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Isolation, Characterization and Quantification of Stilbenes from Some *Carex* Species

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Abstract: Plants of the *Carex* genus (Family: Cyperaceae) have attracted recent attention as potential food additives due to their high levels of potential bioactive compounds. In this study, the stilbene contents of five unexplored *Carex* species were investigated: *Carex capillacea*, *Carex hirta*, *Carex buchananii*, *Carex cuprina*, *Carex glauca*. High-performance liquid chromatography coupled to mass spectrometry (HPLC–MS) and NMR spectroscopy (NMR) were used to identify the structures. A novel stilbene oligomer, carexinol A, was isolated together with five previously known stilbenes: resveratrol-diglucoside, miyabenol A and C, kobophenol A and α-viniferin. Furthermore, this is the first report of resveratrol diglucoside in Carex genus.

Keywords: Cyperaceae; *Carex*; stilbenes; roots; Carexinol A; NMR spectroscopy.

1. Introduction

Stilbenes are non-flavonoid polyphenols with resveratrol as a basic sub-unit. They can be found in numerous plants distributed in several botanical families [1]. Stilbenes are constituents of vine and wine and may be involved in health benefits brought by moderate wine consumption [2]. Stilbenes are very interesting for their biological properties, such as potential antioxidative activity on human low density proteins [3], antimicrobial activity [4], inhibition of human platelet aggregation [5] and as cancer-chemopreventive natural products [6]. Complex stilbenes are present in very small amounts in vine [7]. Thus, we began the evaluation of other botanical families in order to produce large quantities of these compounds for biological studies. Studies have shown that members of the *Carex* genus produce biologically active stilbenes including resveratrol oligomers [8].

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In the current study, from a first screening realized among a few Cyperaceae species of the *Carex* genus five species were selected: *Carex capillacea*, *Carex buchananii*, *Carex hirta*, *Carex glauca* and *Carex cuprina*. To our best knowledge, these five *Carex* species have not been previously investigated. The focus of this study on chemical composition of the *Carex* genus is a qualitative and quantitative approach to stilbene evaluation. Six major stilbenes were identified and quantified from roots: (E)-resveratrol 3,5-O-ß-diglucoside (**1**), (E)-miyabenol C (**3**), kobophenol A (**4**), (+)-α-viniferin (**5**), (E)-miyabenol A (**6**), and a new molecule identified as a stilbene tetramer: carexinol A (**2**).

2. Materials and Methods

2.1. Reagents and standards

The methanol and acetonitrile were obtained from Scharlab® S.L. (Sentmenat, Spain), trifluoroacetic acid (TFA) from Sigma-Aldrich® and deutered solvents from Eurisotop. Water was purified using an Elga water purification system (Bucks, UK) with a resistivity of no less than 18 MΩ/cm.

2.2. Plant material

Harvests were made in the botanical garden of Talence (France) in 2011. After harvest, plants were washed thoroughly to remove earth. The different plant parts were separated: roots, stems, seeds and leaves. In this paper, only root extracts were characterized. The samples were kept in - 20°C before analysis.

2.3. Extraction of plant samples

We used about 500 mg of frozen material for each sample. Each sample was extracted by grinding with (5 x 2) mL of methanol at room temperature. Mortar and pestle were washed with 2 mL of methanol. This extraction was renewed twice. The plant debris was extracted once more by stirring with 100 mL methanol in a 250 mL flask for 24 h in a cold room at + 4 °C. After centrifugation at 750 g for 5 min, the supernatant of each extract was recovered. The methanol extracts of each sample were pooled and concentrated first with a rotary vacuum evaporator. Then the smaller obtained volume (less than 10 mL) was evaporated to dryness with a centrifugal vacuum evaporator Savant® (Farmingdale, USA) speedvac® SVC200. The dried extracts were first re-dissolved in 300 µL methanol and then diluted with 700 µL of distilled water by vortexing and ultrasonic. Each extract is then pre-purified on a solid phase extraction (SPE) minicolumn Supelco® (Bellefonte, USA) Supelcean® LC18 3 mL. The sample was loaded onto the mini-column and washed first with 2 mL water and then eluted twice with 2 mL 90% methanol. The recovered solution contains polyphenols (flavonoids and stilbenes). Each extract was evaporated to dryness with the same centrifugal vacuum evaporator. Before HPLC analyses, the dried extract is re-dissolved in 500 µL HPLC grade 50% methanol by vortexing and ultrasonic and finally filtered through a Millipore® (Billerica, USA) 0.45 µm nylon filter cartridge.

