Two Epimeric Flavalignans from *Trichilia catigua* (Meliaceae) with Antimicrobial Activity

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A mixture of flavalignan cinchonains Ia and Ib was isolated from the bark of *Trichilia catigua*. The structures were established on the basis of spectroscopic data of the natural products and their methylated derivatives including 2D NMR experiments, and compared with data in the literature. These flavalignans exhibited antibacterial activity against *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

Introduction

Trichilia catigua A. Juss. (Meliaceae) is a tree widely distributed in Brazil and commonly known as "Catuaba" or "Catiguá" (Klein, 1984). Its bark has been used in popular medicine as physical and mental tonic and especially as a sexual stimulant. The crude extract ingredients of the Catuama® herbal medicine are from *T. catigua* and another three well-known medicinal plants. They are indicate for physical and mental fatigue, neuromuscular asthenia and weakness disorders and its analgesic effect (Vas *et al.*, 1997) and vasorelaxant action (Calixto *et al.*, 1997).

This paper describes the structures and antibacterial activity of a mixture containing the two epimeric flavalignan cinchonains **1** and **2** which were isolated from the EtOAc extract of the stem bark of *Trichilia catigua*. The spectral data of cinchonains Ia and Ib and their methylated (**1a** and **2a**) derivatives, including 2D ^{1}H–^{1}H-COSY and ^{1}H–^{13}C-COSY–$^nJ_{CH}$ (HMQC, $n = 1$ and HMBC, $n = 2$ and 3) NMR experiments and comparison with literature data (Nonaka *et al.*,1982) were used to identify the structures in the mixture and for the unambiguous ^{1}H and ^{13}C NMR assignments.

Material and Methods

General experimental procedures and plant material

NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker DRX-AVANCE 400 spectrometer and on a Bruker 500 (^{1}H: 200 MHz; ^{13}C: 50 MHz), using TMS as an internal standard or by reference to solvent signals. EIMS: direct inlet at 70 eV on a Shimadzu QP-2000 spectrometer. IR spectra was measured on a Perkin-Elmer FT-16 PC spectrometer on KBr pellets. Elemental analysis was performed on a Perkin-Elmer 2400 Elemental Analyzer A sample of the bark from *Trichilia catigua* A. Juss. was a gift from Laboratory Catarinense S. A., Joinville, SC, who produce and market the Catuama herbal medicine in Brazil.

Extraction and isolation

Air-dried bark (400 g) was extracted with aqueous ethanol (80%) at room temperature for 20 days. The hydroalcoholic extract was concentrated to give a reddish-brown residue (34.5 g) which was then dissolved in EtOH–H_2O (3:7 v/v) and fractionated into hexane– (2.5 g), CH_2Cl_2– (4.8 g), EtOAc– (12.3 g) and aqueous (14.9 g) frs. The EtOAc fr. was chromatographed on a Sigel 60 col-

umn and eluted with *n*-hexane–EtOAc–methanol gradient. Fractions 45–58 (TLC, *n*-hexane–ethyl acetate 2:3, detection $FeCl_3$ reagent) were refractionated by Sephadex LH-20 using a methanol–chloroform system. The final purification was achieved on a flash chromatograph, which afforded a mixture of compounds **1** and **2** (980 mg) as a brown amorphous powder.

Mixture of the epimeric flavalignans **1** *and* **2**

IR ν_{max}/cm^{-1} 3362, 1746, 1612, 1520, 1446, 1360, 1198; EIMS *m/z* 452 $[M]^{\cdot+}$ (2%), 434 (5), 288 (15), 245 (20), 229 (15), 110 (100); ^1H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) Table I.

Mixture of the methyl ether derivatives **1a** *and* **2a**

The mixture of compounds **1** and **2** (50 mg) was treated with excess freshly prepared diazomethane in dry diethyl ether at room temperature to furnish the hexamethyl ether derivatives **1a** and **2a**. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$): Table II.

Bacterial strains and antimicrobial activity

The following species of bacteria were used to test the antimicrobial activity: *Bacillus cereus*, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923. Mueller-Hinton agar and broth (Difco Laboratories) were used for bacterial growth. All bacterial cultures were incubated under aerobic conditions.

