Antioxidant Neolignans from *Cordia americana*

Authors Lucía R. Fernández[1], Adriana Cirigliano[1], María P. Fabani[2], Beatriz Lima[2], Sebastián Alberti[1], Fernando Kramer[3], Alejandro A. Tapia[2], Gabriela Cabrera[1], Jorge A. Palermo[1], Marianela Sánchez[1]

Affiliations The affiliations are listed at the end of the article

Key words
- *Cordia americana*
- Boraginaceae
- neolignans
- bicyclo[2.2.2]octene
- yunnaneic acids
- rufescenolides
- antioxidant activity

Abstract

Five new neolignans with a bicyclo[2.2.2]octene framework were isolated from an ethanolic extract of the bark of *Cordia americana*. The structures and relative configurations of the compounds were elucidated by a combination of spectroscopic methods. All the isolated compounds showed good antioxidant activities in the DPPH radical scavenging (0.5–100 µg/mL) and Ferric-reducing antioxidant power (FRAP, 1–100 µg/mL) assays. One of the compounds displayed mild fungistatic activity at 0.1 µmol/spot against *Fusarium virguliforme* while, at the same time, all compounds were inactive against several strains of Gram (+) and Gram (−) bacteria at all assayed concentrations (10–1000 µg/mL).

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

received February 25, 2013
revised July 15, 2013
accepted October 13, 2013

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DOI http://dx.doi.org/10.1055/s-0033-1351052
Published online
Planta Med © Georg Thieme Verlag KG Stuttgart · New York · ISSN 0032-0943

Correspondence
Dra. Marianela Sánchez
UMYMFOR
Departamento de Química Orgánica
Facultad de Ciencias Exactas y Naturales
Universidad de Buenos Aires, Ciudad Universitaria
Pabellón 2
1428 Buenos Aires
Argentina
Phone: + 54 11 45 76 33 46 int. 245
Fax: + 54 11 45 76 33 46
msanchez@qo.fcen.uba.ar

Introduction

The genus *Cordia*, which belongs to the Boraginaceae family, encompasses approximately 250 species and has a wide range of uses in traditional medicine [1]. *Cordia americana* (L.) Gottschling & J. S. Mill. is a tree commonly found in South American tropical rainforests including South of Brazil, North of Argentina, and South of Paraguay. The leaves have been widely used in traditional medicine to treat diseases related to inflammation and as a wound healer [2]. This species was previously classified as *Patagonula americana* [1] and the isolation of two cordiachromes and a cinnamaldehyde derivative from its petrol ether heartwood extract has been reported [3]. Lignan-type compounds are usually found in the genus *Cordia* [4] and rosmarinic acid was previously identified as the major metabolite of *C. americana* [5, 6].

As part of a systematic survey of the native flora of the province of Misiones (Argentina), we decided to investigate this plant as a source of bioactive secondary metabolites.

Herein, we report the isolation and structural elucidation of five new neolignans (**1**–**5**) from an ethanolic extract of the bark of *C. americana*. In addition, the antioxidant capacity of the isolated compounds was assayed, as well as their antifungal power against two strains of phytopathogenic fungi and their antibacterial activity against several strains of Gram (+) and Gram (−) bacteria.

Results and Discussion

Fractionation of the bark ethanolic extract of *C. americana* led to the isolation of rosmarinic acid and five new neolignans (**Fig. 1**).

The molecular formula of compound **1** was established as $C_{28}H_{26}O_{11}$ on the basis of its HRMS ESI/APCI (*m/z*: 537.14182 [M – H]$^-$), which indicated sixteen degrees of unsaturation. The NMR data of compound **1** are shown in **Table 1**. The ^{13}C NMR and DEPT-HSQC spectra showed the presence of three carbonyl groups, sixteen signals corresponding to sp^2 carbons, four oxygenated methynes, and four additional aliphatic carbons: three methynes and one methylene. The ^1H NMR spectrum showed the presence of two coupled doublets at δ_H 7.45 and 6.28 with a coupling constant of 15.9 Hz, typical of a *trans*-disubstituted double bond. In the aromatic region, characteristic AMX signal patterns suggested the presence of two catechol moieties. The presence of a methoxyl group was evidenced by a 3H singlet at δ_H 3.70. The COSY spectrum revealed that the methylene group (δ_C 37.8; δ_H 3.01 and 3.07) was directly coupled with a double doublet methyne at δ_H

