Personal pdf file for

Giselle Tamayo-Castillo, Víctor Vásquez, María Isabel Ríos, María Victoria Rodríguez, Godofredo Solano, Susana Zacchino, Mahabir P. Gupta

With compliments of Georg Thieme Verlag

www.thieme.de

Isolation of Major Components from the Roots of *Godmania aesculifolia* and Determination of Their Anti-fungal Activities

DOI 10.1055/s-0033-1351025
Planta Med 2013; 79: 1749–1755

For personal use only.
No commercial use, no depositing in repositories.

Publisher and Copyright:
© 2013 by
Georg Thieme Verlag KG
Rüdigerstraße 14
70469 Stuttgart
ISSN 0032-0943

Reprint with the
permission by
the publisher only

Thieme

Isolation of Major Components from the Roots of *Godmania aesculifolia* and Determination of Their Antifungal Activities

Authors Giselle Tamayo-Castillo[1,2], Víctor Vásquez[1], María Isabel Ríos[1], María Victoria Rodríguez[3], Godofredo Solano[1,2], Susana Zacchino[3], Mahabir P. Gupta[4]

Affiliations The affiliations are listed at the end of the article

Key words
- *Godmania aesculifolia*
- Bignoniaceae
- lapachol
- chavicol diglycoside
- *Trichophyton* spp.
- *Microsporum* spp.
- *Aspergillus* spp.
- *Candida*
- *Cryptococcus neoformans*

Abstract

From the methanol root extract of *Godmania aesculifolia*, a species selected in a multinational OAS program aimed at discovering antifungal compounds from Latin American plants, a new chavicol diglycoside (**1**), the known 3,4-dihydroxy-2-(3-methylbut-2-en-1-yl)-3,4-dihydronaphthalen-1(2*H*)-one (**2**), and lapachol (**3**) were isolated and characterized by 1D and 2D NMR and MS techniques. Only **3** exhibited fairly good activity against a panel of clinical isolates of *Cryptococcus neoformans* (MIC_{50} between 7.8 and 31.2 µg/mL) and moderate activities against *Candida* spp. and non-*albicans Candida* spp.

Abbreviations

MIC:	minimum inhibitory concentration
OAS:	Organization of the American States
ATCC:	American Type Culture Collection
CONICIT:	National Council of Science and Technology, Costa Rica
amu:	atomic mass units
CLSI:	Clinical and Laboratory Standards Institute
DMAP:	dimethylaminopyridine
DMSO:	dimethylsulfoxide
ELSD:	evaporative light scattering detector
SCW:	sterility control well
GCW:	growth control well
LTW:	lapachol test well
CFU:	colony forming units
MIC_{80}:	minimum concentration that inhibits 80% of the fungal growth
MIC_{50}:	minimum concentration that inhibits 50% of the fungal growth
AIDS:	acquired immunodeficiency syndrome

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

received February 8, 2013
revised July 24, 2013
accepted October 5, 2013

Bibliography
DOI http://dx.doi.org/10.1055/s-0033-1351025
Planta Med 2013; 79: 1749–1755 © Georg Thieme Verlag KG Stuttgart · New York · ISSN 0032-0943

Correspondence
Prof. Dr. Giselle Tamayo-Castillo
Bioprospecting Unit
Instituto Nacional de Biodiversidad
P. O. Box 22–3100
Santo Domingo de Heredia, Heredia
Costa Rica
Phone: + 506 25 07 82 47
Fax: + 506 25 07 82 64
gtamayo@inbio.ac.cr

Introduction

Opportunistic fungal infections have been a major medical problem during the past two decades especially involving immunocompromised patients such as neonates, cancer patients receiving chemotherapy, organ transplant and burn patients, apart from those with AIDS [1–3].

Of all available drugs for the treatment of systemic and superficial mycoses, only a limited number are efficacious. They belong to five antifungal classes, namely azoles, polyenes, pyrimidines, allylamines, and the recently available echinocandins. However, some of these drugs show toxicity, produce recurrence or lead to the development of resistance due, in part, to the intensive prophylactic use of antifungal drugs [4,5]. As a consequence, there is a general consensus that a next generation of new structures with potent antifungal properties, which could be useful for future development, are urgently needed.

It is known that *Candida* spp. are among the leading causes of nosocomial blood stream infections worldwide and, although *C. albicans* was in the past the usual species associated with invasive infections, at present non-*albicans Candida* spp., such as *C. tropicalis, C. glabrata, C. parapsilopsis, C. krusei*, and others, represent more than half of the isolates of candidiasis in clinical practice nowadays [3]. In turn, *C. neoformans* remains an important life-threatening complication for immune compromised hosts [6], specifically for patients who have received transplants of solid organs.

As part of a multinational OAS project, plants from Costa Rica were evaluated for their antifun-

Electronic reprint for personal use

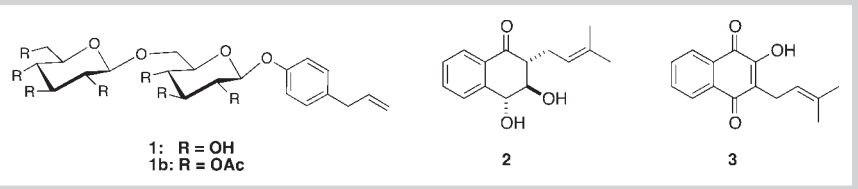

Fig. 1 Chemical structures of compounds **1–3**.

Table 1 ^{1}H, ^{13}C, and 2D-NMR data of **1b** in $CDCl_3$ and C_6D_6.