Agar diffusion test: Bacterial cultures, which had grown overnight, were diluted to a final concentration of approximately 10^6 CFU/ml. The bacterial suspension was spread over the surface of Mueller-Hinton agar and discharged into plates (each plate having five wells of 7 mm diameter made previously in the agar). The wells were filled with 2.5 mg of each of the extracts. The plates were incubated at 36 °C for 20 h. A positive result was defined as a zone of 9 mm or more in diameter of inhibited growth of bacterial strain. Thus, the result of the diffusion test can be interpreted as inactive for inhibition zone smaller than 9 mm or no inhibition or, partially active for inhibition zone between 9 and 12 mm, active for inhibition zone between 13 and 18 mm and very active for inhibition zone greater than 18 mm (Smania *et al.*, 1995).

Minimal inhibitory and minimal bactericidal concentrations: A mixture of the flavolignans **1** and **2** was tested against 4 bacterial species for the determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC). The bacterial suspensions (10^6 CFU/ml), in Mueller-Hinton broth, were inoculated into tubes containing serial dilutions of test compounds and were then incubated at 36 °C for 20 h. The MIC was defined as the lowest concentration of each substance which had been able to completely inhibit the growth of each bacterial strain. The result was expressed in mg/ml. For the MBCs, a subculture was made on plates (Mueller-Hinton agar) from each of the tubes without apparent growth. The MBC was considered as the lowest concentration of the compounds that yielded over 99.9% reduction in the number of colony-forming units (Smania *et al.*, 1995).

Results and Discussion

The ^1H and ^{13}C NMR spectra of the mixture (**1** and **2**) showed several duplicate signals, suggesting the presence of two stereoisomeric compounds. The multiplicity for each carbon signal was deduced by comparative analysis of the HBBD- and DEPT-^{13}C NMR spectra (Table I). This analysis in combination with the low-resolution mass spectrum (*m/z* 452 $[M]^{\cdot+}$, $C_{24}H_{20}O_9$, 15 degrees of unsaturation), elemental analysis (CHN: C = 63.16% and H = 4.41%) and ^1H NMR allowed the deduction of the molecular formula $(C)_{11}(C{=}O)(CH)_{10}(CH_2)_2(O)_2(OH)_6 = C_{24}H_{20}O_9$ for **1** and **2**.

The presence of a flavan-3-ol skeleton was deduced from the ^1H and ^{13}C NMR by observation of the pair of signals at δ_H 4.81/4.85 (*br s*, H-2), δ_H 4.25/4.18 (*m*, H-3) and δ_H 2.95/2.88 (*m*, H-4) attributable to a flavan-3-ol C-ring. The ABX-type aromatic signals at δ_H 6.99/6.83 (*d*, *J* = 1.9 Hz, H-2′), δ_H 6.77/6.69 (*d*, *J* = 8.1 Hz, H5′) and δ_H 6.79/6.61 (*dd*, *J* = 1.9 and 8.1 Hz, H-6′) corresponding to a B-ring, (Table I), were recognized as related to those of epicatechin (Agrawal, 1989) mainly on the basis of the brad singlet attributed to H-2 (Nonaka *et al.*, 1982). The singlet signals in

Table I. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data for compounds **1** and **2** (CD_3OD, δ in ppm), including 2D HMQC and HMBC heteronuclear correlations.