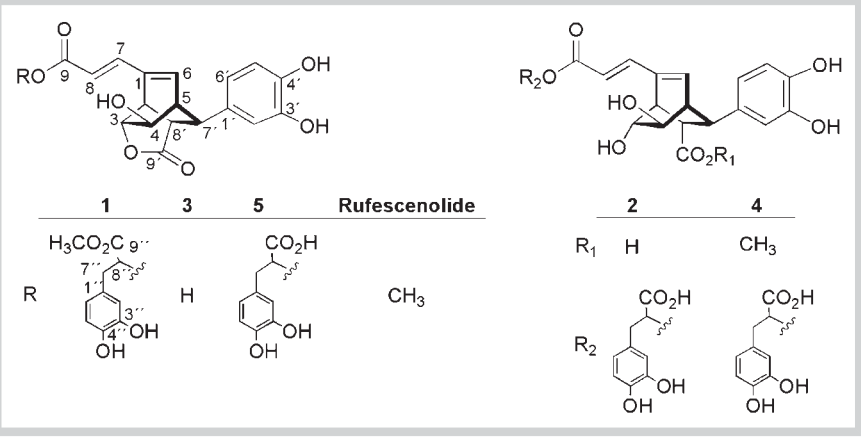

Fig. 1 Chemical structures of compounds **1–5**.

Table 1 NMR data for compound **1** in CD_3OD.

	δ ^{13}C	δ ^{1}H	COSY (^{1}H-^{1}H)	HMBC (^{1}H-^{13}C)	NOESY
1	135.3	–	–		
2	40.2	4.11 (1H, brt, *J* = 4.7)	H-3	C-1, C-4	H-3, H-7, H-8, H-8′
3	84.0	4.25 (1H, dt, *J* = 4.7, 1.0)	H-2, H-8′	C-2, C-4, C-5, C-8′, C-9′	H-2, H-4
4	74.9	4.00 (1H, d, *J* = 3.5)	H-2, H-6, H-7′	C-2, C-3, C-5, C-6, C-7′	H-3, H-5, H-7′
5	48.7	3.30 (1H, m)	H-4, H-6, H-7′	C-1	H-4, H-6, H-2′, H-6′, H-7′
6	142.1	6.55 (1H)*	H-2	C-2, C-3, C-4, C-5, C-7′	H-5, H-7
7	144.5	7.45 (1H, d, *J* = 15.9)	H-6, H-8	C-1, C-2, C-6, C-8, C-9	H-6
8	116.9	6.28 (1H, d, *J* = 15.9)	H-7	C-1, C-9	H-2
9	167.9	–	–		
1′	134.6	–	–		
2′	116.0	6.43 (1H, d, *J* = 2.2)	H-6′	C-1′, C-4′, C-6′, C-7′	H-5, H-7′, H-8′
3′	146.1	–	–		
4′	145.3	–	–		
5′	116.2	6.65 (1H, d, *J* = 8.1)	H-6′	C-1′, C-3′, C-4′	H-6′
6′	120.3	6.34 (1H, dd, *J* = 8.1, 2.2)	H-2′, H-5′	C-2′, C-4′, C-7′	H-5′, H-7′, H-8′
7′	45.5	3.15 (1H, brt, *J* = 2.4)	H-4, H-6, H-2′, H-6′, H-8′	C-2, C-4, C-5, C-6, C-1′, C-2′, C-6′, C-7′, C-9′	H-4, H-5, H-2′, H-6′, H-8′
8′	45.6	2.68 (1H, m)	H-2, H-3	C-3, C-5, C-1′, C-7′, C-9′	H-2, H-2′, H-6′, H-7′
9′	180.8	–	–		
1′′	128.6	–	–		
2′′	117.5	6.70 (1H, d, *J* = 1.5)	H-6′′	C-4′′, C-6′′, C-7′′	H-7′′, H-8′′
3′′	146.2	–	–		
4′′	145.4	–	–		
5′′	116.3	6.69 (1H, d, *J* = 7.9)	H-6′′	C-1′′, C-3′′, C-6′′	
6′′	121.8	6.56 (1H)*	H-2′′, H-5′′	C-2′′, C-4′′, C-7′′	H-7′′
7′′	37.8	3.01 (1H, dd, *J* = 14.3, 7.8) 3.07 (1H, dd, *J* = 14.3, 5.0)	H-8′′	C-1′′, C-2′′, C-6′′, C-8′′, C-9′′	H-2′′, H-6′′, H-8′′
8′′	74.8	5.22 (1H, dd, *J* = 7.8, 5.0)	H-7′′	C-9, C-1′′, C-7′′, C-9′′	H-2′′, H-6′′
9′′	172.0	–	–		
OMe	52.7	3.70 (3H, s)	–	C-9′′	

* Partially overlapped

5.22 (δ_C 74.8). In turn, the latter proton in the HMBC spectrum clearly correlated with two carbonyls at δ_C 172.0 and 167.9. Furthermore, the methylene protons showed correlations with aromatic carbons at δ_C 128.6, 121.8 and 117.5 belonging to one of the catechol moieties and also with the carbonyl at δ_C 172.0, which in turn correlated with the OMe singlet. All these correlations (**Fig. 2**) suggested the presence in the structure of an α-hydroxy-dihydrocaffeic methyl ester moiety, which is typical of salvianic acid A derivatives [7].

