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Antifungal Triterpenoid Saponins from *Lecaniodiscus cupanioides*

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Abstract: Phytochemical investigation of the stems of *Lecaniodiscus cupanioides* Planch (Sapindaceae) afforded two triterpenoid saponins identified as 3-O- [α-L-arabinofuranosyl-(1→3)- α-L-rhamnopyranosyl- (1→2) - α -L-arabinopyranosyl-]-hederagenin (1) and 3-O-[α -L-arabinopyranosyl- (1→3)- α -L-rhamnopyranosyl (1→2)- α -L-arabinopyranosyl-]-hederagenin (2). The structures of the compounds were determined based on chemical investigations and comprehensive NMR spectroscopic studies including ^{1}H, ^{13}C, DEPT, COSY, HMQC, HMBC, MS and comparison with literature data. The compounds exhibited antifungal activity against *C. albicans*, *C. neoformans* and *A. fumigatus*.

Key words: *Lecaniodiscus cupanioides*, Sapindaceae, triterpenoid saponin, hederagenin, antifungal activity

INTRODUCTION

Almost every culture within the wide diversity of the world´s population and habitats uses local plants within its environs as medicines in one form or another. Chemical and biological investigations of folkloric medicinal plants with the reputation of curative potential have provided the world with many of the common clinical drugs and herbal remedies of today (Hamburger and Hostettmann, 1991). It has been reported that several compounds derived from plant species could be regarded as important drugs currently in use and that about 25% of the pharmaceuticals prescribed by doctors in the developed world have their antecedents in chemicals produced by flowering plants (Balandrin *et al*., 1985). There is also a growing interest in the acquisition of botanical enterprises by multinational pharmaceutical companies in recent years (Leaders, 1996).

Lecaniodiscus cupanioides Planch (Sapindaceae) is a tree sometimes planted as a shade-tree and as an ornamental. It appears as a weed in rice fields in Nigeria. The bark is used for cough and broncho-pneumonial infections (Burkill, 2000). Ethnobotanical information revealed its use as galactogen, laxative and febrifuge and has autonomic effects such as lacrimation and skeletal muscle relaxant activity in rats (Sandberg and Croulund, 1977). It is also used as an aphrodisiac and cases of sexual asthenia (Ghana Herbal Pharmacopoeia, 1992). The aqueous root extract of this plant was reported to have central nervous system depressant activity (Yemitan and Adeyemi, 2005). Preliminary screening of extracts from marine organisms and plants for antifungal activities in our laboratory revealed that the stem extract of *L. cupanioides* had promising antifungal properties. Incidence of fungal infection is increasing worldwide and despite treatment, mortality remains very high. Presently, few antifungal agents are available and their use may be limited by harmful side effects (Lorthoraly *et al*., 1999; Andriole, 1999). The present study thus deals with the isolation and characterization of the saponins from *L. cupanioides* and their antifungal effects.

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MATERIALS AND METHODS

^{1}H and ^{13}C-NMR Spectra were measured and reported in ppm by using the residual solvent peak as an internal standard. ESI-FTMS analysis was measured on a Bruker-Magnex BioAPEX 30ES ion cyclotron HR HPLC-FT spectrometer by direct injection into an electrospray interphase. Semi-preparative HPLC was carried out on a Waters 510 system with a gradient programmer.

Plant Material

The stems of *L. cupanioides* were collected at Sango, Ogun State, Nigeria and authenticated by Mr. Odewo of the Forestry Research Institute of Nigeria (FRIN) Ibadan, voucher specimen FHI.105, 353 was deposited at the herbarium.

