Antimalarial, Cytotoxic, and Antifungal Alkaloids from *Duguetia hadrantha*

Ilias Muhammad,*,† D. Chuck Dunbar,† Satoshi Takamatsu,† Larry A. Walker,†,‡ and Alice M. Clark†,§

National Center for Natural Products Research and Departments of Pharmacognosy and Pharmacology, Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, Mississippi 38677

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Bioassay-guided isolation of *Duguetia hadrantha* yielded two new 4,5-dioxo-1-azaaporphinoids, hadranthine A (**1**) and hadranthine B (**2**), together with the known alkaloids imbiline-1 (**3**), sampangine (**4**), and 3-methoxysampangine (**5**), whose structures were determined primarily from 2D-NMR $^1H-^{13}C$ HMBC, and $^1H-^{15}N$ HMBC experiments. This is the first report of the co-occurrence of the copyrine alkaloids **4** and **5**, as well as the first report of either copyrine or imbiline type alkaloids from a *Duguetia* species. Compounds **1**, **4**, and **5** demonstrated in vitro antimalarial activity against *Plasmodium falciparum* (W-2 clone), while **2** was inactive. Instead, **2** showed in vitro cytotoxicity to selected human cancer cell lines (IC_{50} = 3−6 μg/mL against SK-MEL, KB, BT-549, and SK-OV-3), and **4** was also cytotoxic to human malignant melanoma (IC_{50} = 0.37 μg/mL). Sampangine (**4**) also inhibited cell aggregation with a MIC value of <0.15 μg/mL, while 3-methoxysampangine (**5**) was only weakly active.

The genus *Duguetia* comprises about 80 species native to tropical America. The center of distribution is the Amazon basin and Guiana, but the genus is represented also in South America south to Sao Paulo, Brazil, and Paraguay.[1] Chemical investigations of various *Duguetia* species from Colombia, Bolivia, and Brazil have revealed alkaloids, including benzyltetrahydroisoquinolines, aporphines, and oxoaporphines from *D. spixiana*, *D stelichantha*, *D. eximia*, *D. obovata*, and *D. calycina*.[2−7] Some of these benzylisoquinoline-derived aporphines and their dimers exhibited antimalarial, cytotoxic, and antimicrobial activities.[8−10]

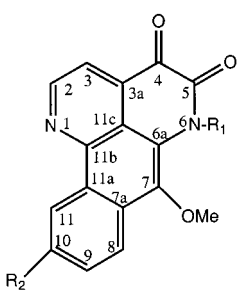
Duguetia hadrantha (Diels.) R. E. Fries. (Annonaceae), a large tree found in an open sandy forest near Loreto, Peru, has not previously been the subject of phytochemical analysis. An ethanolic extract of the stem bark showed sufficient antimalarial and antifungal activities to warrant bioassay-guided fractionation. This led to the isolation of antimalarial, antifungal, and cytotoxic 4,5-dioxo-1-azaaporphine (**1**−**3**) and copyrin (**4**, **5**) alkaloids. To our knowledge, this is the first report of dioxoazaaporphine and copyrine alkaloids from this genus.

Results and Discussion

Re-extraction of the active dried EtOH extract by percolation with *n*-hexane, followed by CH_2Cl_2, resulted in localization of the antimalarial and antifungal activities in the CH_2Cl_2 fraction. Column chromatography, followed by centrifugal preparative thin layer chromatography (see Experimental Section) of the CH_2Cl_2 fraction, resulted in the isolation of five alkaloids (**1**−**5**), of which hadranthine A (**1**), imbiline-1 (**3**),[11] sampangine (**4**),[12] and 3-methoxysampangine (**5**)[13] were found to be active antimalarial constituents. Compounds **3**−**5** were identified by comparison of their physical and spectral data with those previously reported.