	$CDCl_3$		C_6D_6	
Position	**δ_C**	**δ_H (J Hz)**	**δ_C**	**δ_H (J Hz)**
1	155.5 s		155.8 s	
2/6	116.8 d	6.89 d (8.7)	115.2 d	7.03 d (8.5)
3/5	130.1 d	7.14 d (8.7)	129.9 d	7.17 d (8.5)
4	135.2 s		134.7 s	
5/3	116.8 d	6.89 d (8.7)	115.2 d	7.03 d (8.5)
6/2	130.1 d	7.14 d (8.7)	129.9 d	7.17 d (8.5)
7	39.5 t	3.33 d (6.7)	39.3 t	3.26 d (5.6)
8	137.7 d	5.92 m	137.6 d	5.94 m
9	115.9 t	5.03 m	115.5 t	5.02 m
1′	99.2 d	5.01	98.8 d	4.87
2′	71.4 d	5.19 dd (7.7, 9.4)	71.3 d	5.54 dd (7.9, 9.5)
3′	72.8 d	5.24 t (9.5)	72.7 d	5.42 t (9.4)
4′	69.0 d	4.93 m	68.9 d	5.02 m
5′	74.0 d	3.83 m	73.9 d	3.53 m
6′	68.0 t	3.66 dd 3.86 dd	67.4 t	3.54 q 3.77 d (9.8)
1′′	100.6 d	4.55 d (9.2 Hz)	100.2 d	4.36 dd (7.7, 5.5)
2′′	71.3 d	4.96 dd (8.02, 9.2)	71.4 d	5.32 dd (8.36, 5.7)
3′′	73.0 d	5.11 t (9.4)	73.0 d	5.32
4′′	68.4 d	5.03 m	68.2 d	5.30
5′′	71.9 d	3.54 dq (9.8, 2.4)	71.8 d	3.02
6′′	61.9 t	4.08 dd (10.0, 2.3) 4.21 dd (4.8, 7.5)	60.9 t	3.98dd (10.4, 2.0) 4.16dd (8.1, 2.0)
2′-acetyl				
▸ CO	170.5 s	–	168.9 s	–
▸ CH_3	–	–	–	1.64 s
3′-acetyl				
▸ CO	170.1 s		169.8 s	
▸ CH_3	–	–	–	
4′-acetyl				
▸ CO	169.6 s		169.1 s	
▸ CH_3	–	–	–	1.65 s
2′′-acetyl				
▸ CO	169.4 s		169.7 s	
▸ CH_3	–	–	–	–
3′′-acetyl				
▸ CO	170.1 s		169.7 s	
▸ CH_3	–	–	–	–
4′′-acetyl				
▸ CO	169.3 s	–	169.4 s	–
▸ CH_3	20.6 t	1.87 s	–	–
6′′-acetyl				
▸ CO	170.5 s	–	169.9 s	–
▸ CH_3	20.7 t	2.69 s	–	–

gal activity. From this preliminary screening, we selected *Godmania aesculifolia* (Kunth) Standl. roots (Bignoniaceae) for the isolation of major components and the determination of their antifungal activities. There are few reports on the presence of flavonoids and triterpenes in *G. aesculifolia* [7,8]. In this paper, the antifungal-guided fractionation of *G. aesculifolia* extracts led to the isolation of a new compound and two known compounds not previously reported in this species, one of them lapachol. Extracts and compounds were tested on a fungal panel of standardized yeasts, hialohyphomycetes and dermatophytes. In addition, in view of the contradictory anti-yeasts activities reported for lapachol [9,10], we tested it on an extended panel of clinical isolates of the most susceptible yeasts with standardized procedures to unequivocally establish its antifungal activity.

Electronic reprint for personal use

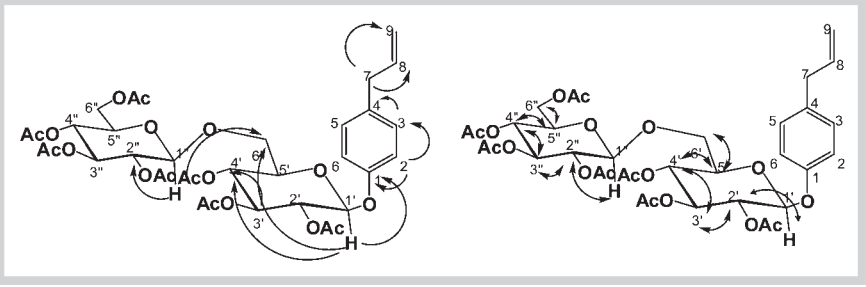

Fig. 2 Selected HMBC correlations (single head arrow) and dqfCOSY correlations (double head arrow) in C_6D_6 for compound **1b**.

Table 2 Minimum inhibitory concentrations (MIC) values (µg/mL) of methanolic extracts from the roots of *Godmania aesculifolia* and compounds isolated from them: chavicol diglycoside (**1**), 3,4-dihydroxy-2-(3-methyl-2-butenyl)-1(2*H*)-naphtalenone (**2**), and lapachol (**3**).