Extraction and Isolation

The stems of *L. cupanioides* were chopped into small bits and dried using an electric oven at 40°C for 3 days. The dried stems were ground in a roller mill. About 1 kg of powdered plant material was extracted exhaustively using Soxhlet apparatus with 96% ethanol (4 L) for 48 h. The filtrate was concentrated under reduced pressure using rotatory evaporator until a semi-solid residue (5.1%w/w) was obtained. The extract was subjected to vacuum liquid chromatography over Si gel column, eluted successively with n-hexane-$CHCl_3$-MeOH in a step gradient by using different ratios to give eleven fractions (V1-V11). Fraction V8 (4.3 g) obtained on elution with $CHCl_3$-MeOH (7:3) was chromatographed over a Si gel column (4.0×70 cm) (EtOAc-Acetone-AcOH-H_2O, 6:2:1:1) isocratically to give eleven subfractions (S1-S11). Fractions S8 (0.31 g) and S9 (0.06 g) gave semi-pure 1 and fractions S10 (0.07 g) and S11 (0.09 g) gave semi-pure 2 which were purified by preparative TLC (Merck 60Å Si gel, 20×20 cm, 1000 μm), developed with EtOAc-Acetone-AcOH-H_2O (6:2:1:1). Final purification was by reverse phase HPLC (Luna 5 μM, C8 100Å, 250×21.20 mm) using CH_3CN-H_2O (40:60) as an eluent (flow rate of 5 mL min^{-1} gradiently and UV detection at 225 um) yielded 15 mg of 1 (t_R = 40 min) and 12 mg of 2 (t_R = 40 min).

Compound 1

Colourless crystals; $[\alpha]_D^{25}$: +11.2° (c 0.5, EtOH); ESI-HRMS m/z 882.3 ($[M-H]^-$); 750 [M-arabinose-H]$^-$; 604 [M-(arabinose+rhamnose)-H]$^-$; 471.3[M-(2arabinose+ rhamnose) -H]$^-$ (calc. for [$C_{46}H_{74}O_{16}$ -1], 882.49767400).

^{1}H NMR (400.13 MHZ, C_6D_5N) δ 0.79 (3H, s, H-24), 0.80 (3H, s, H-26), 0.86 (3H, s, H-29), 0.88 (3H, s, H-30), 0.99 (3H, s, H-25), 1.11 (3H, s, H-27), 5.33 (1H, br s, H-12), 4.16 (1H, m, H-3α), 4.94 (1H, d, J = 7.6 Hz, H-1′), 6.25 (1H, d, J = 7.8 Hz, H-1′′), 6.05 (1H, d, J = 7.9 Hz, H-1′′′).

^{13}C NMR data (100 MHZ, C_6D_5N) δ 38.4 (C-1, t), 25.7 (C-2, t), 80.7 (C-3, d), 43.0 (C-4, s), 47.3 (C-5, d), 17.6 (C-6, t), 32.3 (C-7, t), 39.1 (C-8, s), 47.6 (C-9, d), 36.3 (C-10, s), 23.0 (C-11, t), 122.0 (C-12, d), 144.2 (C-13, s), 41.5 (C-14, s), 27.7 (C-15, t), 23.2 (C-16, t), 46.0 (C-17 s), 41.1 (C-18, d), 45.8 (C-19, t), 30.3 (C-20, s), 33.6 (C-21, t), 32.6 (C-22, t), 63.5 (C-23, s), 13.5 (C-24, q), 15.5 (C-25, q), 16.8 (C-26, q), 25.5 (C-27, q), 179.0 (C-28, s), 32.7 (C-29, q), 23.1 (C-30, q), 104.1 (C-1′, d), 74.7 (C-2′, d), 74.6 (C-3′, d), 69.1 (C-4′, d), 65.6 (C-5′, t), 100.5 (C-1′′, d), 71.2 (C-2′′, d), 78.7 (C-3′′, d), 71.8 (C-4′′, d), 68.8 (C-5′′, d), 18.0 (C-6′′, q), 110.3 (C-1′′′, d), 81.8 (C-2′′′, d), 78.2 (C-3′′′, d), 87.5 (C-4′′, d), 62.1 (C-5′′, t).

Compound 2

Colourless crystals; $[\alpha]_D^{25}$: +15.4° (c 0.5, EtOH); ESI-HRMS m/z 882.2 ($[M-H]^-$), $^-$ (calc. for [$C_{46}H_{74}O_{16}$ -1], 882.49767400).

^{1}H NMR (400.13 MHZ, C_6D_5N): δ 1.16 (3H, br s, H-24), 0.95 (3H, s, H-25), 1.05 (3H, s, H-26), 1.57 (3H, s, H-6′′), 1.25 (3H, s, H-27), 1.00 (3H, s, H-30), 0.95 (1H, s, H-29). 4.90 (1H, d, J = 7.8 Hz, H-1′), 6.30 (1H, d, J = 7.8 Hz, H-1′′), 6.15 (1H, d, J = 7.6 Hz, H-1′′′).