The molecular formula $C_{18}H_{14}N_2O_4$ for **1** was established by HRMS. The UV spectrum demonstrated extended conjugation indicative of a 1-azaaporphine alkaloid. The ^1H and ^{13}C NMR spectra of **1** were in close agreement with the known 4,5-dioxo-1-azaaporphine imbiline-1 (**3**),[11] except for the presence of the OMe group at C-10. Structure **1** was unambiguously established by detailed 2D NMR studies, including $^1H-^1H$ COSY, $^1H-^{13}C$ HMQC, $^1H-^{13}C$ HMBC, and $^1H-^{15}N$ HMBC experiments. The three aromatic protons were assigned by a COSY experiment which established the system $-C_8H{=}C_9H-C_{10}(OMe){=}C_{11}H-$ in **1**. The HMBC experiment established the assignments of the two OMe− groups at C-7 and C-10, and the N-Me at N-6 position by three-bond correlations between δ_{C-10} 160.3, δ_{C-7} 145.6, and H-8; δ_{C-11} 105.7 and H-9; δ_{C-10} 160.3 and C-10-OMe; δ_{C-7} 145.6 and C-7-OMe; and δ_{C-5} 158.1, δ_{C-6a} 121.0, and N-6-Me. The placement of the two nitrogen atoms at N-1 and N-6 was confirmed by a $^1H-^{15}N$ HMBC experiment, which demonstrated the correlations between the signals at δ_N 321.0 (N-1), δ_{H-2} 9.11, and δ_{H-3} 8.22; and δ_N 135.6 (N-6) and δ_H 3.95 (N-6-Me). As a control, a $^1H-^{15}N$ HMBC experiment was performed on imbiline-1 (**3**), which showed similar correlations (i.e., δ_{N-1} 321.9, δ_{H-2} 9.05, and δ_{H-3} 8.05; and δ_{N-6} 135.4 and δ_{H-6-Me} 3.83) with those of **1**.

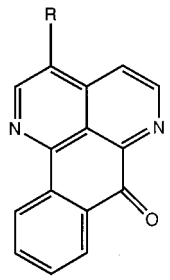
The ^1H and ^{13}C NMR spectral data of **2**, $C_{16}H_{10}N_2O_3$, were found to be generally similar to those observed for **1**, save for the differences associated with the absence of N-Me and OMe groups at N-6 and C-10 positions, respectively.



1 R_1=Me R_2=OMe

2 R_1=H R_2=H

3 R_1=Me R_2=H



4 R=H

5 R=OMe

* To whom correspondence should be addressed. Tel: (662) 915-1051. Fax: (662) 915-7989. E-mail: milias@olemiss.edu.
† National Center for Natural Products Research, Thad Cochran Research Center.
‡ Department of Pharmacology.
§ Department of Pharmacognosy.

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Table 1. Antimalarial Activity of Compounds **1** and **3**−**5**

	P. falciparum (W-2)[a]		*P. falciparum* (D-6)[b]	
compound	IC_{50} (ng/mL)	SI[c]	IC_{50} (ng/mL)	SI[c]
1	120	>40	120	>40
3	280	>17	550	>8.7
4	68	>70	420	>11
5	95	>50	280	>17
chloroquine[d]	140	>34	19	>251
artemisinin[d]	4.4	>1082	5	>952

[a] Chloroquine-resistant clone. [b] Chloroquine-sensitive clone. [c] Selectivity index = IC_{50} (Vero cells)/IC_{50} *Plasmodium falciparum*. [d] The IC_{50} values determined for chloroquine and artemisinin, using microdilution assay,[32] were 29.5 and 0.5 ng/mL against clone W-2 and 1.3 and 1.8 ng/mL against clone D-6, respectively.

Table 2. Cytotoxic Activity of Compounds **1**−**5**

	IC_{50} (μg/mL)			
compound	SK-MEL[a]	KB[b]	BT-549[c]	SK-OV-3[d]
1	>10	*e*	>10	>10
2	3.0	6.4	6.6	3.6
3	2.0	>10	>10	5.0
4	0.37	5.3	6.4	4.1
5	>10	*e*	*e*	>10
doxorubicin	<1.1	<1.1	<1.1	<1.1
5-fluorouracil	6.3	>10	*e*	>10

[a] SK-MEL: human malignant melonoma. [b] KB: human epidermoid carcinoma. [c] BT-549: human ductal carcinoma. [d] SK-OV-3: human ovary carcinoma. [e] Inactive.

