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ANTIFUNGAL LIGNANS FROM THE ARILS OF *VIROLA OLEIFERA*

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Key Word Index—*Virola oleifera*; Myristicaceae; arils; 2,7′-cyclolignans; lignan-7-ols; epoxylignan; antifungal activity.

Abstract—Arils of *Virola oleifera* (Myristicaceae) contain two 2,7′-cyclolignans: galbulin, and 4-hydroxy-5,3′,4′-trimethoxy-2,7′-cyclolignan; five lignan-7-ols: oleiferin-B, 3,4,3′,4′-tetramethoxylignan-7-ol, oleiferin-F, oleiferin-G, and oleiferin-H and one epoxylignan: verrucosin. Four of the isolates are new. All of the lignan-7-ols and verrucosin showed antifungal activity against *Cladosporium sphaerospermum* at a minimum amount of 25 μg, and the lignan-7-ols oleiferin-B and oleiferin-G showed antifungal activity against *C. cladosporoides* at a minimum amount of 10 μg.

INTRODUCTION

Virola species are typically found in the tropical forests, mainly in the Amazon. *Virola oleifera* is one of the few species existing in the Atlantic forest in the southern region of Brazil. This species has been popularly used due to its wound healing, anti-inflammatory, and anti-rheumatic properties [1]. Its leaves have been previously analysed and were reported to accumulate several lignan-7-ols [2]. A large number of lignans and neolignans have been described from different Myristicaceae species [3–6] and some have been suggested as protective agents for the tissues in which they accumulate [7]. Lignans and neolignans are structurally diverse, and antifungal, antitumour, and antioxidant activities have been described [8, 9]. We describe here the isolation and structural determination of two 2,7′-cyclolignans, five lignan-7-ols, and one epoxylignan from *V. oleifera* arils. Additionally, their antifungal activities against *Cladosporium cladosporoides* and *C. sphaerospermum* were evaluated.

RESULTS AND DISCUSSION

Flash chromatography of the dichloromethane extract from the arils of *V. oleifera* and subsequent purification by preparative TLC and HPLC yielded galbulin (**1**), **2**, oleiferin-B (**3**), **4**, **5**, **6**, **7**, and verrucosin (**8**). The compounds **1** [10], **3** [2], **4** [6], and **8** [12], were previously isolated and identified by comparison with reported spectroscopic data.

Compound **2** ($C_{21}H_{26}O_4$) showed a significant mass spectrum fragment ion at *m/z* 286 $[M-C_4H_8]^+$, typical for the retro Diels–Alder fragmentation of 2-7′ cyclolignans [13], and the base peak at *m/z* 255, suggested the presence of a veratryl-guaiacyl-methine moiety. The hydroxyl group was confirmed by the IR spectrum, which showed a broad band at 3521 cm^{-1}. The ^1H NMR spectrum further supported the proposed 2,7′-cyclolignan skeleton, showing a doubly benzylic methine proton at δ 3.38 (*d*, *J* = 10.3 Hz, H-7′), and two methyl protons as a pair of doublets at δ 0.84 (*d*, *J* = 5.9 Hz, Me-8′) and δ 1.07 (*d*, *J* = 5.9 Hz, Me-8). These chemical shifts and the sign of the optical rotation indicated that this lignan has the same configuration as galbulin (8*R*,7′*S*,8′*S*). In the aromatic region of the ^1H NMR spectrum of **2**, the singlet at δ 6.23 could be assigned to H-3, while the singlet at δ 6.53 could be assigned to H-6. Thus the structure of **2** was established as (8*R*,7′*S*,8′*S*)-4-hydroxy-5,3′,4′-trimethoxy-2,7′-cyclolignan.

