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# Antifungal constituents from roots of *Virola surinamensis*

Norberto P. Lopes[a], Massuo J. Kato[a,]*, Massayoshi Yoshida[b]

[a]*Instituto de Química, Universidade de São Paulo, C.P. 26077, Av. Prof. Lineu Prestes, CEP 05599-970, São Paulo SP, Brazil*
[b]*Instituto de Química, Universidade Estadual Paulista, C.P. 355, CEP 14800-900, Araraquara SP, Brazil*

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**Abstract**

Chromatographic fractionation of the dichloromethane extract from roots of *Virola surinamensis* yielded two new natural products, 3-*epi*-juruenolide C and 2′-hydroxy-7,4′-dimethoxyisoflavone, as well as various known steroids, lignans, isoflavones, flavonoids and diarylpropanes. Of these, α,2′-dihydroxy-4,4′-dimethoxydihydrochalcone, biochanin A and 2′-hydroxy-7,4′-dimethoxyisoflavone displayed antifungal activity against *Cladosporium cladosporioides* at a minimum amount of 5 μg, whereas 7-hydroxyflavanone and 7-hydroxy-4′-methoxyisoflavone exhibited an antifungal activity 10-fold higher than the positive control Nystatin. © 1999 Elsevier Science Ltd. All rights reserved.

*Keywords: Virola surinamensis*; Myristicaceae; Flavonoids; Lignans; γ-Lactones; Antifungal activity

## 1. Introduction

*Virola surinamensis* (Rol.) Warb. is a myristicaceous tree that occurs in 'várzea' forest and in river banks of the Amazon. Previous work carried out on seedling leaves, leaves and seeds collected at different localities described the occurrence of arylpropanoids, propiophenones, lignans, neolignans, γ-lactones, polyketides and flavonoids (Barata, Baker, Gottlieb, & Rúveda, 1978; Lopes et al., 1994; Lopes, Blumenthal, Cavalheiro, Kato, & Yoshida, 1996; Blumenthal, Silva, & Yoshida, 1997). Phytochemical studies on Amazonian Myristicaceae have also been carried out with leaves, fruits and wood (Kato, 1995; Martinez & Cuca, 1995). A survey of the chemical composition of eighteen *Virola* species so far investigated revealed the predominance of lignans and neolignans in leaves and fruits, while alkaloids and flavonoids were detected in the wood (Blumenthal, 1994). The scarcity of information on the chemical composition of roots of neotropical myristicaceous species prompted us to extend our research to this tissue as a first step towards understanding the ecophysiological role of secondary metabolites in different tissues of the Myristicaceae. In addition to these structural determinations, the antifungal activities of isolated compounds against *Cladosporium cladosporioides* were evaluated, in order to obtain some clue as to their roles in the roots of *V. surinamensis*.

## 2. Results and discussion

The dichloromethane extract from roots of *V. surinamensis*, submitted to CC followed by prep. TLC or prep. HPLC yielded, besides sitosterol and stigmasterol, veraguensin (**1a**) (Barata & Baker, 1973), galbelgin (**2a**) (Sumathykutty & Rao, 1991), methoxy-galbelgin (**2b**) (Lopes et al., 1996), grandisin (**2c**) (Barbosa-Filho, Silva, Yoshida, & Gottlieb, 1989), guaiacin (**3a**) (Majumber, Chatterjee, & Sengupta, 1972), galbulin (**3b**) (Hughes & Ritchie, 1954), cagayanin (**3c**) (Kuo, Lin, & Wu, 1989), juruenolide C (**4**) (Lopes et al., 1994), *epi*-juruenolide C (**5**), α,2′-dihydroxy-4,4′-dimethoxydihydrochalcone (**6**) (Martinez & Cuca, 1987), 7-hydroxyflavanone (**7**) (Bohlmann & Jakupovic, 1979), 7-hydroxy-4′-methoxyisoflavone (**8a**) (Chang, Suzuki, Kumai, & Tamura, 1969), biochanin A (**8b**) (Santos, Carvalho, & Braz-Filho, 1995), 2′-hydroxy-7,4′-dimethoxyisoflavone (**8c**), virolane (**9a**) (Lima, França, Diaz, & Gottlieb, 1975) and virolanol C (**9b**) (von Rotz, Cuca, & Martinez, 1990). The above known compounds were individually identified by comparison with reported spectroscopic data.