2.4. Stilbenes isolation

2.4.1 Analytical HPLC-DAD

Analyses were carried out on an Agilent® (Santa Clara, USA) 1100 HPLC system coupled with a diode array detector (DAD). 20 µL of each sample was injected for each chromatographic analysis.

Polyphenolic extracts were separated on a Bischoff® (Stuttgart, Germany) Prontosil® reverse-phase C18 column (5 μm packing, 4 mm i.d. × 250 mm) protected with a guard column of the same material. Solvents used for the separation were 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). The elution program at 1 mL/min was a linear gradient of 10 to 90% of (B) in 60 min. The chromatograms were monitored at 286 and 306 nm and the spectra (200-600 nm) continuously recorded.

2.4.2. Preparative HPLC

The purification was carried on a Varian® (Palo Alto, USA) HPLC system coupled with a double UV-visible wavelength detector. The solvents and the gradient were the same than in the analytical conditions. The flow was 3 mL/min with a Bischoff® Prontosil® Eurobond® reverse-phase C18 column (5 μm packing, 8 mm i.d. × 250 mm) protected with a guard column of the same material for the semi-preparative conditions. In order to enhance the production of pure compounds, the flow was 18 mL/min with a Bischoff® Ultrasep® Eurobond® reverse-phase C18 column (5 μm packing, 20 mm i.d. × 250 mm) protected with a guard column (20 mm i.d. × 50 mm) of the same material for the preparative conditions. The injections were 0.1 to 2 mL depending on the semi-preparative and preparative conditions.

2.5. Identification of stilbenes

2.5.1. LC-MS measurements

The analyses were carried on an Agilent® 1200 HPLC system coupled with a DAD and a mass spectrometer Bruker® Esquire® 3000+ (ionization mode: Electrospray (ESI), analyzer: ion trap). The chromatography conditions were the same as for analytical HPLC (same solvents, same column, same flow and gradient).

2.5.2. NMR measurements

NMR spectra for all compounds were performed on a Bruker® 600 MHz spectrometer (Billerica, USA). Deuterated acetone was used as solvent for NMR experiments (except for resveratrol diglucoside which was performed in methanol-d_4). One-dimensional NMR (proton ^{1}H, carbon ^{13}C) and two-dimensional NMR measurements (including COSY, HSQC, HMBC and ROESY) were performed in order to identify the compounds. At least 1 mg of each pure compound was required for structural elucidation.

2.5.3. Polarimetric measurements

Optical rotations were determined in methanol at 20°C on a JASCO® (Jasco-France, Bouguenais, France) P-2000 polarimeter using sodium emission wavelength.

2.6. Stilbenes quantification

Because no commercial standard exists, quantification of individual stilbenes were performed using isolated compounds. Quantification was performed by HPLC-DAD analysis on an Agilent® 1100 Series system by using the same HPLC-DAD conditions previously described. Extracts were dissolved at 1 mg/mL in methanol and 20 μL of these solutions were injected. The identity of each peak was verified in parallel with LC-MS for each *Carex* sample. Stilbene concentrations were

determined by reporting the measured integration area of each compound. Mean values of each extract were calculated from three replicates. For the calibrations, the same volume of injection was used. Seven solutions including blank were prepared for each pure isolated stilbenoid compound (0, 0.001, 0.005, 0.01, 0.02, 0,05 and 0.1 mg/mL in 50% methanol). Each concentration was injected five times.

3. Results and Discussion

3.1. Structure elucidation

Extracts were prepared from roots of five *Carex* species: *C. capillacea*, *C. buchananii*, *C. hirta*, *C. glauca* and *C. cuprina* cultivated in the botanical garden of Talence (France). The genus *Carex* has been divided into many sections primarily on the basis of morphological data and data from cladistic [9].