Compound **5** ($C_{20}H_{24}O_5$), $[\alpha]_D^{21}$ +12.6 ($CHCl_3$, *c* 0.235) was obtained as an oil. The ^1H NMR chemical shifts [δ 0.71 (*d*, *J* = 6.8 Hz, Me-8′), 0.95 (*d*, *J* = 6.8 Hz, Me-8), 2.33 (*d*, *J* = 7.9 Hz, H-7′), and 4.30 (*d*, *J* = 9.0 Hz, H-7)] were quite similar to those of **3** and **4**, suggesting that **5** is a substitutional isomer of **3** and **4**. The presence of methylenedioxyphenyl and methoxyphenyl groups in **5** were confirmed by the singlets at δ 5.83 and 3.74, respectively. The hydroxyl group was inferred by its mass spectrum, which displayed a $[M]^+$ at *m/z* 344, accompanied by a characteristic $[M-H_2O]^+$ at *m/z* 326. The structure of **5** with

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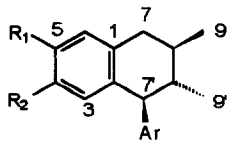
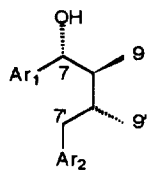


1 R_1=R_2=OMe; Ar=Ve

2 R_1=OMe; R_2=OH; Ar=Ve

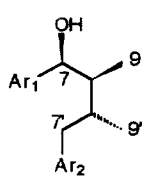


3 Ar_1=Ve, Ar_2=Pi

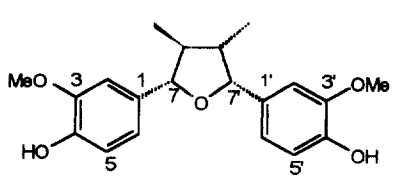
4 Ar_1=Ar_2=Ve

5 Ar_1=Pi, Ar_2=Gu

6 Ar_1=Ve, Ar_2=Gu



7 Ar_1=Ve, Ar_2=Gu



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Gu: Guaiacyl (4-hydroxy-3-methoxyphenyl)

Pi: Piperonyl (3,4-methylenedioxyphenyl)

Ve: Veratryl (3,4-dimethoxyphenyl)

a benzyl alcohol unit attached to the piperonyl group was determined based on the fragment ion at *m/z* 151, which represents [PiCHOH]$^+$. The ^{13}C NMR spectrum revealed two sets of aromatic nuclei and showed the presence of 3,4-methylenedioxyphenyl and 4′-hydroxy-3′-methoxyphenyl moieties. Based on this evidence, **5** was determined as rel-(7*R*,8*S*,8′*R*)-4′-hydroxy-3′-methoxy-3,4-methylenedioxylignan-7-ol, for which the name oleiferin-F is suggested.

Oleiferin-G (**6**) has the molecular formula $C_{21}H_{28}O_5$, from analysis of its ^{1}H NMR and mass spectra, which also indicated the presence of veratryl and guaiacyl units. Indeed, the base peak of the mass spectrum at *m/z* 167 indicated the presence of a dimethoxybenzyl alcohol unit. As expected, in the presence of a trace of acid, **6** formed the dehydration product **2**. The homonuclear COSY spectrum indicated the coupling between the methine proton at δ 1.74 to methyl protons at δ 1.03, and to the oxygenated methine proton at δ 4.38. Additionally, the methine proton at δ 1.48 was coupled to the methyl protons at δ 0.81 and to the benzylic methylene protons at δ 2.39. The aromatic proton at δ 6.61 (*d*, J = 1.8 Hz, H-2) was coupled to the proton at δ 6.70 (*dd*, J = 1.8, 8.1 Hz, H-6), which was also coupled to the proton at δ 6.77 (*d*, J = 8.1 Hz, H-5). As expected, the protons at δ 6.43 (*d*, J = 1.8 Hz, H-2′), δ 6.51 (*dd*, J = 1.8, 8.1 Hz, H-6′) and δ 6.71 (*d*, J = 8.1 Hz, H-5′) were coupled to each other. The homonuclear long-range COSY spectrum also showed the coupling between the carbinolic proton at δ 4.38 and the aromatic proton at δ 6.61 (H-2). Thus, the structure for **6** was proposed as rel-(7*R*,8*S*,8′*R*)-4′-hydroxy-3,4,3′-trimethoxylignan-7-ol.