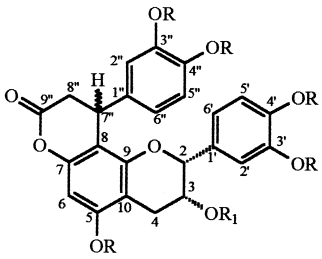
	^{1}H–^{13}C HMQC–$^1J_{CH}$				^{1}H–^{13}C HMBC–$^nJ_{CH}$	
	1		**2**		**1** and **2**	
C/H	^{1}H	^{13}C	H	^{13}C	$^2J_{CH}$	$^3J_{CH}$
2	4.81 (br s)	79.61	4.85 (br s)	80.08		
3	4.25 (m)	66.49	4.18 (m)	66.91		
4	2.95 (m)	28.51	2.88 (m)	28.27		
5		157.17		157.18	H-6	
6	6.22 (s)	96.32	6.23 (s)	96.44		
7		151.94		151.95	H-6	H-7″
8		106.03		106.13	H-7″	H-6
9		153.33		153.40		2H-4, H-7″
10		105.21		105.21	2H-4	H-3, H-6
1′		129.90		129.98	H-2	H-5′
2′	6.99 (d, *J* = 1.9)	115.03	6.83 (d, *J* = 1.9)	114.99		
3′		146.17		146.27		H-5′
4′		147.51		147.60		H-2′, MeO-4′
5′	6.77 (d, *J* = 8.1)	116.03	6.69 (d, *J* = 8.1)	115.94	H-6′	
6′	6.79 (dd, *J* = 8.1, 1.9)	119.39	6.61 (dd, *J* = 8.1, 1.9)	119.20		H-2′
1″		134.26		133.78	H-7″	2H-8″; H-5″
2″	6.55 (d, *J* = 2.0)	114.99	6.63 (d, *J* = 2.0)	115.32		H-6″
3″		148.78		148.78		H-5″, MeO-3″
4″		148.78		148.49		H-2″; H-6″, MeO-4″
5″	6.63 (d, *J* = 8.6)	116.51	6.63 (d, *J* = 8.6)	116.56		
6″	6.45 (dd, *J* = 8.6; 2.0	119.19	6.54 (dd, *J* = 8.6; 2.0)	119.32	H-2″, H-7″	
7″	4.54 (dd, *J* = 6.8; 1.4)	34.54	4.43 (dd, *J* = 6.8; 1.4)	34.18		H-2″, H-6″
8″	2.98	37.53	2.86	37.30	H-7″	
9″		167.35		167.71	2H-8″	H-7″

Values in parentheses indicate coupling constants in Hz.

the aromatic region at δ_H 6.22/6.23 (H-6) suggested the A-ring is tri-substituted. The presence of an epicatechin moiety was confirmed by the ^{13}C NMR spectra (HBBD and DEPT) of **1** and **2** through the signals at δ_C 79.61/80.08 (CH-2), δ_C 66.49/66.91 (CH-3) and δ_C 28.51/28.27 (CH_2-4) in combination with the correspondent values of **1a** and **2a** (Table II) δ_C 77.83/78.02 (CH-2), 65.22/65.62 (CH-3) and δ_C 27.72/27.19 (CH_2-4)] and compared with values reported in the literature[6] for epicatechin and catechin. In addition, the ^{13}C NMR spectra of the mixture of **1** and **2** revealed the presence of signals corresponding to a carboxyl function (δ_C 170.83/170.83, C-9″), two methine (δ_C 34.54/34.18, CH-7″), two methylene (δ_C 37.53/37.30, CH_2-8″) carbon atoms and an additional 3,4-dihydroxyphenyl moiety [δ_C 135.38/135.27 (C-1″), δ_C 114.99/115.32 (CH-2″), δ_C 145.95/146.16 (C-3″), δ_C 145.70/146.21 (C-4″), δ_C 116.51/116.56 (CH-5″) and δ_C 119.19/119.32 (CH-6″)]. The carbonyl ester group was supported by the IR spectrum revealing a strong band at υ_{max} 1746 cm^{-1}.