	Hialohyphomycetes			Dermatophytes					
	Ca	**Sc**	**Cn**	**Afu**	**Afl**	**An**	**Mg**	**Tr**	**Tm**
G. a.	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	**500**	**250**	**250**
1	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
2	> 250	> 250	> 250	> 250	> 250	> 250	**250**	**62.5**	**32**
3	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250	> 250
Amph B	0.78	0.25	0.78	0.50	0.25	0.78	0.50	0.25	0.78
Terbinafine	1.56	0.39	1.56	3.12	0.39	1.56	3.12	0.39	1.56
Ketoconazole	**0.5**	0.25	**0.5**	**0.5**	0.25	**0.5**	**0.5**	0.25	**0.5**

Ca: *Candida albicans* ATCC 10231, *Sc: Saccharomyces cerevisiae* ATCC 9763, Cn: *Cryptococcus neoformans* ATCC 32264, Afu: *Aspergillus fumigatus* ATCC 26934, Afl: *Aspergillus flavus* ATCC 9170, An: *Aspergillus niger* ATCC 9029, Mg: *Microsporum gypseum* C 115 2000, Tr: *Trichophyton rubrum* C 113 2000, Tm: *Trichophyton mentagrophytes* ATCC 9972

Results and Discussion

The bioassay-guided fractionation of MeOH extract from the roots of *G. aesculifolia* led to the isolation of the new chavicol diglycoside (**1**) and the known compounds 3,4-dihydroxy-2-(3-methylbut-2-en-1-yl)-3,4-dihydronaphthalen-1(2*H*)-one (**2**) and lapachol (**3**) (see **Fig. 1**). Compound **1** was obtained as a white solid and presented a molecular weight of 458.1788 amu, which corresponds to the formula $C_{21}H_{30}O_{11}$. It was peracetylated to yield **1b,** with a molecular weight of 752.2527 amu, consistent with the presence of seven acetyl groups. The ^{1}H-NMR and ^{13}C-NMR signals (in $CDCl_3$ and C_6D_6) of **1b** are shown in **Table 1**. The first deuterated solvent was used for the determination of coupling constants while the second allowed resolution of signals and obtention of additional information on coupling constants. The aglycon unit is a *p*-substituted aromatic ring, which is in agreement with the AA'BB' pattern observed in the ^{1}H-NMR spectrum. Substituted carbons at δ 155.8 and 134.7 ppm (in C_6D_6) indicated the presence of an oxygenated C-1 and an allyl group at C-4, respectively. This moiety showed ^{1}H-NMR signals at δ 5.02 (m), 5.94 (dq), and 3.26 (d) ppm. The appearance of two anomeric signals at δ 4.87/98.8 and 4.36/100.2 ppm is consistent with the presence of two sugar units (see **Fig. 2**). HMBC correlation of the anomeric proton at δ 4.87 ppm (H-1′) with the carbon signal at 155.8 ppm showed that the first sugar unit is joined to C-1. A β(1,6)-linkage between the two sugar units could be determined on the basis of the long correlation observed between the second anomeric proton at δ 4.36 ppm (H-1″) and the methylene carbon at 67.4 ppm (C-6′). The coupling constants measured in C_6D_6 for H-1′ of about 8 Hz (obtained indirectly from H-2′) and in $CDCl_3$ for H-1″ of 9.2 Hz are in agreement with both β-linkages (see **Fig. 2**). The remaining coupling constants indicate the presence of glucose units (**Table 1**): H-3′ is observed in C_6D_6 as a triplet with a coupling constant of 9.4 Hz, indicating that H-2′ and H-4′ are in axial orientations. In addition, H2″ and H3″ present coupling constants in $CDCl_3$ of 8.02 and 9.4 Hz, respectively.

Compounds **2** and **3** were identified by comparison of their 1D- and 2D-NMR data with those reported in the literature [11,12].

Regarding antifungal activity, *G. aesculifolia* MeOH root extracts exhibited moderate activity against the dermatophytes *Microsporum gypseum* C115, *Trichophyton rubrum* C110, and *T. mentagrophytes* ATCC 9972 with MICs 250–500 µg/mL. Of the isolated compounds, only compound **3** showed MICs = 250, 62.5, and 31.2 µg/mL (**Table 2**) against *M. gypseum, T. rubrum*, and *T. mentagrophytes*, respectively.

Lapachol (**3**) is a common constituent of plants, particularly of the Bignoniaceae family. It is used as a pH indicator and is commercially available in Brazil as an adjuvant in the treatment of cancer [12–14]. In addition, it has been reported to exhibit broad-spectrum antimicrobial properties [15–17].

Previous studies report contradictory results on the antifungal activity of **3**. In an article on this subject, Gonçalves de Lima et al. [10] observed that **3** was inactive against species of *Candida* and *Cryptococcus* genera while Guiraud et al. [9] found that **3** possessed a potent antifungal activity (MIC = 0.03 µg/mL) against *C. albicans, C. parapsilopsis, C. tropicalis*, and *Cryptococcus neoformans* that was comparable to or lower than ketoconazole. Then Gafner et al. [18] reported that **3** was active against *Cladosporium cucumerinum* and *C. albicans* in bioautographic assays. In contrast, a recent work [19] reported that **3** was inactive with an MIC > 1.03 µmol/ml (> 249 µg/mL) against *C. albicans, C. tropicalis*, and *Crytococcus gatti* and displayed higher activities (0.13–0.26 µmol/mL = 24–48 µg/mL) against *Paracoccidiodes* spp.

In this work, we carried out a new antifungal evaluation of lapachol by using the standardized methodologies recommended by CLSI [20,21]. Initially, **3** was tested against a panel of nine standardized fungal strains that comprised the yeasts *C. albicans, S.*

Electronic reprint for personal use

Table 3 Inhibition percentages of growth of clinical strains of *Candida* spp. at different concentrations (µg/mL) of lapachol. MIC_{100}, MIC_{80}, and MIC_{50} are recorded in µg/mL. For the sake of comparison, the inhibition percentage of the standardized *C. albicans* strain ATCC 10231 by lapachol is included too.