Furthermore, a close comparison of the ^{13}C NMR spectrum of **2** with those of imbiline-1 (**3**), -2, and -3, isolated from *Eupomatia species*,[11] led to the conclusion that indeed **2** is the N-demethyl derivative of **3**. Compound **2** was reported previously as a side product during the synthesis of imbiline-1 (**3**), but its physical and spectral data were not reported.[14] The HMBC experiment on **2** showed the three-bond correlations between δ_{C-4} 179.3 and H-3; δ_{C-7} 142.0, C-7-OMe, and H-8; δ_{C-7a} 130.4, H-9, and H-11; and δ_{C-11a}130.6, H-8, and H-10, confirming the placement of the C-4-carbonyl, the C-7-methoxyl group, and ABCD protons of the O-disubstituted benzene ring. The $^{1}H-^{15}N$ HMBC experiment on **2** demonstrated the correlations between δ_{N-1} 321.5, δ_{H-2} 9.25, and δ_{H-3} 8.26, as observed for **1** and **3**. From the foregoing data the structure of **2**, named hadranthine B, was assigned as shown.

Antimalarial evaluation of the isolated compounds revealed that alkaloids **1** and **3**−**5** are more active against chloroquine-resistant *P. falciparum*[15,16] clone W-2 (IC_{50} = 120, 280, 68, and 95 ng/mL, respectively; Table 1) than chloroquine-sensitive clone D-6, while **2** is inactive against either clone. Because of the apparent selectivity for the W-2 clone and absence of cytotoxicity toward VERO cells, alkaloids **1**, **4**, and **5** warrant further investigation as potential antimalarial lead compounds.

Both hadranthine B (**2**) and sampangine (**4**) showed in vitro cytotoxic activity[16] against SK-MEL, KB, BT-549, and SK-OV-3 cell lines, with IC_{50} values between 3 and 6 μg/mL and 0.37−6.4 μg/mL, respectively, against these cell lines (Table 2). The antimalarial alkaloids **1** and **5** were found to be devoid of cytotoxic activity. Compounds **4** and **5** are responsible for the antifungal activity[17] of the extracts (Table 3). Sampangine (**4**) is known to be strongly antifungal.[18,19] Hadranthine A (**1**) is also active against *Candida albicans* with IC_{50} and MIC values of 4.5 and 20 μg/mL, respectively.

To determine their effects on immune response and inflammation, hadranthine B (**2**), sampangine (**4**), and 3-methoxysampangine (**5**) were also evaluated for their in vitro effects on lymphocyte-associated antigen-1 (LFA-1: CD11a/CD18)/intercellular adhesion molecule-1 (ICAM-1: CD54) mediated aggregation and adhesion using HL-60 and HeLa cell lines[20−22] (Table 4). Sampangine (**4**) inhibited cell aggregation (MIC <0.15 μg/mL, as compared to MIC 0.39 μg/mL for cytochalasin B), but was inactive in the cell adhesion assay (IC_{50} >10μg/mL; vs 0.25 μg/mL for cytochalasin B). 3-Methoxysampangine (**5**) was weakly active in the primary cell aggregation assay, while sampangine (**4**) was >15-fold more cytotoxic than cytochalasin B (as determined by XTT assay).