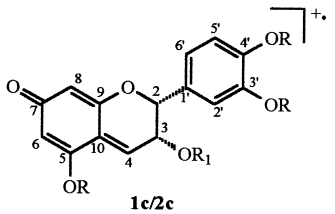
The location of this C_6–C_3 biogenetic moiety involved in a pyranone ring (lactone) linked to the A-ring at carbon C-8 and oxygen atom attached to carbon C-7 was evidenced mainly by heteronuclear long range couplings ($^2J_{CH}$ and $^3J_{CH}$) observed in the HMBC spectra of the mixtures of **1** and **2** (Table I) and their derivatives **1a** and **2a** (Table II). The ^{1}H–^{1}H-NOESY spectrum of **1a** and **2a** was used to assign unambiguously the chemical shifts of each of the hydrogens of the carbons CH-2, CH-3 and CH_2-4 as shown in **1c** and **2c**. The cross-peaks also indicated dipolar-dipolar interactions between H-6 (**1a**/**2a**: δ_H 6.30/6.33) and MeO-5 (**1a**/**2a**: δ_H 3.81/3.80), revealing that the hydrogens corresponding to singlet signals (**1a**/**2a**: δ_H 6.30/6.33, H-6) and methoxyl groups at δ_H 3.81 (**1a**) and δ_H 3.80 (**2a**) are maintaining on *ortho*-relation, in accordance with the structures **1** and **2** or **3** and **5** and excluding the alternatives **5** and **6**.

The extensive use of 2D ^{1}H–^{13}C correlation techniques (HMQC and HMBC, Table I) allowed assignment of the ^{1}H and ^{13}C NMR spectra (Breitmaier *et al.*, 1987) of the C-7″ epimeric structures

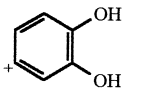


1 H-7"β, R=R$_1$=H
1a H-7"β R=Me, R$_1$=H

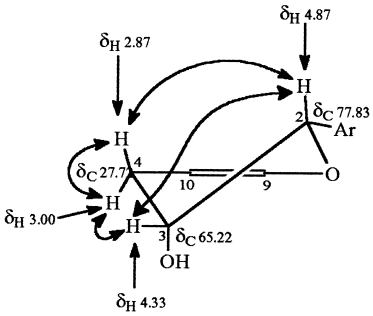
2 H-7"α, R=R$_1$=H
2a H-7"α, R=Me, R$_1$=H



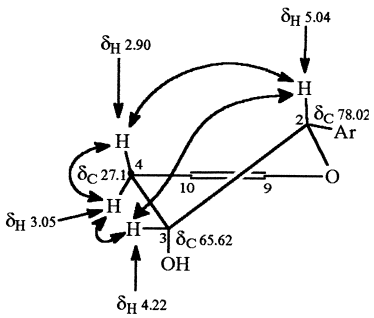
1c/2c
m/z 288



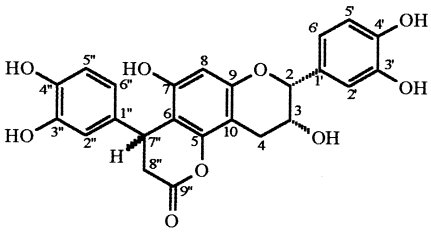
1d/2d
m/z 109



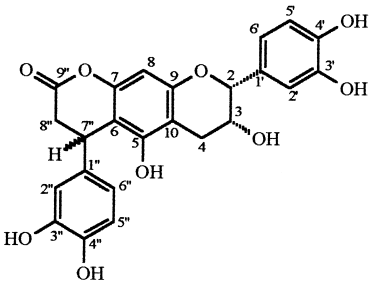
1b



2b



3 H-7"β
4 H-7"α



5 H-7"β
6 H-7"α

to differences in configuration (α and β) of the catechol (3,4-dihydroxyphenyl) substituent on the pyranone ring. The link between the pyranone ring and the epicatechin by a bond between C-8 and O–C-7 of **1** and **2** was deduced by the heteronuclear long range couplings between C-5 (δ_C 157.17/157.18) and H-6 (δ_H 6.22/6.23, $^2J_{CH}$), C-8 (δ_C 106.03/106.13) and both H-6 (δ_H 6.22/6.23, $^3J_{CH}$) and H-7″ (δ_H 4.54/4.43, $^2J_{CH}$), C-7 (δ_C 151.94/151.95) and both H-6 (δ_H 6.22/6.23, $^2J_{CH}$) and H-7″ (δ_H 4.54/4.43, $^3J_{CH}$), C-9 (δ_C 153.33/153.40) and H-7″ (δ_H 4.54/4.43, $^3J_{CH}$) and 2H-4 (δ_H 2.95/2.88, $^3J_{CH}$), C-10 (δ_C 105.21/105.21) and H-3 (δ_H 4.25/4.18, $^3J_{CH}$), H-6 (δ_H 6.22/6.23, $^3J_{CH}$) and 2H-4 (δ_H 2.95/2.88, $^2J_{CH}$), C-1″ (δ_C 135.38/135.27) and H-5″ (δ_H 6.63/6.63, $^3J_{CH}$), H-7″ (δ_H 4.54/4.43,

Table II. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data for compounds **1a** and **2a** ($CDCl_3$, δ in ppm), including 2D HMQC and HMBC heteronuclear correlations.