^{13}C NMR data (100 MHZ, C_6D_5N) δ 39.2 (C-1, t), 26.5 (C-2, t), 81.4 (C-3, d), 43.8 (C-4, s), 47.9 (C-5,d), 18.4 (C-6, t), 33.1 (C-7, t), 39.9 (C-8, s), 48.3 (C-9, d), 37.0 (C-10, s), 23.8 (C-11, t), 122.8 (C-12, d), 144.9 (C-13, s), 42.3 (C-14, s), 28.5 (C-15, t), 24.4 (C-16, t), 46.8 (C-17 s), 39.9 (C-18, d), 46.5 (C-19, t), 31.1 (C-20, s), 34.4 (C-21, t), 33.3 (C-22, t), 64.2 (C-23, s), 14.3 (C-24, q), 16.2 (C-25, q), 17.6 (C-26, q), 26.3 (C-27, q), 180.4 (C-28, s), 33.4 (C-29, q), 23.9 (C-30, q), 104.8 (C-1′, d), 75.2 (C-2′, d), 74.7 (C-3′, d), 69.7 (C-4′, d), 66.4 (C-5′, t), 101.5 (C-1′′, d), 72.2 (C-2′′, d), 83.1 (C-3′′, d), 73.2 (C-4′′, d), 69.9 (C-5′′, d), 18.6 (C-6′′, q), 107.7 (C-1′′′, d), 73.3 (C-2′′′, d), 79.9 (C-3′′′, d), 69.9 (C-4′′′, d), 67.3 (C-5′′′, t).

Acid Hydrolysis of 1 and 2

Each isolate (10 mg) was heated in a mixture of 20% HCl (6.5 mL) and CH_3OH (3.5 mL) under reflux for 7 h. The reaction mixture was concentrated under reduced pressure to remove methanol and diluted with water (2 mL) and extracted with chloroform (10 mL ×3). The H_2O layer was neutralized with Na_2CO_3, filtered and concentrated under reduced pressure and residue which contained sugars were subjected to paper chromatography analysis with standard sugars glucose, rhamnose, arabinose and xylose. (BuOH-HOAc-H_2O (4:1:5) was used as solvent and detection was by aniline/phthalate spray.

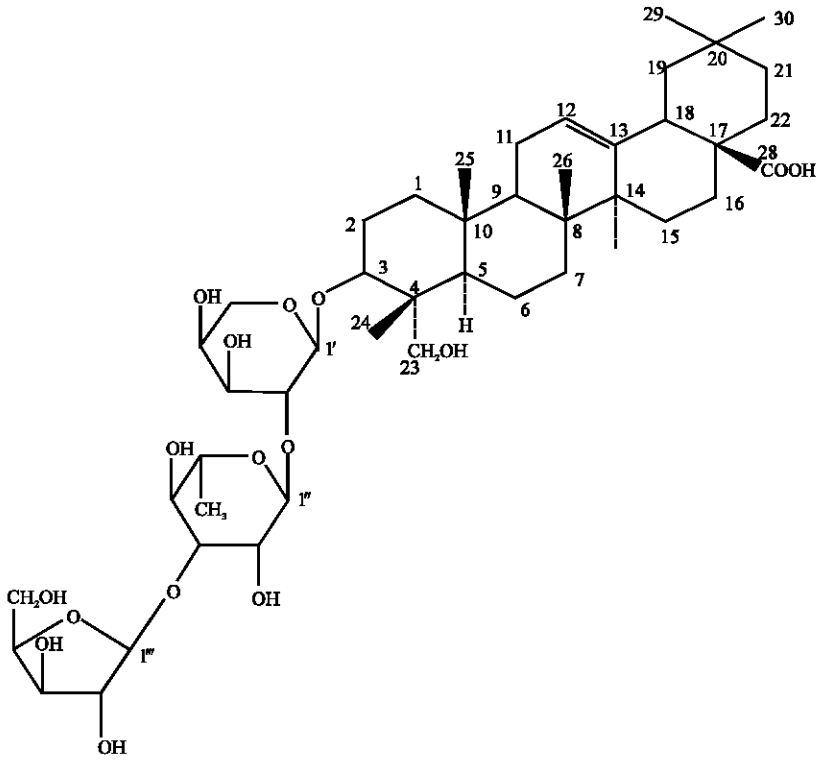
Antifungal Assay

The organisms used in this study were *Candida albicans* (ATCC 90028), *Candida neoformans* (ATCC 90113) and *Aspergillus fumigatus* (ATCC 90906). The antifungal assays were evaluated by agar diffusion method. The antifungal activity was determined by measuring the diameter of zone of inhibition (mm). The determination of MIC was performed using a two fold dilution technique as previously described (Peterson *et al*., 1992). The MIC, μg mL^{-1}, was recorded as the lowest concentration that prevented visible growth (Hamann *et al*., 1993). The antifungal agent amphotericin B was included as positive control in each assay.