Elemental analysis combined with low resolution MS ($M^+$ 298) established a molecular formula $C_{17}H_{14}O_5$ for **8c**. Its $^1H$ NMR spectrum showed a resonance at $\delta$ 8.04 (1H, s) corresponding to the characteristic isoflavone proton and the coupling pattern system for the aromatic protons similar to that for isoflavone 2′-methoxy-formononetin (**8d**) (Jain, Tripathi, Pandey, & Rücker, 1996).

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* Corresponding author.

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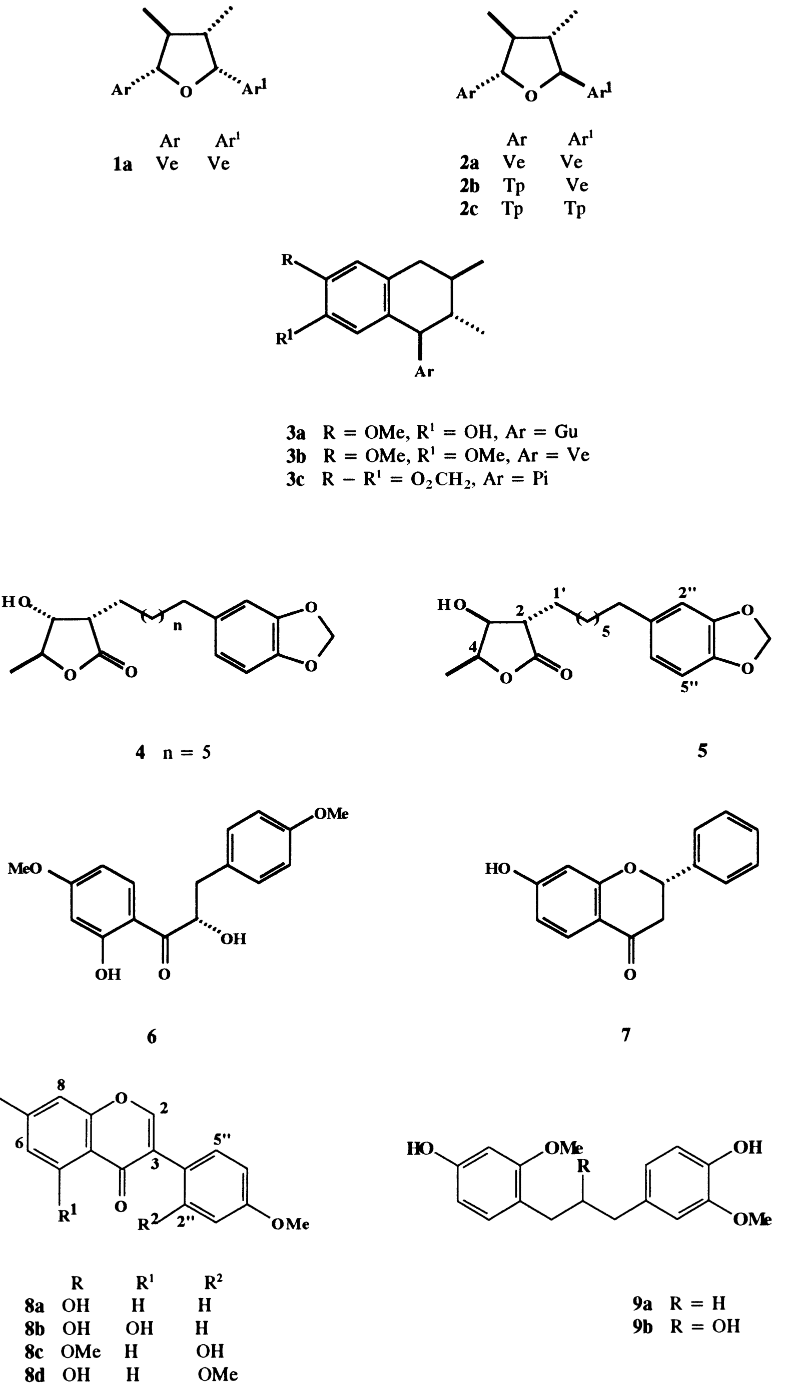

| | Ar | Ar¹ |
|---|---|---|
| **1a** | Ve | Ve |

| | Ar | Ar¹ |
|---|---|---|
| **2a** | Ve | Ve |
| **2b** | Tp | Ve |
| **2c** | Tp | Tp |

**3a** R = OMe, $R^1$ = OH, Ar = Gu
**3b** R = OMe, $R^1$ = OMe, Ar = Ve
**3c** R – $R^1$ = $O_2CH_2$, Ar = Pi

**4** n = 5

**5**

**6**

**7**

| | R | R¹ | R² |
|---|---|---|---|
| **8a** | OH | H | H |
| **8b** | OH | OH | H |
| **8c** | OMe | H | OH |
| **8d** | OH | H | OMe |

**9a** R = H
**9b** R = OH

**Pi** = Piperonyl (3,4-methylenedioxyphenyl)
**Gu** = Guaiacyl (4-hydroxy-3-methoxyphenyl)
**Ve** = Veratryl (3,4-dimethoxyphenyl)
**Tp** = 3,4,5-Trimethoxyphenyl

Structure 1.