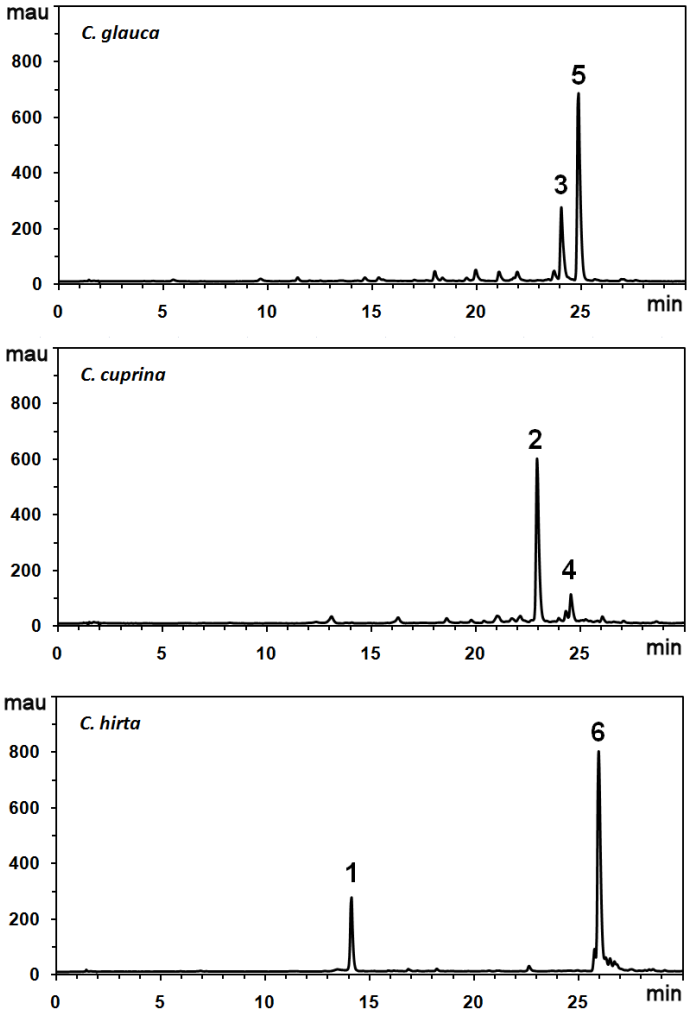

Figure 1. HPLC-DAD chromatograms (306 nm) of three *Carex* extracts.

Carex extracts were analyzed by HPLC-DAD in order to investigate their stilbene constituents. Chromatograms of *C. hirta*, *C. cuprina* and *C. glauca* are presented in Figure 1. These extracts were further purified on reverse-phase preparative scale HPLC using appropriate mixtures of methanol and water. In this way, the six stilbenes were isolated. The isolated resveratrol monomer and oligomers were identified on the basis of MS (Table 1) and NMR (Tables 2 and 3) data.

Compound **1** was isolated from *Carex hirta*, t_R HPLC-DAD = 14.1 min. Experimental mass ESI-MS (positive mode) *m/z* 553; theoretical mass *m/z* 553.2; corresponding formula: $C_{26}H_{33}O_{13}$. $[\alpha]^{20}_D$: + 42° (*c* 0.2, MeOH). ^{1}H- and ^{13}C-NMR data (see Tables 2 and 3) were consistent with those previously described [10].

Compound **2** was isolated from *Carex cuprina*, t_R HPLC-DAD = 22.9 min. Experimental mass ESI-MS (positive mode) *m/z* 941; theoretical mass *m/z* 941.3; corresponding formula: $C_{56}H_{45}O_{14}$. $[\alpha]^{20}_D$: + 87° (*c* 0.2, MeOH). ^{1}H- and ^{13}C-NMR data were reported in Tables 2 and 3. These data were consistent with a new stilbene oligomer.

Compound **3** was isolated from *Carex glauca*, t_R HPLC-DAD = 24.0 min. Experimental mass ESI-MS (positive mode) *m/z* 681; theoretical mass *m/z* 681.2; corresponding formula: $C_{42}H_{33}O_9$. $[\alpha]^{20}_D$: + 90° (*c* 0.2, MeOH). ^{1}H- and ^{13}C-NMR data (see Tables 2 and 3) were consistent with those previously described [4,11].

Compound **4** was isolated from *Carex buchananii* and *Carex cuprina*, t_R HPLC-DAD = 24.5 min. Experimental mass ESI-MS (positive mode) *m/z* 925; theoretical mass *m/z* 925.3; corresponding formula: $C_{56}H_{45}O_{13}$. $[\alpha]^{20}_D$: + 201° (*c* 0.2, MeOH). ^{1}H- and ^{13}C-NMR data (see Tables 2 and 3) were consistent with those previously described [11].

Compound **5** was isolated from *Carex glauca*, t_R HPLC-DAD = 24.9 min. Experimental mass ESI-MS (positive mode) *m/z* 679; theoretical mass *m/z* 679.2; corresponding formula: $C_{42}H_{31}O_9$. $[\alpha]^{20}_D$: + 33° (*c* 0.2, MeOH). ^{1}H- and ^{13}C-NMR data (see Tables 2 and 3) were consistent with those previously described [12].

Compound **6** was isolated from *Carex capillacea* and *Carex hirta*, t_R HPLC-DAD = 26.0 min. Experimental mass ESI-MS (positive mode) *m/z* 907; theoretical mass *m/z* 907.3; corresponding formula: $C_{56}H_{43}O_{12}$. $[\alpha]^{20}_D$: – 69° (*c* 0.2, MeOH). ^{1}H- and ^{13}C-NMR data (see Tables 2 and 3) were consistent with those previously described [4,13,14].