RESULTS AND DISCUSSION

Evidence from ^{13}C NMR chemical shifts showed that both 1 and 2, possess the same triterpenoid skeleton, differing only in the sugar regions. A comparison of the ^{13}C NMR signals due to the compounds with those of reported saponins revealed that they are monodesmosides of 3-O-glycoside and the aglycones as hederagenins (Li *et al*., 1990).

Mineral acid hydrolysis of 1 yielded arabinose and rhamnose as the sugar components. The negative ion HRESI mass spectrum of 1 revealed the molecular ion peaks appearing at m/z 882.3 $[M-H]^-$ corresponding to the molecular formula $C_{46}H_{74}O_{16}$ with fragment peaks appeared at m/z 750.9, 604.0 and 471.3 and indicating the loss of arabinose, arabinose + rhamnose and 2×arabinose+ rhamnose. This sequence indicated that a terminal arabinose moiety is linked to an inner rhamnose that is linked to iuner arabinose and this, in turn, is attached to the C-3 of hederagenin (Mahato *et al*., 1991). This fragmentation pattern confirms interglycosidic linkages in all the sugars of 1. Comparison of ^{13}C NMR spectra of the sugar portions with corresponding methylglycosides revealed that the sugar moieties are of α-L-arabinofuranose, α-L-arabinopyranose and α-L-rhamnopyranose (Gorin and Mazurek, 1975).



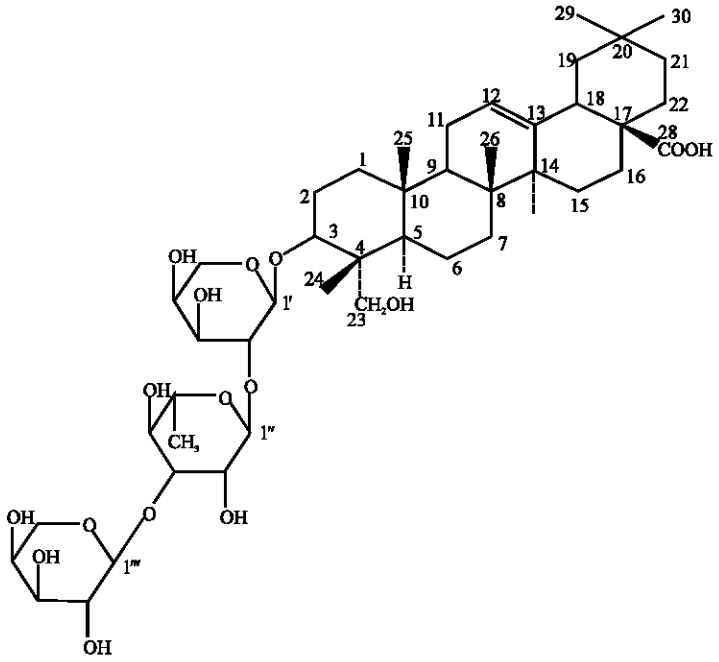
Structure of compound (1)