The MS spectra of **8c** and **8d** revealed the same molecular ion at *m/z* 298. However, the loss of 31 or 17 amu for the characteristic ions at *m/z* 267 and 281 located, respectively, the methoxyl or hydroxyl groups at the 2′-position in isoflavones (Harborne & Mabry, 1982) and thus determined for **8c** the presence of a hydroxyl at the 2′ position. The differential NOE spectrum of **8c** showed an enhancement of H-8 and H-5′ signals when the methoxyl protons at $\delta$ 3.93 and at $\delta$ 3.81 were, respectively, irradiated. These results located the methoxyl groups at C-7 and C-4′ in **8c**, thus the structure could be defined as 2′-hydroxy-7,4′-dimethoxyisoflavone.

The molecular formula $C_{19}H_{26}O_5$ for **5** was determined by elemental analysis and by MS $M^+$ [334 (25%)]. This compound had a $\gamma$-lactone carbonyl absorption at 1758 $cm^{-1}$, a molecular ion at 334 amu and a base peak at 135 amu in its mass spectrum suggesting the same constitution of juruenolide C (**4**). The $^1H$ NMR analysis of **5** indicated a $\gamma$-lactone moiety that showed a doublet at $\delta$ 1.35 (Me-4), a double quartet at $\delta$ 4.61 (H-4), a triplet at $\delta$ 4.18 (H-3) and a multiplet at $\delta \sim 2.5$ (H-2). The literature reports the determination of the relative stereochemistry of 3-hydroxy and 4-methyl groups in five membered ring lactones based on $^{13}C$ chemical shifts observed in NMR spectra, as well as by lanthanide induced $^1H$ NMR chemical shifts and NOE experiments (Vieira et al., 1983; Magri, Kato, & Yoshida, 1996; Chavez & Roque, 1997). A pair of butenolide epimers differing in configuration at C-3 in the lactone ring, was isolated previously from *Iryanthera paraensis* (Magri et al., 1996). The $^{13}C$ NMR spectra of $\alpha$ and $\beta$ epimers from *I. paraensis* showed distinct chemical shifts for Me-4 ($\delta$ 18.1 versus 13.8, respectively) and C-1′ ($\delta$ 23.2 versus 27.2, respectively). In our case, juruenolide C **4** ($\alpha$-OH) and **5** ($\beta$-OH) exhibited 18.1 versus 14.1 $\delta$ and 23.3 versus 27.6 $\delta$ for Me-4 and C-1′, respectively, in their $^{13}C$ NMR spectra. These data support a structure with a $\beta$-OH functionality as depicted for compound **5**. The negative signal for optical rotation of **5** is in accordance with reported data (Vieira et al., 1983; Lopes et al., 1994; Magri et al., 1996; Chavez & Roque, 1997) and confirmed the structure as (2*S*,3*S*,4*S*)-3-*epi*-juruenolide C.

The antifungal activities for individual compounds were evaluated by a TLC bioautography method (Homans & Fuchs, 1970) and minimum amounts required to show an inhibition zone in the TLC plate were determined (Table 1). As can be seen, the neolignans showed no detectable activities, while juruenolides had activity only at 100 µg. The compounds **9a** and **9b** exhibited an inhibition zone in the TLC plate at the same concentration of Nystatin. The $\alpha$,2′-dihydroxy-4,4′-dimethoxydihydrochalcone, biochanin A and 2′-hydroxy-7,4′-dimethoxyisoflavone were active at minimum amount of 5 µg, while the 7-hydroxyflavanone and 7-hydroxy-4′-methoxyisoflavone showed antifungal activities 10-fold higher than the positive control Nystatin (10 µg).