This appears to be the first report of hadranthine A (**1**) and B (**2**), as well as the first co-occurrence of sampangine (**4**) and 3-methoxysampangine (**5**), from a natural source. Compounds **4** and **5** were isolated previously from *Cananga odorata*[12] (family Annonaceae) of Asian and *Cleistopholis patens*[13] (family Annonaceae) of African origin, respectively. Both **4** and **5** were later synthesized and evaluated for antimycotic and antimycobacterial activity.[18,19] Imbiline and copyrine alkaloids have previously been reported from *Eupomatia bennettii* and *E. laurina*[11] (family Eupomataceae). Interestingly, other *Duguetia* species yield mostly benzyltetrahydroisoquinoline and aporphine alkaloids, while *D. hadrantha* has yielded the imbiline and copyrine alkaloids. This report of their occurrence in *Duguetia hadrantha* (family Annonaceae) suggests a close chemotaxonomic relation. Thus, the alkaloidal profile of *D. hadrantha* appeared to be more similar to those of the annonaceous plants *C. patens* and *C. odorata* than to those of other species of the same genus. Hadranthine A (**1**) and B (**2**), and the copyrines **4** and **5**, possess an obvious structural relationship with other 1-aza-4,5-dioxoaporphines (imbilines 1−3) and 1-aza-7-oxoaporphines (eupomatidines 1−3) previously isolated from *Eupomatia* species.[11] The biogenetic pathway for **2** could be envisaged to involve oxidation of the C-4(5) bond,[23] reduction ($-N_6{=}C_{6a}-$ bond as envisaged in sampangine-7-*O*-glucosides[24]), and C-7-*O*-methylation of the precursor **4** to give the 1-aza-4,5-dioxoaporphine, hadranthine B (**2**). Routes can also be envisaged for the formation of **1** from such a 1-aza-7-oxoaporphine precursor.

Finally, it is intriguing to note that the antimalarial activity of Peruvian *D. hadrantha* stem bark is contributed, in part, by the well-known antifungal copyrins (sampangine and 3-methoxysampangine), suggesting a possible common molecular target.

Experimental Section

General Experimental Procedure. Melting points (uncorrected) were recorded on an Electrothermal 9100 instrument. UV spectra were obtained in MeOH, using a Hewlett-Packard 8452A spectrophotometer, and IR spectra were taken as KBr disks on a Ati Mattson (Genesis Series) FTIR spectrophotometer. The NMR spectra were recorded on a Bruker Avance DRX-500 instrument at 500 MHz (^{1}H) and 125 MHz (^{13}C) in $CDCl_3$ or C_5D_5N, using TMS as internal standard. Multiplicity determinations (DEPT) and 2D NMR spectra (COSY, HMQC, HMBC) were run using standard Bruker pulse programs. The ^{15}N NMR spectra were recorded at 50.7 MHz with chemical shift relative to liquid NH_3 by calibrating nitromethane to 380.2 ppm. High-resolution MS were obtained by direct injection using Bruker Bioapex-FTMS with electrospray ionization (ESI); TLC analyses were carried out on Si gel F254 plates, with the solvent system CH_2Cl_2−MeCN (95:5). For flash column chromatography, Si gel G (J.T. Baker, 40 μm Flash) was used and a CH_2Cl_2−MeCN mixture as a solvent system. Centrifugal preparative TLC (CPTLC, using Chromatotron, Harrison Research Inc. Model 8924): 1 or 2 mm Si gel

Table 3. Antimicrobial Activity of Compounds **1** and **3**−**5**

compound	IC_{50}/MIC (μg/mL)[a]			
	C. albicans	*C. neoformans*	*S. aureus*	MR *S. aureus*
1	4.5/20	*b*	*b*	*b*
3	9.0/50	50/NT	*b*	*b*
4[c]	0.15/0.39	0.15/0.78	5.5/NT	9.0/12.5
5[c]	3.0/20	0.65/3.125	15/25	20/NT
amphotericin B	0.011/0.020	0.045/0.156	*b*	*b*
rifampicin	NT	NT	0.01/0.020	0.0025/0.3125

[a] IC_{50}/MIC values after 48 h of incubation at 37 °C. [b] Inactive. [c] The MIC values determined for **4** and **5** by Peterson et al.[18] were 1.56 and 0.78 μg/mL against *C. albicans* and *C. neoformans*, respectively.