	Lapachol (3) (µg/mL)									Amph
Strain	**250**	**125**	**62.5**	**31.25**	**15.62**	**7.8**	**3.9**	MIC_{100}	MIC_{80}	MIC_{50}	MIC_{100}
C. albicans ATCC 10231	69.49 ± 5.41	49.33 ± 7.63	35.79 ± 10.53	31.26 ± 2.89	27.28 ± 2.10	19.676 ± 4.58	15.72 ± 0.57	> 250	> 250	125	0.78
C. albicans C 125	100	31.30 ± 8.09	33.76 ± 9.00	27.47 ± 11.67	16.86 ± 0.97	15.18 ± 1.01	15.53 ± 2.33	250	250	250	0.78
C. albicans C 126	80.27 ± 9.01	73.87 ± 6.50	52.74 ± 5.33	47.66 ± 2.54	42.90 ± 0.56	45.85 ± 2.58	35.62 ± 4.13	> 250	250	31.25	1.56
C. albicans C 127	66.53 ± 6.96	33.57 ± 1.50	28.85 ± 8.69	11.57 ± 3.97	16.64 ± 5.91	14.43 ± 2.47	14.69 ± 7.84	> 250	> 250	250	0.78
C. albicans C 129	100	81.06 ± 6.71	46.84 ± 8.91	43.82 ± 5.41	35.23 ± 2.69	36.02 ± 5.21	31.42 ± 3.51	> 250	125	62.5	1.56
C. albicans C 130	46.35 ± 12.23	30.44 ± 1.77	30.43 ± 6.21	7.30 ± 2.69	7.70 ± 4.86	4.59 ± 2.37	4.91 ± 2.47	> 250	> 250	> 250	0.78
C. tropicalis C 131	34.32 ± 1.25	17.26 ± 1.18	11.21 ± 3.43	5.06 ± 0.30	4.46 ± 1.10	3.75 ± 3.41	0	> 250	> 250	> 250	0.78
C. krusei C 117	90.50 ± 2.00	87.86 ± 14.14	35.13 ± 6.61	7.20 ± 1.98	4.72 ± 0.41	5.41 ± 1.44	0	> 250	125	125	0.39
C. glabrata C 115	33.39 ± 18.45	34.40 ± 5.52	26.55 ± 7.97	13.13 ± 7.54	7.92 ± 1.51	4.31 ± 2.56	2.55 ± 1.58	> 250	> 250	> 250	0.39
C. parapsilopsis	43.83 ± 1.80	42.48 ± 7.92	37.66 ± 7.06	32.50 ± 5.13	30.25 ± 1.25	22.20 ± 5.86	0	> 250	> 250	> 250	0.78
C. kefir C 123	27.47 ± 1.39	10.18 ± 6.28	15.95 ± 0.88	6.40 ± 0.33	4.63 ± 1.98	4.68 ± 0.40	2.73 ± 0.92	> 250	> 250	> 250	0.78
C. lusitaneae C 132	61.78 ± 5.57	39.81 ± 2.70	17.33 ± 1.84	16.28 ± 1.31	17.84 ± 3.94	19.27 ± 3.36	10.25 ± 1.38	> 250	> 250	250	0.39

C = CEREMIC, Reference Center on Mycology; ATCC: American Type Culture Collection; Amph: amphotericin B

cerevisiae, and *C. neoformans*, the filamentous fungi *A. niger*, *A. flavus*, and *A. fumigatus* and the dermatophytes *M. gypseum*, *T. mentagrophytes*, and *T. rubrum*. Results of MIC values are shown in **Table 2**. Lapachol appears to be active only against the three tested dermatophytes with MICs between 31.2 and 250 µg/mL, with *T. mentagrophytes* being the most susceptible species.

To corroborate the antifungal activity of **3** against *Candida* spp. and *C. neoformans*, it was retested against eleven other *Candida* strains, including five clinical isolates of *C. albicans* and the six non-*albicans Candida* spp., all of them provided by CEREMIC (see General experimental procedures) and against eleven strains of *Cryptococcus neoformans* from Malbrán Institute (Buenos Aires, Argentina). MIC_{80} and MIC_{50} were also determined. The evaluation with a less stringent endpoint such as MIC_{80} and MIC_{50} has shown to consistently represent the *in vitro* activity of compounds [20] and often provides a better correlation with other measurements of antifungal activity [22, 23].

Results showed (**Table 3**) that **3** inhibited the eleven *Candida* strains at all concentrations tested, but with percentages of inhibition lower than 100% in 10 out of the 11 strains tested. It only inhibited 100% of growth of *C. albicans* (strain C 125) at 250 µg/mL [MIC (= MIC_{100}) = 250 µg/mL]. These results are consistent with those shown in **Table 2** where **3** was inactive (MIC > 250 µg/mL) against the standardized ATCC strain of *C. albicans*.

On the other hand, when MIC_{80} or MIC_{50} were analyzed, **3** produced a 50% inhibition of the growth for six out of the eleven strains of the panel with concentrations between 31.2 and 250 µg/mL and inhibited 80% of the growth of four strains between 125–250 µg/mL.

Finally, **3** displayed a noteworthy activity against the clinical isolates of *C. neoformans* (**Table 4**) inhibiting 50% of growth of eight *C. neoformans* clinical isolates at concentrations as low as 7.8 and 15.6 µg/mL and only one at 31.2 µg/mL.