The HMBC experiment showed that the carbonyl group at δ_C 167.9 correlated with the *trans*-disubstituted double bond protons. Moreover, the δ_H 7.45 proton had correlations with a quaternary carbon at δ_C 135.3 ppm and with an sp^2 methyne carbon at δ_C 142.1 (δ_H 6.55) consistent with the presence of a diunsaturated ester. Based on a careful examination of the COSY data, a cyclic connectivity pattern was established between H-4 (δ_H 4.00), H-5 (δ_H 3.30), H-7′ (δ_H 3.15), H-8′ (δ_H 2.68), H-2 (δ_H 4.11) and H-3 (δ_H 4.25). Besides, HMBC correlations between H-2, H-3 and C-4 (δ_C 74.9) were consistent with the closure of a six-membered ring. A vicinal coupling between the resonances at δ_H 6.55 (H-6) and δ_H 3.30 (H-5), together with an HMBC correlation between H-7 (δ_H 7.45) and C-2 (δ_C 40.2) established the presence

of a rigid bicyclo[2.2.2]octene core. The structure of this bicyclic framework was supported by HMBC correlations between C-5 (δ_C 48.7) with protons at δ_H 4.25 (H-3) and δ_H 2.68 (H-8′), and by long-range, W-couplings of the resonance at δ_H 6.55 (H-6) with δ_H 3.15 (H-7′), δ_H 4.00 (H-4), and δ_H 4.11 (H-2).

In the HMBC spectrum, the unassigned carbonyl group (δ_C 180.8) showed a correlation with H-3 (δ_H 4.25), typical of lactones. Both H-8′ (δ_H 2.68) and H-7′ (δ_H 3.15) gave HMBC correlations with the lactone carbonyl, which connected C-3 and C-8′. Furthermore, correlations of δ_H 3.15 (H-7′) with δ_C 134.6, δ_C 120.3 and δ_C 116.0 clearly indicated that the remaining aromatic ring was bound to the rigid core at C-7′. All these facts led to the proposal of structure **1**. A compound with a similar lactone core in which C-9 is a methyl ester, rufescenolide, was recently isolated from *Cordia rufescens* [8], and for that reason compound **1** was named rufescenolide B. The relative configuration of **1** was determined by the correlations observed in a phase-sensitive NOESY experiment (**Fig. 2**). In particular, strong NOEs between H-4 and H-7′ indicated an *endo*-type relationship between them. Additional correlations between H-5 and the aromatic protons H-2′ (δ_H 6.43) and H-6′ (δ_H 6.34) were in accordance with this assignment.

The ^{1}H spectra of compounds **1** and **2** were very similar. The main differences between them were the absence of the methoxyl group in **2** and that the signal at δ_H 4.25 (δ_C 84.0, C-3) was replaced by a double doublet at δ_H 4.17 (δ_C 67.5). The HRMS ESI/APCI of compound **2** was consistent with the molecular formula $C_{27}H_{26}O_{12}$ (*m/z*: 541.13504 [M – H]$^-$) indicating fifteen degrees of unsaturation, one less than in compound **1**. All this information supported the opening of the lactone ring. The NMR data showed strong similarities between the proposed structure and yunnaneic acid D [9], except that in **2**, C-3 is a secondary alcohol instead of a ketone. The correlations observed in a NOESY spectrum confirmed the same relative configuration for the bicyclo [2.2.2]octene core as in compound **1**. Compound **2** was named yunnaneic acid I to keep consistency with previously reported compounds [9,10].

By examination of the ^{1}H and ^{13}C spectra of compound **3**, the lack of the α-hydroxy-dihydrocaffeic methyl ester moiety was evident, while the bicyclo[2.2.2]octene and the lactone ring were retained. These facts suggested that in **3**, C-9 was a carboxylic acid. This assumption was confirmed by the HRMS ESI/APCI spectrum (*m/z*: 343.08130 [M – H]$^-$, $C_{18}H_{16}O_7$). Therefore the previously mentioned rufescenolide [8] is the methyl ester of compound **3**. Even though rufescenolide was not found in the present study, its C-9 free acid form was isolated, and thus compound **3** was named rufescenolide C to keep consistency with the nomenclature.