Oleiferin-H (**7**) has the same molecular formula as **6** and was characterized as an epimer of **6**, differing only in the configuration of carbinolic proton. The ^{1}H NMR spectrum showed signals assignable to carbinolic protons: δ 4.38 (*d*, J = 9.1 Hz, H-7) for **6**, δ 4.23 (*d*, J = 9.0 Hz, H-7) for **7**; and signals for methyl protons at δ 0.81 (*d*, J = 6.8 Hz, Me-8′), δ 1.03 (*d*, J = 6.8 Hz, Me-8) for **6**, and δ 0.73 (*d*, J = 6.6 Hz, H-9′), δ 0.89 (*d*, J = 6.6 Hz, H-9) for **7**. Based on this evidence and the opposite signs of optical rotations, **6** and **7** have the same structure, differing in the stereochemistry at H-7; thus, the structure for **7** is rel-(7*S*,8*S*,8′*R*)-4′-hydroxy-3,4,3′-trimethoxylignan-7-ol.

In a preliminary screening, the dichloromethane extract of arils displayed activity against the pathogenic fungus *Cladosporium cladosporoides*, and the saprophyte fungus *C. sphaerospermum*. Antifungal activities for the individual isolates were evaluated in a TLC bioautographic method [14], and the minimum amounts required to show an inhibition zone in the TLC plate were determined (Table 1).

The cyclolignans **1**, and **2** were inactive against *C. cladosporoides* and *C. sphaerospermum*. Among lignan-7-ols, **4** and **6** were the most active against *C. cladosporoides* showing the minimum inhibitory amount of 10 μg. Interestingly, stereoisomer **7** was inactive against the same fungus. A similar pattern was observed against *C. sphaerospermum*, in which **7** proved to be significantly less active than **3**–**6** and **8**.

Although some antifungal activities against *C. cladosporoides* and *C. sphaerospermum* were observed for lignans occurring in the arils of *V. oleifera*, broad protection against all micro-organisms under natural conditions cannot be expected, if we consider that as

Table 1. Bioautography assay with *Cladosporium cladosporoides* and *C. sphaerospermum* for lignans **1**–**8**

Lignans	Antifungal activities*	
	C. cladosporoides	*C. sphaerospermum*
1	i	i
2	i	i
3	25	25
4	10	25
5	25	25
6	10	25
7	i	150
8	50	25
Nystatin (positive control)	5	5
Niconazole (positive control)	0.8	0.8
Ketoconazole (positive control)	1.0	1.0

* Minimum amount (μg) required for the inhibition of fungal growth on TLC plates.
i = inactive at 150 μg.

soon as those fruits not removed by birds fall to the ground, they are infected by micro-organisms which rot the seeds, thereby precluding germination [15].

EXPERIMENTAL

General. The ^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) spectra were recorded on a Brüker AC-200 spectrometer, and the ^{1}H NMR (300 MHz) spectra was recorded on a Brüker DPX-300 spectrometer, using TMS as int. standard. EIMS were measured at 70 eV on a HP5990/5988 A spectrometer. IR: KBr, OR measured at λ = 588 nm. Bioautography was performed on TLC (silica gel, pre-coated plates, Art. 5715-Merck). Elemental analysis were performed on Perkin–Elmer CHN Elemental Analyser 2400.

Plant material. Arils of *V. oleifera* (Schott) A. C. Smith were collected in the region of Vale do Ribeira, Atlantic Forest (São Paulo State, Brazil) in October 1994, and identified by Dr João Baitelo (Instituto Florestal de São Paulo). A voucher specimen (Lopes 090) has been deposited in the Herbarium of Instituto de Biociências, Universidade de São Paulo.

Micro-organisms. Cladosporium cladosporoides (Fresen de Vries) SPC 140, and *C. sphaerospermum* (Pemzig) SPC 491, have been maintained at Instituto de Botânica [14].

Isolation. Arils of *V. oleifera* (16 g) were dried, milled and extracted with CH_2Cl_2 at room temp. The concd extract was suspended in hot MeOH and kept overnight at 0 . The fatty material was filtered and the mother liquor concd under vacuum to yield 5.0 g. The CH_2Cl_2 extract submitted to CC (silica gel 30 g) eluted with CH_2Cl_2, followed by prep. TLC and HPLC, gave **1** (4.9 mg), **2** (1.6 mg), **3** (18.5 mg), **4** (28.4 mg), **5** (5.9 mg), **6** (36.8 mg), **7** (0.7 mg), and **8** (0.7 mg).