Table 1. Chromatographic data (peak number and retention time) and *m/z* values of stilbenes isolated in *Carex* species.

Peak	t_R (mn)	MH^+ (m/z)	Stilbenes
1	14.1	553	resveratrol diglucoside
2	22.9	941	carexinol A
3	24.0	681	(E)-miyabenol C
4	24.5	925	kobophenol A
5	24.9	679	(+)-α-viniferine
6	26.0	907	(E)-miyabenol A

Peaks are identified as follows: (E)-resveratrol 3,5-O-ß-diglucoside (**1**), carexinol A (**2**), (E)-miyabenol C (**3**), kobophenol A (**4**), (+)-α-viniferin (**5**) and (E)-miyabenol A (**6**) (Fig. 2). The Chromatographic data (peak number and retention time) and the *m/z* values of each isolated stilbenes are shown in Table 1. Using NMR and HPLC-ESI-MS compounds **3** to **6** were identified as known stilbenes from *Carex* species: (E)-miyabenol C (**3**), kobophenol A (**4**) and (+)-α-viniferin (**5**) and (E)-miyabenol A (**6**). Miyabenol A (**6**) was first isolated as constituent of *Carex fedia* var. *Miyabei* [4]. Kobophenol A (**4**) was isolated from *Carex kobomugi* together with miyabenol C (**3**) and ε-viniferin [15] and was recently identified in *Carex folliculata* [8]. Finally, (+)-α-viniferin (**5**) was previously purified from the root of *Carex humilis* [16]. All these compounds present potential biological properties. (+)-α-viniferin is an inhibitor of cyclooxygenase activity of prostaglandin H2 synthase [16]. Miyabenol A has antibiotic activities against Gram positive bacteria [4] and anti-inflammatory effect on lipopolysaccaride-induced nitric oxide production [17]. Kobophenol A has antioxidant and antibacterial activities [8]. Moreover kobophenol A has neuroprotective effects against the withdrawal of tropic support, nitrosative stress, and mitochondrial damage in SH-SY5Y neuroblastoma cells [18]. Miyabenol C shows antimicrobial activities [11] and protein kinase C inhibitor activity [19].