Compound **4** showed an almost identical set of spectra as compound **2**, except that C-9′ was a methyl ester. For this reason, compound **4** was identified as the methyl ester of **2**, and correspondingly named yunnaneic acid J. Compound **5**, on the basis of its NMR spectra, was identified as the C-9″ free acid form of compound **1** and thus named rufescenolide D. The NMR data of compounds **2–5** are shown in **Table 2**.

There are only a few previous reports of neolignans having related structures with a bicyclo[2.2.2]octene framework, which include yunnaneic acids A–D and F from *Salvia yunnanensis* [9, 10], helisterculins A–B from *Helicteres isora* [11], and rufescenolide from *Cordia rufescens* [8]. Due to the existence of previous trivial names, the name rufescenolide was kept for those compounds (**1, 3**, and **5**) that had the lactone moiety between C-9′ and C-3, while the remaining compounds (**2** and **4**) were named as yunnaneic acids I and J, respectively. The numbering system of the structures was that of yunnaneic acids to keep uniformity with previously reported compounds [9].

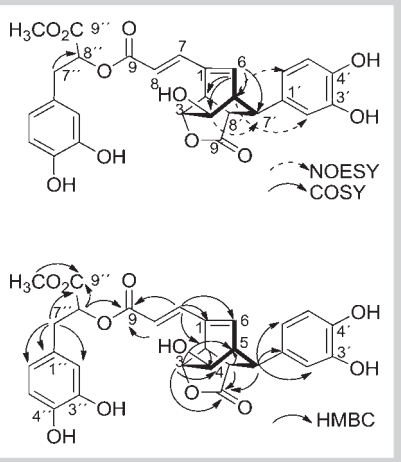

Fig. 2 2D NMR key correlations for compound **1**.

Compounds **1–5** were evaluated for antioxidant capacity by the following methods: DPPH radical scavenging and Ferric-reducing antioxidant power assays (FRAP). DPPH is widely used for quickly assessing the ability of polyphenols to transfer labile H atoms to radicals, a likely mechanism of antioxidant protection. **Fig. 40S** (see Supporting Information) shows that the scavenging activity against DPPH free radicals for compounds **1** and **4** was similar to the reference compound (catechin), while **2, 3** and **5** were less active, but still in the same order of magnitude. These results suggest that the structural differences between these compounds do not significantly influence their H atom-donating capacity. The EC_{50} found for compounds **1–5** were 3.6, 10.5, 10.3, 5.1 and 19.1 µg/mL respectively. Compound **1** showed the lowest EC_{50} value, being more active than catechin (EC_{50} 4.1 µg/mL).

FRAP measures the reducing capability of the compounds, evaluating the conversion of a Fe^{3+}/ferricyanide complex to Fe^{2+}. The reducing power of compounds **1–5** grew stronger as the concentrations increased (**Fig. 41S**, see Supporting Information). At a given dose, the reducing power of **1** and **4** was distinctively higher than that of the other compounds, even though the reducing power of compounds **2, 3** and **5** was still comparable to that of catechin.

The antibiotic activity of the compounds was tested against several strains of human pathogens. Since the measured MIC values were all higher than 250 µg/mL, the compounds were considered not active against this panel of bacteria.

The isolated compounds were tested against two strains of *Fusarium* involved in soybean sudden death syndrome, which is a great concern in Argentina due to its role as one of the main soybean producing countries. Compound **5** showed an inhibition halo of 11 mm in diameter at 50 µg (0.1 µmol/spot) after 48 h against *F. virguliforme*, but the halo vanished after 72 h. All the tested compounds were inactive against *F. solani*.

In conclusion, five new neolignan derivatives (**1–5**), all biogenetically related to rosmarinic acid, were isolated from the ethanolic extract of the bark of *C. americana*. All of the compounds displayed good antioxidant activity. However, despite their phenolic nature, none of the isolated compounds had antibacterial or antifungal capacity, and only compound **5** showed mild fungistatic activity against *F. virguliforme*.

Table 2 NMR data for compounds **2–5** in CD_3OD.

