (*s*)
29	16.6	16.7	1.10 (*s*)	16.7	1.07 (*s*)	16.7	1.12 (*s*)
30	65.9	65.9	4.30, 4.20	65.9	4.30, 4.20	66.1	4.30, 4.19
20-OH			5.94 (*s*)		5.93 (s)		5.94 (*s*)
Ara (*p*)-1	104.9	105.8	4.80 (d, 7.1)	105.1	4.87 (d, 5.6)	105.9	4.76 (d, 7.1)
2	77.0	77.1	4.51	75.1	4.65	77.4	4.50
3	83.8	83.6	4.24	81.0	4.44	83.8	4.22
4	68.5	68.7	4.51	68.4	4.43	68.8	4.50
5	65.9	65.9	4.20, 3.70	65.1	4.15, 3.66	66.0	4.21, 3.70
Glc(*p*)-1	105.7	105.1	5.16 (d, 7.8)	101.7	5.61 (d, 8.0)	105.2	5.15 (d, 7.6)
2	75.2	75.4	3.97 (t, 8.0)	75.5	5.75 (dd, 8.0, 9.2)	75.4	3.97 (t, 8.0)
3	78.0	78.2	4.22	76.6	4.43	78.2	4.22
4	71.4	71.6	4.20	71.9	4.26	71.6	4.18
5	78.0	78.5	3.96	79.0	4.01	78.7	3.96
6	62.5[d]	62.7	4.52, 4.34	62.5	4.51, 4.30	62.7	4.50, 4.32
Ara (*f*)-1	110.3	110.4	6.12 (d, 2.7)	109.2	6.13 (d, 2.1)	110.4	6.13 (d, 2.1)
2	83.5	83.9	5.06	84.0	4.98	83.9	5.00
3	78.4	78.0	4.89	78.5	4.84	78.9	4.69
4	84.9	85.1	4.81	85.4	4.81	81.4	4.89
5	62.1[d]	62.2	4.34, 4.26	62.5	4.30, 4.19	65.4	4.82, 4.73
p-Coum-1				126.4			
2,6				130.9	7.61 (d, 8.5)		
3,5				116.7	7.10 (d, 8.5)		
4				161.3			
α-C				115.8	6.94 (d, 15.9)		
β-C				145.7	7.98 (d, 15.9)		
O═C				167.6			
Malonyl-1						168.6	
2						43.4	3.73 (s)
3						170.3	

[a] For ^{1}H NMR data, coupling patterns well resolved are expressed with multiplicity and coupling constants in Hz in parentheses. [b] Data from Higuchi et al.[14] [c] Signals arising from the *cis-p*-coumaroyl form: ^{1}H NMR: δ 5.59 (d, J = 8.0, Glc H-1), 5.70 (dd, J = 8.0, 9.2 Hz, Glc H-2), 8.04 (d, J = 8.3 Hz, Coum-2,6), 7.10 (d, J = 8.3 Hz, Coum-3,5), 6.89 (d, J = 12.8 Hz, Coum-α-H), 6.45 (d, J = 12.8 Hz, Coum-β-H); ^{13}C NMR: δ 126.7 (Coum-1), 133.9 (Coum-2,6), 115.97 (Coum-3,5), 160.5 (Coum-4), 166.7 (C═O). [d] Signals may be interchanged within each column.

Hz), and NOESY (mixing time 800 ms) spectra of **1**; Bruker Avance DRX-400 (400 MHz) for the ^{1}H and ^{13}C NMR, $^{1}H-^{1}H$ COSY, and HMQC spectra of **2** and **3**; and Bruker Avance DRX-500 (500 MHz) for the gradient HMBC (optimized for J coupling at 10 Hz) spectra of **2** and **3**. FABMS were performed on a ZAB HS instrument. Column chromatography was run using Si gel (40 μm, J. T. Baker) and reversed-phase Si gel (C_{18}, 40 μm, J. T. Baker). TLC was performed on Si gel sheets (Alugram Sil G/UV_{254}, Macherey−Nagel, Germany) and reversed-phase plates (RP_{18} F_{254S}, Merck, Germany), unless otherwise noted.

Plant Material. Stems of *Colubrina retusa* L. (Rhamnaceae) were collected in Venezuela. A voucher specimen of this plant is deposited at the National Center for the Development of Natural Products (voucher no. V 13021).

Extraction and Isolation. The dried stems (500 g) were ground to a coarse powder and extracted with 95% EtOH (2.5 L × 4) at 37 °C for 3 h. Removal of the solvent under 45 °C

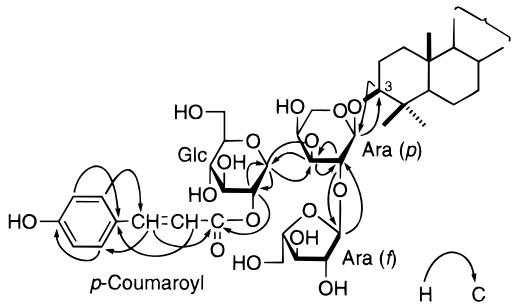


Figure 2. Key HMBC correlations of **2**.