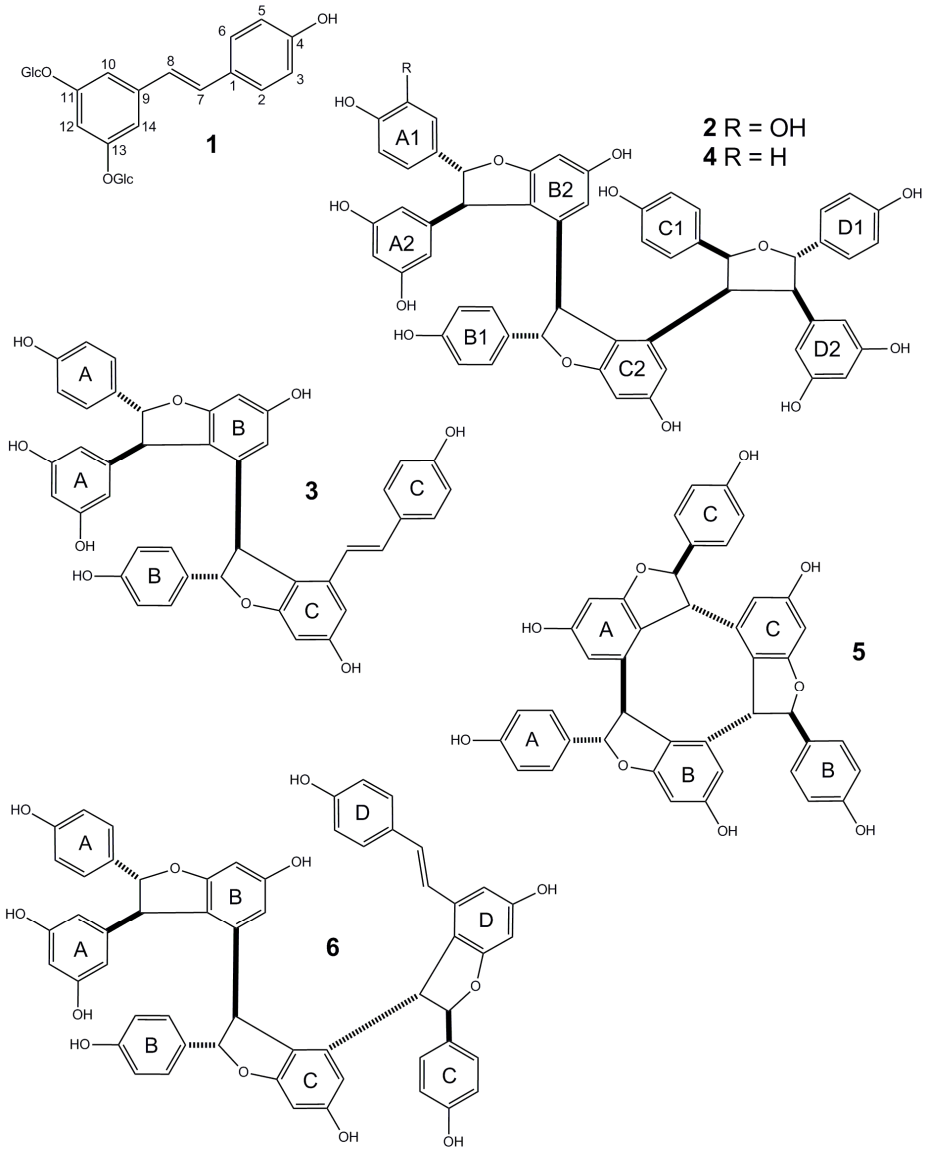

Figure 2. Chemical structures of identified stilbenes.

Concerning compound **1**, HPLC-ESI-MS (positive mode) showed an ion at *m/z* 553 allowing the formula $C_{26}H_{33}O_{13}$ (calcd. $C_{26}H_{32}O_{13}$ for: 553.2). The one dimensional NMR data of **1** showed characteristic signals of a resveratrol structure (Tables 2 and 3). Indeed the resonance set, between δ 6.7 and 7.4 ppm, composed of three systems of two olefinic and seven aromatic protons, was typical for resveratrol [20]. Furthermore the resonance set, between δ3.7 and 4.0 ppm, was attributed to two β-glycosyl units linked to an aromatic ring and one doublet at δ4.94 ppm (2H, J = 7.2 Hz) was attributed to the two anomeric protons. The overlapped signals of the two glycosyl units indicated that they were on symmetrical positions. The assignment of the resveratrol moiety as *trans* was due to the presence of the coupling constant of the olefinic proton signals at δ 6.89 and 7.08 (each, J = 16.4 Hz). Finally, NMR and MS data indicated that compound **1** was (E)-resveratrol 3,5-O-ß-diglucoside previously identified in *Vitis vinifera* cell suspension cultures [10]. To our acknowledgment, this compound is identified for the first time in the *Carex* genus.

Table 2. [1]H-NMR data of identified stilbenes at 300 K.