Results reported here allow to establish the antifungal properties of components of *G. aesculifolia* and provide reliable or unequivocal results on the antifungal activity of lapachol against important human opportunistic pathogenic fungi such as *Candida* spp. and *C. neoformans*.

Materials and Methods

General experimental procedures

For preparative HPLC, a custom-made Varian chromatograph with two detectors (UV Knauer and ELSD Sedex) was used. Separations were performed on a flanged MODCOL, filled with C-18 Kromasil (50 × 250 mm, 10 µm, volume of 500 mL). Final purifications were also performed on a Waters 996 chromatograph (Millenium32®), with a Waters PDA detector equipped with a Waters Prep-Nova Pak HR-C18 column, 60 Å, 7.8 × 300 mm. PTLCs were prepared in a Camag system (layer thickness: 2 mm), using silica gel 60_{GF} (Merck). Melting points were measured on a Barnsted electrothermal 9100 apparatus. The NMR spectra of **1**–**3** were recorded on a Varian Mercury 400BB MHz spectrometer using the residual solvent signals as references. ^{1}H-NMR and ^{13}C-NMR assignments were determined by gHSQC, gHMQC, gHMBC, ^{1}H-^{1}H-COSY, and dqf-COSY. HR-ESI-MS were performed in the Mass Spectrometry Facility of the University of Illinois at Urbana-Champaign, USA.

Electronic reprint for personal use

Table 4 Inhibition percentages of growth of clinical strains of *Cryptococcus neoformans* at different concentrations (µg/mL) of lapachol. MIC_{100}, MIC_{80}, and MIC_{50} are recorded in µg/mL.

	Lapachol (µg/mL)								Amph	Itra	Vori
Strain	250	125	62.5	31.25	15.62	7.8	3.9	MIC_{100}	MIC_{80}	MIC_{50}	MIC_{100}	MIC_{100}	MIC_{100}
IM 983040	100	100	92.30 ± 7.38	69.27 ± 1.84	64.02 ± 066	28.43 ± 8.77	17.81 ± 1.03	125	15.62	15.62	0.13	< 0.015	< 0.015
IM 972724	100	100	86.57 ± 3.10	77.47 ± 3.47	17.54 ± 1.98	15.21 ± 5.44	0	125	62.5	31.25	0.06	0.25	< 0.015
IM 042074	90.74 ± 1.48	85.01 ± 1.47	79.21 ± 0.86	78.14 ± 4.07	76.14 ± 2.70	39.52 ± 2.06	30.25 ± 5.31	> 250	62.5	15.62	0.25	< 0.015	< 0.015
IM 983036	88.92 ± 1.54	78.69 ± 1.07	78.05 ± 2.00	74.40 ± 8.94	67.35 ± 10.31	57.73 ± 2.30	20.36 ± 2.36	> 250	62.5	7.8	0.13	< 0.015	< 0.015
IM 00319	100	87.99 ± 0.70	87.99 ± 2.14	80.12 ± 3.01	76.19 ± 2.13	73.22 ± 3.10	40.43 ± 2.45	250	31.25	7.8	0.25	< 0.015	< 0.015
IM 972751	100	100	95.59 ± 0.20	64.10 ± 0.20	49.65 ± 0.23	32.73 ± 4.66	26.45 ± 3.54	125	62.5	15.62	0.25	< 0.015	0.030
IM 031631	100	99.35 ± 0.62	94.70 ± 13.65	69.53 ± 1.48	67.16 ± 2.11	12.04 ± 6.83	1.74 ± 2.13	125	62.5	15.62	0.13	0.25	0.25
IM 031706	100	100	78.76 ± 2.84	69.19 ± 11.14	63.32 ± 8.38	57.75 ± 7.13	12.04 ± 0.06	125	62.5	7.8	0.25	0.50	0.50
IM 961951	100	56.65 ± 4.02	56.32 ± 1.38	56.10 ± 2.68	54.25 ± 2.74	50.87 ± 1.75	20.11 ± 0.01	250	250	7.8	0.06	< 0.015	0.015

Amph: amphotericin B; Itra: itraconazole; Vori: voriconazole; IM: Malbrán Institute

Plant material

G. aesculifolia was collected in Lomas de Barbudal Biological Reserve (Guanacaste) in August 2000 (collection LA2583, voucher INB0003433719). Voucher specimens were deposited at INBio's Herbarium for future reference, and the plant species was identified by the taxonomist Luis G. Acosta. Collection of plant species was authorized by Res. 288.99-OFAU; Res. 288.2003 OFAU, CM 001OT-2005, and CM INBio 012–2006 OT.