Table 4. Cell Proliferation, Aggregation, and Adhesion Activity[a] of Compounds **2**, **4**, and **5**

compound	cell aggn. MIC (μg/mL) (A)	cell prolifn. XTT IC_{50} (μg/mL) (B)	specific index (B)/(A)	cell adhsn. IC_{50} (μg/mL) (C)	specific index (B)/(C)
2	4.2	>12.5	3.0	NT	
4	<0.15	2.0	>13.3	>10	<0.2
5	12.5	7.2	0.6	NT	
cytochalasin B	0.39	>30	76.9	0.25	>120

[a] Assay system for inhibitors of LFA-1/ICAM-1-mediated aggregation combined with XTT assay as a primary assay. Following LFA-1/ICAM-1-mediated adhesion, assay was performed with HL-60 cells and HeLa cells as a secondary assay.

GF Chromatotron rotors (Analtech, Inc.) using a N_2 flow rate of 4 mL min^{-1}. The isolated compounds were visualized by observing under UV-254 nm, followed by development in an iodine chamber or spraying with Dragendorff's reagent.

Plant Material. The stem bark of *D. hadrantha* was collected in July 1996 from Loreto (Maynas), Peru, and identified by Dr. Sidney T. McDaniel, Institute for Botanical Exploration, Mississippi State University. A voucher specimen (IBE 11713) has been deposited in the Herbarium of The University of Mississippi.

Extraction and Bioassay. The powdered air-dried stem bark of *D. hadrantha* (0.5 kg) was extracted by percolation with 95% EtOH (3 × 2 L), and the combined extracts were evaporated under reduced pressure and then freeze-dried (yield 23 g). A portion of the active dried EtOH extract (20 g) was re-extracted by percolation with *n*-hexane, followed by CH_2Cl_2, and finally the residual extract was washed with MeOH (each 200 mL × 3 times). The combined *n*-hexane, CH_2Cl_2, and MeOH fractions were separately filtered and dried, which afforded 4.5, 9.5, and 5.5 g, respectively. Antimalarial and antifungal screenings (vide infra) of these fractions showed that the activity resided in the CH_2Cl_2-soluble fraction (IC_{50} <0.5 μg/mL [selectivity index >9.0] against *P. falciparum* (D-6 clone); IC_{50} <2.0 μg/mL against *C. neoformans*).

Isolation of Alkaloids. The active CH_2Cl_2 fraction (9 g) was flash chromatographed on Si gel (350 g), using initially CH_2Cl_2 (1 L), followed by 5−7% MeCN−CH_2Cl_2 as eluent to afford **3** as red needles [200 mg; mp 246−248 °C (crystallized from CH_2Cl_2−*n*-hexane); lit.[11] mp 212−214 °C (crystallized from EtOH as red prisms)], followed by mixtures of compounds **1**, **4**, and **5** (400 mg). These three components were separated by CPTLC (2 mm Si gel rotors), using 20% EtOAc in *n*-hexane as solvent, which afforded **1** [50 mg; R_f 0.5, Si gel, solvent CH_2Cl_2−MeCN (95:5)], followed by a mixture of **4** and **5** (180 mg), which was separated by another CPTLC (1 mm Si gel rotors) using CH_2Cl_2−MeCN (95:5) as eluant, to give **4** as orange needles (30 mg; mp 215−217 °C, lit.[12] mp 210 °C), followed by **5** as yellow prisms [40 mg; mp 193−195 °C (crystallized from CH_2Cl_2−MeCN); lit.[13] mp 213−215 °C (crystallized from *n*-hexane−EtOAc as yellow needles]. Further elution of the CC with 10% MeCN−CH_2Cl_2 yielded **2** (60 mg; R_f 0.33) as orange plates. The spectral data of **3**−**5** were in agreement with those reported in the literature.[11−13] The identity of **4** was confirmed by direct comparison with an authentic sample of sampangine.