	2		3		4		5	
	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H
1	141.9	–	135.7	–	141.6	–	135.5	–
2	42.9	3.49 (1H, m)	40.5	4.10 (1H, brt, *J* = 5.0)	43.4	3.66 (1H, m)	40.3	4.05 (1H, brt, *J* = 5.0)
3	67.5	4.17 (1H, dd, *J* = 7.5, 2.7)	84.2	4.24 (1H, dt, *J* = 5.0, 1.1)	78.2	3.45 (1H, m)	84.4	4.24 (1H, brdt, *J* = 5.0, 1.0)
4	71.7	4.00 (1H, dd, *J* = 7.5, 2.9)	75.0	3.97 (1H, d, *J* = 3.6)	80.0	3.77 (1H, m)	75.0	3.97 (1H, d, *J* = 3.5)
5	48.6	2.94 (1H, m)	48.3	3.24 (1H, m)	48.3	2.85 (1H, m)	48.7	3.25 (1H, m)
6	139.8	6.57 (1H, brd, *J* = 6.6)	138.0	6.37 (1H, brd, *J* = 6.8)	141.7	6.57 (1H)*	140.6	6.44 (1H, brd, *J* = 6.6)
7	144.4	7.42 (1H, d, *J* = 15.8)	140.3	7.26 (1H, d, *J* = 15.8)	142.7	7.36 (1H, d, *J* = 15.8)	146.1	7.37 (1H, d, *J* = 15.8)
8	117.6	6.18 (1H, d, *J* = 15.8)	122.8	6.22 (1H, d, *J* = 15.8)	117.4	6.16 (1H, d, *J* = 15.8)	118.4	6.24 (1H, d, *J* = 15.8)
9	169.0	–	173.8	–	168.8	–	168.6	–
1′	138.2	–	134.8	–	138.0	–	134.6	–
2′	115.4	6.55 (1H, d, *J* = 2.1)	116.0	6.43 (1H, d, *J* = 2.2)	115.5	6.58 (1H)*	116.0	6.41 (1H, d, *J* = 2.2)
3′	146.1	–	146.1	–	144.9	–	146.1	–
4′	144.8	–	145.3	–	146.1	–	145.3	–
5′	116.2	6.62 (1H, d, *J* = 8.1)	116.1	6.63 (1H, d, *J* = 8.2)	116.2	6.62 (1H, d, *J* = 8.1)	116.1	6.63 (1H, d, *J* = 8.1)
6′	119.8	6.44 (1H, dd, *J* = 8.1, 2.1)	120.3	6.34 (1H, dd, *J* = 8.2, 2.2)	119.9	6.46 (1H, dd, *J* = 8.1, 2.2)	120.3	6.33 (1H, dd, *J* = 8.1, 2.2)
7′	44.5	3.29 (1H, m)	45.8	3.12 (1H, brt, *J* = 2.4)	44.2	3.50 (1H, dd, *J* = 6.8, 1.2)	45.7	3.12 (1H, m)
8′	52.5	2.38 (1H, dd, *J* = 6.8, 2.1)	45.8	2.64 (1H, m)	49.0	2.33 (1H, dd, *J* = 6.8, 2.0)	45.8	2.66 (1H, m)
9′	177.9	–	181.1	–	176.2	–	181.0	–
1′′	131.1	–	–	–	130.9	–	131.2	–
2′′	117.4	6.77 (1H, d, *J* = 1.9)	–	–	117.4	6.75 (1H, d, *J* = 2.0)	117.5	6.76 (1H, d, *J* = 2.0)
3′′	145.9	–	–	–	146.0	–	146.0	–
4′′	144.7	–	–	–	144.8	–	144.8	–
5′′	116.2	6.67 (1H, d, *J* = 8.1)	–	–	116.2	6.67 (1H, d, *J* = 8.0)	116.2	6.66 (1H, d, *J* = 8.1)
6′′	121.7	6.56 (1H, dd, *J* = 8.1, 1.9)	–	–	121.7	6.61 (1H, dd, *J* = 8.0, 2.0)	121.7	6.62 (1H, dd, *J* = 8.1, 2.0)
7′′	38.7	3.09 (1H, dd, *J* = 14.3, 3.3) 2.93 (1H, m)	–	–	38.6	3.11 (1H, dd, *J* = 14.3, 3.2) 2.93 (1H, dd, *J* = 14.3, 9.7)	38.8	3.11 (1H, m) 2.91 (1H, dd, *J* = 14.3, 9.8)
8′′	77.7	5.08 (1H, dd, *J* = 9.8, 3.3)	–	–	77.5	5.10 (1H, dd, *J* = 9.7, 3.2)	78.0	5.10 (1H, dd, *J* = 9.8, 3.2)
9′′	177.8	–	–	–	177.6	–	177.4	–
OMe	–	–	–	–	52.4	3.67 (3H, s)	–	–

* Partially overlapped

It is important to notice that the purity of isolated compound **3** was about 87% (calculated by NMR). Therefore the results on biological activity should be considered not conclusive. For the other compounds, the purity was higher than 97%.