Extraction and Isolation

Dried and finely powdered roots (0.283 kg) of *G. aesculifolia* were macerated in 2.5 L 2 × 8 h of EtOH 95% (FANAL). After filtration, the EtOH extract was concentrated to dryness under reduced pressure at < 40 °C yielding 15.2 g. Twelve grams of the latter were detannified using 45 g of polyamide resin DPA-6S (Supelco) and the resulting extract was adsorbed on a filled Diaion HP-20 (37 g) (Supelco) column (5.5 × 9.0 cm), thoroughly washed with deionized water and finally desorbed with 1.2 L of MeOH (Spectrum). The resulting MeOH extract was evaporated to dryness to obtain 3.3 g of extract, from which 1.9 g was fractionated on a preparative Varian HPLC collecting 48 fractions of 180 mL every 2 min (flow rate 90 mL/min) using the following step gradient elution program: EtOH : H_2O 10 : 90 (10 min), 20% EtOH (10 min), 30% EtOH (15 min), 50% EtOH (20 min), 70% EtOH (15 min), 90% EtOH (15 min), and finally EtOH 100%. Fractions 10–13 contained compound **1** (298.2 mg, 15.7% yield, 40% EtOH : H_2O) as the major component, fractions 20–25 and 26–33 contained mainly compounds **2** (147.7 mg, 7.7% yield, 50% EtOH : H_2O), and compound **3** (217.3 mg, 11.4% yield, 70% EtOH : H_2O), respectively. Fractions 10–13 (298.2 mg) were fractionated on a column of 25 × 3 cm filled with (130 g) RP-C18 40–63 µm (Lichrosorb; Merck), eluted using 50 mL of water and then increments of 10% v/v of mixtures of methanol and water, yielding 8 fractions of 100 mL. Fraction 6 (114.7 mg, 1 : 1 MeOH : H_2O) was subsequently purified on a PTLC [$CHCl_3$: MeOH : H_2O 65 : 35 : 5; R_f 0.32; $CHCl_3$ (Laboratorios Arvi)] obtaining 94.2 mg of pure **1** as white crystals (m. p. 151–152 °C, 97% purity). PTLC of fractions 20–25 (147.4 mg) afforded **2** (30.0 mg, $CHCl_3$:MeOH 9 : 1, R_f: 0.31, 97% purity). PTLC of fractions 26–33 (217 mg) afforded **3** (10 mg, $CHCl_3$: MeOH 95 : 5 R_f: 0.77, 98% purity).

Peracetylation of **1**: To assist structure elucidation, 10 mg of **1** were peracetylated with 25 mL of acetic anhydride (Aldrich), 0.5 mL of pyridine (Aldrich) and 3–5 crystals of DMAP (Aldrich), refluxed for 24 h, concentrated in a rotatory evaporator and redissolved in $CHCl_3$. The organic phase was washed with water, 5% HCl (100 mL) and with $NaHCO_3$ 0.2% (100 mL, Aldrich), and finally with water (100 mL) and evaporated on a rotatory evaporator. The resulting residue (20 mg) was further purified on a PTLC (15 mg, $CHCl_3$: MeOH 9 : 1, R_f: 0.83, m. p. 190–191 °C), yielding **1b** with 97% purity.

Chavicol diglycoside (**1**): White crystals, TOF-MS-ESI: 481.1686 $[M + Na]^+$ calculated for ($C_{21}H_{30}O_{11}$: 458.1788; $C_{21}H_{30}O_{11}Na$: 481.16859) m. p. 151–152 °C.

Peracetyl chavicol diglycoside (**1b**): White crystals. TOF-MS-ESI: 775.2425 $[M + Na]^+$ calculated for ($C_{35}H_{44}O_{18}$: 752.2527; $C_{35}H_{44}O_{18}Na$: 775.24253); m. p. 190–191 °C; ^{1}H-NMR (400 MHz, $CDCl_3$, and C_6D_6): ▶ **Table 1**; ^{13}C-NMR (100 MHz, $CDCl_3$ and C_6D_6) ▶ **Table 1**.

3,4-Dihydroxy-2-(3-methylbut-2-en-1-yl)-3,4-dihydronaphthalen-1(2H)-one (**2**): Colorless oil, ^{1}H NMR (400 MHz, Chloroform-d): δ = 8.00 (dd, J = 7.9, 1.3 Hz, H-8), 7.76 (d, J = 7.9 Hz, H-5), 7.63 (td, J = 7.6, 1.4 Hz, H-6), 7.41 (t, J = ddd, J = 7.9, 7.6, 0.9 Hz, H-7),

Electronic reprint for personal use

5.15 (ddt, *J* = 8.1, 6.8, 1.4 Hz, H-2′), 4.82 (d, *J* = 9.2 Hz, H-4), 3.84 (dd, *J* = 11.5, 9.2 Hz, H-3), 2.77 (m, H-1′), 2.63 (dt, *J* = 11.5, 4.7 Hz, H-2), 1.70 (s, H-4′), 1.64 (s, H-5′); ^{1}H NMR (400 MHz, methanol-d4): δ = 7.91 (dd, *J* = 7.9, 1.1 Hz, H-8), 7.79 (dbr, *J* = 7.82, H-6), 7.66 (td, *J* = 7.7, 1.4 Hz, H-5), 7.41 (tbr, J = 7.30 H-7), 5.12 (ddd, *J* = 7.8, 5.1, 1.2 Hz, H-2′), 4.67 (d, *J* = 8.8 Hz, H-4), 3.68 (dd, *J* = 10.9, 8.8 Hz, H-3), 2.77 (m, H-1′), 2.61 (m, H-2), 1.70 (s, H-4′), 1.64 (s, H-5′); ^{13}C NMR (101 MHz, methanol-d4): δ = 199.7 (C-1), 145.4 (C-10), 135.3 (C-6), 134.5 (C-3′), 132.5 (C-9), 128.7 (C-7), 127.6 (C-5), 127.4 (C-8), 122.0 (C-2′), 74.9 (C-3), 74.4 (C-4), 55.0 (C-2), 26.1 (C-4′), 25.1 (C-1′), 18.1 (C-5′).