The ^{13}C NMR spectrum of 1 demonstrated 46 carbon resonances in partial agreement with the molecular formula $C_{46}H_{74}O_{16}$ indicating three sugar moieties, two pentoses and one hexose while the remaining 30 signals assigned to the aglycone. The ^{13}C NMR spectrum showed anomeric carbon signals at δ_C 111.0, 104.9 and 101.3 which were consistent with the presence of trisaccharide chain. The DEPT spectrum displayed 7 methyl, 13 methylene, 18 methine and 8 quarternary carbon atoms. The ^1H and ^{13}C NMR and HMBC data indicated the presence of a trisubstituted double bond δ_H 5.33 ppm (1H, br s, H-12) and δ_C 144.2 and 122.0 ppm, characteristic of a Δ^{12} double bond in an oleanane skeleton (Ahmad *et al*., 1993; Silverstein and Webster, 1997). The appearance of signal at δ_C 179.0 is due to the presence of CO_2H group and seven methyl groups: δ_H 0.99 ppm (3H, s, H-24)/δ_C 13.5 ppm (C-24), δ_H 0.80 ppm (3H, s, H-25)/δ_C 15.5 ppm (C-25), δ_H 0.88 ppm (3H, s, H-26)/δ_C 16.8 ppm (C-26), δ_H 1.46 ppm (3H, s, H-5′′)/δ_C 18.10 ppm (C-5′′), δ_H 0.88 ppm (3H, s, H-30)/δ_C 23.1 ppm (C-30), δ_H 1.11 ppm (3H, s, H-27)/δ_C 25.5 ppm (C-27) and δ_H 0.80 ppm (3H, s, H-29)/δC 32.7 ppm (C-29).

The points of attachment of the sugar units in 1 were determined from the ^{13}C NMR chemical shifts (Table 1). The C-3 of the aglycone resonated at δ_C 80.7, thus showing +7.0 ppm desheilding as compared to hederagenin (Li *et al*., 1990). This is an indication that the sugar moieties are attached at this carbon. The upfield shift of C-2 signal by 1.90 ppm and the ^3J-HMBC correlation of H-3 (δ 4.16) to C-1′ (δ 104.1) also confirmed the above proposed site of glycosidation. The presence of hydroxy group attached to C-23 is evidenced by signal in the downfield region of the ^{13}C NMR spectrum, δ_C 63.5 ppm. A comparison of the chemical shift of C-2′ of arabinose (C-2′, δ 74.7) with that of methyl arabinose (C-2, δ 71.8) allowed the assigument of a 1→2 linkage between arabinose and rhamnose. The down field ^{13}C NMR chemical shift of C-3′′ of rhamnose at 78.7 and small upfield shift of C-4′′ of rhamnose at 71.8 indicated 1→3 linkage between rhamnose and the terminal arabinose. The

nature of the interglycosidic linkage was further confirmed by long-range connectivity information obtained from HMBC spectrum which showed ^{3}J-HMBC interaction of protons at δ 4.45 (H-2′) and δ 4.64 (H-3′′) with anomeric carbons at δ 100.5 (C-1′′) and δ 110.3 (C-1′′′) in agreement with presence of (1→2) and (1→3) linkages between arabinose and rhamnose and rhamose and the terminal arabinose, respectively. From these results the structure of 1 was elucidated as 3-O- [α-L-arabinofuranosyl- (1→3)-α-L-rhamnopyranosyl- (1→2)-α-L-arabinopyranosyl-]-hederagenin.

Compound 2 afforded hederagenin as the aglycone and arabinose and rhamnose as sugar components on acid hydrolysis. A comparison of the ^{13}C NMR spectra of the sugar portions with corresponding methylglycosides suggested that the sugar moieties are of 2 units of α-L-arabinopyranose and the other α-L-rhamnopyranose (Gorin and Mazurek, 1975) indicating a different sugar composition from 1. The negative ion HRESI mass spectrum of 2 exhibited the molecular ion peak at m/z 882.2 $[M-H]^-$ corresponding to the empirical molecular formula $C_{46}H_{74}O_{16}$. The ^{1}H and ^{13}C NMR chemical shifts were compared with the literature reports for triterpenoidal sapogenins/saponins (Li *et al*., 1990), which confirmed the identity of the aglycone as hederagenin. The olefinic resonances at δ_C 144.9 and 122.8 corresponding to quaternary and methine behaviour, revealed the presence of unsaturation at C-12 in an oleanane skeleton (Tori *et al*., 1974). The appearance of a signal at δ_C 180.4 is ascribed to the carbon of CO_2H group. The DEPT spectrum of 2 displayed 7 methyl, 13 methylene, 18 methine and 8 quarternary carbon atoms. There are seven methyl groups δ_H 1.16 ppm (3H, s, H-24)/δ_C 14.3 ppm (C-24), δ_H 0.95 ppm (3H, s, H-25) /δ_C 16.2 ppm (C-25), δ_H 1.05 ppm (3H, s, H-26)/δ_C 17.6 ppm (C-26), δ_H 1.57 ppm (3H, s, H-6′′) /δ_C 18.60 ppm (C-6′′), δ_H 1.25 ppm (3H, s, H-27)/δ_C 26.3 ppm (C-27), δ_H 1.00 ppm (3H, s, H-30) /δ_C 23.9 ppm (C-30) and δ_H 0.95 ppm (3H, s, H-29)/δC 33.4 ppm (C-29). The presence of hydroxy group attached to C-23 was evidenced by signal in the downfield region of the ^{13}C NMR spectrum, δ_C 64.2 ppm. The anomeric carbon signals resonated at δ 107.7, 104.8 and 101.5 indicating the presence of three sugar moieties. Mass spectral fragmentation pattern and results of acid hydrolysis tend to suggest identical structure for 1 and 2.