Since the lactonized bicyclo[2.2.2]octene core of the rufescenolides was only detected, to date, in neolignans from two *Cordia* species including the present work, we propose that this structural feature may be considered a chemotaxonomical marker, a fact that merits revisiting previously studied species belonging to this genus.

Materials and Methods

General experimental procedures

Optical rotations were measured on a Perkin-Elmer 343 polarimeter. ^{1}H and ^{13}C NMR spectra were performed either on Bruker Avance-2 (500 MHz) or AC-200 (125 MHz) instruments, using CD_3OD as the solvent. Proton chemical shifts were referenced to the residual signal of CD_3OD at δ 3.35, and ^{13}C NMR were referenced to the central peak of CD_3OD at 49.0 ppm. Homonuclear ^{1}H connectivities were determined by COSY experiments. DEPT-HSQC allowed the determination of carbon multiplicities as well as one-bond proton-carbon connectivities, and HMBC allowed the determination of long-range proton-carbon connectivities. The relative configurations were determined by gradient-enhanced NOESY experiments. All 2D NMR experiments were performed using standard pulse sequences. HRESI mass spectra were recorded using a MicrOTOF QII Bruker mass spectrometer. Reversed-phase dry column flash chromatography was carried out on octadecyl functionalized silica gel (Aldrich Chemical Co). Sephadex LH-20 was obtained from GE Healthcare. TLCs were carried out on Merck Silicagel 60 F_{254} plates. TLC plates were sprayed with 2% vanillin in concentrated H_2SO_4 and/or $FeCl_3$ 2% in EtOH. All solvents were distilled prior use.

Plant material

Specimens of *C. americana* were collected at Santa Inés (Garupá), Misiones, Argentina in January 2010. A voucher specimen (Nº 1446) was identified by Prof. Manuela E. Rodriguez (Universidad

Nacional de Misiones) and is stored at the herbarium of Cátedra Sistemática Teórica (UNAM-Argentina).

Extraction and isolation

Fresh bark material (450 g) was extracted with EtOH (1.5 L × 3) for 3 days at room temperature. The aqueous extract obtained after evaporation of the solvent was partitioned between EtOAc and H_2O affording aqueous (PCM) and organic (PCP) phases. PCM (350 mL) was eluted through a column of Amberlite XAD-12 (5 × 57 cm), washed with water (400 mL × 3), and eluted with MeOH (1.5 L). The methanolic eluate was concentrated and the syrupy residue (602 mg) was resuspended in EtOAc. The EtOAc soluble portion (351 mg) was subjected to dry column flash chromatography on silica (7 × 3.5 cm) eluting with an EtOAc-MeOH gradient (1 : 0, 9 : 1, 7 : 3, 0 : 1; 50 mL each). Fr. 1 (25.3 mg) was purified by reversed-phase preparative TLC with EtOH-H_2O (3 : 7) as the eluant to yield **1** (7.4 mg). Fr. 4 (114.7 mg) was resuspended in MeOH-Me_2CO (1 : 1) and the soluble fraction (85.3 mg) was permeated through a Sephadex LH-20 column (3 × 21 cm) (eluted with MeOH; 800 mL, 15 mL fractions). Fr. 26–30 (11.4 mg) was further purified by reversed-phase preparative TLC with EtOH-H_2O (2 : 8) to yield **2** (2.8 mg).

PCP (4.66 g) was partitioned between MeOH-H_2O (9 : 1) and cyclohexane to yield lipophylic (1.00 g) and polar (3.66 g) subextracts. The latter was subjected to dry column flash chromatography on silica (7 × 4 cm) [eluted with a CH_2Cl_2-EtOAc-MeOH gradient (8 : 2 : 0, 6 : 4 : 0, 4 : 6 : 0, 2 : 8 : 0, 0 : 1 : 0, 0 : 9 : 1, 0 : 8 : 2, 0 : 7 : 3; 400 mL each; E1–E8)]. E3 (518.2 mg) was further subjected to reversed-phase dry column flash chromatography (7 × 3.3 cm) [eluted with an EtOH-H_2O gradient (1 : 9, 2 : 8, 3 : 7, 1 : 0; 50 mL each, E3.1–E3.4)]. E3.2 (71.2 mg) was permeated through a Sephadex LH-20 column (3 × 21 cm) using MeOH as the eluant (600 mL, 15 mL fractions). After TLC comparison, fractions were pooled into two groups: S1 (Frs.14–17) and S2 (Frs. 22–33). S1 (12.1 mg) was purified by reversed-phase preparative TLC using EtOH-H_2O (2 : 8) to yield **3** (5.1 mg). Rosmarinic acid (2.7 mg) was obtained from S2 (12.5 mg) by reversed-phase preparative TLC with EtOH-H_2O (3 : 7).