Hadranthine-A (7,10-Dimethoxy-6-methyl-4,5-dihydronaphthol[1,2,3-*ij*][2,7]-naphthyridine-4,5-(6*H*)-dione) (1): light purple plates (CH_2Cl_2); mp 288−290 °C; UV (MeOH) λ_{max} (log ϵ) 208 (4.35), 244 (3.90), 315 (3.75), 328 (3.80), 450 br (3.65) nm; IR (KBr) ν_{max} 2985−2820, 1694 (C=O), 1670 (C=O), 1615, 1575, 1480, 1440, 1430, 1375, 1350, 1330, 1300, 1285, 1265, 1100 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.11 (1H, d, J = 5.3 Hz, H-2), 8.55 (1H, d, J = 2 Hz, H-11), 8.22 (1H, d, J = 5.3 Hz, H-3), 8.13 (1H, br d, J = 8 Hz, H-8), 7.35 (1H, dd, J = 2, 8 Hz, H-9), 4.05 (3H, s, C-10-OMe), 3.95 (3H, s, 6-N-Me), 3.90 (3H, s, C-7-OMe); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.1 (s, C-4), 160.3 (s, C-10), 158.1 (s, C-5), 148.4 (d, C-2), 145.6 (s, C-7), 145.3 (s, C-11b), 132.7 (s, C-11a), 132.4 (s, C-3a), 125.1 (s, C-7a), 125.0 (d, C-8), 121.2 (d, C-9), 121.0 (s, C-6a), 119.9 (d, C-3), 118.9 (s, C-11c), 105.7 (d, C-11), 63.5 (q, C-7-OMe), 56.2 (q, C-10-OMe), 36.2 (q, 6-N-Me); FT-HRMS *m*/*z* 323.1033 $[MH]^+$ (calcd for $C_{18}H_{14}N_2O_4$, 323.1026).

Hadranthine-B (7-Methoxy-4,5-dihydronaphthol[1,2,3-*ij*][2,7]naphthyridine-4,5-(6*H*)-dione) (2): orange plates (MeCN−CH_2Cl_2); mp 214−216 °C; UV (MeOH) λ_{max} (log ϵ) 204 (4.3), 242 (3.85), 282 sh (3.10), 305 (3.65), 316 (3.68), 460 (3.55) nm; IR (KBr) ν_{max} 3411 (N−H), 2950, 2820, 1690, (C=O), 1670 (C=O), 1624, 1541, 1528, 1457, 1316, 1259, 1018 cm^{-1}; ^{1}H NMR (C_5D_5N, 500 MHz) δ 13.3 (br s, N−H), 9.25 (1H, d, J = 5.5 Hz, H-2), 9.44 (1H, br d, J = 7.5 Hz, H-11), 8.26 (1H, d, J = 5.5 Hz, H-3), 8.15 (1H, br d, J = 7.5 Hz, H-8), 7.79 (1H, td, J = 2.3, 7.5 Hz, H-10), 7.67 (1H, td, J = 2.3, 7.5 Hz, H-9), 3.95 (3H, s, C-7-OMe); ^{13}C NMR (C_5D_5N, 125 MHz) δ 179.3 (s, C-4), 157.2 (s, C-5), 149.5 (d, C-2), 146.5 (s, C-11b), 142.0 (s, C-7), 134.8 (s, C-3a), 130.7 (d, C-9), 130.6 (s, C-11a), 130.4 (s, C-7a), 127.9 (d, C-10), 126.1 (d, C-11), 123.1 (d, C-8), 122.8 (s, C-6a), 119.8 (d, C-3), 118.0 (s, C-11c), 62.4 (q, C-7-OMe); HRMS *m*/*z* 279.0764 $[MH]^+$ (calculated for $C_{16}H_{10}N_2O_3$, 279.0764).