	^{1}H–^{13}C HMQC–$^1J_{CH}$				^{1}H–^{13}CHMBC–$^nJ_{CH}$	
	1a		**2a**		**1a** and **2**[a]	
C/H	^{1}H	^{13}C	^{1}H	^{13}C	$^2J_{CH}$	$^3J_{CH}$
2	4.87 (br s)	77.83	5.04 (br s)	78.02		H-2′, H-6′
3	4.33 (m)	65.22	4.22 (m)	65.62	H-2, 2H-4	
4	3.00 (H-4β)	27.72	3.05 (H-4β)	27.19		
	2.87 (H-4α)		2.90 (H-4α)			
5		157.84		157.91		2H-4, MeO-5
6	6.30 (s)	92.30	6.33 (s)	92.11		
7		150.85		150.87	H-6	H-7″
8		105.34		104.70	H-7″	H-6, 2H-8″
9		150.43		150.43	H-6	2H-4, H-7″
10		103.93		103.47	2H-4	H-3; H-6
1′		129.90		129.98	H-2	H-5′
2′	6.99 (d, 1.9)	109.08	6.75 (d, 1.9)	108.63		H-2, H-6′
3′		148.70		148.70		H-5′, MeO-3′
4′		147.51		147.60		H-2′, MeO-4′
5′	6.91 (d, 8.2)	110.00	6.81 (d, 8.0)	110.21		
6′	7.04 (dd, 8.2, 1.9)	117.95	6.76 (dd, 8.0, 1.9)	117.64	H-5′	H-2′
1″		134.26		133.78	H-7″	2H-8″; H-5″
2″	6.70 (d, 1.9)	110.86	6.72 (d, 2.0)	110.86		H-6″
3″		148.78		148.78		H-5″, MeO-3″
4″		148.78		148.49		H-2″; H-6″, MeO-4″
5″	6.73 (d, 8.1)	110.97	6.68 (d, 8.3)	110.97		
6″	6.63 (dd, 8.3, 2.0)	117.77	6.65	118.19		H-2″, H-7″
7″	4.60	34.09	4.57	33.69	2H-8″	H-2″, H-6″
8″	3.03	36.13	3.03	36.64	H-7″	
9″		167.35		167.71	2H-8″	H-7″

Values in parentheses indicate coupling constants in Hz.

$^2J_{CH}$) and 2H-8″ (δ_H 2.98/2.86, $^3J_{CH}$) and C-9″ (δ_C 170.83/170.83) and H-7″ (δ_H 4.54/4.43, $^3J_{CH}$) and 2H-8″ (δ_H 2.98/2.86, $^2J_{CH}$). Other heteronuclear long range couplings are also summarized in Table I.

These deductions were confirmed by the HMBC spectrum of the mixture of the methyl ether derivatives **1a** and **2a**, revealing heteronuclear long range couplings between C-5 (**1a**/**2a**: δ_C 157.84/157.91) and 2H-4 (**1a**/**2a**: δ_H 3.00, 2.87/3.05, 2.90, $^3J_{CH}$) and MeO-5 (**1a**/**2a**: δ_H 3.80/3.81, $^3J_{CH}$), C-9 (**1a**/**2a**: δ_C 150.43/150.43) and 2H-4 (**1a**/**2a**: δ_H 3.00, 2.87/3.05, 2.90, $^3J_{CH}$) and H-7″ (**1a**/**2a**: δ_H 4.60/4.57, $^3J_{CH}$) and C-10 (**1a**/**2a**: δ_C 103.93/103.47) and 2H-4 (**1a**/**2a**: δ_H 3.00, 2.87/3.05, 2.90 $^2J_{CH}$), H-3 (**1a**/**2a**: δ_H 4.33/4.22, $^3J_{CH}$) and H-6 (**1a**/**2a**: δ_H 6.33/6.30, $^3J_{CH}$). The percentages of **1a** (40%) and **2a** (60%) in the mixture were estimated on the basis of the intensity of the signals corresponding to H-2 (**1a**/**2a**: δ_H 4.87/5.04) in the ^{1}H NMR spectrum.