E4 (441.5 mg) was subjected to column chromatography (3.5 × 23 cm) with CH_2Cl_2-MeOH 9 : 1 (3.0 L, 15 mL fractions). Fr. 139–173 (210.0 mg) was partitioned between EtOAc and H_2O. The aqueous fraction was evaporated to dryness and the resulting residue (180.0 mg) was resuspended in MeOH-Me_2CO (1 : 1). The soluble fraction (120.0 mg) was permeated through a Sephadex LH-20 column with MeOH (3 × 21 cm, 800 mL, 15 mL fractions). Frs. 14–17 and 22–33 yielded compounds **4** (11.2 mg) and **5** (35.0 mg), respectively.

New isolates

Rufescenolide B (**1**): Brown oil; $[\alpha]_D^{25}$ + 71.2 (*c* 0.01425, MeOH); UV λ_{max} (MeOH) 213 nm (ε 17366), 275 (24028); IR (KBr) ν_{max} 3376, 1716, 1630, 1524, 1438, 1280, 1177, 983 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 1**; ESI-MS *m/z* [M – H]$^-$ 537.14182 (calcd. for $C_{28}H_{25}O_{11}$, 537.14024).

Yunnaneic acid I (**2**): Brown oil; $[\alpha]_D^{25}$ + 48.4 (*c* 0.0475, MeOH); UV λ_{max} (MeOH) 218 nm (ε 7809), 275 (9417); IR (KBr) ν_{max} 3371, 1696, 1620, 1594, 1528, 1279, 1195, 1064 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 2**; ESI-MS *m/z* [M – H]$^-$ 541.13504 (calcd. for $C_{27}H_{25}O_{12}$, 541.13515).

Rufescenolide C (**3**): Yellow oil; $[\alpha]_D^{25}$ + 141.4 (*c* 0.145, MeOH); UV λ_{max} (MeOH) 218 nm (ε 3855), 259 (5659); IR (KBr) ν_{max} 3397, 1777, 1641, 1604, 1533, 1386, 1174, 985 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 2**; ESI-MS *m/z* [M – H]$^-$ 343.08130 (calcd. for $C_{18}H_{15}O_7$, 343.08233).

Yunnaneic acid J (**4**): Brown oil; $[\alpha]_D^{25}$ + 137.9 (*c* 0.00725, MeOH); UV λ_{max} (MeOH) 220 nm (ε 44235), 268 (60073); IR (KBr) ν_{max} 3345, 1763, 1705, 1627, 1594, 1524, 989 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 2**; ESI-MS *m/z* [M – H]$^-$ 555.15338 (calcd. for $C_{28}H_{27}O_{12}$, 555.15080).

Rufescenolide D (**5**): Brown oil; $[\alpha]_D^{25}$ + 5.18 (*c* 0.135, MeOH); UV λ_{max} (MeOH) 215 nm (ε 14431), 270 (17954); IR (KBr) ν_{max} 3410, 1767, 1701, 1625, 1594, 1282, 980 cm^{-1}; ^1H and ^{13}C NMR data, see **Table 2**; ESI-MS *m/z* [M – H]$^-$ 523.12653 (calcd. for $C_{27}H_{23}O_{11}$, 523.12459).

Bioassays

Antioxidant activity

The free radical scavenger effect of the compounds was assessed by the fade of a methanolic solution of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) [12]. Compounds were assayed at concentrations of 0.5, 1, 5, 10, 20, 50, 75 and 100 µg/mL. Scavenging activities were evaluated spectrophotometrically at 517 nm using the absorbance of the DPPH radical as a reference. Catechin (Sigma-Aldrich, ≥ 98%) was used as a reference compound (EC_{50} 4.1 µg/mL). The loss of color indicated the free radical scavenging efficiency of the substances. DPPH antioxidant capacity was calculated as follows:

$$\% \text{ Scavenging effect} = [1 - (A_{sample} - A_{blank})/A_{DPPH}] \times 100$$

The compound concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated from the graph plotting inhibition percentage at A_{517} against the compound concentration.