Antimalarial/Parasite LDH Assay. The in vitro antimalarial assay procedure[16] utilized at the NCNPR, University of Mississippi, is an adaptation of the parasite lactate dehydrogenase (pLDH) assay developed by Makler et al.,[25,26] using a 96-well microplate assay protocol with two *P. falciparum* clones [Sierra Leone D6 (chloroquine-sensitive) and Indochina W2 (chloroquine-resistant)]. The primary screening involves determination of pLDH inhibition (percentage) of each sample tested at 15.9 and 1.59 μg/mL for extracts and pure compounds, respectively. The IC_{50} values are determined only for samples that inhibit parasite growth by >50% for one of the clones. The antimalarial agents chloroquine and artemisinin are used as positive controls, while DMSO is the negative (vehicle) control.

Cytotoxicity Assay. The in vitro cytotoxic activity was determined against four human cancer cell lines, SK-MEL, KB, BT-549, and SK-OV-3 (Table 3), obtained from American Type Culture Collection (ATCC, Rockville, MD). For initial (primary) evaluation, extracts and fractions are screened at a single concentration (100 μg/mL). Follow-up secondary assays are conducted at three concentrations (10, 3.3, and 1.1 μg/mL), using a culture-treated 96-well microplate.[16] The level of toxicity of each sample is determined by measuring the effect on a fibroblast cell line from African green monkey kidney (VERO; nontransformed). For secondary assays, IC_{50} values are determined from logarithmic graphs of growth inhibition values. The cytotoxic agents doxorubicin and 5-fluorouracil are

used as positive controls, while DMSO is used as negative (vehicle) control.

Antimicrobial Assay. The preliminary antimicrobial activities of the crude extracts/fractions and the IC_{50}/MIC values of compounds **1**−**5** were determined by using a modified 96-well microplate assay protocol.[17] The test organisms used were ATCC strains of *Candida albicans* B311 (# 90028), *Cryptococcus neoformans* (# 90113), *Staphylococcus aureus* (# 6535), and methicillin-resistant *S. aureus* (#33591). Amphotericin B and rifampin were used as positive controls, with DMSO as a negative control.

Cell Aggregation Assay. Effects on cell aggregation were determined as previously described.[27] HL-60, the myelomonocytic cell line, was suspended at a density of 1×10^6 cells/mL, and 150 μL of the cell suspension was added to each well of a 96-well plate. After incubation with a test sample for 10 min, phorbol myristate acetate (PMA, 10 ng/mL) was added. Plates were placed in a CO_2 incubator, and aggregation of the cells was observed microscopically 16 h after the PMA addition. The known cell aggregation inhibitor cytochalasin B, anti-LFA-1, and anti-ICAM-1 monoclonal antibody were used as positive controls.

XTT Assay for Cytotoxicity. Following the cell aggregation assay, the XTT (3′-1[(phenylamono)carbonyl]-3,4-tetrazoliumbis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) assay was performed.[28,29] Briefly 25 μL of XTT-phenazine methosulfate (PMS) solution (1 mg/mL XTT solution supplemented by 25 μM of PMS) was added to the cells in each well on the microplates. After incubating for 4 h at 37 °C, absorbance at 450 nm was measured by microplate reader (reference absorbance at 630 nm).

Cell Adhesion Assay.[29] HL-60 cells were stained with a CFSE (carboxyfluorescein diacetate succinimyl ester, Molecular Probes).[30] CFSE-labeled HL-60 cells and potential inhibitors were added to the wells of microplates that contained confluent monolayers of HeLa cells, a carcinoma cell line that expresses ICAM-1. PMA (50 ng/mL) was added to stimulate the HL-60 cells to convert LFA-1 to its high avidity binding state.[31] The cultures were incubated in a CO_2 incubator for 45 min at 37 °C. Nonadherent HL-60 cells were washed off, the remaining cells were solubilized with 1% Triton X-100, and fluorescence was quantitated using a CytoFluor 2350 fluorescence measurement system (Millipore) with excitation wavelength of 496 nm and emission at 519 nm. Anti-ICAM-1 monoclonal antibody and cytochalasin B were used as a positive control.

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