Structure of compound (2)

Table 1: ^{13}C (125 MHZ, C_6H_5N) NMR shifts for compounds 1-2

	1		2	
Position	δC	HMBC (C→H)	δC	HMBC (C→H)
C-3 sugars				
Ara I				
1′	104.1	H-3, H-5′, H-2′	104.8	H-3, H-5′, H-2′
2′	74.7	H-1′′, H-1′	75.2	H-1′′, H-1′
3′	74.6		74.7	
4′	69.1		69.7	
5′	65.6	H-4′	66.4	H-4′
Rham				
1′′	100.5	H-2′′,H-2′,H-5′′	101.5	H-2′′,H-2′,H-5′′
2′′	71.2	H-3′′,	72.2	
3′′	78.7	H-1′′′	83.1	
4′′	71.8		73.2	
5′′	68.8		69.9	
6′′	18.0	H-5′′	18.6	H-5′′
Ara II				
1′′′	110.3	H-3′′, H-2′′′	107.7	H-3′′, H-2′′′
2′′′	81.8		73.3	
3′′′	78.2		79.9	
4′′′	87.5		69.9	
5′′′	62.1	H-4′′′	67.3	H-1′′′, H-4′′′

The sugar linkages in 2 were determined using the glycosidation rule (Tori *et al*., 1974; Seo *et al*., 1978; Mahato *et al*., 1991). The ^{13}C NMR spectrum of 2 (Table 1) showed significant displacement of signals for C-3 (+7.7 ppm) of the aglycone, for C-2′ (+3.4 ppm) of the arabinopyranosyl moiety and for C-3′′ for inner rhamnopyranosyl moiety (+11.8 ppm) in comparison to the reported values for hederagenin (Tori *et al*., 1974) and methyl pyranoside due to glycosidation at these positions. The nature of the glycosidic linkage was further confirmed by the HMBC spectrum, which showed ^2J-HMBC and ^3J-HMBC interaction of protons at δ 4.47 (H-2′) and δ 4.63 (H-3), respectively with anomeric carbon C-1′ (δ 104.1) and ^3J-HMBC interaction of proton at δ 4.47 (H-2′) with C-1′′ (δ 100.5). The C-3′′ proton at δ 4.64 also showed ^2J-HMBC and ^3J-HMBC to C-2′′ (δ 81.8) and C-1′′′ (δ 110.3), respectively. However in the ^{13}C NMR spectrum, different resonance signals were obtained for the terminal α-L-arabinopyranosyl. It was observed that in 2 the signal of C-5′′′ due to arabinose was displaced downfield by 5.2 ppm and signal C-4′′′ was displaced upfield by 15.6 ppm. This disclosed that the terminal arabinose has C-5′′′ attached directly to oxygen to form a pyranose and not a furanose as it was the case with 1. Consequently, 2 was elucidated as 3-O- [α-L-arabinopyranosyl- (1→3)-α-L-rhamnopyranosyl (1→2)-α-L-arabinopyranosyl-]-hederagenin. Although the two compounds isolated were known compounds (Encarnacion *et al*., 1981) but the method of isolation and analysis of structures were different.

Compounds 1 and 2 were evaluated for antifungal activity in the agar well- diffusion assay. Compound 1 exhibited antifungal activity against *C. albican*, *C. neoformans* and *A. fumigatus* with IC_{50} of 4.5 μg mL^{-1}, 15.0 μg mL^{-1} and MIC 10.0 μg mL^{-1}, respectively and 2 exhibited similar activities with IC_{50} of 8.5 μg mL^{-1}, 10.0 μg mL^{-1} and MIC >25.0 μg mL^{-1}, respectively.

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