Lapachol (**3**): Red orange crystals, ^{1}H NMR (400 MHz, methanol-d4): δ= 8.02 (m, 2H H-5 H-8), 7.76 (td, *J* = 7.5, 1.5 Hz, H-6), 7.71 (td, *J* = 7.5, 1.5 Hz, H-5), 5.18 (dddd, *J* = 7.3, 5.9, 2.8, 1.4 Hz, H-2′), 3.25 (d, *J* = 7.3 Hz, H-1′), 1.78 (s, H-4′), 1.76 (s, H-5′); ^{13}C NMR (101 MHz, Methanol-d4): δ = 186.4 (C-4), 182.9 (C-1), 156.8 (C-2), 135.6 (C-6), 134.3 (C-3′), 134.1 (C-7) 133.9 (C-10), 131.7 (C-9), 127.2 (C-5), 126.9 (C-8), 124.8 (C-3), 121.8 (C-2′), 26.1 (C-5′), 23.4 (C-1′), 18.2 (C-4′).

Antifungal evaluation

Microorganisms and media: Standardized strains from the American Type Culture Collection (ATCC), and CEREMIC, Reference Center of Mycology, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario, Argentina were used on initial screenings: *C. albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *C. neoformans* ATCC 32264, *Aspergillus flavus* ATCC 9170, *A. fumigatus* ATTC 26934, *A. niger* ATCC 9029, *Trichophyton rubrum* C 110, *T. mentagrophytes* ATCC 9972, and *M. gypseum* C 115.

Clinical isolates from CEREMIC included 11 strains of *Candida* spp. (5 of them *C. albicans* and 6 non-*albicans Candida* spp.) and 9 strains of *C. neoformans*. The number of each voucher specimen is presented in ◉ **Tables 3** and **4**. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Inocula of cells or spore suspensions were obtained according to reported procedures and adjusted to 1–5 × 10^{3} cells/spores with CFU/mL [20, 21]. Standard drugs were purchased from Sigma Co.

Antifungal susceptibility testing: MIC of each extract or compound was determined using broth microdilution techniques following the guidelines of the CLSI for yeasts and for filamentous fungi (M-27 A2 and M 38-A,) in 96-well microtiter plates [20, 21]. MIC values were determined in RPMI-1640 (Sigma Chemical Co.) buffered to pH 7.0 with MOPS. The starting inocula were approximately 1–5 × 10^{3} CFU/mL. Microtiter trays were incubated at 35 °C for yeasts and hialohyphomycetes and at 28–30 °C for dermatophyte strains in a moist, dark chamber, and MICs were visually recorded at 48 h for yeasts, and at a time according to the control fungus growth, for the rest of the fungi. Ketoconazole (Janssen Pharmaceutical) (> 98%), terbinafine hydrochloride (Novartis, Argentina) (> 98%), and amphotericin B (Sigma Chem. Co.) (~ 80%) were used as standard drugs.

Extracts or compounds stock solutions were diluted twofold with RPMI from 1000–0.98 or 250–0.98 µg/ml, respectively (final volume = 100 µL), and a final DMSO concentration ≤ 1%. To each well, 100 µL inocula suspension was added with the exception of the sterility control where sterile water was added to the well instead. MIC was defined as the minimum inhibitory concentration of the extract or compound, which resulted in total inhibition of the fungal growth.

Determination of MIC_{80} and MIC_{50}: LTWs were prepared with stock solutions of **3** in DMSO diluted with RPMI to final concentrations of 250–0.98 µg/mL. An inoculum suspension (100 µL) was added to each well (final volume in the well = 200 µL). A GCW (containing medium, inoculum, the same amount of DMSO used in LTW, but compound free) and a SCW (sample, medium, and sterile water instead of inoculum) were included for each strain tested. Microtiter trays were incubated in a moist, dark chamber at 35 °C, 48 h for *Candida* and *Cryptococcus* strains. Microplates were read in a VERSA Max microplate reader (Molecular Devices). Amphotericin B, ketoconazole, voriconazole (> 98%) and itraconazole (> 98%) were used as positive controls (100% inhibition). Tests were performed in duplicate. Inhibiton of growth for each concentration of lapachol was calculated as follows:

% of inhibition: 100 – (OD405 LTW – OD405SCW)/OD405 GCW – OD405 LCW)

MIC_{80} and MIC_{50} were defined as the lowest concentration of a compound that showed 80% or 50% reduction of the growth control, respectively.

Supporting information

NMR spectra (1D and 2D experiments) for compound **1** are available as Supporting Information.

Acknowledgements

We wish to thank Luis Guillermo Acosta (INBio) for the identification of the plant specimens. This research was financed by the National Council of Science and Technology, CONICIT (FORINVES 011–05), the Organization of American States OAS (SEDI/AICD/106/01), and SENACYT, Panamá. We are grateful to Renee Kontnik (ICCB, Harvard Medical School) for facilitating MS measurements. SZ acknowledges ANPCyT (PICT 2010/0608) and CONICET, Argentina for a post-doctoral fellowship (MVR).

Conflict of Interest

The authors declare no conflict of interest.