In our previous investigations on the composition of secondary metabolites in *Virola surinamensis* from Combú Island, we found the predominance of lignans in leaves and seeds (Lopes et al., 1996). In this study, roots afforded 16 aromatic compounds in which flavonoids were the major compounds (on a dry weight basis). The profile of secondary metabolism of the roots is similar to that of woody tissue from *Virola* species (Blumenthal, 1994). The flavonoids isolated from roots of *V. surinamensis* showed high antifungal activities against *Cladosporium cladosporioides* in the bioautography assay (Homans & Fuchs, 1970). This fungus belongs to normal mycoflora of the grains (Pitt et al., 1994) or the commercial hatcheries (Eckman & Morgan-Jones, 1979) and there is no described relationship with *V. surinamensis* plants, as far as there is a large spore number it has been adopted in our bioassay (Young, personal communication). The occurrence of flavonoids in roots tissues and its role in both defense against pathogen and modulation for *Rhizobium* bacteria are a well documented phenomena (Kurz, 1989; Denarie, Debelle, & Rosenberg, 1992; Recourt et al., 1992).

Table 1
Bioautographic assay with *Cladosporium cladosporioides*

| Compounds | Antifungal activities (µg) |
|---|---|
| **1a** | i |
| **2a** | i |
| **2b** | 200 |
| **2c** | 200 |
| **3a** | i |
| **3b** | i |
| **3c** | i |
| **4** | 100 |
| **5** | 100 |
| **6** | 1 |
| **7** | 5 |
| **8a** | 1 |
| **8b** | 5 |
| **8c** | 5 |
| **9a** | 10 |
| **9b** | 10 |
| Nystatin | 10 |

i = inactive at 200 µg.

## 3. Experimental

### 3.1. General

Prep. TLC was carried out on Si gel PF-254 (Merck) and CC on Si gel 60H (0.005–0.045 mm) (Merck). Optical rotations were measured on Polamat A-Carl Zeiss. The $^1H$ NMR (200 MHz) and $^{13}C$ NMR (50 MHz) spectra were recorded on a Bruker-AC 200 in $CDCl_3$ with TMS as internal standard. EIMS was obtained at 70 eV on HP 5988-A.

### 3.2. Plant material

Roots of *Virola surinamensis* were collected in February, 1995 at Combú Island (01°30.175′S; 048°27.709′W), near Belém, Pará State, Brazil. A dry voucher sample (Lopes-037) has been deposited in the S.P.F.-Herbário do Instituto de Biociências da Universidade de São Paulo.

### 3.3. Microorganisms

*Cladosporium cladosporioides* (Fresen de Vries) SPC 140 has been mantained at the Instituto de Botânica, Secretaria de Agricultura do Estado de São Paulo.

### 3.4. Isolation of constituents

Air-dried and powdered roots (340.0 g) were extracted with $CH_2Cl_2$ at room temperature and yielded 3.0 g of extract. The extract was submitted to CC (Si-gel 170.0 g) and eluted with hexane–EtOAc at increasing polarities to give 10 pooled frs (A–J). Fractions B (240.2 mg) and G (96.7 mg) were recrystallized in MeOH and MeOH/hexane, respectively, to afford a mixture of sitosterol and stigmasterol (120.7 mg) from fr. B and 7-hydroxy-4′-methoxyisoflavone **8a** (35.0 mg) from fr. G. The following frs submitted to prep. TLC (silica gel) furnished the indicated compounds: fr. C (95.9 mg): 7-hydroxyflavanone **7** (4.9 mg), galbulin **3b** (12.9) and cagayanin **3c** (1.0 mg); fr. F (121.8 mg): α,2′-dihydroxy-4,4′-dimethoxydihydrochalcone **6** (20.0 mg), grandisin **2c** (1.8 mg) and veraguensin **1a** (2.2 mg) and fr. H (130.3 mg): biochanin A **8b** (10 mg) and α,2′-dihydroxy-4,4′-dimethoxydihydrochalcone **6** (28.5 mg). Fr. D (125.3 mg), submitted to prep. HPLC (Si-60 column hexane:HOAc, 7:3), gave juruenolide C **4** (34.5 mg), *epi*-juruenolide C **5** (5.0 mg) and guaiacin **3a** (9.7 mg). Frs E (90.7) and I (114.0 mg) submitted to prep. TLC (silica gel, hexane: EtOAc), followed by prep. HPLC (RP-8 column, 10 mm, 250 × 4.6 mm column, MeOH:$H_2O$, 4:1) afforded galbelgin **2a** (14.2 mg), methoxy-galbelgin **2b** (2.0 mg), 2′-hydroxy-7,4′-dimethoxyisoflavone **8c** (20.0 mg), virolane **9a** (14.0 mg) and virolanol C **9b** (5.4 mg).