H	**1***	**2****	**3****	**4****	**5****	**6****
2a	7.38 *d* (8)	7.04 *d* (2)	7.15 *d* (8)	7.33 *d* (8)	7.22 *d* (8)	6.81 *d* (8)
3a	6.78 *d* (8)		6.83 *d* (8)	6.88 *d* (8)	6.82 *d* (8)	6.63 *d* (8)
5a	6.78 *d* (8)	6.85 *d* (8)	6.83 *d* (8)	6.88 *d* (8)	6.82 *d* (8)	6.63 *d* (8)
6a	7.38 *d* (8)	6.82 *dd* (2;8)	7.15 *d* (8)	7.33 *d* (8)	7.22 *d* (8)	6.81 *d* (8)
7a	6.89 *d* (16)	5.43 *d* (2)	5.37 *d* (5)	5.50 *brs*	5.94 *d* (10)	5.11 *d* (6)
8a	7.08 *d* (16)	4.36 *d* (2)	4.62 *d* (5)	4.31 *brs*	4.70 *d* (10)	4.12 *d* (6)
10a	6.94 *d* (2)	6.01 *d* (2)	6.15 *d* (2)	6.01 *d* (2)		5.90 *d* (2)
12a	6.75 *d* (2)	6.02 *t* (2)	6.20 *t* (2)	6.03 *t* (2)	6.24 *d* (2)	6.09 t (2)
14a	6.94 *d* (2)	6.01 *d* (2)	6.15 *d* (2)	6.01 *d* (2)	6.05 *d* (2)	5.90 *d* (2)
2(6)b		6.20 *d* (8)	6.49 *d* (8)	6.19 d (8)	7.02 *d* (8)	6.58 *d* (8)
3(5)b		6.50 *d* (8)	6.64 *d* (8)	6.50 *d* (8)	6.72 *d* (8)	6.42 *d* (8)
7b		4.99 *d* (4)	5.18 *brs*	4.98 *d* (4)	6.07 *brs*	5.08 *d* (2)
8b		3.50 *d* (4)	4.30 *d* (2)	3.48 *d* (4)	3.97 *brs*	4.68 *d* (2)
10b						
12b		6.49 *d* (2)	6.32 *d* (2)	6.51 *d* (2)	6.22 *d* (2)	6.31 *d* (2)
14b		5.95 *d* (2)	6.05 *d* (2)	5.95 *d* (2)	6.00 *d* (2)	5.97 *d* (2)
2(6)c		6.45 *d* (8)	7.10 *d* (8)	6.40 *d* (8)	7.05 *d* (8)	6.61 *d* (8)
3(5)c		6.59 *d* (8)	6.74 *d* (8)	6.58 *d* (8)	6.78 *d* (8)	6.64 *d* (8)
7c		5.21 *d* (4)	6.87 *d* (16)	5.04 *d* (4)	4.91 *d* (8)	5.11 *d* (2)
8c		3.37 *dd* (6;4)	6.58 *d* (16)	3.28 *dd* (6;4)	4.62 *d* (8)	4.31 *d* (2)
10c						
12c		5.99 *d* (2)	6.32 *d* (2)	5.99 *d* (2)	6.25 *d* (2)	6.18 *d* (2)
14c		6.43 *d* (2)	6.65 *brs*	6.41 *d* (2)		6.05 *d* (2)
					6.72 *brs*	
2(6)d		7.10 *d* (8)		7.07 *d* (8)		7.00 *d* (8)
3(5)d		6.78 *d* (8)		6.76 *d* (8)		6.71 *d* (8)
7d		5.17 *d* (11)		5.14 *d* (11)		6.87 *d* (16)
8d		3.20 *dd* (11;6)		3.09 *dd* (11;6)		6.63 *d* (16)
10d		5.81 *d* (2)		5.79 *d* (2)		
12d		6.09 *d* (2)		6.08 *t* (2)		6.34 *d* (2)
14d		5.81 *d* (2)		5.79 *d* (2)		6.67 *d* (2)
Glucose						
1′-1″	4.94 *d* (7)					
2′-2″	3.36-3.56					
3′-3″	3.36-3.56					
4′-4″	3.36-3.56					
5′-5″	3.36-3.56					
6′-6″	3.94 *dd* (12;2)					
	3.70 *dd* (12;6)					

*methanol-d_4; **acetone-d_6.

Table 3. ^{13}C-NMR data of identified stilbenes at 300 K.

C	1*	2**	3**	4**	5**	6**
1a	128.2	136.3	133.0	135.0	131.3	132.6
2a	129.4	112.5	127.4	126.3	127.7	128.3
3a	115.7	146.0	116.2	115.9	115.6	115.9
4a		145.5	158.1	157.6	158.4	157.9
5a	115.7	116.3	116.2	115.9	115.6	115.9
6a	129.4	117.0	127.4	126.3	127.7	128.3
7a	129.6	92.9	94.4	92.1	89.7	95.1
8a	125.5	58.2	57.0	57.8	52.5	56.1
9a	140.6	147.9	147.3	147.3	147.0	147.3
10a	108.8	106.8	106.8	106.2	120.9	107.0
11a	157.7	159.9	160.8	159.0	159.0	159.8
12a	104.1	102.0	102.0	101.5	96.2	102.4
13a	157.5	159.9	160.8	159.0	157.0	159.8
14a	108.8	106.8	106.8	106.2	105.1	107.0
1b		133.7	133.0	133.1	131.5	132.9
2(6)b		127.4	127.1	126.7	127.8	127.9
3(5)b		116.0	115.3	115.4	115.2	115.7
4b		157.4	157.2	156.9	157.1	157.9
7b		93.6	92.0	93.0	86.1	92.5
8b		52.4	50.9	52.0	46.2	49.9
9b		144.7	142.6	148.0	139.9	142.8
10b		120.3	120.0	119.4	118.8	119.2
11b		162.1	160.7	161.4	158.0	162.9
12b		96.1	96.0	96.2	97.2	96.7
13b		160.7	158.3	160.5	162.4	160.2
14b		108.4	107.4	107.8	108.2	124.1
1c		134.5	131.5	131.4	132.0	133.4
2(6)c		127.8	128.4	127.1	128.3	127.4
3(5)c		115.4	115.3	114.8	115.5	116.0
4c		156.0	157.2	155.6	157.5	157.9
7c		85.0	130.9	84.4	95.4	93.1
8c		52.7	122.4	52.1	55.6	51.5
9c		136.7	136.5	132.9	141.0	142.9
10c		124.4	121.0	120,0	119.7	120.0
11c		160.9	162.5	160.3	161.0	162.1
12c		95.8	96.8	95.3	96.2	96.7
13c		158.8	159.2	153.8	161.0	160.2
14c		110.6	104.1	109.9	105.8	107.2
1d		134.6		134.1		129.2
2(6)d		129.0		128.4		128.9
3(5)d		116.1		115.4		116.9
4d		157.7		157.1		158.0
7d		85.4		84.8		131.6
8d		62.1		61.8		122.5
9d		139.6		136.7		136.4
10d		109.3		108.6		120.9
11d		158.3		158.0		162.2
12d		103.4		102.9		97.1
13d		158.3		158.0		159.5
14d		109.3		108.6		104.4
Glucose						
1′-1″	101.6					
2′-2″	74.2					
3′-3″	77.2					
4′-4″	71.3					
5′-5″	77.5					
6′-6″	62.4					