All these data are in accordance with the structures **1a** and **2a** for the methyl derivatives and, consequently, **1** and **2** for components of the mixture isolated from *T. catigua*. In addition, the peaks at *m/z* 288 (20%) and 109 (100%) observed in the mass spectrum of the mixture of **1** and **2** can be justified by fragments **1c**/**2c** (explained by elimination of a caffeiloxy moiety incorporated in the pyranone ring linked to the A-ring) and **1d**/**2d** (corresponding to a catechol moiety) are in accordance with the proposed structures.

With the relative configuration of the chiral carbons CH-2 (H-2β) and CH-3 (H-3β) of established epicatechin moiety [2α-(3′,4′-dihydroxyphenyl) and 3α-hydroxy, *cis*-configuration], all that was left was to determine the relative stereochemistry of the remaining chiral carbon atom CH-7″. The ^{1}H NMR spectrum of the mixture of the methyl ether derivatives **1a** and **2a** revealed the major distinction in the differences between the chemical shifts of the hydrogens H-2β [$\Delta\delta_H$= 4.87 (**1a**) – 5.04

(**2a**) = −0.17 ppm] and of the methoxy groups MeO-3″ (**1a**/**2a**: δ_H 3.57/3.58). These significant differences can justified by relative stereochemistry of the hydrogen H-2β and the 3″,4″-dimethoxyphenyl at carbon CH-7″: a) H-2β and H-7″β (3″,4″-dimethoxyphenyl at α-position, **1a**) in a same plane; b) H-2β and H-7″α (3″,4″-dimethoxyphenyl at β-position, **2a**). Inspection of molecular models indicates that the chiral carbon CH-7″ of **2a** (H-7″α) may adopt conformation with the aromatic ring of the 3″,4″-dimethoxyphenyl moiety deshielding the H-2β (δ_H 5.04) and of the 3′,4′-dimethoxyphenyl shielding the methoxyl group MeO-3″ of **1a** (δ_H 3.57) and **2a** (δ_H 3.58) by anisotropic effects. These data were used to postulate the relative configurations of the methyl ether derivatives, as shown in **1a** and **2a**, and consequently the two epimeric components (**1** and **2**) of the mixture obtained from *T. catigua.* Thus, these compounds were identified as cinchonains Ia (**1**) and Ib (**2**) isolated previously from *Cinchona succeruba* (Nonaka *et al.*, 1982) and *Phyllocladus trichomanoides* (Foo, 1987) and their structures are in accordance with revision by Chen et *al*. (1993).

Of the three tested extracts, AcOEt extract was the one that presented the best inhibitory activity against bacterial growth. This extract inhibited the growth of the Gram-positive as *B. cereus* and *S. aureus* with inhibition zone of 29 mm and 32 mm respectively, and Gram-negative species as *E. coli* and *P. aeruginosa* with inhibition zone of 18 mm and 30 mm respectively. From this extract were obtained the flavalignanas **1** and **2**, which results of MIC and MBC are presented in the Table III. The two compounds were more actives against the Gram-positive than the Gram-negative bacteria. The values obtained for MIC and MBC were close to one another (either the same or the MBC was twofold the MIC). These results suggest that the flavalignans isolated show a bactericidal effect.

Table III. Minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) values (mg/ml) of the mixture flavolignans **1** and **2** against four bacterial species.

B. cereus		*E. coli*		*P. aeruginosa*		*S. aureus*	
MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
0.31	0.62	0.62	1.25	0.62	1.25	0.31	0.31

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