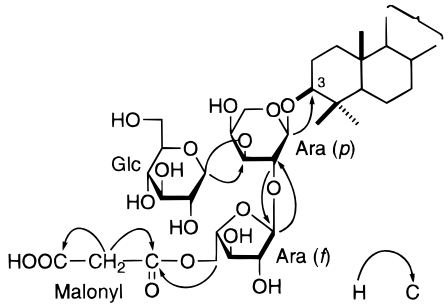


Figure 3. Key HMBC correlations of **3**.

yielded an EtOH extract (12.5 g). The EtOH extract (11.6 g) was suspended in H_2O (1.5 L), extracted with $CHCl_3$ (1 L × 3) and then with 1-BuOH (saturated with H_2O, 1 L × 3). The combined 1-BuOH layers were evaporated to dryness in vacuo (45 °C) to give a yellow residue (5.94 g). This residue (5.0 g) was subjected to column chromatography on Si gel using $CHCl_3$−MeOH−H_2O (70:10:1 to 20:10:1) as the eluting solvent (6 L), followed by MeOH (1.5 L). Fractions of 25 mL each were collected. Similar fractions were pooled according to TLC to yield a total of 17 fractions (A−Q). Part of fraction L (300 mg) was chromatographed on Si gel with $CHCl_3$−MeOH [7:2 (450 mL) to 2:1 (300 mL)] to yield **1** (196 mg). Fraction I (120 mg) was column chromatographed on Si gel using $CHCl_3$−MeOH−H_2O (55:10:1, 520 mL), followed by a reversed-phase column (C_{18}) using 70% MeOH (300 mL) to furnish **2** (10.8 mg). Fraction N (130 mg) was chromatographed on reversed-phase Si gel (C_{18}) (65% MeOH, 500 mL) to yield **3** (5.6 mg).

Saponin 1: needles from MeOH, $[\alpha]^{25}_D$ −43° (*c* 1.0, MeOH) $[\alpha]_D$ −39.9° (*c* 1.1, MeOH, lit.[14])}; ^{1}H and ^{13}C NMR spectra, Table 1; FABMS (negative) *m*/*z* 898 $[M]^-$, 736 $[M - Glc\ (162)]^-$.

Saponin 2: powder, $[\alpha]^{25}_D$ −23° (*c* 0.90, MeOH); ^{1}H and ^{13}C NMR spectra, Table 1; FABMS (negative) *m*/*z* 1044 $[M]^-$, 766 $[M - p\text{-coumaroyl} - Ara(f)]^-$.

Saponin 3: powder, $[\alpha]^{25}_D$ −24° (*c* 0.48, MeOH); ^{1}H and ^{13}C NMR spectra, Table 1; FABMS (negative) *m*/*z* 984 $[M]^-$, 898 $[M - malonyl]^-$, 766 $[M - malonyl - Ara(f)]^-$.

Acid Hydrolysis of 1. Saponin **1** was spotted on a Si gel plate (Si 250 F_{254}, J. T. Baker) and hydrolyzed in situ by exposure to HCl vapor at 80 °C for 15 min. The TLC plate was then developed with $CHCl_3$−MeOH−AcOH−H_2O (14:6:2:1) and sprayed with 10% H_2SO_4 for detection. Glucose and arabinose were detected with R_f values of 0.09 and 0.15, respectively.

Alkaline Hydrolysis of 2 and 3. A solution of each saponin (about 0.5 mg) in 1% KOH (0.1 mL) was kept at room temperature for 30 min. The reaction mixture was directly subjected to TLC analysis using $CHCl_3$−MeOH−H_2O (30:10:1) as a developing system and 10% H_2SO_4 as a color reagent. Saponin **1** was detected, with an R_f value of 0.72, from the solutions of both **2** and **3**. For detection of the acid group, the reaction mixture of each of **2** and **3** was acidified with 2N HCl and evaporated to dryness under a stream of N_2. The residue was dissolved in a drop of MeOH. Each MeOH solution was spotted on TLC along with the reference acid (i.e., *p*-coumaric acid for compound **2** and malonic acid for compound **3**). The plate was developed in $CHCl_3$−MeOH (6:1) (hydrolysis product of compound **2**) or C_6H_6−EtOAc−HCOOH (3:6:1) (hydrolysis product of compound **3**). *p*-Coumaric acid, with an R_f value of 0.47, was detected by UV in the reaction mixture of **2**, while malonic acid, with an R_f value of 0.62 (bromocresol purple as a spray reagent), was found in that of **3**.

Antifungal Assay. Inhibitory activity against *C. albicans*, *C. neoformans*, and *A. fumigatus* was assayed using a modification of the protocol recommended by the National Committee on Clinical Laboratory Standards and adapted to a 96-well microtiter plate format. The yeast-like fungi were grown to a desired concentration in appropriate nutrient broth (SDB for *C. albicans*, Mycophil for *C. neoformans*) and then seeded into wells. For *A. fumigatus*, spores were gently scraped from fungus growth. Plant extracts and column fractions were added to the wells at final concentrations of 1000, 200, and 40 μg/mL; while pure compounds are 50, 10, and 2 μg/mL. After an appropriate incubation period (24 h, 37 °C for *C. albicans*; 48 h, 30 °C for *C. neoformans* and *A. fumigatus*), the inhibitory activity of the sample was assessed as the minimum inhibitory concentration, the lowest concentration tested in which no growth was observed. Amphotericin B was used as a positive control.

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References and Notes

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