Affiliations

[1] Unidad Estratégica de Acción de Bioprospección, Instituto Nacional de Biodiversidad (INBio), Santo Domingo de Heredia, Heredia, Costa Rica
[2] Escuela de Química & CIPRONA, Universidad de Costa Rica, Ciudad Universitaria Rodrigo Facio, San Pedro de Montes de Oca, San José, Costa Rica
[3] Farmacognosia y Biología Vegetal, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional Rosario, Rosario, Argentina
[4] Centro de Investigaciones Farmacognósticas de la Flora Panameña, CIFLORPAN, Universidad de Panamá, Panamá, Panamá

References

1 *Barrett D.* From natural products to clinically useful antifungals. Biochim Biophys Acta 2002; 1587: 224–233
2 *Kauffman CA.* Clinical efficacy of new antifungal agents. Curr Opin Microbiol 2006; 9: 483–488
3 *Pfaller M, Diekema D.* Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 2007; 20: 133–163
4 *Isham N, Bradley M, Ghannoum M.* Susceptibility profile of dermatophytes shows no development of terbinafine resistance. Abstr Intersci Conf Antimicrob Agents Chemother 1999; 39: 549
5 *Mukherjee PK, Leidich SD, Isham N, Leitner I, Ryder NS, Ghannoum MA.* Clinical *Trichophyton rubrum* strain exhibiting primary resistance to terbinafine. Antimicrob Agents Chemother 2003; 47: 82–86
6 *Brouwer A, Rajanuwong A, Chierakul W, Griffin GE, Larsen RA, White NJ, Harrison TS.* Combination antifungal therapies for HIV-associated

Electronic reprint for personal use

cryptococcal meningitis: a randomised trial. Lancet 2004; 363: 11 764–11 767
7 *Wollenweber E, Dorr M, Gomez LDP.* Exudate flavonoids in *Godmania aesculifolia* (Bignoniaceae). Biochem Syst Ecol 1996; 24: 481–482
8 *Stermitz FR, Arslanian RL, Castro O.* Flavonoids from the leaf surface of *Godmania aesculifolia* (Bignoniaceae). Biochem Syst Ecol 1992; 20: 481
9 *Guiraud P, Steiman R, Campos-Takaki GM, Seigle-Murandi F, Simeon de Buochberg M.* Comparison of antibacterial and antifungal activities of lapachol and beta-lapachone. Planta Medica 1994; 60: 373–374
10 *Gonçalves de Lima O, D'Alburquerque I, Gonçalves de Lima C, Dalia Maia M.* Substâncias antimicrobianas de plantas superiores – Comunicação XX: atividade antimicrobiana de alguns derivados do lapachol em comparacão com a xiloidona, nova ortonaftoquinona natural isolada de extratos do cerne do Pau d'Arco rôxo, *Tabebuia avellaneda* Lor. Ex Griseb. Revista do Instituto de Antibióticos (Recife) 1962; 4: 3–17
11 *Sun JS, Geiser AH, Frydman B.* A preparative synthesis of lapachol and related naphthoquinones. Tetrahedron Lett 1998; 39: 8221–8224
12 *Peraza-Sanchez SR, Chavez D, Chai HB, Shin YG, Garcia R, Mejia M, Fairchild CR, Lane KE, Menendez AT, Farnsworth NR, Cordell GA, Pezzuto JM, Kinghorn AD.* Cytotoxic constituents of the roots of *Ekmanianthe longiflora*. J Nat Prod 2000; 63: 492–495
13 *Lira AM, Araujo AAS, Basilio IDJ, Santos BLL, Santana DP, Macedo RO.* Compatibility studies of lapachol with pharmaceutical excipients for the development of topical formulations. Thermochim Acta 2007; 457: 1–6
14 *Ossowski T, Goulart MOF, de Abreu FC, Santana AEG, Miranda PRB, Costa CO, Liwo A, Falkowski P, Zarzeczanska D.* Determination of the pK(a) values of some biologically active and inactive hydroxyquinones. J Braz Chem Soc 2008; 19: 175–183
15 *Ali RM, Houghton PJ, Hoo TS.* Antifungal activity of some Bignoniaceae found in Malaysia. Phytother Res 1998; 12: 331–334
16 *Kuete V, Eyong KO, Folefoc GN, Beng VP, Hussain H, Krohn K, Nkenqfack AE.* Antimicrobial activity of the methanolic extract and of the chemical constituents isolated from *Newbouldia laevis*. Pharmazie 2007; 62: 552–556
17 *De Moura K, Emery F, Neves-Pinto C, Pinto MCFR, Dantas AP, Salomão K, de Castro SL, Pinto AV.* Trypanocidal activity of isolated naphtoquinones from *Tabebuia* and some heterocyclic derivatives: A Review from an Interdisciplinary Study. J Braz Chem Soc 2001; 12: 325–338
18 *Gafner S, Wolfender JL, Nianga M, Stoeckli-Evans H, Hostettmann K.* Antifungal and antibacterial naphtoquinones from *Newboldia alevis* roots. Phytochemistry 1996; 42: 1315–1320
19 *Azevedo Souza M, Johann S, Lima LA, Campos FF, Mendes IC, Beraldo H, de Souza-Fagundes EM, Cisalpino PS, Rosa CA, Alves TM, de Sá NP, Zani CL.* The antimicrobial activity of lapachol and its thiosemicarbazone and semicarbazone derivatives. Mem Inst Oswaldo Cruz 2013; 108: 342–351
20 *CLSI.* Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A3, 3rd edition. Wayne: Clinical and Laboratory Standards Institute; 2008: 1–25
21 *CLSI.* Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard M38-A2, 2nd edition. Wayne: Clinical and Laboratory Standards Institute; 2008: 1–35
22 *Ernst E, Roling E, Petzold R, Keele DJ, Klepser ME. In vitro* activity of micafungin (FK-463) against *Candida* spp.: microdilution, time-kill, and postantifungal-effect studies. Antimicrob Agents Chemother 2002; 46: 3846–3853
23 *Klepser M, Ernst EJ, Lewis RE, Ernst ME, Pfaller MA.* Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. Antimicrob Agents Chemother 1998; 42: 1207–1212

Electronic reprint for personal use