(8R,7′S,8′S)-4-*Hydroxy*-5,3′,4′-*trimethoxy*-2,7′-*cyclolignan* (**2**). White solid. ($C_{21}H_{26}O_4$ requires 73.68% C, 7.60% H; found 73.47% C, 7.56% H). RR_t = 7.72 min in HPLC (ODS-Hypersil column 5 μm, 100 × 2.1 mm i.d., with gradient starting from MeOH–H_2O 1:1 (1 min) changing to 67:33 (8 min), 87:13 (6 min), and finally 100:0 (15 min); flow rate at 0.3 ml min^{-1}, with detection at 254+280 nm) (condition A). $[\alpha]_D^{21}$ −14.2° ($CHCl_3$, *c* 0.42). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.84 (3H, *d*, *J* = 5.9 Hz, Me-8′), 1.07 (3H, *d*, *J* = 5.9 Hz, Me-8), 1.58 (1H, *m*, H-8′), 1.66 (1H, *m*, H-8), 2.59 (1H, *dd*, *J* = 10.3, 15.8 Hz, H-7ax.), 2.72 (1H, *dd*, *J* = 5.1, 15.8 Hz, H-7eq.), 3.38 (1H, *d*, *J* = 10.3 Hz, H-7′), 3.78 (3H, *s*, OMe-), 3.79 (3H, *s*, OMe-), 3.83 (3H, *s*, OMe-), 6.23 (1H, *s*, H-6), 6.53 (1H, *s*, H-2), 6.56 (1H, *d*, *J* = 2.2 Hz, H-2′), 6.67 (1H, *dd*, *J* = 2.2, 8.1 Hz, H-6′), 6.78 (1H, *d*, *J* = 8.1 Hz, H-5′). MS *m/z* (rel. int.): 342 ([M]$^+$, 16), 286 (5), 285 (14), 256 (18), 255 (100), 205 (4), 204 (13), 189 (26), 137 (3).

Oleiferin-F (**5**). Yellow oil. ($C_{20}H_{24}O_5$ requires 69.77% C, 6.98% H; found 69.38% C, 6.93% H); $[\alpha]_D^{21}$ +12.6 ($CHCl_3$, *c* 0.235). RR_t = 9.79 min in HPLC (condition A). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.71 (3H, *d*, *J* = 6.8 Hz, H-9′), 0.95 (3H, *d*, *J* = 6.8 Hz, H-9), 1.47 (1H, *m*, H-8), 1.72 (1H, *m*, H-8′), 2.33 (2H, *d*, *J* = 7.9 Hz, H-7′), 3.74 (3H, *s*, OMe), 4.30 (1H, *d*, *J* = 9.0 Hz, H-7), 6.37 (1H, *d*, *J* = 2.0 Hz, H-2′), 6.51 (1H, *dd*, *J* = 2.0, 7.9 Hz, H-6′), 6.58 (1H, *d*, *J* = 2.2 Hz, H-2), 6.67 (1H, *dd*, *J* = 2.2, 7.9 Hz, H-6), 6.76 (1H, *d*, *J* = 7.9 Hz, H-5′), 6.78 (1H, *d*, *J* = 7.9 Hz, H-5). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 130.0 (C-1), 107.6 (C-2), 146.3, 148.4 (C-3, C-3′), 145.2, 147.4 (C-4, C-4′), 109.6, 113.9 (C-5, C-5′), 121.7 (C-6), 78.1 (C-7), 41.1 (C-8), 9.9 (C-9), 129.7 (C-1′), 111.3 (C-2′), 122.2 (C-6′), 43.8 (C-7′), 35.4 (C-8′), 14.1 (C-9′), 55.9 (OMe), 100.4 (OCH_2O). MS *m/z* (rel. int.): 344 ([M]$^+$, 5) 326 (50), 162 (10), 151 (100), 135 (36).

Oleiferin-G (**6**). Yellow oil. ($C_{21}H_{28}O_5$ requires 70.00% C, 7.78% H; found 69.64% C, 7.79% H). RR_t = 8.42 min in HPLC (condition A). $[\alpha]_D^{21}$ +13.3° ($CHCl_3$, *c* 0.825). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 0.81 (3H, *d*, *J* = 6.8 Hz, H-9′), 1.03 (3H, *d*, *J* = 6.8 Hz, H-9), 1.48 (1H, *m*, H-8′), 1.74 (1H, *m*, H-8), 2.39 (2H, *d*, *J* = 7.7 Hz, H-7′), 3.75 (3H, *s*, OMe), 3.78 (3H, *s*,