Ferric-reducing antioxidant power was measured by the direct reduction of $Fe^{3+}(CN^-)_6$ to $Fe^{2+}(CN^-)_6$ and was determined by measuring the absorbance resulting from the formation of the Perl's Prussian Blue complex following the addition of excess ferric ions (Fe^{3+}) [13]. Different concentrations of compounds and catechin (1, 10, 20, 50, 75, 100 µg/mL) in 0.75 mL of distilled water were mixed with 1.25 mL of 0.2 M, pH 6.6 sodium phosphate buffer, and 1.25 mL of potassium ferricyanide (1%). The mixture was incubated at 50 °C for 20 min. Then, the reaction mixture was acidified with 1.25 mL of trichloroacetic acid (10%). Finally, 0.5 mL of $FeCl_3$ (0.1%) was added, and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates greater reduction capability [14].

Antimicrobial activity

Strains from the American Type Culture Collection (ATCC), Malbrán Institute (MI), Pasteur Institute (PI) and Laboratorio de Microbiología (LM, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina) were used: *Escherichia coli* ATCC 25922, *Escherichia coli*-LM1, *Escherichia coli*-LM2, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* sp.-LM, *Salmonella enteritidis*-MI, *Yersinia enterocolítica*-PI, *Staphylococcus aureus* methicillin sensitive ATCC 29213 and *Staphylococcus aureus* methicillin resistant ATCC 43300. Bacteria were grown on Mueller-Hinton agar medium.

Cultures less than 30 h old were transferred to sterile Mueller-Hinton broth and incubated at 37 °C until the growth reached turbidity equal to or greater than that of 0.5 McFarland standards. The culture was adjusted with sterile physiological solution to give a final organism density of 5×10^5 CFU/mL [15, 16]. Antibacterial activity was evaluated with the agar dilution method using Mueller-Hinton agar medium. Stock solutions of the compounds in DMSO were reduced to get serial twofold dilutions that were added to each medium resulting in concentrations ranging from 10 to 1000 µg/mL. The final concentration of DMSO in the assay did not exceed 1 %. The minimum inhibitory concentration (MIC) was defined as the lowest concentration showing no visible bacterial growth after incubation time. The antimicrobial agent cefotaxime (Argentia Pharmaceutica, 100 %) was included in the assays as a positive control. The MICs found for this compound were 12.5 µg/mL against *S. enteritidis* and 0.5 µg/mL against the rest of the assayed bacteria. The plates were incubated for 24 h at 37 °C. Tests were carried out in triplicate.

Antifungal activity

Fusarium virguliforme (Centro de Referencia de Micología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario Nº CCC220.05) and *Fusarium solani* (Instituto Spegazzini, Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata Nº LPSC868) were cultured in 3 % (w/v) malt extract-agar medium (Oxoid Ltd.) in 9 cm petri dishes at 25 °C and in darkness. In order to obtain the spores, fungi were cultured for 7–10 days. Harvesting was carried out by suspending the spores in sterilized water [17].

Direct bioautography on TLC was employed as the method for detecting fungitoxic substances [18]. A concentration level of 50 µg/spot of each assayed compound was used. Benomyl (Sigma-Aldrich, 95 %), which was used as a positive control, showed an inhibition zone of 30 mm at a concentration level of 6 µg/spot (0.02 µmol/spot) and 12 mm at 0.6 µg/spot (0.002 µmol/spot). Each assayed compound was deposited in a thin-layer normal-phase silica plate separating each spot from the other by 3 cm in order to establish the inhibition halos. The plate was dipped in the suspension of spores and was incubated at 25 °C in darkness and controlled humidity. The inhibition zone diameter was visualized after 48 h or 72 h.

Supporting information

NMR spectra of new compounds are available as Supporting Information.

Acknowledgements

This research was supported by grants from CONICET (PIP Nº 516–2009), UBACYT (Nº 20020100100123 -Prog 2011–2014), CICITCA-UNSJ, and ANPCYT (PICT 2010-Nº 1808). We thank Ing. José Gallardo and Lic. Gernot Eskuche (UMYMFOR–CONICET) for recording some of the NMR spectra. L. R. F., M. P. F. and B. L. thank CONICET for fellowships. G. C., J. A. P. and M. S. are researchers from CONICET.

Conflict of Interest

The authors declare no conflict of interest.

Affiliations

[1] UMYMFOR, Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina
[2] Instituto de Biotecnología – Cátedras Bioquímica Agrícola y Química Orgánica-Departamento de Ingeniería Agronómica, Universidad Nacional de San Juan, San Juan, Argentina
[3] Departamento de Farmacia, Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Posadas, Argentina

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