* methanol-d_4; **acetone-d_6.

Compound **2** was obtained as a pale brown solid. HPLC-ESI-MS (positive mode) showed an ion at *m/z* 941 allowing the formula $C_{56}H_{45}O_{14}$ (calcd. $C_{56}H_{45}O_{14}$ for: 941.3). Assignments of all ^{1}H and

^{13}C signals for **2** were made by analyzing COSY, HSQC, and HMBC two-dimensional NMR spectroscopic data. The ^{1}H and ^{13}C data (Tables 2 and 3) are consistent with a linear-type of resveratrol tetramer. These data show the presence of three 4-hydroxyphenyl groups (B1, C1 and D1), two 3,5-dihydroxyphenyl groups (A2 and D2), a 3,4-dihydroxyphenyl groups (A1), two 3,5-dioxygenated-1,2-disubstituted benzene ring (B2 and C2), two sequences of dihydrobenzofuran protons (H-7a/H-8a, H-7b/H-8b) and a terahydrofuran system (H-8c/H-7c/H-7d/H-8d). The assignments all NMR resonances for **2** were achieved as shown in Table 4 by making use of HMBC spectrum. The relative stereochemical configuration of **2** was determined from ROESY experiments. The presence of NOEs between H-7a/H-10(14)a and H-8a/H-2(6)a indicates a *trans* dihydrobenzofuran system for unit A. NOEs between H-7b/H-14b and H-8b/H-2(6)b support a similar relationship for the dihydrobenzofuran system of unit B. NOE correlations between H-7c/H-8c, H-7c/H-8d and H-8c/H-8d indicate the relative stereochemistry of the tetrahydrofuran system. Cross NOEs between H-8a, H-8b and H-8c show the spatial vicinity of these protons. These results suggest the relative stereochemistry to be that shown in Figure 2. This configuration is also supported by relatively lower chemical shifts of H-10(14)a and H-2(6)b, 6.01 and 6.20 ppm respectively, which result from overlapping of rings A2 and B1. Taking into account NMR and MS data, it was concluded that compound was a new resveratrol oligomer called carexinol A.

Table 4. Major HMBC correlations of carexinol A (**2**).

C	**HMBC**	**C**	**HMBC**
1a	5a, 7a, 8a	1c	3(5)c, 7c, 8c
2a	6a, 7a	2(6)c	2(6)c, 3(5)c, 7c
3a	5a	3(5)c	2(6)c, 3(5)c
4a	2a, 6a	4c	2(6)c, 3(5)c
5a	-	7c	2(6)c, 8c
6a	2a, 7a	8c	7c, 14c, 8d
7a	2a, 6a, 8a	9c	7c, 8c, 8d
8a	7a, 10(14)a	10c	8c,12c, 14c, 7b, 8b
9a	7a, 8a	11c	7b, 8b
10(14)a	8a, 12a, 10(14)a	12c	14c
11(13)a	10(14)a	13c	12c, 14c
12a	10(14)a	14c	8c, 12c
1b	3(5)b, 7b, 8b	1d	7d, 8d, 3(5)d
2(6)b	2(6)b, 3(5)b, 7b	2(6)d	7d, 2(6)d, 3(5)d
3(5)b	2(6)b, 3(5)b	3(5)d	2(6)d, 3(5)d
4b	2(6)b, 2(6)b	4d	2(6)d, 3(5)d
7b	2(6)b, 8b	7d	2(6)d, 8d
8b	7b, 14b	8d	7d, 10(14)d, 8c
9b	7b, 8b	9d	7d, 8d, 8c
10b	7a, 8a, 8b, 12b, 14b	10(14)d	12d
11b	7a, 8a, 12b	11(13)d	10(14)d
12b	14b	12d	10(14)d
13b	12b, 14b		
14b	8b, 12b		

3.2 Total contents of stilbenes

The concentrations of stilbenes in *Carex* extracts were determined by HPLC-DAD measurements at 306 nm (wavelength corresponding to the average λ_{max} of identified stilbenes) using an external calibration method with pure stilbenes. Concerning calibrations, the linearity was verified in the range of concentrations used and volume of injection (0 – 0.1 mg/mL, 20 µL) for each compound. The calibration data are given in table 5.