OMe), 3.88 (3H, *s*, OMe), 4.38 (1H, *d*, $J = 9.1$ Hz, H-7), 5.34 (1H, *br s*, OH), 6.39 (1H, *d*, $J = 1.8$ Hz, H-2′), 6.48 (1H, *dd*, $J = 1.8$, 8.0 Hz, H-6′), 6.61 (1H, *d*, $J = 1.8$ Hz, H-2), 6.70 (1H, *d*, $J = 1.8$, 8.1 Hz, H-6), 6.76 (1H, *d*, $J = 8.0$ Hz, H-5′), 6.78 (1H, *d*, $J = 8.1$ Hz, H-5). ^{13}C NMR ($CDCl_3$, 50 MHz): δ 137.6 (C-1), 110.9 (C-2), 146.2, 150.3 (C-3, C-3′), 144.3, 148.4 (C-4, C-4′), 114.5, 116.5 (C-5, C-5′), 119.4 (C-6), 78.1 (C-7), 41.2 (C-8), 10.0 (C-9), 132.7 (C-1′), 110.0 (C-2′), 123.3 (C-6′), 41.9 (C-7′), 35.7 (C-8′), 15.4 (C-9′), 55.6 (OMe), 55.8 (OMe), 56.0 (OMe). MS *m/z* (rel. int.): 360 $[M]^+$ (1), 342 (3), 167 (100), 151 (11), 137 (21).

Oleiferin-H (**2e**). Yellow oil. $RR_t = 9.06$ min, in HPLC (condition A). $[\alpha]_D^{31}$ −15.2 ($CHCl_3$, *c* 0.44). ^{1}H NMR (300 MHz $CDCl_3$): δ 0.73 (3H, *d*, $J = 6.6$ Hz, Me-9′), 0.89 (3H, *d*, $J = 6.6$ Hz, Me-9), 1.53 (1H, *m*, H-8′), 1.67 (1H, *m*, H-8), 2.30 (2H, *d*, $J = 8.6$ Hz, H-7′), 3.66 (3H, *s*, OMe), 3.68 (3H, *s*, OMe), 3.81 (3H, *s*, OMe), 4.23 (1H, *d*, $J = 9.0$ Hz, H-7), 6.32 (1H, *d*, $J = 1.8$ Hz, H-2′), 6.42 (1H, *dd*, $J = 1.8$, 7.0 Hz, H-6′), 6.52 (1H, *dd*, $J = 1.9$, 7.8 Hz, H-6), 6.65 (1H, *d*, $J = 1.9$ Hz, H-2), 6.71 (1H, *d*, $J = 7.0$ Hz, H-5′), 6.72 (1H, *d*, $J = 7.8$ Hz, H-5). MS *m/z* (rel. int.): 360 $[M]^+$ (2), 342 (23), 237 (18), 167 (100), 137 (24).

Verrucosin (**8**). Yellow oil. $[\alpha]_D^{31}$ −30.0 ($CHCl_3$, *c* 0.900). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.60 (3H, *d*, $J = 6.9$ Hz, H-9′), 0.99 (3H, *d*, $J = 6.9$ Hz, H-9), 1.85 (1H, *m*, H-8), 3.79 (3H, *s*, OMe), 3.85 (3H, *s*, OMe), 4.33 (1H, *d*, $J = 9.3$ Hz, H-7), 5.04 (1H, *d*, $J = 8.7$ Hz, H-7′) 6.88 (2H, *d*, $J = 7.8$ Hz, H-5/H-5′), 6.98 (2H, *dd*, $J = 1.7$, 7.8 Hz, H-6/H-6′), 7.02 (2H, *d*, $J = 1.7$, H-2/H-2′).

Antifungal assay. Antifungal activities for isolated compounds were determined by a bioautographic method on TLC plates [14]. Different vols of a CH_2Cl_2 soln (10 mg ml^{-1}) of extracts or isolated compounds were applied on pre-coated TLC plates. After evapn of the solvents, suspensions of *Cladosporium cladosporoides* and *C. sphaerospermum* were sprayed followed by incubation at 25° in the dark. After 48 hr clearly visible inhibition zones indicated the minimum inhibitory activities for individual compounds (Table 1).

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