### 3.5. (2S,3S,4S)-3-Hydroxy-4-methyl-2-[(7′-piperonyl)-n-heptyl]-γ-lactone (epi-juruenolide C) 5

Viscous oil. (Found: C, 68.52; H, 7.82. $C_{19}H_{26}O_5$ requires: C, 68.26; H, 7.78%.) $[\alpha]_D = -6°$ (MeOH). IR $\nu_{max}$ $cm^{-1}$: 3456, 2929, 2858, 1758 (C=O), 1489, 1464, 1445. EIMS 70 eV, *m/z* (rel. int.): 334 $[M]^+$ (25), 326 (8), 148 (26), 135 (100). $^1H$ NMR (200 MHz, $CDCl_3$): δ 2.4–2.6 (1H, m, H-2), 4.18 (1H, t, $J = 4.8$ Hz, H-3), 4.61 (1H, dq, $J = 4.8$, 7.1 Hz, H-4), 1.35 (3H, d, $J = 7.1$ Hz, Me-4), 1.70 (2H, m, H-1′), 1.2–1.6 (10H, m, H-2′, H-3′, H-4′, H-5′, H-6′), 2.50 (2H, t, $J = 7.8$ Hz, H-7′), 6.65 (1H, d, $J = 1.6$ Hz, H-2″), 6.75 (1H, d, $J = 7.9$ Hz, H-5″), 6.58 (1H, dd, $J = 7.8$, 1.6 Hz, H-6″). $^{13}C$ NMR (50 MHz, $CDCl_3$): δ 177.5 (C-1), 49.1 (C-2), 73.8 (C-3), 78.1 (C-4), 14.7 (Me-4), 27.1 (C-1′), 28.3 (C-2′), 28.9 (C-4′), 29.1 (C-3′), 29.2 (C-5′), 31.6 (C-6′), 35.5 (C-7′), 136.6 (C-1″), 108.1 (C-2″), 147.4 (C-3″), 145.3 (C-4″), 108.8 (C-5″), 121.0 (C-6″), 100.6 ($OCH_2O$).

### 3.6. 2′-Hydroxy-7,4-dimethoxyisoflavone (8c)

White amorphous solid. (Found: C, 68.82; H, 4.68. $C_{17}H_{14}O_5$ requires: C, 68.46; H, 4.70%.) $RR_f$ 0.50 (pre-coated plate, silica 60 $F_{254}$, art. Merck 5808, $CHCl_3$/MeOH, 4:1, 1% isopropanol, biochanin $RR_f$ 0.40). UV $\lambda_{max}^{MeOH}$ nm (log ε): 247.5 (4.7), 262.5 (4.6), 281.5 (4.4), 314.0 (3.7); + NaOH 247.0, 272.5, 291.5, 325.0. EIMS 70 eV, *m/z* (rel. int.): 298 $[M]^+$ (15), 281 (11), 164 (17), 151 (100). $^1H$ NMR (200 MHz, $CDCl_3$): δ 8.04 (1H, s, H-2), 8.25 (1H, d, $J = 9.0$ Hz, H-5), 7.04 (1H, dd, $J = 9.0$, 2.3 Hz, H-6), 6.90 (1H, d, $J = 2.3$ Hz, H-8), 7.06 (1H, d, $J = 8.5$ Hz, H-6′), 6.53 (1H, dd, $J = 8.5$, 2.6 Hz, H-5′), 6.64 (1H, d, $J = 2.6$ Hz, H-3′), 9.32 (1H, s, OH-2′), 3.81 (3H, s, OMe-4′), 3.93 (3H, s, OMe-7). $^{13}C$ NMR (50 MHz, $CDCl_3$): δ 154.5 (C-2), 124.8 (C-3), 178.7 (C-4), 117.5 (C-4a), 127.8 (C-5), 115.7 (C-6), 164.7 (C-7), 104.3 (C-8), 157.9 (C-8a), 112.8 (C-1′), 157.9 (C-2′), 99.6 (C-3′), 161.9 (C-4′), 107.6 (C-5′), 130.1 (C-6′), 55.9 (OMe-4′), 55.3 (OMe-7).

### 3.7. Antifungal assay

Antifungal activities for isolated compounds were determined by bioautographic method on TLC plates (Homans & Fuchs, 1970). Different volumes of a $CH_2Cl_2$ solutions of isolated compounds containing 200, 100, 10, 1 and 0.1 μg were applied on pre-coated TLC plates. After evaporation of solvent, a suspension of *Cladosporium cladosporioides* was sprayed on the plate, which was then incubated at 25°C in the dark. After 48 h clearly visible inhibition zones indicated the minimum inhibitory activities for individual compounds. Nystatin (10 μg) was used as a positive control.

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