Table 5. Calibration parameters of the HPLC quantification method of the stilbenoid compounds.

Compound	Regression equation * Y = aC + b	Correlation coefficient (r^2)	LOD µg/mL	LOQ µg/mL
resveratrol diglucoside	Y = 181057C + 24.504	0.99927	0.745	2.166
carexinol A	Y = 1317C – 0.224	0.99469	1.815	5.655
(E)-miyabenol C	Y = 8481.6C + 7.447	0.99891	1.621	3.353
kobophenol A	Y = 1840.4C + 0.558	0.99509	1.885	5.578
(+)-α-viniferine	Y = 5169.9C + 2.698	0.99925	1.138	2.575
(E)-miyabenol A	Y = 17634C + 3.531	0.99958	0.675	1.75

where C is the concentration of compound in mg/mL and Y is the peak area. LOD is the limit of detection, LOQ is the limit of quantification. Confidence limit 95%, n = 5 repeats, N = 7 concentrations, volume of injection = 20 µL.

The average content of the six stilbenes in the different *Carex* species are shown in Table 6. The total concentrations of identified stilbenes were thus determined at 3.5 mg/g for *C. capillacea*, 7.6 mg/g for *C. buchananii*, 2.8 mg/g for *C. hirta*, 8.9 mg/g *C. glauca* and 5.0 mg/g for *C. cuprina*. From all data reported above, we can state that *Carex* species are rich in stilbenes. These *Carex* species can be distinguished by studying the stilbene content. Indeed, some stilbenes seem characteristic of specific species (resveratrol diglucoside was detected only in *C. hirta*, miyabenol C and α-viniferin only in *C. glauca* and carexinol A only in *C. cuprina*).

Table 6. Average content of stilbenes in root *Carex* species (mg/g).

Stilbenes	*C. capillacea*	*C. hirta*	*C. cuprina*	*C. glauca*	*C. buchananii*
resveratrol diglucoside (**1**)	-	0.7 ± 0.1	-	-	-
carexinol A (**2**)	-	-	2.8 ± 0.2	-	-
(E)-miyabenol C (**3**)	-	-	-	3.4 ± 0.1	-
kobophenol A (**4**)	-	-	2.2 ± 0.2	-	3.7 ± 0.2
(+)-α-viniferine (**5**)	-	-	-	5.5 ± 0.1	-
(E)-miyabenol A (**6**)	3.5 ± 0.3	2.1 ± 0.1	-	-	3.9 ± 0.1

Concerning individual compounds, resveratrol diglucoside (**1**) is the less abundant component and was only detectable in *Carex hirta*. This molecule is already known in *Vitis vinifera* [10] but had never been identified in *Carex* genus and Cyperaceae family. The new stilbene tetramer carexinol A (**2**) was only identified in *Carex cuprina*. Miyabenol C (**3**) and α-viniferin (**5**) are detected in *Carex glauca*. These molecules are already known in *Carex kobomugi* [11] and *Carex humilis* [16]. Kobophenol A (**4**) was detected in *Carex cuprina* and *Carex buchananii*. This molecule is already known in *Carex kobomugi* [11] and *Carex folliculata* [8]. Miyabenol A (**6**) was detected in *Carex hirta* and *Carex buchananii*. This molecule is already known in *Carex fedia* [4].

To conclude, in order to isolate complex stilbenes, our study shows that *Carex* genus seems one of the most promising in the Cyperaceae family. The presence of stilbenes is certainly not restricted to an organ but is globally much more important in roots according to our preliminary investigations of aerial parts of the plants (data not shown). Analyses by HPLC-MS and RMN allowed the identification of numerous molecules: a carexinol A (which is a new compound), (E)-miyabenol A, (E)-miyabenol C, kobophenol A, (+)-α-viniferin and (E)-resveratrol 3,5-O-ß-diglucoside already known in vine. These stilbenes obtained in great quantities are going to allow us to make biological tests to estimate their pharmacological potentialities.

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